Genetic Polymorphisms of the Human PNPLA3 Gene Are Strongly Associated with Severity of Non-Alcoholic Fatty Liver Disease in Japanese

Takahisa Kawaguchi[1,2], Yoshio Sumida[3], Atsushi Umemura[4], Keitaro Matsuo[5], Meiko Takahashi[1], Toshinari Takamura[6], Kohichiroh Yasui[7], Toshiji Saibara[8], Etsuko Hashimoto[9], Miwa Kawanaka[10], Sumio Watanabe[11], Sumio Kawata[12], Yasuharu Imai[13], Miki Kokubo[1], Toshihide Shima[4], Hyohun Park[4], Hideo Tanaka[5], Kazuo Tajima[5], Ryo Yamada[1], Fumihiko Matsuda[1,2]*, Takeshi Okanoue[4] for the Japan Study Group of Nonalcoholic Fatty Liver Disease (JSG-NAFLD)

1 Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan, **2** Institut National de la Sante et de la Recherche Medicale (INSERM) Unite U852, Kyoto University Graduate School of Medicine, Kyoto, Japan, **3** Center for Digestive and Liver Diseases, Nara City Hospital, Nara, Japan, **4** Center of Gastroenterology and Hepatology, Saiseikai Suita Hospital, Suita, Japan, **5** Division of Epidemiology and Prevention, Aichi Cancer Center, Nagoya, Japan, **6** Department of Disease Control and Homeostasis, Kanazawa University, Graduate School of Medical Science, Kanazawa, Japan, **7** Department of Molecular Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan, **8** Department of Gastroenterology and Hepatology, Kochi Medical School, Kochi, Japan, **9** Department of Internal Medicine and Gastroenterology, Tokyo Women's Medical University, Tokyo, Japan, **10** Center of Liver Diseases, Kawasaki Hospital, Kawasaki Medical School, Okayama, Japan, **11** Department of Gastroenterology, Juntendo University School of Medicine, Tokyo, Japan, **12** Department of Gastroenterology, Yamagata University School of Medicine, Yamagata, Japan, **13** Department of Internal Medicine, Ikeda Municipal Hospital, Ikeda, Japan

Abstract

Background: Nonalcoholic fatty liver disease (NAFLD) includes a broad range of liver pathologies from simple steatosis to cirrhosis and fibrosis, in which a subtype accompanying hepatocyte degeneration and fibrosis is classified as nonalcoholic steatohepatitis (NASH). NASH accounts for approximately 10–30% of NAFLD and causes a higher frequency of liver-related death, and its progression of NASH has been considered to be complex involving multiple genetic factors interacting with the environment and lifestyle.

Principal Findings: To identify genetic factors related to NAFLD in the Japanese, we performed a genome-wide association study recruiting 529 histologically diagnosed NAFLD patients and 932 population controls. A significant association was observed for a cluster of SNPs in *PNPLA3* on chromosome 22q13 with the strongest *p*-value of 1.4×10^{-10} (OR = 1.66, 95%CI: 1.43–1.94) for rs738409. Rs738409 also showed the strongest association ($p=3.6\times10^{-6}$) with the histological classifications proposed by Matteoni and colleagues based on the degree of inflammation, ballooning degeneration, fibrosis and Mallory-Denk body. In addition, there were marked differences in rs738409 genotype distributions between type4 subgroup corresponding to NASH and the other three subgroups ($p=4.8\times10^{-6}$, OR = 1.96, 95%CI: 1.47–2.62). Moreover, a subgroup analysis of NAFLD patients against controls showed a significant association of rs738409 with type4 ($p=1.7\times10^{-16}$, OR = 2.18, 95%CI: 1.81–2.63) whereas no association was obtained for type1 to type3 ($p=0.41$). Rs738409 also showed strong associations with three clinical traits related to the prognosis of NAFLD, namely, levels of hyaluronic acid ($p=4.6\times10^{-4}$), HbA1c ($p=0.0011$) and iron deposition in the liver ($p=5.6\times10^{-4}$).

Conclusions: With these results we clearly demonstrated that Matteoni type4 NAFLD is both a genetically and clinically different subset from the other spectrums of the disease and that the *PNPLA3* gene is strongly associated with the progression of NASH in Japanese population.

