Mak HY, Hoare S, Henttu PM, Parker MG (1999) Molecular determinants of the estrogen receptor–coactivator interface. Mol Cell Biol 19:3895–3903

Nayernia K, Li M, Jaroszynski L, Khusainov R, Wulf G, Schwandt I, Korabiowska M, Michelmann HW, Meinhardt A, Engel W (2004) Stem cell based therapeutical approach of male infertility by teratocarcinoma derived germ cells. Hum Mol Genet 13:1451–1460

Nicholas CR, Xu EY, Banani SF, Hammer RE, Hamra FK, Reijo Pera RA (2009) Characterization of a Dazl–GFP germ cell-specific reporter. Genesis 47:74–84

Payer B, Chuva de Sousa Lopes SM, Barton SC, Lee C, Saitou M, Surani MA (2006) Generation of stella–GFP transgenic mice: a novel tool to study germ cell development. Genesis 44:75–83

Richards JS (1994) Hormonal control of gene expression in the ovary. Endocr Rev 15:725–751

Richards JS, Jonassen JA, Rolfes AI, Kersey K, Reichert LE Jr (1979) Adenosine 3′,5′-monophosphate, luteinizing hormone receptor, and progesterone during granulosa cell differentiation: effects of estradiol and follicle-stimulating hormone. Endocrinology 104:765–773

Ristevski S (2005) Making better transgenic models: conditional, temporal, and spatial approaches. Mol Biotechnol 29:153–163

Tanaka SS, Nagamatsu G, Tokitake Y, Kasa M, Tam PPL, Matsui Y (2004) Regulation of expression of mouse interferon-induced transmembrane protein like gene-3, Ifitm3 (mil-1, fragilis), in germ cells. Dev Dyn 230: 651–659

Toda K, Takeda K, Okada T, Akira S, Saibara T, Kaname T, Yamamura K, Onishi S, Shizuta Y (2001) Targeted disruption of the aromatase P450 gene (Cyp19) in mice and their ovarian and uterine responses to 17β-oestradiol. J Endocrinol 170:99–111

Toda K, Okada Y, Zubair M, Morohashi KI, Saibara T, Okada T (2004) Aromatase-knockout mouse carrying an estrogen-inducible enhanced green fluorescent protein gene facilitates detection of estrogen actions in vivo. Endocrinology 145:1880–1888

Toda K, Hayashi Y, Okada T, Morohashid K, Saibarae T (2005) Expression of the estrogen-inducible EGFP gene in aromatase-null mice reveals differential tissue responses to estrogenic compounds. Mol Cell Endocrinol 229:119–126

Toda K, Hayashi Y, Ono M, Saibara T (2012) Impact of ovarian sex steroids on ovulation and ovulatory gene induction in aromatase-null mice. Endocrinology 153:386–394

Tsien RY (1998) The green fluorescent protein. Annu Rev Biochem 67:509–544

Vidal F, Sage J, Cuzin F, Rassoulzadegan M (1998) Cre expression in primary spermatocytes: a tool for genetic engineering of the germ line. Mol Reprod Dev 51:274–280

West JA, Viswanathan SR, Yabuuchi A, Cunniff K, Takeuchi Y, Park I-H, Sero JE, Zhu H, Perez-Atayde A, Frazier AL, Surani MA, Daley GQ (2009) A role for Lin28 in primordial germ-cell development and germ-cell malignancy. Nature 460:909–914

Yeom YI, Fuhrmann G, Ovitt CE, Brehm A, Ohbo K, Gross M, Hübner K, Schöler HR (1996) Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells. Development 122:881–894

Yoshimizu T, Sugiyama N, Felice MD, Yeom YI, Ohbo K, Masuko K, Obinata M, Abe K, Schöler HR, Matsui Y (1999) Germline-specific expression of the Oct-4/green fluorescent protein (GFP) transgene in mice. Dev Growth Differ 41:675–684

Zambrowicz BP, Harendza CJ, Zimmermann JW, Brinster RL, Palmiter RD (1993) Analysis of the mouse protamine 1 promoter in transgenic mice. Proc Natl Acad Sci USA 90:5071–5075

J Gastroenterol
DOI 10.1007/s00535-013-0911-1

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Type 2 diabetes mellitus is associated with the fibrosis severity in patients with nonalcoholic fatty liver disease in a large retrospective cohort of Japanese patients

Takashi Nakahara · Hideyuki Hyogo · Masato Yoneda · Yoshio Sumida · Yuichiro Eguchi · Hideki Fujii · Masafumi Ono · Takumi Kawaguchi · Kento Imajo · Hiroshi Aikata · Saiyu Tanaka · Kazuyuki Kanemasa · Kazuma Fujimoto · Keizo Anzai · Toshiji Saibara · Michio Sata · Atushi Nakajima · Yoshito Itoh · Kazuaki Chayama · Takeshi Okanoue · Japan Study Group of Nonalcoholic Fatty Liver Disease (JSG-NAFLD)

Received: 3 September 2013 / Accepted: 7 November 2013

Abstract
Background The prevalence of nonalcoholic fatty liver disease (NAFLD) and metabolic syndrome have been increasing worldwide. The associations between metabolic factors and the histologic severity of NAFLD have not yet been clarified. Therefore, we studied the relationships between relevant metabolic factors and the histological severity of NAFLD.
Methods In a cross-sectional multicenter study conducted in Japan, we examined 1,365 biopsy-proven NAFLD patients. The frequencies of underlying lifestyle-related diseases and their relationships to the NAFLD histology were investigated.
Results The hepatic fibrosis stages (Stage 0/1/2/3/4) were 22.6/34.1/26.7/14.5/2.1 (%) in the male patients, and 16.2/31.7/23.9/21.6/6.6 (%) in the female patients. Dyslipidemia was present in 65.7 % (hypertriglyceridemia, 45.3 %; increased low-density lipoprotein cholesterol, 37.5 %; decreased high density lipoprotein cholesterol, 19.5 %) of patients. Hypertension was present in 30.2 %, and diabetes mellitus (DM) in 47.3 %. The fibrosis stage increased with age, especially in postmenopausal females. The body mass index was positively correlated with the fibrosis stage. Deterioration of glucose control was positively correlated

Electronic supplementary material The online version of this article (doi:10.1007/s00535-013-0911-1) contains supplementary material, which is available to authorized users.

T. Nakahara · H. Hyogo · H. Aikata · K. Chayama
Department of Medicine and Molecular Sciences, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

M. Yoneda (✉) · K. Imajo · A. Nakajima
Division of Gastroenterology, Yokohama City University Graduate School of Medicine, Yokohama, Japan
e-mail: dryoneda@yahoo.co.jp

M. Yoneda
Center for Liver Diseases, University of Miami Miller School of Medicine, Miami, USA

Y. Sumida · Y. Itoh
Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Graduate School of Medical Science, Kyoto, Japan

Y. Eguchi
Liver Center, Saga Medical School, Saga, Japan

H. Fujii
Department of Hepatology, Graduate School of Medicine, Osaka City University, Osaka, Japan

M. Ono · T. Saibara
Department of Gastroenterology and Hepatology, Kochi Medical School, Kochi, Japan

T. Kawaguchi · M. Sata
Division of Gastroenterology, Department of Medicine and Digestive Disease Information and Research, Kurume University School of Medicine, Kurume, Japan

S. Tanaka · K. Kanemasa
Center for Digestive and Liver Disease, Nara City Hospital, Nara, Japan

K. Fujimoto · K. Anzai
Internal Medicine, Saga Medical School, Saga, Japan

T. Okanoue
Hepatology Center, Saiseikai Suita Hospital, Suita, Japan

Published online: 26 November 2013

with the fibrosis stage, this correlation being more prominent in females. Multivariate analysis identified age and DM as significant risk factors for advanced fibrosis. No significant correlation of the fibrosis stage was observed with hypertension. There was a negative correlation between the serum triglyceride levels and the fibrosis stage. *Conclusions* DM appeared to be a significant risk factor for advanced fibrosis in patients with NAFLD, and would therefore need to be properly managed to prevent the progression of NAFLD.

Keywords NAFLD · Histology · Diabetes mellitus · Retrospective study

Abbreviations

NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
IR	Insulin resistance
DM	Diabetes mellitus
NAFL	Nonalcoholic fatty liver
BMI	Body mass index
CT	Computed tomography
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
GGT	Gamma glutamyl transpeptidase
ChE	Cholinesterase
HDL	High density lipoprotein
LDL	Low-density lipoprotein
FPG	Fasting plasma glucose
HbA1c	Hemoglobin A1c
FFA	Free fatty acid
CRP	C-reactive protein
IRI	Immunoreactive insulin
HOMA-IR	Homeostasis model assessment-insulin resistance
SD	Standard deviation
IGT	Impaired glucose tolerance
NGT	Normal glucose tolerance

Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most commonly encountered chronic liver disease in the world. According to Japanese annual health check reports, 9–30 % of Japanese adults suffer from NAFLD [1–3]. Since it is now known that almost 10–20 % of individuals with NAFLD have nonalcoholic steatohepatitis (NASH), the prevalence of NASH is estimated to be 1–3 % in the adult Japanese population, similar to the prevalence reported from Western countries.

Nonalcoholic fatty liver disease includes a wide spectrum of liver diseases, ranging from nonalcoholic fatty liver (NAFL), a benign and non-progressive condition, to NASH, which can progress to liver cirrhosis and hepatocellular carcinoma even in the absence of a history of significant alcohol consumption [4–7]. Furthermore, NASH is considered to be the hepatic manifestation of metabolic syndrome, and has been shown to be associated with obesity, insulin resistance (IR) and abnormalities of glucose and lipid metabolism [8–16]. Importantly, the rates of nonalcoholic fatty liver (NAFL) and NASH are expected to continue to grow with the developing pandemic of obesity and diabetes mellitus, to become global public health concerns.

Owing to the difficulties in diagnosing NAFLD (NAFL and/or NASH) and referral bias, it has been difficult to determine the prognostic factors in patients with NAFLD. NAFLD is a complex disease with multiple etiopathogenetic factors, including obesity, type 2 DM, dyslipidemia, hypertension, and other diseases associated with metabolic dysregulations. Recent reports have suggested that DM is an independent risk factor for NAFLD [17–19]. Despite the high prevalence and potentially serious nature of this disease, relatively little is known about the metabolic factors that might be associated with the histological severity of NAFLD.

The purpose of this study was to conduct a retrospective investigation of the association between metabolic factors and the histologic severity of NAFLD in a large cohort of Japanese patients with NAFLD.

Patients and methods

Patient population

A total of 1,365 biopsy-proven NAFLD patients seen between 2001 and 2012 were enrolled from institutes affiliated with the Japan Study Group of NAFLD (JSG-NAFLD), represented by the following nine hepatology centers in Japan: Hiroshima University, Kyoto Prefectural University of Medicine, Yokohama City University, Kochi Medical School, Saga Medical School, Osaka City University, Nara City Hospital, Kurume University, and Saiseikai Suita Hospital. A portion of the patients (76.8 %; 1,048 out of 1,365) had also been involved in the previous JSG-NAFLD study [20, 21]. Informed consent was obtained from each patient, and the study was conducted in conformity with the ethical guidelines of the 7th revision of the Declaration of Helsinki (in October 2008) [22] and the approval of the ethics and research committees of the hospitals. In all patients, the current and past daily alcohol intake was less than 20 g per day; details regarding alcohol consumption were obtained independently by at least two physicians and confirmed by close family members. None

of the patients were receiving any medications that could cause NASH. Among the patients, those with the following disorders were excluded: secondary causes of steatohepatitis, drug-induced liver disease, alcoholic liver disease, viral hepatitis, autoimmune hepatitis, primary biliary cirrhosis, α1-antitrypsin deficiency, hemochromatosis, Wilson's disease, and biliary obstruction. [23].

Study design

A complete physical examination was performed on each patient within 1 month prior to the liver biopsy, as reported previously [24]. The body mass index (BMI) was calculated as the weight (kg) divided by height (m)-squared. Obesity was defined as a BMI of greater than 25, according to the criteria of the Japan Society for the Study of Obesity [25]. Computed tomography (CT) was used to determine the visceral fat area at the level of the umbilicus [26], as previously reported [24]. Dyslipidemia was diagnosed based on serum cholesterol levels higher than 220 mg/dl and/or high-density lipoprotein cholesterol levels lower than 40 mg/dl and/or triglyceride levels over 150 mg/dl. Hypertension was diagnosed if the patient was on antihypertensive medication and/or had a resting recumbent blood pressure of $\geq$ 130/85 mmHg on at least two occasions. Hyperuricemia was diagnosed based on serum uric acid levels higher than 7.0 mg/dl. DM was diagnosed according to the 2006 World Health Organization (WHO) criteria [27].

Venous blood samples were taken in the morning following overnight fasting for 12 h. The laboratory evaluation in all patients included a blood cell count, hemoglobin, platelet count; and the serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), AST/ALT ratio, lactate dehydrogenase (LDH), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT), cholinesterase (ChE), total bilirubin, direct bilirubin, albumin, total cholesterol, triglycerides, high density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, fasting plasma glucose (FPG), hemoglobin A1c (HbA1c), immunoreactive insulin (IRI), ferritin, uric acid, free fatty acid (FFA), and hyaluronic acids, were measured periodically during the treatment using the standard techniques of clinical chemistry laboratories.

Insulin resistance was calculated by the homeostasis model assessment-insulin resistance (HOMA-IR) using the following formula: HOMA-IR = fasting insulin (μU/ml) × plasma glucose (mg/dl)/405 [28].

Pathology

Patients enrolled in this study underwent percutaneous liver biopsy under ultrasonic guidance after obtaining informed consent. Formalin-fixed, paraffin-embedded liver sections were stained routinely with hematoxylin-eosin, silver reticulin, and Masson trichrome. All the specimens were examined by an experienced pathologist who was unaware of the clinical and biochemical data of the patients. Histological diagnosis for NAFLD was performed according to the methods of Matteoni et al. [6]. Grading and staging was classified according to Brunt et al. [29] and Kleiner et al. [30], as previously reported. In brief, steatosis was graded as follows: grade 1 (5–33 % of hepatocytes affected), grade 2 (34–66 % of hepatocytes affected), or grade 3 (> 66 % of hepatocytes affected). Necroinflammation was graded from grade 0 (absent) to 3 (1, occatonal ballooned hepatocytes and no or very mild inflammation; 2, ballooning of hepatocytes and mild-to-moderate portal inflammation; 3, intra-acinar inflammation and portal inflammation). Fibrosis was staged from grade 0 (absent) to 4 (1, perisinusoidal/pericellular fibrosis; 2, periportal fibrosis; 3, bridging fibrosis; 4, cirrhosis).

Statistical analyses

The data were statistically analyzed using R software, version 3.0.0. Continuous variables were expressed as mean ± standard deviation (SD). Qualitative data are expressed as numbers, with percentages shown in parentheses.

Statistically significant differences in the quantitative data were determined using the *t* test or Mann–Whitney *U* test. Multivariate analysis was carried out by logistic regression. Differences were considered to be statistically significant at *P* values of less than 0.05.

Results

Patient characteristics

A total of 1,365 biopsy-proven patients with NAFLD were enrolled in this study. The demographic and clinical characteristics of the male and female NAFLD patients are shown in Supplemental Table 1. Of the total, 709 were males. The mean age of the patients was 51.0 ± 14.9 years (45.7 ± 15.1 and 56.8 ± 12.4 years for males and females, respectively). Whereas no significant differences were observed in the BMI, blood pressure, waist circumference, and visceral fat area between the male and female patients, the subcutaneous fat area and L/S ratio were significantly higher in the female patients. Statistically significant differences were observed in the white blood cell count, hemoglobin, and serum levels of transaminases, AST to ALT ratio, LDH, ALP, GGT, ChE, total and direct bilirubin, albumin, triglycerides, HDL cholesterol, fasting

Table 1 Prevalences of metabolic abnormalities in NAFLD patients

Variable	Percentage
BMI ≥ 25	73.0
Hypertension	39.9
Dyslipidemia	65.7
Hypertriglyceridemia	45.3
Hyper-LDL cholesterolemia	37.5
Hypo-HDL cholesterolemia	19.5
DM	47.3
Hyperuricemia	30.2

glucose, HbA1c, ferritin, uric acid, and hyaluronic acid between the male and female patients, as shown in Supplemental Table 1.

The frequencies of the metabolic abnormalities in the NAFLD patients are shown in Table 1. Obesity, as defined by the criteria of the Japan Society for the Study of Obesity, was seen in 73.0 % of the NAFLD patients, hypertension was found in 39.9 %, dyslipidemia in 65.7 % (hypertriglyceridemia, 45.3 %; hyper-LDL cholesterolemia, 37.5 %; hypo-HDL cholesterolemia, 19.5 %), type 2 diabetes in 47.3 %, and hyperuricemia in 30.2 % of the patients.

Distribution of the metabolic factors by the histological findings

The fibrosis stages (Stage 0/1/2/3/4) were 22.6/34.1/26.7/14.5/2.1 (%) in males, and 16.2/31.7/23.9/21.6/6.6 (%) in females, respectively. The distribution of the fibrosis stage in the different age groups in both genders is shown in Supplementary Fig. 1. Whereas the percentage of patients with advanced fibrosis (Stage 3 and 4) increased gradually with age in both genders, significant increase was seen after the age of 60 years in the females.

The prevalences of obesity (BMI ≥ 25) for each fibrosis stage are shown in Supplementary Fig. 2. The percentages of patients with obesity for each fibrosis stage (Stage 0/1/2/3/4) were 61.3/73.3/79.9/86.4/80.0 (%) in males, and 57.1/72.9/74.4/75.9/74.4 (%) in females, respectively. The prevalence of obesity showed a linear increase with progression of the fibrosis stage in the male NAFLD patients. However, no such increase was observed in the female NAFLD patients between Stage 1 and Stage 4.

The prevalences of dyslipidemia for each fibrosis stage are shown in Figs. 1 and 2. The percentages of patients with hypertriglyceridemia for each fibrosis stage (Stage 0/1/2/3/4) were 56.3/57.7/54.8/51.0/26.7 (%) in males, and 34.0/39.5/39.1/30.2/12.2 (%) in females, respectively. The percentages of patients with hyper-LDL cholesterolemia for each fibrosis stage (Stage 0/1/2/3/4) were 38.6/36.2/41.3/30.4/18.2 (%) in males, and 43.4/38.0/43.6/36.8/11.1 (%) in females, respectively. The prevalence rates of dyslipidemia (hypertriglyceridemia and hyper-LDL cholesterolemia) decreased with progression of the fibrosis stage, especially in Stage 4.

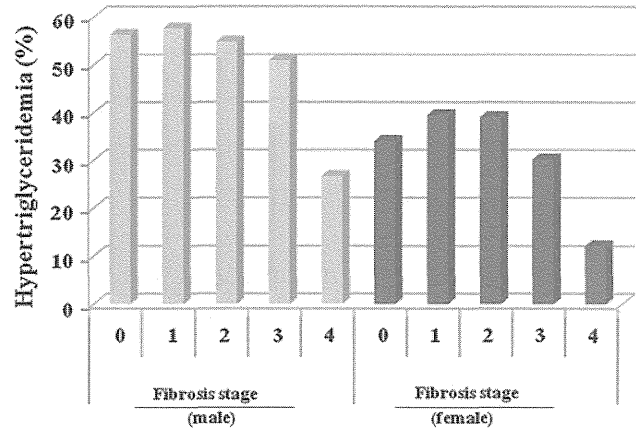


Fig. 1 Prevalence of hypertriglyceridemia for each stage of fibrosis. The *horizontal axis* shows the fibrosis stage and the *longitudinal axis* shows the percentage of patients with hypertriglyceridemia

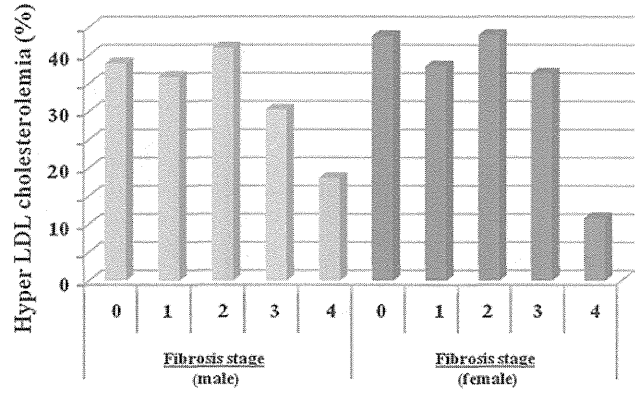


Fig. 2 Prevalence of hyper-LDL cholesterolemia for each stage of fibrosis. The *horizontal axis* shows the fibrosis stage and the *longitudinal axis* shows the percentage of patients with hyper-LDL cholesterolemia

The prevalence of hypertension for each fibrosis stage was shown in Fig. 3. The percentages of patients with hypertension for each fibrosis stage (Stage 0/1/2/3/4) were 17.9/34.0/40.3/51.4/42.9/35.3 (%) in males, and 35.3/50.0/47.7/50.0/23.9 (%) in females respectively.

The prevalences of impaired glucose tolerance, including DM, for each fibrosis stage are shown in Fig. 4. The percentages of patients with DM for each fibrosis stage (Stage 0/1/2/3/4) were 23.7/32.8/53.7/65.8 (%) in males, and 34.7/45.2/60.9/64.7 (%) in females, respectively. The percentages of patients with impaired glucose tolerance (IGT) in each fibrosis stage (Stage 0/1/2/3/4) were 6.6/18.5/17.6/16.2 (%) in males, and 15.3/10.6/14.1/14.1 (%) in females, respectively. The percentages of patients with

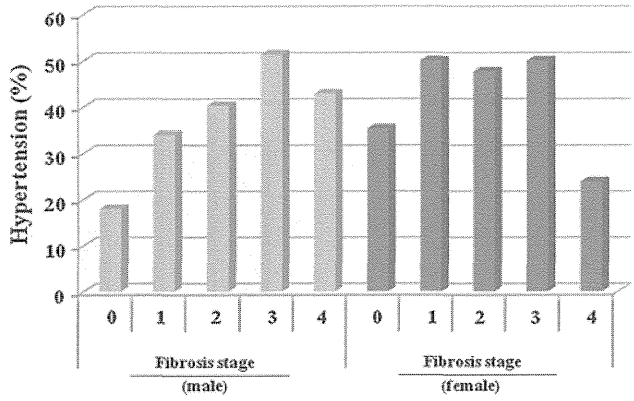


Fig. 3 Prevalence of hypertension for each stage of fibrosis. The *horizontal axis* shows the fibrosis stage and the *longitudinal axis* shows the percentage of patients with hypertension

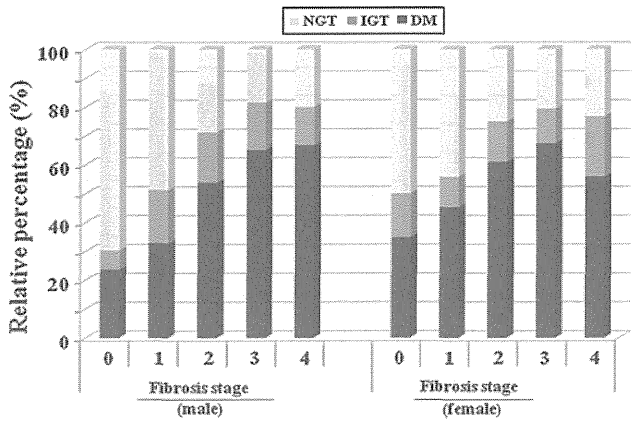


Fig. 4 The glucose tolerance pattern for each fibrosis stage in male and female NAFLD patients. The *horizontal axis* shows the fibrosis stage and the *longitudinal axis* shows the normal glucose tolerance, impaired glucose tolerance, or DM

normal glucose tolerance (NGT) were 69.7/48.7/28.7/17.9 (%) in males, and 50.0/44.2/25.0/21.2 (%) in females, respectively. The percentage of patients with DM increased with progression of the fibrosis stage in both male and female NAFLD patients.

Factors associated with advanced fibrosis

Factors associated with advanced fibrosis were examined (Table 2). NAFLD patients with advanced fibrosis were older, more likely to be female, and obese. The BMI, visceral fat area, and liver/spleen (L/S) ratio were significantly higher in NAFLD patients with advanced fibrosis. Furthermore, significant increases of the serum level of AST, AST/ALT ratio, ALP, GGT, total and direct bilirubin, fasting glucose, HbA1c, IRI, HOMA-IR, ferritin, FFA, and hyaluronic acid, and decreases of hemoglobin, platelet count, ChE, albumin, total cholesterol, triglycerides, LDL cholesterol, and uric acid were observed in the patients with advanced fibrosis. In cases with high fasting plasma glucose levels, HOMA-IR does not reflect insulin resistance exactly, and was assumed to be a reference level. To investigate the factors that might be related to the progression to advanced fibrosis, univariate analysis was performed between NAFLD patients with advanced fibrosis and those with no or mild fibrosis, as shown in Table 3. The results of the analysis revealed obesity (BMI $\geq$ 25), hypertension, hypotriglyceridemia, hyper-LDL cholesterolemia, DM, and hyperuricemia as risk factors for advanced fibrosis. Multivariate analysis identified older age, low serum triglyceride and DM as risk factors for advanced fibrosis.

Discussion

Many factors have been reported to be implicated in the pathogenesis of NAFLD, including obesity, DM, dyslipidemia and hypertension. However, it is still unclear how the metabolic factors might affect the pathogenesis and progression of NAFLD [11, 20, 31–34]. Therefore, identifying the risk factors for the deterioration of NAFLD would be useful for designing therapeutic strategies not only for the liver itself, but also for these metabolic diseases. Whereas a large number of papers have reported the differences in the clinical features between NAFL and NASH, comparisons of the clinical features by the histological severity are scarce. In this study, we retrospectively investigated the associations between metabolic factors and the histologic severity of NAFLD in a large cohort of 1,365 biopsy-proven NAFLD patients, considered as one of the largest-scale studies in the world to date.

The first important finding of our study was that the severity of fibrosis advanced gradually with age in the male patients with NAFLD, while it increased only in those women over 60 years of age. This gender difference may be attributable to menopause in females [35, 36].

The second important finding of our study was the association between obesity and fibrosis severity in NAFLD patients. We compared the prevalence of obesity and the histological severity of NAFLD. As shown in Supplementary Fig. 2, whereas the prevalence of obesity increased with the progression of fibrosis in males, the prevalence remained at approximately 70 % in all age groups of females.