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* E-mail: fumi@genome.med.kyoto-u.ac.jp

Introduction

Nonalcoholic fatty liver disease (NAFLD) includes a broad range of pathologies from fatty liver (simple steatosis), steatonecrosis, and steatohepatitis to cirrhosis [1–3]. NAFLD often accompanies other lifestyle-related pathologies of metabolic syndrome such as diabetes mellitus, hypertension and dyslipidemia, and the number of NAFLD patients is increasing worldwide along with the escalation in the incidence of metabolic syndrome [4]. Prevalence of NAFLD is considered as approximately 8% in Japanese and 6–35% in Europeans [4,5]. The majority of NAFLD

shows simple steatosis with a good prognosis, but approximately 10–30% of NAFLD histologically diagnosed as nonalcoholic steatohepatitis (NASH) shows hepatocyte degeneration (ballooning hepatocyte), necrosis, inflammation and fibrosis, with a higher frequency of liver-related death both in Japanese and European populations [6,7]. Insulin resistance and oxidative stress are considered to be key players in the progression of NASH [8,9]. However, the progression of NASH has been considered to be complex involving multiple genetic factors interacting with the environment and lifestyle, because only a portion of NAFLD patients develops NASH.

The first Genome-wide association (GWA) study searching for such genetic factors identified the *PNPLA3* gene as a major genetic determinant for the predisposition to NAFLD in Hispanic, African American and European American populations according to liver fat contents [10], which was subsequently confirmed in Europeans and Asians according to liver biopsy. Association of *PNPLA3* with not only fatty liver and TG content, but also inflammation and fibrosis were shown in the subsequent studies, so *PNPLA3* may be widely associated with the development of NAFLD [11–13]. More recently, another GWA study reported the association of four additional genes with NAFLD in Europeans [14]. Also, a candidate gene-based approach revealed the association between NAFLD and the apolipoprotein C3 gene in Indians [15]. However, the precise role of such genes in the development of NASH still remains to be elucidated. In addition, no GWA study has been reported for Asian populations to date although the genetic components and their relative contribution may be different between ethnicities.

The Japan NASH Study Group was founded in 2008 aiming at the identification of genetic determinants predisposing to NASH in the Japanese population. Here we report the first GWA study of NAFLD in the Japanese using DNA samples of patients with liver histology-based diagnoses recruited through this multi-institutional research network.

Results

Genome-wide Association Analysis of NAFLD in Japanese

We conducted a GWA study using DNA samples of 543 patients with NAFLD and 942 controls. After quality controls of genotyping results (see materials and methods for details), a total of 529 patients consisting of four NAFLD subgroups according to Matteoni's classification [2] (type1; 100, type2; 73, type3; 29, type4; 327) and 932 controls were subjected to statistical analyses (Table 1). This index pathologically classifies NAFLD according to the degree of inflammation, hepatocyte degeneration, and the existence of fibrosis and Mallory-Denk body in the liver. Genome scan results of 932 DNA samples collected for other genetic studies were used as general Japanese population controls [16]. After standard quality control procedure as described in materials and methods, genotype distributions of 484,751 autosomal SNP markers were compared between the NAFLD cases and control subjects by exact trend test. A slight inflation of p-values was observed by genomic control method ($\lambda = 1.04$) (Figure S1).

We identified six SNP markers located at chromosome 22q13 showing genome-wide significance ($p<1.04\times10^{-7}$) (Figure 1). Among them, four SNPs, namely, rs2896019, rs926633, rs2076211 and rs1010023, located in the *PNPLA3* gene and in strong linkage disequilibrium (LD) ($r^2>0.93$), returned p-values smaller than 1×10^{-9} ($p = 1.5\times10^{-10}$, 7.5×10^{-10}, 1.4×10^{-9} and 1.5×10^{-9}, respectively) (Table 2). Rs738407 and rs3810662 also located in *PNPLA3* showed significant but weaker associations ($p = 1.0\times10^{-7}$ and 1.0×10^{-7}, respectively) than the above four SNP markers. Rs738491, rs2073082, rs3761472, rs2235776, rs2143571 and rs6006473 were in the neighboring *SAMM50* gene which is outside of the linkage disequilibrium (LD) block where the top SNP markers were distributed (Figure 2). These markers were in moderate LD with each other ($r^2>0.42$) and showed p-values between 3.9×10^{-6} and 6.4×10^{-7} but did not reach genome-wide significance (Table S1). Rs738409, the SNP which showed the strongest association with NAFLD in the first GWA study [10], was not included in the SNP array used in our study. This SNP was therefore genotyped using Taqman technology in the same case and control samples that were used for genome scan. Rs738409 showed the strongest association with the disease ($p = 1.4\times10^{-10}$, OR = 1.66, 95%CI: 1.43–1.94) among all the SNP markers examined in this study. The association remained after the correction for population stratification with EIGENSTRAT [17] ($p = 2.3\times10^{-11}$). Although a peak consisting of a cluster of SNPs was observed at the *HLA* locus on chromosome 6 (minimal p-value of 4.10×10^{-7} for rs9262639 located at the 3′ of *C6orf15* gene), the association disappeared when EIGENSTRAT was applied ($p>1.6\times10^{-3}$). We consider this as a result of population stratification between the cases and controls.