It has been reported that 42–72 % of patients with NAFLD, including NASH, have dyslipidemia [37, 38]. Consist with these reports, dyslipidemia was present in 65.7 % of patients in our study, including hypertriglyceridemia in 45.3 %, increased serum low-density lipoprotein cholesterol in 37.5 %, and decreased serum high-density

Table 2 Comparison for the demographic and clinical characteristics between patients with mild (Stage 0–2) and advanced (Stage 3, 4) fibrosis with NAFLD

Variable	All cases (n = 1,365)	Stage 0–2 (n = 1,062)	Stage 3, 4 (n = 303)	P value	P value (after adjustment for age/sex)
Age	51 ± 14.9	49 ± 15.0	57 ± 12.8	<0.0001	
Gender (male/female)	709/656	591/471	118/185	<0.0001	
Clinical and anthropometric measure					
Body mass index (kg/m^2)	27.9 ± 4.8	27.7 ± 4.8	28.6 ± 4.7	0.0006	<0.0001
BMI ≥ 25 (%)	73.0	71.2	79.5	0.0054	<0.0001
Waist circumference (cm^2)	96.7 ± 13.5	96.1 ± 12.4	98.1 ± 15.4	0.2372	0.0239
Subcutaneous fat area (cm^2)	220.7 ± 103.9	221.1 ± 110.7	219.7 ± 87.1	0.4631	0.4865
Visceral fat area (cm^2)	151.9 ± 65.9	144.4 ± 56.8	168.5 ± 80.3	0.0025	0.0007
L/S ratio	0.75 ± 0.30	0.73 ± 0.29	0.81 ± 0.32	0.0013	0.3528
Blood pressure sys. (mmHg)	127 ± 16.9	127 ± 15.7	124 ± 21.3	0.5343	0.0867
Blood pressure dia. (mmHg)	77 ± 11.1	77 ± 11.0	76 ± 11.5	0.6701	0.6802
Laboratory studies					
White blood cells (/μl)	6,330 ± 1,616.9	6,348 ± 1,583.4	6,272 ± 1,717.7	0.7037	0.6377
Hemoglobin (g/dl)	14.5 ± 1.6	14.6 ± 1.6	14.2 ± 1.6	<0.0001	0.6617
Platelet count (×10^4/μl)	22.4 ± 10.0	23.7 ± 10.4	18.0 ± 6.7	<0.0001	<0.0001
AST (IU/l)	57 ± 38.9	52 ± 36.0	72 ± 44.6	<0.0001	<0.0001
ALT (IU/l)	88 ± 60.3	87 ± 60.2	92 ± 60.4	0.1319	0.0003
AST/ALT	0.72 ± 0.3	0.67 ± 0.3	0.89 ± 0.4	<0.0001	<0.0001
LDH (IU/l)	210 ± 55.1	209 ± 56.7	213 ± 49.9	0.1446	0.5835
ALP (IU/l)	258 ± 111.0	250 ± 103.5	284 ± 130.1	<0.0001	0.0003
GGT (IU/l)	91 ± 103.4	88 ± 103.2	101 ± 103.4	<0.0001	0.0023
ChE (IU/l)	374 ± 106.5	383 ± 104.7	345 ± 107.3	<0.0001	0.0004
Bilirubin, total (mg/dl)	0.89 ± 0.39	0.86 ± 0.36	0.97 ± 0.45	0.0024	0.7731
Bilirubin, direct (mg/dl)	0.21 ± 0.16	0.19 ± 0.13	0.26 ± 0.22	<0.0001	0.2974
Albumin (g/dl)	4.46 ± 0.43	4.50 ± 0.39	4.29 ± 0.50	<0.0001	<0.0001
Total cholesterol (mg/dl)	209 ± 41.9	212 ± 41.5	200 ± 41.6	<0.0001	<0.0001
Triglyceride (mg/dl)	164 ± 102.6	170 ± 107.7	145 ± 79.7	<0.0001	0.0226
HDL cholesterol (mg/dl)	51 ± 15.7	51 ± 16.2	51 ± 13.9	0.3545	0.0297
LDL cholesterol (mg/dl)	130 ± 37.9	133 ± 37.2	123 ± 38.8	<0.0001	0.0009
Fasting plasma glucose (mg/dl)	114 ± 37.8	111 ± 36.1	123 ± 41.4	<0.0001	0.0001
HbA1c (NGSP) (%)	6.32 ± 1.2	6.26 ± 1.2	6.67 ± 1.4	<0.0001	0.0003
IRI (μIU/ml)	15.2 ± 18.5	13.7 ± 11.7	20.1 ± 31.4	<0.0001	<0.0001
HOMA-IR	4.89 ± 10.0	3.98 ± 5.6	6.84 ± 15.5	<0.0001	0.0012
Ferritin (ng/ml)	260.2 ± 475.8	255.8 ± 522.4	275.4 ± 254.5	0.5642	0.5421
Uric acid (mg/dl)	5.9 ± 1.5	6.0 ± 1.5	5.7 ± 1.3	0.0297	0.8563
Free fatty acid (μEq/l)	0.41 ± 0.3	0.36 ± 0.3	0.56 ± 0.3	<0.0001	<0.0001
Hyaluronic acid (ng/ml)	64.3 ± 168.9	42.2 ± 66.9	145.8 ± 329.9	<0.0001	<0.0001

Comparison between patients with mild (Stage 0–2) and advanced (Stage 3, 4) fibrosis using the Chi-square test for binary variables and logistic regression of group indicator on continuous variables

lipoprotein cholesterol in 19.5 % of patients. However, as the third important finding of our study, dyslipidemia tended to decrease in prevalence as the fibrosis stage progressed. Multivariate analysis revealed a negative correlation between the serum triglyceride levels and the fibrosis stage (OR = 0.5687, 95 % CI 0.394–0.821). This result may reflect a deterioration of lipid metabolism with the progression of liver fibrosis towards liver cirrhosis.

The fourth finding of our study was the recognition of a relationship between hypertension and the fibrosis severity in NAFLD patients. In our NAFLD population, hypertension was present in 30.2 %. Whereas no obvious trends in

Table 3 Multiple regression analysis to identify predictive factors for the advanced fibrosis

Variable	All cases	Stage 0–2	Stage 3, 4	Univariate odds ratio (95 % CI)	*P* value	Multivariate odds ratio (95 % CI)	*P* value
Age (mean)	51.0	49.2	57.5	1.042 (1.032–1.053)	<0.0001	1.036 (1.021–1.051)	<0.0001
Female (%)	48.1	44.4	61.1	1.967 (1.516–2.553)	<0.0001	1.180 (0.787–1.768)	0.423
BMI ≥ 25 (%)	73.0	71.2	79.5	1.566 (1.149–2.133)	0.0045	1.568 (0.991–2.481)	0.0545
Hypertension (%)	39.9	38.0	47.3	1.468 (1.063–2.027)	0.0198	0.943 (0.641–1.387)	0.7640
Hypertriglyceridemia (%)	45.3	82.7	34.7	0.566 (0.432–0.739)	<0.0001	0.663 (0.453–0.970)	0.0343
Hyper-LDL cholesterolemia (%)	37.5	39.6	30.7	0.676 (0.496–0.920)	0.0129	0.885 (0.596–1.313)	0.5444
Hypo-HDL cholesterolemia (%)	19.5	19.7	18.9	0.836 (0.671–1.343)	0.7680		–
DM (%)	47.3	42.1	64.9	2.544 (1.948–3.320)	<0.0001	2.387 (1.603–3.553)	<0.0001
Hyperuricemia (%)	30.2	32.1	24.4	0.684 (0.485–0.965)	0.0308	1.058 (0.693–1.617)	0.793

the prevalence of hypertension were observed in females, comparison of the relationship between the prevalence of hypertension and the stage of fibrosis, except for Stage 4, revealed a tendency towards increase in the prevalence of hypertension with progression of the fibrosis stage. In general, blood pressure is considered to have an effect on the rate of progression of NAFLD. Systolic and diastolic blood pressures have been reported to be correlated with the liver fat content, and patients with systolic hypertension were reported to be correlated with the liver fat contents, and patients with systolic hypertension were reported to show a two-fold higher risk of development of NAFLD [39]. As shown in Fig. 3, the decrease in the rate of hypertension in NAFLD patients with Stage 4 liver fibrosis might be, at least in part, attributable to the hyperdynamic circulation, characterized by peripheral vasodilation and increased portal resistance, observed in patients with liver cirrhosis [40, 41].

Impaired glucose tolerance is well known to accompany NAFLD. While it appears clear that abnormal glucose tolerance, including DM, is a risk factor for NAFLD and vice versa, the relationship between abnormal glucose tolerance and the histological severity of NAFLD is still unknown. The fifth finding in our study was that the prevalence of DM increased with progression of the fibrosis stage (Fig. 4). Multivariate analysis identified DM as an independent risk factor for advanced fibrosis (OR = 2.8573, 95 % CI 1.941–4.207). In vitro, high glucose and high insulin concentrations, which are often observed in patients with NAFLD, were shown to stimulate connective tissue growth factor expression, which is known as one of the important mechanisms involved in the progression of hepatic fibrosis [42]. Furthermore, the cirrhotic condition is suspected to facilitate the development of hyperinsulinemia and hyperglycemia via the deteriorated liver function [43, 44]. Taken together, it would be reasonable to consider DM as both a cause and result of NAFLD [45].

In conclusion, we have reported the prevalences of lifestyle-related diseases, such as obesity, dyslipidemia, hypertension, and DM, in NAFLD patients according to the stage of fibrosis. Multivariate analysis identified DM as a significant risk factor for advanced fibrosis. Accordingly, impaired glucose tolerance, including DM, should be properly evaluated and managed for preventing the progression of NAFLD, even in the early stages of NASH.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Hamaguchi M, Kojima T, Takeda N, Nakagawa T, Taniguchi H, Fujii K, et al. The metabolic syndrome as a predictor of nonalcoholic fatty liver disease. Ann Int Med. 2005;143:722–8.
2. Kojima S, Watanabe N, Numata M, Ogawa T, Matsuzaki S. Increase in the prevalence of fatty liver in Japan over the past 12 years: analysis of clinical background. J Gastroenterol. 2003;38:954–61.
3. Amarapurkar DN, Hashimoto E, Lesmana LA, Sollano JD, Chen PJ, Goh KL, Asia-Pacific Working Party on NAFLD. How common is non-alcoholic fatty liver disease in the Asia-Pacific region and are there local differences? J Gastroenterol Hepatol. 2007;22:788–93.
4. Teli MR, James OF, Burt AD, Bennett MK, Day CP. The natural history of nonalcoholic fatty liver: a follow-up study. Hepatology. 1995;22:1714–9.
5. Tokushige K, Hashimoto E, Horie Y, Taniai M, Higuchi S. Hepatocellular carcinoma in Japanese patients with nonalcoholic fatty liver disease, alcoholic liver disease, and chronic liver disease of unknown etiology: report of the nationwide survey. J Gastroenterol. 2011;46:1230–7.
6. Matteoni CA, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ. Nonalcoholic fatty liver disease: spectrum of clinical and pathological severity. Gastroenterology. 1999;116:1413–9.
7. James O, Day C. Non-alcoholic steatohepatitis: another disease of affluence. Lancet. 1999;353:1634–6.
8. Ono M, Saibara T. Clinical features of nonalcoholic steatohepatitis in Japan: evidence from the literature. J Gastroenterol. 2006;41:725–32.

9. Marchesini G, Brizi M, Bianchi G, Tomassetti S, Bugianesi E, Lenzi M, et al. Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. Diabetes. 2001;50:1844–50.
10. Hui JM, Farrell GC. Clear messages from sonographic shadows? Links between metabolic disorders and liver disease, and what to do about them. J Gastroenterol Hepatol. 2003;18:1115–7.
11. Marchesini G, Bugianesi E, Forlani G, Cerrelli F, Lenzi M, Manini R, et al. Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. Hepatology. 2003;37:917–23.
12. Angulo P. Nonalcoholic fatty liver disease. N Engl J Med. 2002;346:1221–31.
13. Farrell GC, Larter CZ. Nonalcoholic fatty liver disease: from steatosis to cirrhosis. Hepatology. 2006;43:S99–112.
14. Angulo P. Obesity and nonalcoholic fatty liver disease. Nutr Rev. 2007;65:S57–63.
15. Neuschwander-Tetri BA, Clark JM, Bass NM, Van Natta ML, Unalp-Arida A, Tonascia J, et al. Clinical, laboratory and histological associations in adults with nonalcoholic fatty liver disease. Hepatology. 2010;52:913–24.
16. Larter CZ, Chitturi S, Heydet D, Farrell GC. A fresh look at NASH pathogenesis. Part 1: the metabolic movers. J Gastroenterol Hepatol. 2010;25:672–90.
17. Fracanzani AL, Valenti L, Bugianesi E, Andreoletti M, Colli A, Vanni E, et al. Risk of severe liver disease in nonalcoholic fatty liver disease with normal aminotransferase levels: a role for insulin resistance and diabetes. Hepatology. 2008;48:792–8.
18. Rafiq N, Bai C, Fang Y, Srishord M, McCullough A, Gramlich T, et al. Long-term follow-up of patients with nonalcoholic fatty liver. Clin Gastroenterol Hepatol. 2009;7:234–8.
19. Hossain N, Afendy A, Stepanova M, Nader F, Srishord M, Rafiq N, et al. Independent predictors of fibrosis in patients with nonalcoholic fatty liver disease. Clin Gastroenterol Hepatol. 2009;7:1224–9.
20. Yoneda M, Fujii H, Sumida Y, Hyogo H, Itoh Y, Ono M, et al. Platelet count for predicting fibrosis in nonalcoholic fatty liver disease. J Gastroenterol. 2011;46:1300–6.
21. Sumida Y, Yoneda M, Hyogo H, Yamaguchi K, Ono M, Fujii H, et al. A simple clinical scoring system using ferritin, fasting insulin and type IV collagen 7S for predicting steatohepatitis in nonalcoholic fatty liver disease. J Gastroenterol. 2011;46:257–68.
22. The World Medical Association, Inc. Declaration of Helsinki; Ethical principle for medical research involving human subject. Seoul: WMA General Assembly; 2008.
23. Iwamoto K, Kanno K, Hyogo H, Yamagishi S, Takeuchi M, Tazuma S, et al. Advanced glycation end products enhance the proliferation and activation of hepatic stellate cells. J Gastroenterol. 2008;43:298–304.
24. Hyogo H, Tazuma S, Arihiro K, Iwamoto K, Nabeshima Y, Inoue M, et al. Efficacy of atorvastatin for the treatment of nonalcoholic steatohepatitis with dyslipidemia. Metabolism. 2008;57:1711–8.
25. Matsuzawa U, Inoue S, Ikeda Y, Sakata T, Saito Y, Sato Y, Japanese Society for the Study of Obesity, et al. New criteria of obesity (in Japanese). J Jpn Soc Study Obes. 2000;6:18–28.
26. Ricci C, Longo R, Gioulis E, Bosco M, Pollesello P, Masutti F, et al. Noninvasive in vivo quantitative assessment of fat content in human liver. Noninvasive in vivo quantitative assessment of fat content in human liver. J Hepatol. 1997;27:108–13.
27. World Health Organization. Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia: report of a WHO/IDF consultation. Geneva: World Health Organization; 2006.
28. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia. 1985;28:412–9.
29. Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol. 1999;94:2467–74.
30. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology. 2005;41:1313–21.
31. Paul Angulo. Nonalcoholic fatty liver disease. N Engl J Med. 2002;18:1221–31.
32. Ludwig J, Viggiano TR, McGill DB, Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. Mayo Clin Proc. 1980;55:434–8.
33. Liou I, Kowdley KV. Natural history of nonalcoholic steatohepatitis. J Clin Gastroenterol. 2006;40(Suppl 1):S11–6.
34. Abdelmalek MF, Diehl AM. Nonalcoholic fatty liver disease as a complication of insulin resistance. Med Clin North Am. 2007;91:1125–49.
35. Kamada Y, Kiso S, Yoshida Y, Chatani N, Kizu T, Hamano M, et al. Estrogen deficiency worsens steatohepatitis in mice fed high-fat and high-cholesterol diet. Am J Physiol Gastrointest Liver Physiol. 2011;301:1031–43.
36. Zhu L, Brown WC, Cai Q, Krust A, Chambon P, McGuinness OP, et al. Estrogen treatment after ovariectomy protects against fatty liver and may improve pathway-selective insulin resistance. Diabetes. 2013;62:424–34.
37. Eguchi Y, Hyogo H, Ono M, Mizuta T, Ono N, Fujimoto K, et al. Prevalence and associated metabolic factors of nonalcoholic fatty liver disease in the general population from 2009 to 2010 in Japan: a multicenter large retrospective study. J Gastroenterol. 2012;47:586–95.
38. Harrison SA, Oliver D, Arnold HL, Gogia S, Neuschwander-Tetri BA. Development and validation of a simple NAFLD clinical scoring system for identifying patients without advanced disease. Gut. 2008;57:1441–7.
39. Donati G, Stagni B, Piscaglia F, Venturoli N, Morselli-Labate AM, Rasciti L, et al. Increased prevalence of fatty liver in arterial hypertensive patients with normal liver enzymes: role of insulin resistance. Gut. 2004;53:1020–3.
40. Schrier RW, Arroyo V, Bernardi M, Epstein M, Henriksen JH, Rodés J. Peripheral arterial vasodilation hypothesis: a proposal for the initiation of renal sodium and water retention in cirrhosis. Hepatology. 1988;8:1151–7.
41. Vallance P, Moncada S. Hyperdynamic circulation in cirrhosis: a role for nitric oxide? Lancet. 1991;337:776–8.
42. Paradis V, Perlemuter G, Bonvoust F, Dargere D, Parfait B, Vidaud M, et al. High glucose and hyperinsulinemia stimulate connective tissue growth factor expression: a potential mechanism involved in progression to fibrosis in nonalcoholic steatohepatitis. Hepatology. 2001;34:738–44.
43. Megyesi C, Samols E, Marks V. Glucose tolerance and diabetes in chronic liver disease. Lancet. 1967;18:1051–6.
44. Petrides AS, DeFronzo RA. Glucose and insulin metabolism in cirrhosis. J Hepatol. 1989;8:107–14.
45. Arase Y, Kobayashi M, Suzuki F, Suzuki Y, Kawamura Y, Akuta N, et al. Effect of type 2 diabetes on risk for malignancies includes hepatocellular carcinoma in chronic hepatitis C. Hepatology. 2013;57:964–73.

Journal of Gastroenterology and Hepatology

JGHF

doi:10.1111/jgh.12271

NUTRITION-RELATED LIVER DISORDERS: NAFLD

Characteristics and diagnosis of NAFLD/NASH

Etsuko Hashimoto, Makiko Taniai and Katsutoshi Tokushige

Department of Internal Medicine and Gastroenterology, Tokyo Women's Medical University, Tokyo, Japan

Key words
diagnosis, non-alcoholic fatty liver diseases (NAFLD), non-alcoholic steatohepatitis (NASH).

Accepted for publication 23 April 2013.

Correspondence
Dr Etsuko Hashimoto, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan. Email: drs-hashimoto@mti.biglobe.ne.jp

Abstract

Non-alcoholic fatty liver disease (NAFLD) is considered to be a hepatic manifestation of metabolic syndrome. NAFLD has become an important public health issue because of its high prevalence. NAFLD consists of two clinicopathological entities: simple steatosis, which generally follows a benign non-progressive clinical course, and non-alcoholic steatohepatitis (NASH), which may progress to cirrhosis and hepatocellular carcinoma. The diagnosis of NAFLD is based on the following three criteria: non-alcoholic, detection of steatosis either by imaging or by histology, and appropriate exclusion of other liver diseases. Alcoholic liver disease can occur when daily alcohol consumption exceeds 20 g in women or 30 g in men. Thus, non-alcoholic indicates lower levels of these alcohol consumptions. However, there is still no clear consensus regarding the threshold alcohol consumption for defining non-alcoholic liver disease. Then, there is the strong recommendation for a change in the nomenclature, such as use of the term metabolic fatty liver and metabolic steatohepatitis. NASH has emerged as a clinicopathological entity, and even now, a liver biopsy remains the gold standard for making a definitive diagnosis. However, liver biopsy has several drawbacks. In general practice, NAFLD is a convenient-to-use term for the diagnosis and management of these patients, and serum biomarkers that indicate the severity of fibrosis serve as clinically useful tools for the identification of NAFLD in patients with bridging fibrosis or cirrhosis. In the future, improved understanding of the pathogenesis of NASH and new technologies may contribute to the diagnostic process and provide reliable, non-invasive alternatives to liver biopsy.

Changes in diet and lifestyle have resulted in a dramatic increase in the prevalence of obesity and metabolic syndrome in Western countries and many Asian countries. This has resulted in a significant increase in the incidence of non-alcoholic fatty liver disease (NAFLD), which is considered to be a hepatic manifestation of metabolic syndrome. NAFLD has become an important public health issue because of its high prevalence. NAFLD consists of two clinical entities: simple steatosis and non-alcoholic steatohepatitis (NASH). Currently, NAFLD is the most common cause of chronic liver disease in these countries. In this review, we summarize the current concepts relating to the characteristics and diagnosis of NAFLD/NASH.

Nomenclature of NAFLD/NASH

NAFLD is characterized by excessive accumulation of fat, or steatosis, in the liver in individuals with a history of a little or no alcohol consumption. While simple steatosis accounts for 80–90% cases of NAFLD, NASH accounts for the remaining 10–20%. Simple steatosis is mostly a benign non-progressive clinical entity, while NASH can progress to cirrhosis or even hepatocellular carcinoma (HCC). NASH is histologically characterized by hepatic steatosis associated with evidence of liver cell injury (ballooning degeneration) and inflammation, steatohepatitis, and varying degrees of fibrosis; these histological features are indistinguishable from those of alcoholic hepatitis. NASH has emerged as a distinct clinicopathological entity,[1–6] and even now, a liver biopsy still remains the "gold standard" for making a definitive diagnosis.

Traditionally, fatty disorders of the liver have been classified as alcoholic or non-alcoholic (Fig. 1). Primary NAFLD/NASH is associated with obesity, diabetes, or dyslipidemia, and the so-called insulin resistance or metabolic syndrome. Secondary NAFLD/NASH is rare and may be associated with many conditions such as polycystic ovary syndrome, endocrine diseases, sleep apnea, and pancreatoduodenal resection, etc. According to the practice guideline proposed by American Association for the Study of Liver Diseases, steatogenic medications are not included as a cause of NAFLD; however, historically, drug-induced fatty liver has been included under NAFLD. Therefore, the classification is still confusing. Using the term "non-alcoholic" to describe fatty liver disease associated with all other etiologies than alcohol consumption renders the condition heterogeneous. Then, there is the strong recommendation for a change in the nomenclature, such as use of the term metabolic fatty liver and metabolic steatohepatitis.[2,5,6] Thus, there is no consensus yet on the best way to classify fatty disorders of the liver.

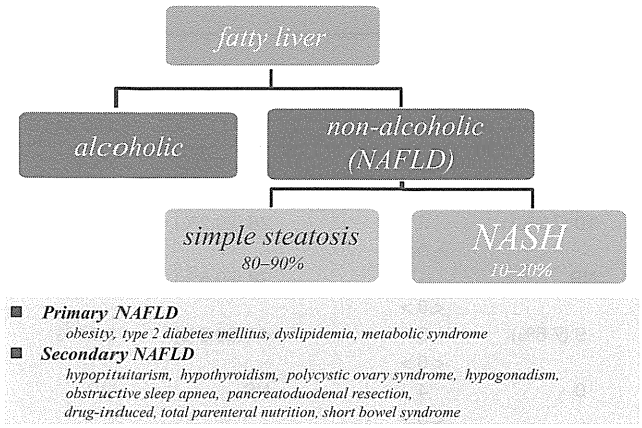


Figure 1 Fatty liver is classified as alcoholic or non-alcoholic. Then, non-alcoholic fatty liver disease (NAFLD) is divided into simple steatosis and non-alcoholic steatohepatitis (NASH) based on the histological features. NAFLD/NASH is classified into two categories depending on the causes: primary and secondary.

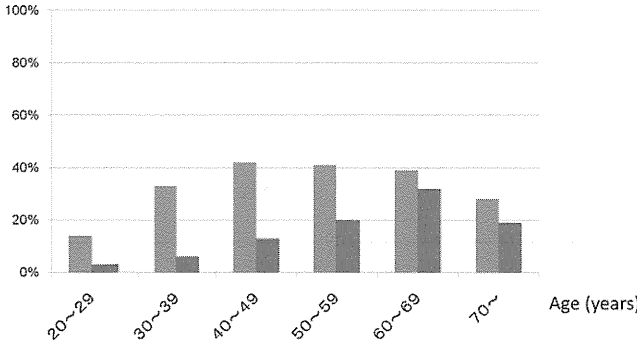


Figure 2 The prevalence of non-alcoholic fatty liver disease (NAFLD) by age and sex. Among men, the prevalence of NAFLD is around 40% in each age group. In women, the prevalence gradually increased with age.[9] A cross-sectional study: Japanese adults, $n = 5075$. (■) Male; (■) female (Source: Adapted from Eguchi *et al.*[9] with permission).

Clinical features of NAFLD/NASH

Epidemiology. According to data from annual health check-ups, 10–40% of Japanese adults have ultrasonography (US) -diagnosed NAFLD.[7–9] NASH is observed in 10–20% of cases of NAFLD, while the estimated prevalence of NASH is 1–8%. Age and gender differences in both the prevalence and severity of NAFLD/NASH are well known, which may just reflect the differences in the prevalence of obesity and metabolic syndrome in the general population[9–11] (Figs 2,3). The prevalence of NAFLD increased with the severity of risk factors; it was 10–20% in non-obese individuals, around 50% in those with a body mass index (BMI) more than 25 kg/m^2 but less than 30 kg/m^2, and around 80% in those with a BMI over 30 kg/m.[2,7,9] The prevalence of NAFLD was around 50% in type 2 diabetes and around 50% in patients with dyslipidemia. The prevalence of NAFLD also shows ethnic differences; it is higher in Hispanics followed by white and

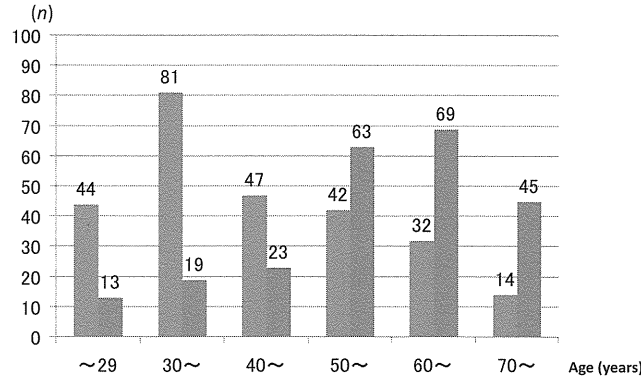


Figure 3 The age and sex distribution of the patients with biopsy-proven non-alcoholic steatohepatitis. Women were significantly more common above 50 years of age. (■) Male $n = 260$; (■) female $n = 232$ (Source: Adapted from Hashimoto *et al.*[11]).

lower in African Americans.[12] Family members of subjects with NAFLD are also at increased risk because of genetic background.[13–15]

Clinical features. NAFLD patients are usually asymptomatic until the condition progresses to liver cirrhosis. Therefore, NAFLD is often detected based on the presence of hepatic steatosis on abdominal US during routine health checkups or clinical visits for other diseases among non-alcoholic individuals. Most patients with NAFLD have insulin resistance; obesity, diabetes, or dyslipidemia. While NAFLD could be the result of insulin resistance, a causal role of NAFLD in insulin resistance has also been reported. Thus, there could be a vicious cycle involving these diseases. NAFLD is no longer considered to be a primary liver disease but rather as a part of metabolic syndrome.[16] Blood chemistry shows mild elevation of transaminases, and also other evidence for liver dysfunction in the cirrhotic stage.