Impact of *PNPLA3* Polymorphisms to the Pathogenicity of NAFLD

We next examined whether or not the seven SNPs in the *PNPLA3* gene were associated with the pathogenic status of NAFLD. The genotype distributions of these SNPs were compared by Jonckheere-Terpstra test among the four subgroups of NAFLD patients categorized by Matteoni's classification (type1 to type4). There was a significant increase in the frequency of the risk allele from Matteoni type1 to type4 for all of the seven SNPs (p-values ranging from 3.6×10^{-6} to 0.0017) (Table 2). Among them, rs738409 again showed the strongest association ($p = 3.6\times10^{-6}$) as seen in the simple case/control analysis. On the other hand, there was no significant association between control and Matteoni type1 ($p = 0.76$).

In order to clarify how rs738409 influences the pathogenicity of NAFLD, we performed pairwise comparisons of genotype distributions in the four subgroups of NAFLD patients. There were marked differences in genotype distributions between type4 subgroup and the other three subgroups by multivariable logistic regression adjusted for age, sex and body mass index (BMI) ($p = 2.0\times10^{-5}$, OR = 2.18, 95%CI: 1.52–3.18 between type1 and type4; $p = 1.4\times10^{-3}$, OR = 1.81, 95%CI: 1.26–2.62 between type2 and type4; $p = 0.027$, OR = 1.85, 95%CI: 1.07–3.19 between type3 and type4) (Figure 3). On the other hand, no significant associations were obtained for type1 to type3 in any combinations. When we performed the same analysis between type4 and the pooled genotypes of type1 to type3, we again obtained a significant difference ($p = 4.8\times10^{-6}$, OR = 1.96, 95%CI: 1.47–2.62).

We further examined the specific association of rs738409 with type4 subgroup by using the case/control association results of the initial genome scan. 529 NAFLD patients were divided into 202 patients with type1 to type3 and 327 patients with type4, and genotype distributions of rs738409 in each subgroup were compared with those of 932 control subjects. Exact trend test returned an extremely strong association of rs738409 with type4 subgroup ($p = 1.7\times10^{-16}$, OR = 2.18, 95%CI: 1.81–2.63) whereas no association was obtained for type1 to type3 subgroups ($p = 0.41$).

Table 1. Clinical characteristics according to the histological classification.