Natural history. The long-term prognoses of NAFLD, including histologically diagnosed simple steatosis, NASH and cirrhotic NASH have been reported from population-based studies as well as hospital-based cohort studies (Table 1).[17–28] According to these studies, the prognoses vary widely among these conditions.[17–28] Longitudinal histological studies have confirmed the benign clinical course of simple steatosis, although a few studies have reported the development of cirrhosis in some patients with "simple steatosis."[29] Progression to fibrosis in NASH appears to occur more frequently among patients whose baseline liver biopsies demonstrate greater necroinflammatory changes.[30]

It has been reported that as compared with individuals in the general population, those with NAFLD show a lower-than-expected survival with a standardized mortality ratio of 1.34 to 1.69 because of increases in the risk of cardiovascular diseases and liver-related death.[17,18,20,23] The most common causes of death in patients with NAFLD are cardiovascular disease and malignancy, followed by liver-related death. However, overall, NAFLD appears to be slowly progressive, with liver-related morbidity and mortality occurring only in a minority of subjects. The reported risk

Table 1 Studies on Long-Term Mortality in NAFLD and NASH

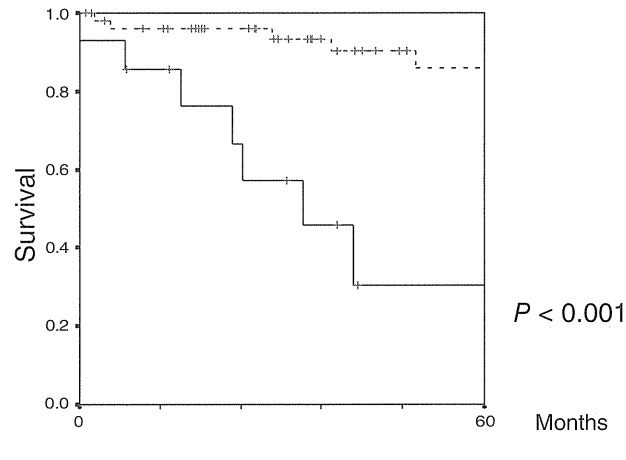
Author	Diagnosis	*n*	Average F/U (years)	Cirrhosis* prevalence *n* (%)	HCC* *n* <at baseline>	Death	
						Overall *n* (%)	Liver-related/ overall (%)
Adams *et al.*[17]	NAFLD**	420	7.6	21 (5%)	2 < 0 >	53 (12.6%)	13.2%
Ekstedt *et al.*[18]	NAFLD***	129	13.7	10 (7.8%)	3 < 0 >	26 (20.2%)	7.7%
Rafiq *et al.*[19]	NAFLD***	131	18.5	NR	1 < 0 >	78 (59.5%)	15.4%
Söderberg *et al.*[20]	NAFLD***	118	21	9 (7.6%)	5 < 0 >	47 (39.8%)	19.1%
Sørensen *et al.*[21]	NAFLD**	1800	6.2	0	4 < 0 >	NR	NR
Teli *et al.*[22]	Simple*** Steatosis	40	9.6	0	0 < 0 >	14 (35.0%)	0.0%
Dam-Larsen *et al.*[23]	Simple*** Steatosis	170	20.4	2 (1.2%)	0 < 0 >	48 (28.2%)	2.1%
Evans *et al.*[24]	NASH***	26	8.7	1 (4%)	0 < 0 >	4 (15%)	0.0%
Hui *et al.*[25]	Cirrhotic-NAFLD***	23	7.0	100%	0 < 0 >	6 (26%)	83.3%
Sanyal *et al.*[26]	Cirrhotic-NAFLD***	152	10	100%	13 < 3 >	29 (19.1%)	69.0%
Yatsuji *et al.*[27]	Cirrhotic-NAFLD***	68	3.4	100%	21 < 14 >	19 (27.9%)	78.9%
Söderberg *et al.*[20]	Cirrhotic-NAFLD***	9	21	100%	3 < 0 >	8 (88.9%)	50.0%
Ascha *et al.*[28]	Cirrhotic-NAFLD**	195	3.2	100%	25 < 0 >	NR	NR

*At the end of the follow-up period. **The diagnosis was made by imaging or liver biopsy. ***The diagnosis was made by liver biopsy.
F/U, follow up; HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NR, not reported.

factors for the development of cirrhosis are older age, presence of diabetes, and morbid obesity.[29]

We conducted a comparative analysis of the natural history of 68 patients with biopsy-proven cirrhotic NASH and 69 age- and sex-matched patients with liver cirrhosis associated with hepatitis C virus infection (LC-C).[27] The mean age of the patients with cirrhotic NASH was 62.7 years. Patients with cirrhotic NASH showed a similar survival rate to that of the patients with LC-C (75.2% and 73.8%, respectively), although the rate of development of HCC was lower (5-year HCC development rate: 11.3% for cirrhotic NASH *vs* 30.5% for LC-C). The leading cause of death in patients with cirrhotic NASH was HCC, followed by liver failure[27] (Fig. 4). All previous studies have confirmed that patients with cirrhotic NASH exhibit a similar clinical course to those with LC-C, and the reported rates of development of HCC in these patients were similar to our data (around 10% at 5 years).[20,25–28]

Characteristics of HCC in NAFLD/NASH. Concerning the risk factors for the development of HCC, we identified advanced fibrosis, older age, histological low-grade inflammation, and low aspartate aminotransferase (AST) levels as the risk factors for presence of HCC.[31] It is well known that when NASH progresses to the end stage, the necroinflammatory changes and serum transaminase levels gradually decline; therefore, presence



Month

month	0	12	36	60
NASH without HCC	54	47	29	20
NASH with HCC	14	10	4	1

Figure 4 The survival of cirrhotic non-alcoholic steatohepatitis (NASH) patients with or without hepatocellular carcinoma (HCC. HCC) was a significant risk factor for death of cirrhotic NASH patients.[27] (——) NASH with HCC; (·····) NASH without HCC (Source: Adapted from Yatsuji *et al.*[27]).

of low-grade inflammation and a low serum AST level may indicate the end stage of cirrhotic NASH. Accordingly, the risk factors for the development of HCC in patients with NASH are the features of end-stage NASH and older age.

We compared the clinical features of 34 NASH-related HCC (NASH-HCC) patients and 56 age-, sex-, and treatment-matched patients with hepatitis C virus infection-related HCC (HCV-HCC).[32] As expected, there was a significantly higher prevalence of obesity, diabetes, and dyslipidemia in the NASH-HCC group. Serum transaminases were significantly higher in the HCV-HCC group, while the serum gamma-glutamyl transferase level was significantly higher in the patients with NASH-HCC. The 5-year survival rate was 55.2%, and the 5-year recurrence rate after curative treatment was 69.8% in patients with NASH-HCC. The survival and recurrence rates were similar in the two groups. HCC in NASH may also be of multicentric origin, similar to the case of HCC associated with viral hepatitis.

According to previous studies, 10–75% of all NASH-related HCCs occur in patients with non-cirrhotic NASH.[33] The high incidence of HCC arising from non-cirrhotic NASH may be partly due to the fact that the diagnosis of NASH is based on histology, and liver tissue can only be obtained by liver biopsy or surgery in patients with preserved liver function. Moreover, end-stage cirrhotic NASH cannot be diagnosed with any confidence because of its "burned out" histology. These points may introduce significant bias. Further studies are required to clarify the true incidence of HCC arising from non-cirrhotic NASH.

How is NAFLD/NASH diagnosed?

The diagnosis of NAFLD is based on the presence of the following three criteria: non-alcoholic, detection of steatosis either by imaging or by histology, and appropriate exclusion of other liver diseases.[1–6] NASH is diagnosed based on the presence of steatohepatitis on liver biopsy. Given the lack of surrogate markers yet for the diagnosis of NAFLD, it is important to exclude other liver diseases such as alcoholic liver diseases, viral hepatitis, autoimmune liver diseases, and metabolic or hereditary liver diseases. However, the prevalence of NAFLD is extremely high, NAFLD is often complicated by other liver diseases such as viral hepatitis, etc., and NAFLD exacerbates liver damage and reduces the response to treatments. Epidemiological studies have shown that alcoholic liver disease can occur when the daily alcohol consumption exceeds 20 g in women and 30 g in men. Then, NAFLD is diagnosed when the alcohol consumption is lower than the aforementioned in the respective sexes. Serum transaminases are not helpful for the diagnosis of steatosis because 50–80% of patients with hepatic steatosis have normal transaminase levels. In stage 3 fibrosis, fibrosis markers such as hyaluronic acid, etc., are elevated, and in the cirrhotic stage, reduction of the platelet count and evidence of liver dysfunction such as elevation of the serum bilirubin and ammonia, etc., are noted.

Imaging modalities. Abdominal US is currently the most common method employed for qualitative assessment of hepatic steatosis because it is non-invasive, widely available, cheap, and provides useful information. Presence of hepatic steatosis on abdominal US is usually defined based on the presence of at least two of the following findings: increased hepatorenal contrast, liver brightness, deep attenuation, and vascular blurring (Fig. 5). However, the diagnosis by US has several limitations; it is subjective, operator-dependent, shows poor sensitivity for the detection of mild steatosis, and is a poor tool for quantifying the steatosis. Both computed tomography (CT) and magnetic resonance imaging (MRI) seem to be more objective and more sensitive techniques for the quantification of steatosis, but MRI is still less widely available and much more expensive. For the diagnosis of steatosis by CT, the liver-to-spleen attenuation ratio is measured, and the diagnosis of steatosis is made when the ratio is less than 0.9. Of course, CT also has limitations with respect to the diagnosis of steatosis, including poor sensitivity for the detection of mild steatosis, X-ray exposure of the patients, and unavailability for patients with hemosiderosis. Unfortunately, none of these imaging modalities is useful for the diagnosis of NASH.

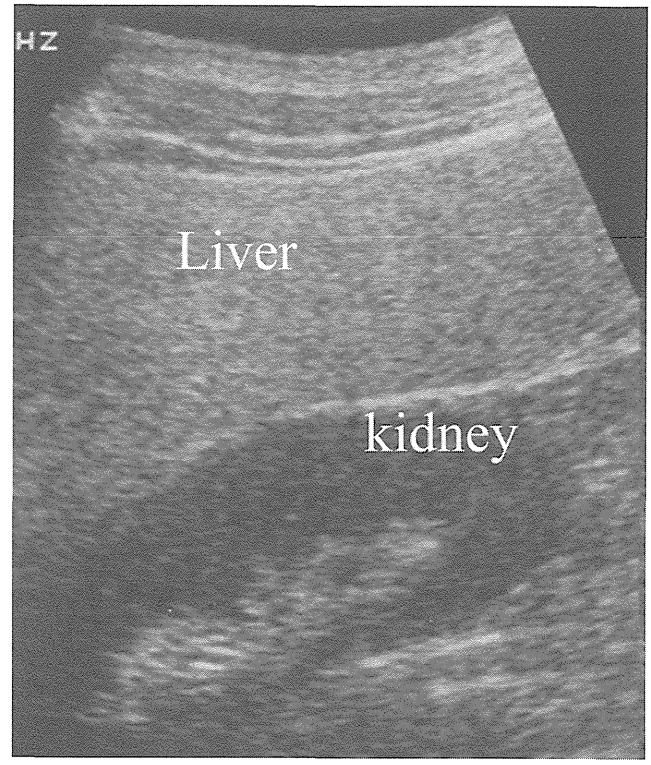


Figure 5 This is an image of ultrasonography. Hepatic steatosis leads to increased hepatorenal contrast, liver brightness, deep attenuation, and vascular blurring. Ultrasonography is an acceptable first-line screening procedure for detection of steatosis in clinical practice.

Concerning interference with the detection of steatosis by advanced fibrosis, the decrease in the detection sensitivity is marked for both US and CT.[34] The sensitivity of US and CT for advanced fibrosis is also decreased markedly in patients with severe steatosis and obese patients, being more marked for US. An awareness of these disadvantages of the common imaging modalities would be useful for a more precise diagnosis of hepatic steatosis and fibrosis in patients with NAFLD.

Liver biopsy. The principal histological features of NASH are as follows: presence of macrovesicular steatosis, ballooning

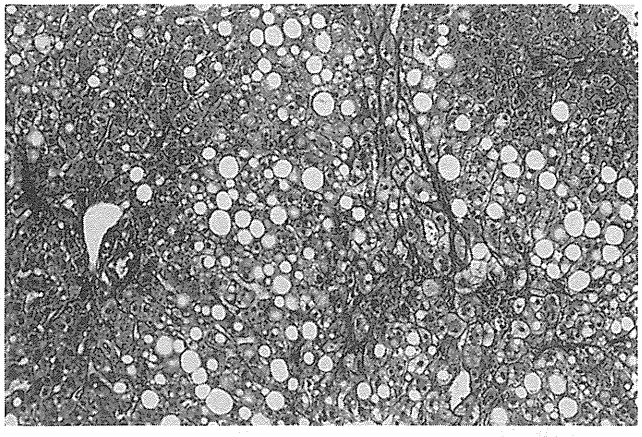


Figure 6 This is a liver biopsy with Mallory staining for fibrosis. Macrovesicular steatosis and prominent pericellular fibrosis around the central vein are present (▼), while portal fibrosis is mild (↓).

degeneration of the hepatocytes, and mixed lobular inflammation. These characteristic pathological features with Mallory hyaline and pericellular fibrosis are predominantly seen around the central veins (zone 3) (Fig. 6). Atypical features have been reported in pediatric cases and morbidly obese cases, such as more periportal steatosis (zone 1), little or no ballooning or Mallory hyaline, and more portal-based chronic inflammation and fibrosis.

Three important pathological classifications have been proposed for NAFLD: Matteoni's classification, Brunt's classification, and the NALFD activity score (NAS).[35–37]

In 1999, Matteoni *et al.*[35] described a classification system that served to distinguish between NASH and non-NASH. They divided 132 NAFLD patients into four categories: type 1, steatosis alone; type 2, steatosis with lobular inflammation only; type 3, steatosis with hepatocellular ballooning; and type 4, type 3 plus either Mallory–Denk bodies or fibrosis. They confirmed the benign clinical course of patients with type 1 or 2 NAFLD and the progressive clinical course of patients who had either type 3 or 4 NAFLD. As a result of these differences, these authors defined type 1 and type 2 histological forms of NAFLD as "non-NASH," and type 3 and type 4 as NASH. However, this classification did not include an assessment of the severity or pattern of NASH, such as the degree of steatosis, inflammation, location of these changes (i.e. lobular or portal), or the degree of fibrosis.

In the same year as Matteoni's classification system was published, Brunt *et al.*[36] proposed a semiquantitative grading and staging system for NASH. This classification was applicable to only NASH and not to the entire spectrum of NAFLD.

In 2005, the NASH Clinical Research Network Pathology Committee developed and validated a histological scoring system based on Brunt's classification, NAS, as a semiquantitative instrument by which to judge treatment responses or disease progression in clinical studies.[37] The NAS system addresses the full spectrum of NAFLD and is applicable to both adult and pediatric NAFLD patients. The score is determined as the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3), and ballooning degeneration (0–2). A score of ≥ 5 correlated with the diagnosis of NASH made independently by an experienced pathologist without using the score; likewise, scores of less than 3 were correlated with "not NASH," and scores of 3 or 4 were regarded as borderline. In regard to fibrosis, stage 1 referred to perisinusoidal fibrosis in zone 3 (perivenular area: delicate [1A] and dense [1B]), and detection of portal fibrosis without perisinusoidal fibrosis was defined as 1C. Stage 2 was characterized by perisinusoidal and portal/periportal fibrosis. Stage 3 was defined as bridging fibrosis and stage 4 as cirrhosis. Although the authors reminded us that the NAS system was never intended to be used for the diagnosis of NASH, NAS has frequently been used as a surrogate method for establishing the diagnosis of NASH. Then, they assessed the relation between NASH diagnosed by NAS and pathological diagnosis of steatohepatitis (in this case, NASH) and found that the definitive diagnosis of NASH was not always correlated with threshold values of the NAS.[38] They concluded that clinical pathologists should be encouraged not to use NAS as a categorical approach for the diagnosis of NASH.

Younossi *et al.*[39] assessed the ability to predict the long-term liver-related mortality based on the pathological characteristics. The study cohort consisted of 209 patients with biopsy-proven NAFLD who were followed up for at least 5 years. The results of their multivariate analysis identified only fibrosis as an independent predictor of liver-related mortality. According to the findings of this study, assessment of the severity of hepatic fibrosis is essential for determining the prognosis in patients with NASH.[40]

Indication of liver biopsy. NASH has emerged as a distinct clinicopathological concept, and even now, biopsy evaluation is considered the "gold standard" for a definitive diagnosis. However, liver biopsy has several drawbacks; it is an expensive and invasive procedure and is fraught with the possibility of sampling error and variability in pathologist interpretation. Moreover, given the extremely high prevalence of NAFLD, a liver biopsy would be poorly suited as a diagnostic test for NASH. Accordingly, at present, liver biopsy may only be considered in NAFLD patients who are considered to be at an increased risk of developing NASH with advancing fibrosis or are suspected to have coexisting other chronic liver diseases.[5,6] In general practice, NAFLD is a convenient-to-use term for the diagnosis and management of these patients, and serum biomarkers that indicate the severity of fibrosis serve as clinically useful tools for the identification of NAFLD in patients with bridging fibrosis or cirrhosis.

Non-invasive assessment of NASH and advanced fibrosis in NAFLD. Recently, several biochemical markers and imaging modalities have been reported for predicting NASH and the severity of hepatic fibrosis.[41–47] An ideal biomarker should be simple to measure, accurate, reproducible, inexpensive, and readily available. In general, while most of the biomakers and scoring systems show similar accuracy for the detection of advanced fibrosis, their accuracy is weak for the diagnosis of mild fibrosis. The NAFLD Fibrosis Score is a widely validated scoring system for predicting the severity of fibrosis that is based on six readily assessable clinical variables (age, BMI, hyperglycemia, platelet count, albumin, AST/alanine aminotransferase ratio).[46]

Several imaging techniques have also been advocated as non-invasive diagnostic tests for NASH. US-based transient elastography or FibroScan has shown promising results for assessment of

the severity of liver fibrosis and degree of steatosis. However, these modalities are expensive and not widely available.

Pathogenesis of NASH

The development of NASH is thought to initiate from basal steatosis as the first hit, followed by a "second hit" that is capable of inducing necroinflammation; this hypothesis is the so-called "two-hit theory."[48,49] The second hit can include oxidative stress, especially that arising from mitochondrial stress, insulin resistance, inflammatory cytokines, etc. Autophagy may also play an important role in the pathogenesis of NASH. Recently, a new concept to explain the pathogenesis of NASH was reported by Tilg and Moschen, namely, the "multi-parallel hit" hypothesis.[50] This hypothesis, based on reports that endoplasmic reticulum stress and cytokine-mediated stress can induce steatosis as well as necroinflammation, suggests that multiple hits act together in the development of NASH. Steatosis should therefore be considered as a part of the liver's early "adaptive" response to stress rather than as the first hit in disease progression.

I have summarized the characteristics and diagnosis of NAFLD/NASH. There is still no clear consensus regarding the threshold alcohol consumption for defining "non-alcoholic" liver disease. In the future, a change in the nomenclature of NAFLD/NASH might be needed because there are so many obese people who drink much alcohol and show the histological features of steatohepatitis. Liver biopsy currently remains the gold standard for the diagnosis of NASH. In the future, improved understanding of the pathogenesis of NASH and new technologies may contribute to the diagnostic process and provide reliable non-invasive alternatives to liver biopsy.

References

1 Ludwig J, Viggiano TR, McGill DB, Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin. Proc.* 1980; **55**: 434–8.
2 Sanyal AJ; American Gastroenterogical Association. AGA technical review on nonalcoholic fatty liver disease. *Gastroenterology* 2002; **123**: 1705–25.
3 Neuschwander-Tetri BA, Caldwell SH. Nonalcoholic steatohepatitis: summary of an AASLD single topic conference. *Hepatology* 2003; **37**: 1202–19.
4 Farrell GC, Chitturi S, Lau GK *et al.* Guidelines for the assessment and management of non-alcoholic fatty liver disease in the Asia-Pacific region: executive summary. *J. Gastroenterol. Hepatol.* 2007; **22**: 775–7.
5 Ratziu V, Bellentani S, Cortez-Pinto H, Day C, Marchesini G. A position statement on NAFLD/NASH based on the EASL 2009 special conference. *J. Hepatol.* 2010; **53**: 372–84.
6 Chalasani N, Younossi Z, Lavine JE *et al.* The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. *Hepatology* 2012; **55**: 2005–23.
7 Kojima S, Watanabe N, Numata M, Ogawa T, Matsuzaki S. Increase in the prevalence of fatty liver in Japan over the past 12 years: analysis of clinical background. *J. Gastroenterol.* 2003; **38**: 954–61.
8 Amarapurkar DN, Hashimoto E, Lesmana LA *et al.* How common is non-alcoholic fatty liver disease in the Asia-Pacific region and are there local differences? *J. Gastroenterol. Hepatol.* 2007; **22**: 788–93.
9 Eguchi Y, Hyogo H, Ono M *et al.* Prevalence and associated metabolic factors of nonalcoholic fatty liver disease in the general population from 2009 to 2010 in Japan: a multicenter large retrospective study. *J. Gastroenterol.* 2012; **47**: 586–95.
10 Yatsuji S, Hashimoto E, Tobari M, Tokushige K, Shiratori K. Influence of age and gender in Japanese patients with non-alcoholic steatohepatitis. *Hepatol. Res.* 2007; **37**: 1034–43.
11 Hashimoto E, Tokushige K. Prevalence, gender, ethnic variations, and prognosis of NASH. *J. Gastroenterol.* 2011; **46** (Suppl. 1): 63–9.
12 Browning JD, Szczepaniak LS, Dobbins R *et al.* Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* 2004; **40**: 1387–95.
13 Romeo S, Kozlitina J, Xing C *et al.* Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. *Nat. Genet.* 2008; **40**: 1461–5.
14 Kawaguchi T, Sumida Y, Umemura A *et al.* Genetic polymorphisms of the human PNPLA3 gene are strongly associated with severity of non-alcoholic fatty liver disease in Japanese. *PLoS ONE* 2012; **7**: e38322.
15 Tokushige K, Takakura M, Tsuchiya-Matsushita N, Taniai M, Hashimoto E, Shiratori K. Influence of TNF gene polymorphisms in Japanese patients with NASH and simple steatosis. *J. Hepatol.* 2007; **46**: 1104–10.
16 Targher G, Bertolini L, Padovani R *et al.* Prevalence of nonalcoholic fatty liver disease and its association with cardiovascular disease among type 2 diabetic patients. *Diabetes Care* 2007; **30**: 1212–18.
17 Adams LA, Lymp JF, St Sauver J *et al.* The natural history of nonalcoholic fatty liver disease: a population-based cohort study. *Gastroenterology* 2005; **129**: 113–21.
18 Ekstedt M, Franzén LE, Mathiesen UL *et al.* Long-term follow-up of patients with NAFLD and elevated liver enzymes. *Hepatology* 2006; **44**: 865–73.
19 Rafiq N, Bai C, Fang Y *et al.* Long-term follow-up of patients with nonalcoholic fatty liver. *Clin. Gastroenterol. Hepatol.* 2009; **7**: 234–8.
20 Söderberg C, Stål P, Askling J *et al.* Decreased survival of subjects with elevated liver function tests during a 28-year follow-up. *Hepatology* 2010; **51**: 595–602.
21 Sørensen HT, Mellemkjaer L, Jepsen P *et al.* Risk of cancer in patients hospitalized with fatty liver: a Danish cohort study. *J. Clin. Gastroenterol.* 2003; **36**: 356–9.
22 Teli MR, Day CP, Burt AD, Bennett MK, James OF. Determinants of progression to cirrhosis or fibrosis in pure alcoholic fatty liver. *Lancet* 1995; **346**: 987–90.
23 Dam-Larsen S, Becker U, Franzmann MB, Larsen K, Christoffersen P, Bendtsen F. Final results of a long-term, clinical follow-up in fatty liver patients. *Scand. J. Gastroenterol.* 2009; **44**: 1236–43.
24 Evans CD, Oien KA, MacSween RN, Mills PR. Non-alcoholic steatohepatitis: a common cause of progressive chronic liver injury? *J. Clin. Pathol.* 2002; **55**: 689–92.
25 Hui JM, Kench JG, Chitturi S *et al.* Long-term outcomes of cirrhosis in nonalcoholic steatohepatitis compared with hepatitis C. *Hepatology* 2003; **38**: 420–7.
26 Sanyal AJ, Banas C, Sargeant C *et al.* Similarities and differences in outcomes of cirrhosis due to nonalcoholic steatohepatitis and hepatitis C. *Hepatology* 2006; **43**: 682–9.
27 Yatsuji S, Hashimoto E, Tobari M, Taniai M, Tokushige K, Shiratori K. Clinical features and outcomes of cirrhosis due to non-alcoholic steatohepatitis compared with cirrhosis caused by chronic hepatitis C. *J. Gastroenterol. Hepatol.* 2009; **24**: 248–54.
28 Ascha MS, Hanouneh IA, Lopez R, Tamimi TA, Feldstein AF, Zein NN. The incidence and risk factors of hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. *Hepatology* 2010; **51**: 1972–8.