Phenotype	Matteoni classification of NAFLD				Control	*p*-value
	Type 1	Type 2	Type 3	Type 4		
Number of samples	100	73	29	327	932	
Sex (Male/Female)	59/41	47/26	13/16	130/197	471/461	0.0023‡
Age (year)	49.7±15.3	51.5±15.3	49.4±14.0	57.6±14.8	48.8±16.3	<0.001
Physical measurement						
BMI	26.2±4.3	27.7±4.8	27.6±3.5	27.7±5.2	–	0.054
Amount of visceral fat (cm^2)	146.8±65.3	154.3±47.7	136.8±53.8	151.7±57.4	–	0.46
Abdominal circumscript (cm)	90.9±9.9	94.1±10.0	88.5±10.2	94.1±11.8	–	0.10
Biochemical trait						
AST (IU/L)	31.1±14.6	36.4±18.5	52.4±35.1	57.7±48.4	–	<0.001
ALT (IU/L)	48.6±30.8	62.8±47.6	81.5±46.9	74.9±48.4	–	<0.001
GGT (IU/L)	71.0±62.5	67.1±66.9	96.1±91.3	76.6±73.9	–	0.25
Albumin (g/dL)	4.5±0.4	4.4±0.3	4.5±0.3	4.3±0.4	–	<0.001
Total bilirubin (mg/dL)	0.9±0.5	0.9±0.5	0.9±0.6	0.8±0.4	–	0.063
Cholinesterase (unit)	389.1±97.0	354.3±97.2	371.1±109.9	348.9±93.2	–	<0.001
Type IV collagen 7S (ng/dL)	3.8±0.7	3.9±0.9	3.9±0.8	5.1±1.7	–	<0.001
Hyaluronic acid (ng/dL)	25.6±22.5	33.6±29.5	31.5±24.0	80.9±84.3	–	<0.001
Triglycerides (mg/dL)	151.9±73.8	154.0±92.1	166.1±86.5	161.2±85.7	–	0.23
Total cholesterol (mg/dL)	209.1±32.8	194.0±38.0	203.0±39.9	200.3±39.0	–	0.093
HbA1c (%)	6.1±1.1	5.9±1.2	6.5±1.8	6.2±1.3	–	0.13
IRI (μg/dL)	9.1±5.4	11.4±9.0	10.4±6.3	14.9±9.9	–	<0.001
FPG (mg/dL)	112.9±33.7	107.3±27.4	109.9±27.7	114.8±33.8	–	0.14
HOMA-IR	2.4±1.5	2.9±2.4	3.0±2.1	4.2±3.0	–	<0.001
hs-CRP (mg/dL)	1078.9±1407	1048.3±1185.0	865.8±658.4	1579.2±2377.9	–	0.027
Adiponectin (μg/mL)	7.4±4.4	8.5±6.6	6.6±2.6	6.9±4.3	–	0.24
Leptin (ng/mL)	9.9±7.4	9.1±6.2	11.3±9.4	12.4±7.9	–	<0.001
Ferritin (ng/mL)	145.8±101.1	176.5±134.0	271.2±307.0	208.3±180.3	–	0.027
Uric acid (mg/dL)	5.9±1.5	5.7±1.2	5.4±1.9	5.7±1.6	–	0.77
PLT ($\times 10^4/\mu L$)	23.0±5.9	22.9±4.9	21.9±6.7	20.2±6.4	–	<0.001
ANA (0/1/2/3/4)	42/17/4/0/0	31/8/4/1/2	15/6/2/0/0	147/76/31/8/12	–	0.015
Clinical history						
Diabetes (NGT/IGT/DM)	36/11/34	24/7/27	12/8/7	103/35/119	–	0.45*
Hyperlipidemia (+/−)	31/68	31/42	9/20	120/206	–	0.60‡
Hypertension (+/−)	64/35	33/40	19/10	155/172	–	0.013‡
Liver biopsy feature						
Brunt grade (1/2/3)	–	–	19/3/2	149/133/44	–	<0.001‡
Brunt stage (1/2/3/4)	–	–	–	123/74/105/24	–	–
Fat droplet (1/2/3/4)	38/32/19/11	14/29/18/7	7/3/10/4	51/99/104/52	–	<0.001
Iron deposition (0/1/2/3/4)	30/14/21/10/1	24/9/12/2/1	10/5/2/2/0	132/56/29/29/11	–	0.16

Measurements are shown as mean ± standard deviation. Categorical values are shown by the count number. *P*-values are calculated by Jonckheere-Terpstra test unless otherwise stated;
‡Chochran-Armitage trend test,
*Kruskal-Wallis test. Abbreviations used for each trait are summarized in materials and methods.
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Association of rs738409 Genotypes with Clinical Traits

The quantitative effects of rs738409 genotypes to clinical traits were examined by multivariable regression adjusted for age, sex and BMI (statistical calculation 1, Table 3). Five categorical ordinals, namely, anti-nuclear antibody (ANA), Brunt grade, Brunt stage, fat deposition and iron deposition, were also tested by an ordinal logistic regression analysis. Potential associations ($p<0.05$) were obtained for 11 traits, namely, aspartate transaminase (AST), alanine aminotransferase (ALT), type IV collagen 7S, hyaluronic acid, hemoglobin A1c (HbA1c), fasting immunoreactive insulin (IRI), fasting plasma glucose (FPG), platelet count (PLT), Brunt grade, fat deposition and iron deposition (Table 3). When the results were further adjusted for Matteoni type (statistical calculation 2), AST, hyaluronic acid, HbA1c, FPG,