29 Adams LA, Sanderson S, Lindor KD, Angulo P. The histological course of nonalcoholic fatty liver disease: a longitudinal study of 103 patients with sequential liver biopsies. *J. Hepatol.* 2005; **42**: 132–8.
30 Argo CK, Northup PG, Al-Osaimi AM, Caldwell SH. Systematic review of risk factors for fibrosis progression in non-alcoholic steatohepatitis. *J. Hepatol.* 2009; **51**: 371–9.
31 Hashimoto E, Yatsuji S, Tobari M *et al.* Hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. *J. Gastroenterol.* 2009; **44** (Suppl. 19): 89–95.
32 Tokushige K, Hashimoto E, Yatsuji S *et al.* Prospective study of hepatocellular carcinoma in nonalcoholic steatohepatitis in comparison with hepatocellular carcinoma caused by chronic hepatitis C. *J. Gastroenterol.* 2010; **45**: 960–7.
33 Yasui K, Hashimoto E, Komorizono Y *et al.* Characteristics of patients with nonalcoholic steatohepatitis who develop hepatocellular carcinoma. *Clin. Gastroenterol. Hepatol.* 2011; **9**: 428–33; quiz e50.
34 Tobari M, Hashimoto E, Yatsuji S, Torii N, Shiratori K. Imaging of nonalcoholic steatohepatitis: advantages and pitfalls of ultrasonography and computed tomography. *Intern. Med.* 2009; **48**: 739–46.
35 Matteoni CA, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology* 1999; **116**: 1413–19.
36 Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am. J. Gastroenterol.* 1999; **94**: 2467–74.
37 Kleiner DE, Brunt EM, Van Natta M *et al.* Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005; **41**: 1313–21.
38 Brunt EM, Kleiner DE, Wilson LA, Belt P, Neuschwander-Tetri BA; (CRN) NCRN. Nonalcoholic fatty liver disease (NAFLD) activity score and the histopathologic diagnosis in NAFLD: distinct clinicopathologic meanings. *Hepatology* 2011; **53**: 810–20.
39 Younossi ZM, Stepanova M, Rafiq N *et al.* Pathologic criteria for nonalcoholic steatohepatitis: interprotocol agreement and ability to predict liver-related mortality. *Hepatology* 2011; **53**: 1874–82.
40 Angulo P. Diagnosing steatohepatitis and predicting liver-related mortality in patients with NAFLD: two distinct concepts. *Hepatology* 2011; **53**: 1792–4.
41 Hashimoto E, Farrell GC. Will non-invasive markers replace liver biopsy for diagnosing and staging fibrosis in non-alcoholic steatohepatitis? *J. Gastroenterol. Hepatol.* 2009; **24**: 501–3.
42 Musso G, Gambino R, Cassader M, Pagano G. Meta-analysis: natural history of non-alcoholic fatty liver disease (NAFLD) and diagnostic accuracy of non-invasive tests for liver disease severity. *Ann. Med.* 2011; **43**: 617–49.
43 Feldstein AE, Wieckowska A, Lopez AR, Liu YC, Zein NN, McCullough AJ. Cytokeratin-18 fragment levels as noninvasive biomarkers for nonalcoholic steatohepatitis: a multicenter validation study. *Hepatology* 2009; **50**: 1072–8.
44 Sumida Y, Yoneda M, Hyogo H *et al.* Japan Study Group of Nonalcoholic Fatty Liver Disease (JSG-NAFLD). A simple clinical scoring system using ferritin, fasting insulin, and type IV collagen 7S for predicting steatohepatitis in nonalcoholic fatty liver disease. *J. Gastroenterol.* 2011; **46**: 257–68.
45 Kaneda H, Hashimoto E, Yatsuji S, Tokushige K, Shiratori K. Hyaluronic acid levels can predict severe fibrosis and platelet counts can predict cirrhosis in patients with nonalcoholic fatty liver disease. *J. Gastroenterol. Hepatol.* 2006; **21**: 1459–65.
46 Angulo P, Hui JM, Marchesini G *et al.* The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. *Hepatology* 2007; **45**: 846–54.
47 Guha IN, Parkes J, Roderick P *et al.* Noninvasive markers of fibrosis in nonalcoholic fatty liver disease: validating the European Liver Fibrosis Panel and exploring simple markers. *Hepatology* 2008; **47**: 455–60.
48 Day CP, James OF. Steatohepatitis: a tale of two "hits." *Gastroenterology* 1998; **114**: 842–5.
49 Stärkel P, Sempoux C, Leclercq I *et al.* Oxidative stress, KLF6 and transforming growth factor-beta up-regulation differentiate non-alcoholic steatohepatitis progressing to fibrosis from uncomplicated steatosis in rats. *J. Hepatol.* 2003; **39**: 538–46.
50 Tilg H, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. *Hepatology* 2010; **52**: 1836–46.

Journal of Gastroenterology and Hepatology

JGHF

doi:10.1111/jgh.12239

NUTRITION-RELATED LIVER DISORDERS: NAFLD

Hepatocarcinogenesis in non-alcoholic fatty liver disease in Japan

Katsutoshi Tokushige, Etsuko Hashimoto and Kazuhiko Kodama

Department of Internal Medicine and Gastroenterology, Tokyo Women's Medical University, Tokyo, Japan

Key words
hepatocellular carcinoma (HCC), non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH).

Accepted for publication 4 March 2013.

Correspondence
Dr Katsutoshi Tokushige, 8-1 Kawada-cho Shinjuku-ku, Tokyo 162-8666, Japan. Email: ktoku@pg7.so-net.ne.jp

Abstract

In Japan, there has been a gradual increase in cases of non-viral chronic liver diseases, including non-alcoholic fatty liver disease (NAFLD), occurring with hepatocellular carcinoma (HCC). First, a national survey investigating the etiology of HCC in Japan was performed. Among HCCs based on non-viral disease, alcoholic liver disease with HCC accounted for 7.2% of all HCCs, followed by chronic liver disease of unknown etiology with HCC (5.1%) and NAFLD with HCC (2.0%). The clinical characteristics of these three HCC groups were clearly different. In our second analysis, the HCC development rates among liver cirrhosis with NAFLD, alcoholic cirrhosis, and cirrhosis with hepatitis C virus (HCV) were compared. HCC development rates were 11.3%/5 years in NAFLD cirrhosis, 30.5%/5 years in HCV cirrhosis, and 12.5%/5 years in alcoholic cirrhosis, suggesting that the hepatocarcinogenesis in NAFLD and alcoholic liver disease were similar but were lower than that in HCV.

Using Cox hazards analysis, older age, higher serum γ-glutamyl transpeptidase level, and higher Child–Pugh score as risk factors of HCC were identified. Finally, clinical data of NAFLD-HCC with the data for HCC with HCV (HCV-HCC) were compared. The percentage of NAFLD-HCC patients with des-gamma-carboxy prothrombin-positive was higher than that with α-fetoprotein-positive. The 5-year survival and recurrence rates for NAFLD-HCC were almost similar to those for HCV-HCC. In Asian countries, the prevalence of NAFLD is increasing. Therefore, elucidating the pathogenesis and clinical features of HCC in patients with NAFLD is indeed an urgent problem.

Introduction

Primary liver cancer is the fifth most common cancer worldwide and the third most common cause of cancer mortality.[1,2] Hepatocellular carcinoma (HCC) accounts for about 90% of primary liver cancers. With respect to the underlying liver disease, the latest nationwide report of the Liver Cancer Study Group of Japan showed that hepatitis C virus (HCV)-related liver disease is the most common underlying cause of HCC.[3] HCV-related HCC accounts for 67% of all HCC cases, followed by 16% for hepatitis B virus (HBV)-related HCC. The incidence of HCV-related HCC has been gradually decreasing in recent years, while the incidence of HCC associated with non-viral chronic liver disease has gradually been increasing.

In Pacific and Asian countries, the prevalence of non-alcoholic fatty liver disease (NAFLD) in the general population is increasing dramatically and ranges from 5% to 40%.[4,5] NAFLD consists of simple steatosis and non-alcoholic steatohepatitis (NASH), while NASH comprises a wide spectrum of conditions from NASH without fibrosis to cirrhosis. Obesity and diabetes mellitus have been established as significant risk factors for HCC by epidemiological observations and experimental studies,[6,7] and there is increasing evidence that NASH is a risk factor for HCC.[8,9] We reported that HCC was a critical factor in the prognosis of NAFLD cirrhosis.[10] Therefore, there is an urgent need to elucidate pathogenesis, clinical features, and treatments for these diseases, especially NAFLD advanced stages and NAFLD-related HCC (NAFLD-HCC).

In this review, we describe the survey of HCC in Japan that my colleagues and I conducted, the rate at which HCC develops from NAFLD, the risk factors for HCC in NAFLD, the clinical features of NAFLD-HCC.

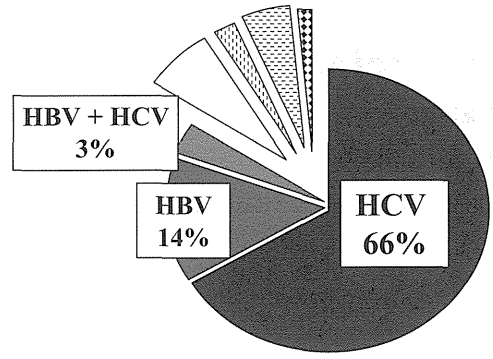
National survey of HCC

We performed a national survey investigating the etiology of HCC in the Japanese population in 2010. The nationwide survey included 14 530 HCC patients diagnosed during 2006–2009,[11] of whom 14.1% were positive for hepatitis B surface (HBs) antigen, 66.3% were positive for HCV-RNA, and 3.7% were positive for both HBs antigen and HCV-RNA. Among those surveyed, 15.8% of patients were diagnosed as having non-HBV, non-HCV HCC.

Among HCCs based on non-viral disease, alcoholic liver disease with HCC (ALD-HCC) accounted for 7.2% of all HCCs, followed by chronic liver disease of unknown etiology with HCC (unknown HCC) (5.1%) and NAFLD with HCC (2.0%) (Fig. 1). The characteristics of these three groups were clearly different from one another (median age was 72 years for NAFLD-HCC, 68 years for ALD-HCC, and 73 years for unknown HCC, $P < 0.01$; female gender was 38%, 4%, and 37%, respectively, $P < 0.01$) (Table 1). Body mass index (BMI) and the prevalence of diabetes, hypertension, and dyslipidemia were significantly higher in patients with NAFLD-HCC than in those with ALD-HCC and unknown HCC. Serum levels of total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ-glutamyl transpeptidase (GTP) were significantly higher in the ALD-HCC group compared with the other groups, while the platelet count and serum albumin level were lowest in the ALD-HCC group. The hemoglobin A_{1C} and fasting blood glucose levels were highest in the NAFLD-HCC group. These data suggested that clinical characteristics of these three HCC groups were clearly different from one another.

Regarding the etiology of HCC in Western countries, NAFLD-HCC has been reported to account for 3.8–13% of all HCCs.[12,13] In comparison with Western countries, the prevalence of NAFLD-HCC is lower in Japan. This is not only due to the low incidence of NAFLD-HCC but also to the high incidence of hepatitis virus-related HCC in Japan. However, the incidence of NAFLD-HCC in Japan is expected to increase in the future because of the rising prevalence of NAFLD associated with obesity and/or diabetes.

To determine whether modest alcohol intake could influence carcinogenesis in patients with unknown HCC, we divided the patients into a no alcohol subgroup (alcohol consumption < 20 g/day) and a modest alcohol intake subgroup (alcohol consumption of 20–70 g/day) (Table 2). Among the no alcohol subgroup, the prevalence of women was markedly higher ($P < 0.001$) at 58%



Alcoholic -HCC	**7.2%**
Etiology unknown-HCC	**5.1%**
NAFLD-HCC	**2.0%**
Autoimmune Liver diseases (PBC + AIH + PSC)*	**1.2%**
Congestive disease	**0.1%**
Metabolic disease**	**0.1%**

Figure 1 National survey in Japan. (2006–2009). Among the 14 530 patients with hepatocellular carcinoma (HCC), 14.1% were positive for hepatitis B surface (HBs) antigen, 66.3% were positive for hepatitis C virus (HCV)-RNA, and 3.7% were positive for both HBs antigen and HCV-RNA. Among the HCC patients with non-viral liver diseases, alcoholic liver disease with HCC (ALD-HCC) (7.2%) was the most common diagnosis, followed by unknown HCC (5.1%). Non-alcoholic fatty liver disease (NAFLD)-HCC (2.0%) was the third most common etiology. (■) HCV; (■) hepatitis B virus (HBV); (■) HBV + HCV; (□) alcoholic; (⊡) NAFLD; (⊟) etiology unknown; (⊞) others. *AIH, autoimmune hepatitis; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis. **Metabolic disease; Wilson disease, hemochromatosis, etc. Adapted from Tokushige *et al.*[11]

Table 1 Comparison among NAFLD-HCC, ALD-HCC, and unknown HCC

	NAFLD-HCC ($n = 292$)	ALD-HCC ($n = 991$)	Unknown-HCC ($n = 614$)	*P* value
Age at diagnosis	72 ± 8.4	68 ± 9.1	73 ± 10.1	< 0.001
Gender (female)	38%	4%	37%	< 0.001
BMI(kg/m^2)	27.0 ± 4.0	23.8 ± 3.7	23.5 ± 4.1	< 0.001
Diabetes	70%	49%	43%	< 0.001
Hypertension	60%	43%	46%	< 0.001
Dyslipidemia	35%	14%	15%	< 0.001
Liver cirrhosis	62%	78%	52%	< 0.001
Albumin (g/dL)	3.8 ± 0.6	3.6 ± 0.6	3.6 ± 0.6	< 0.001
Total bilirubin (mg/dL)	0.9 ± 1.3	1.1 ± 1.9	0.9 ± 1.7	< 0.001
AST (IU/L)	40 ± 36	80 ± 301	43 ± 71	< 0.001
ALT (IU/L)	35 ± 35	45 ± 176	30 ± 44	0.03
γ-GTP (IU/L)	91 ± 202	147 ± 271	88 ± 198	< 0.001
FBS (mg/dL)	119 ± 57	111 ± 63	107 ± 53	< 0.001
HbA_{1c} (%)	6.3 ± 1.4	5.9 ± 1.6	5.7 ± 1.4	< 0.001
Platelet count (× 10^4/mm^3)	14.1 ± 7.4	12.6 ± 8.0	15.2 ± 9.1	< 0.001
AFP (ng/mL)	12 ± 427 557	11 ± 368 512	13.0 ± 94 155	0.284

Adapted from Tokushige *et al.*[11]

γ-GTP, gamma-glutamyl transpeptidase; AFP, α-fetoprotein; ALD, alcoholic liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FBS, fasting blood sugar; HbA_{1C}, hemoglobin A_{1C}; HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease.

Table 2 Comparison between the no alcohol and modest alcohol subgroups of unknown HCC

	No alcohol intake $n = 316$	Modest alcohol intake $n = 214$	*P* value
Age at diagnosis	75.5 ± 10.2	72 ± 9.0	< 0.001
Gender (female)	58%	8%	< 0.001
BMI (kg/m^2)	23.8 ± 4.5	23.5 ± 3.4	0.396
Diabetes	41%	46%	0.214
Hypertension	45%	49%	0.424
Dyslipidemia	15%	15%	0.989
Liver cirrhosis	57%	42%	0.001
Albumin (g/dL)	3.6 ± 0.7	3.8 ± 0.6	0.030
Total bilirubin (mg/dL)	0.9 ± 1.5	0.8 ± 1.2	0.266
AST (IU/L)	44 ± 63	39 ± 73	0.081
ALT (IU/L)	29 ± 45	29 ± 42	0.455
γ-GTP (IU/L)	75 ± 184	103.5 ± 213	0.003
FBS (mg/dL)	106 ± 51	110 ± 56	0.050
HbA_{1c} (%)	5.7 ± 1.3	5.7 ± 1.5	0.307
Platelet count (× 10^4/mm^3)	14.6 ± 9.0	16.8 ± 8.7	0.001
AFP (ng/mL)	13.3 ± 77 396	10 ± 31 196	0.378

Adapted from Tokushige *et al.*[11]
γ-GTP, gamma-glutamyl transpeptidase; AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FBS, fasting blood sugar; HbA_{1c}, hemoglobin A_{1c}; HCC, hepatocellular carcinoma.

versus only 8% in the modest alcohol subgroup. The mean age at diagnosis of HCC was higher in the no alcohol subgroup than in the modest alcohol intake subgroup (75.5 years *vs* 72 years, $P < 0.001$). Between the two subgroups, the modest alcohol intake subgroup showed different clinical features in terms of unknown HCC and showed the same trends in regard to gender, BMI, lifestyle-related diseases, and γ-GTP levels as the ALD-HCC group.

These data suggested that a relatively low alcohol intake may lead to the development of non-viral HCC. The alcohol consumption criteria for diagnosis of alcoholic liver disease vary around the world,[14,15] and the alcohol consumption criterion for alcoholic liver disease proposed by the Japanese Study Group of Alcoholic Liver Disease is more than 70 g/day. Our data suggest that social or modest intake of alcohol might have a more significant role in hepatic carcinogenesis than is presently thought. In the future, more detailed studies will need to be performed, including assessment of alcohol metabolism genotypes.

HCC rate in patients with NAFLD

Kawamura *et al.* reported that rate of HCC was 0.51%/12 years from all NAFLD, including simple steatosis.[16] In NAFLD as a whole, the development of HCC is rare. However, liver fibrosis is the most important factor for development of HCC in any liver disease. To make clear the hepatocarcinogenic power in NAFLD, we compared the HCC development rates among liver cirrhosis (LC) with NAFLD (NAFLD-LC), alcoholic cirrhosis (ALD-LC), and cirrhosis infected with HCV (HCV-LC) in our hospital. HCC development rates were 11.3%/5 years in NAFLD-LC, 30.5%/5 years in HCV-LC, and 12.5%/5 years in ALD-LC (Fig. 2).[10,17] Sanyal *et al.* and Ascha *et al.* reported that the HCC development

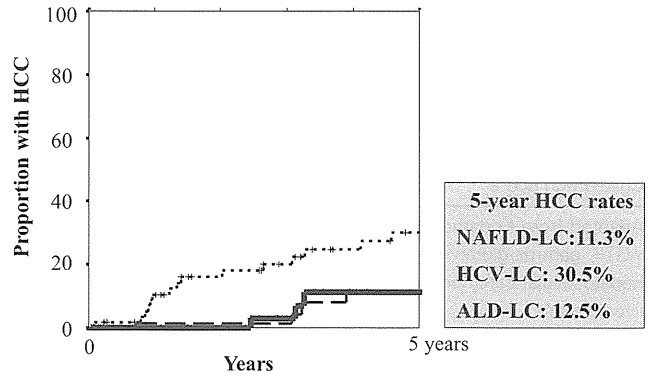


Figure 2 Hepatocellular carcinoma (HCC) rate in non-alcoholic fatty liver disease (NAFLD) cirrhosis (NAFLD-LC), alcoholic liver disease-liver cirrhosis (ALD-LC), and hepatitis C virus (HCV)-liver cirrhosis (HCV-LC). The HCC rates in NAFLD-LC and ALD-LC were similar, and were lower than that in HCV-LC. (——) NAFLD; (— —) ALD; (·····) HCV. Adapted from Yatsuji *et al.* and Kodama *et al.*[10,17]

Table 3 The comparison between NAFLD-HCC ($n = 41$) and NAFLD without HCC ($n = 533$) by multivariate logistic regression model

	Odds ratio	95% CI	*P* value
Age (older)	1.103	1.050~1.159	< 0.001
Gender (male)	4.680	1.803~12.146	0.002
Liver fibrosis	2.718	1.745~4.233	< 0.001
Activity	0.361	0.163~0.802	0.012
ALT	0.974	0.955~0.993	0.007
γ-GTP	1.005	1.001~1.009	0.008

γ-GTP, gamma-glutamyl transpeptidase; ALT, alanine aminotransferase; CI, confidence interval; HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease.

rate in NAFLD cirrhosis was about 10–13% in 5 years and lower than that of HCV-LC in the USA.[18,19] These data almost match the Japanese data. The rates of hepatocarcinogenesis in NAFLD and alcoholic liver disease were almost identical but were lower than that in chronic HCV liver disease.

Risk factors of NAFLD-HCC

To clarify the risk factors of HCC in NAFLD, we compared clinical data between NAFLD-HCC and NAFLD without HCC with a multivariate logistic regression model. Both NAFLD patients with and without HCC were admitted to our hospital between 1990 and 2011. NAFLD was diagnosed by liver biopsy. Age, gender, BMI, diabetes, hypertension, dyslipidemia, blood examinations (total bilirubin, albumin, AST, ALT, alkaline phosphatase [ALP], γ–GTP, platelet, prothrombin time [PT]), and liver histology findings (fibrosis grade, activity grade, and steatosis grade) were analyzed as risk factors of HCC. In the results, older age, male gender, advanced liver fibrosis, lower activity of liver histology, lower ALT level, and higher γ-GTP level were detected as risk factors of HCC in the population with NAFLD-HCC (Table 3). However, this analysis did not include the factor of duration, and liver fibrosis is the most important factor for

Table 4 Risk factors for HCC in the NAFLD-LC(n = 72) according to the Cox hazards model

	Hazard ratio	95% CI	*P* value
Age (older)	1.12	1.014–1.226	0.024
γ-GTP	1.01	1.002–1.022	0.023
Child–Pugh score	3.09	1.374–6.934	0.006

Adapted from Kodama *et al.*[17]
γ-GTP, gamma-glutamyl transpeptidase; CI, confidence interval; HCC, hepatocellular carcinoma; LC, liver cirrhosis; NAFLD, non-alcoholic fatty liver disease.

development of HCC. In the next analysis, we investigated the risk factors for HCC in 72 NAFLD-LC patients with a Cox hazards model. All NAFLD-LC patients were admitted to our hospital between 1990 and 2011. NAFLD-LC was diagnosed by liver biopsy. The patients with NAFLDH-LC were assessed with regard to the development of HCC, and their risk factors for HCC were analyzed. Age, gender, BMI, ascites, varices, encephalopathy, diabetes, hypertension, dyslipidemia, blood examinations (total bilirubin, albumin, AST, ALT, ALP, hypertension, γ-GTP, platelet, PT), and Child–Pugh score were analyzed as risk factors of HCC. Older age, higher serum γ-GTP level, and higher Child–Pugh score were identified as risk factors in NAFLD-LC (Table 4), and older age and Child–Pugh were confirmed by log-rank test.[17] Kawamura *et al.* reported the risk factors for HCC in all NAFLD patients as being old age, AST > 40 IU/mL, advanced fibrosis, and diabetes mellitus.[16] Ascha *et al.* reported that NASH patients with cirrhosis had a greatly increased risk of liver cancer, and even social alcohol consumption appeared to be the most significant factor associated with the risk of HCC.[19] Considering all of these findings, we conclude that older age, male gender, advanced fibrosis, γ-GTP level, which was the marker of oxidative stress, diabetes mellitus, and mild alcohol intake might be important factors in the pathogenesis of HCC in NAFLD.

Clinical features of NAFLD-HCC

Finally, we compared the clinical data of NAFLD-HCC with the data for HCC caused by HCV infection (HCV-HCC) in our hospital. The percentage of NAFLD-HCC patients with des-gamma-carboxy prothrombin-positive results was higher than that of patients with α-fetoprotein-positive results.[20] Yasui *et al.* also showed the same profile of tumor markers in NASH-HCC.[21] In our hospital, the 5-year survival rate in the treated NAFLD-HCC group was 55.2%, and the cumulative HCC recurrence rate at 5 years was 69.8% as opposed to a 5-year survival rate of 50.6% and recurrence rate of 83.1% in the HCV-HCC group.[22] The 5-year survival and recurrence rates for NAFLD-HCC were almost similar to those for HCV-HCC.

Zen *et al.* reported a case of HCC arising in a patient diagnosed with NASH at 62 years old. At 66 years old, her first hepatic tumor appeared. The pathological diagnosis of the first nodule was "pseudolymphoma." When she was 72 years old, three hepatic tumors appeared and were diagnosed as moderately differentiated HCC. At age 73, two more tumors appeared and were diagnosed as well-differentiated HCC and a dyplastic nodule.[23] These results suggested a multicentric occurrence of HCC in NASH, similar to HCC based on viral hepatitis.

We had measured anti-hepatitis B core (HBc) antibody to investigate the influence of HBV on the carcinogenesis of NAFLD-HCC. The difference between the NAFLD-HCC group and HCV-HCC group was not significant, and none of the NAFLD-HCC patients had high HBc antibody titers that would have led to the suspicion that they were HBV carriers. These findings therefore suggested that even if HBV did influence carcinogenesis in NAFLD, the influence would be minimal.

We reported that HCC was a critical factor in the prognosis of NAFLD.[10] Regular screening for HCC is extremely important, especially in NAFLD patients with advanced fibrosis, and the strong possibility of recurrence also warrants close attention.

In conclusion, in Asian countries, the prevalence of NAFLD is increasing dramatically. Elucidating the pathogenesis, clinical features, and treatment of HCC in NAFLD is an urgent problem.

References

1 Bosch FX, Ribes J, Díaz M, Cléries R. Primary liver cancer: worldwide incidence and trends. *Gastroenterology* 2004; **127** (5 Suppl. 1): S5–S16.
2 Sherman M. Hepatocellular carcinoma: epidemiology, risk factors and screening. *Semin. Liver Dis.* 2005; **25**: 143–5.
3 Ikai I, Arii S, Okazaki M *et al.* Report of the 17th nationwide follow-up survey of primary liver cancer in Japan. *Hepatol. Res.* 2007; **37**: 676–91.
4 Chitturi S, Farrell GC, Hashimoto E, Saibara T, Lau GK, Sollano JD, Asia-Pacific Working Party on NAFLD. Non-alcoholic fatty liver disease in the Asia-Pacific region: definitions and overview of proposed guidelines. *J. Gastroenterol. Hepatol.* 2007; **22**: 778–87.
5 Amarapurkar DN, Hashimoto E, Lesmana LA, Sollano JD, Chen PJ, Goh KL, Asia-Pacific Working Party on NAFLD. How common is non-alcoholic fatty liver disease in the Asia-Pacific region and are there local differences? *J. Gastroenterol. Hepatol.* 2007; **22**: 788–93.
6 Starley BQ, Calcagno CJ, Harrison SA. Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection. *Hepatology* 2010; **51**: 1820–32.
7 Siegel AB, Zhu AX. Metabolic syndrome and hepatocellular carcinoma: two growing epidemics with a potential link. *Cancer* 2009; **115**: 5651–61.
8 Shimada M, Hashimoto E, Taniai M *et al.* Hepatocellular carcinoma in patients with non-alcoholic steatohepatitis. *J. Hepatol.* 2002; **37**: 154–60.
9 Bugianesi E. Non-alcoholic steatohepatitis and cancer. *Clin. Liver Dis.* 2007; **11**: 191–207.
10 Yatsuji S, Hashimoto E, Tobari M, Taniai M, Tokushige K, Shiratori K. Clinical features and outcomes of cirrhosis due to non-alcoholic steatohepatitis compared with cirrhosis caused by chronic hepatitis C. *J. Gastroenterol. Hepatol.* 2009; **24**: 248–54.
11 Tokushige K, Hashimoto E, Horie Y, Taniai M, Higuchi S. Hepatocellular carcinoma in Japanese patients with nonalcoholic fatty liver disease, alcoholic liver disease and chronic liver disease of unknown etiology: report of the Nationwide Survey. *J. Gastroenterol.* 2011; **46**: 1230–7.
12 Malik SM, Gupte PA, de Vera ME, Ahmad J. Liver transplantation in patients with nonalcoholic steatohepatitis-related hepatocellular carcinoma. *Clin. Gastroenterol. Hepatol.* 2009; **7**: 800–6.
13 Marrero JA, Fontana RJ, Su GL, Conjeevaram HS, Emick DM, Lok AS. NAFLD may be a common underlying liver disease in patients with hepatocellular carcinoma in the United States. *Hepatology* 2002; **36**: 1349–54.
14 Mandayam S, Jamal MM, Morgan TR. Epidemiology of alcoholic liver disease. *Semin. Liver Dis.* 2004; **24**: 217–32.