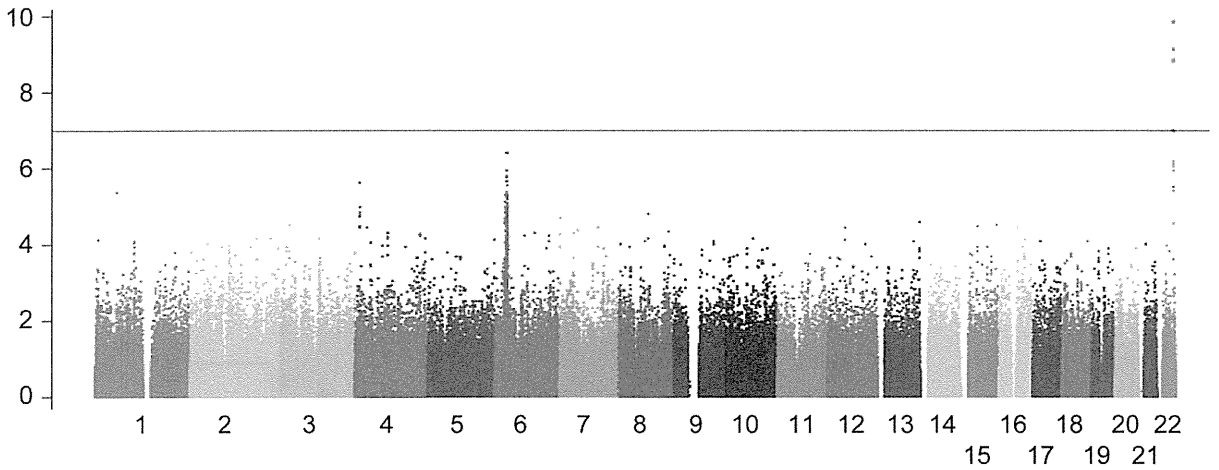

Figure 1. Manhattan plot of the GWA study. Association *p*-values are calculated by exact trend test and plotted along the chromosome in $-\log_{10}$ scale. The horizontal line indicates Bonferroni-adjusted significance threshold ($p = 1.03 \times 10^{-7}$).
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PLT, Brunt grade and iron deposition showed *p*-values smaller than 0.05. The level of serum triglyceride was not significant in the initial analysis, but became significant after being adjusted for Matteoni's type ($p = 0.013$). Among them, only three traits, namely, hyaluronic acid, HbA1c and iron deposition, remained significant ($p < 0.0021$) after Bonferroni's correction for multiple testing (Table 3).

Associations of Previously Reported SNPs with NAFLD

Previous genetic studies identified four chromosomal loci, namely, *LYPLAL1* at 1q41, *GCKR* at 2p23, *NCAN* at 19p12 and *PPP1R3B* at 8p23.1, associated with NAFLD in populations of European descent [14]. We examined whether or not the associations were reproduced in the Japanese population by extracting genotype information of SNP markers corresponding to these four loci. As shown in Table 4, the association of rs780094 in *GCKR* with NAFLD was at the border of significance ($p = 0.011$, OR = 0.82, 95%CI: 0.70–0.91) in the case/control analysis. However, the association was lost when examined between rs780094 genotypes and Matteoni types. There were no associations of rs2228603 in *NCAN* and rs12137855 in *LYPLAL1* with either NAFLD or Matteoni types. Rs4240624 in *PPP1R3B* was not in the SNP array used for this study, and this marker was not polymorphic or at a very low frequency in the Japanese (0 in 90

Table 2. List of the SNP markers in the *PNPLA3* locus at chromosome 22q showing genome wide significance.

		Genotyping Result and Allele Frequency of A2						Statistics		
			NAFLD					NAFLD vs. Control		Matteoni
dbSNPID	A1/A2	Control	Total	Type 1	Type 2	Type 3	Type 4	*p*-value†	OR (95%CI)	*p*-value‡
rs738407	T/C	124/447/361	46/200/283	12/51/37	10/28/35	4/14/11	20/107/200	1.0×10^{-7}	1.56(1.32–1.83)	3.4×10^{-5}
		(0.627)	(0.724)	(0.625)	(0.671)	(0.621)	(0.775)			
rs738409	C/G*	247/468/217	88/236/203	20/59/21	21/30/22	8/11/9	39/136/151	1.4×10^{-10}	1.66(1.43–1.94)	3.6×10^{-6}
		(0.484)	(0.609)	(0.505)	(0.507)	(0.518)	(0.672)			
rs2076211	C/T*	248/473/211	92/242/195	21/58/21	21/30/22	8/11/10	42/143/142	1.4×10^{-9}	1.61(1.38–1.87)	3.2×10^{-5}
		(0.480)	(0.597)	(0.500)	(0.507)	(0.534)	(0.653)			
rs2896019	T/G*	246/473/213	91/234/204	20/57/23	22/29/22	7/12/10	42/136/149	1.5×10^{-10}	1.66(1.42–1.93)	2.6×10^{-5}
		(0.482)	(0.607)	(0.515)	(0.500)	(0.552)	(0.664)			
rs1010023	T/C*	249/473/210	94/239/196	21/57/22	22/29/22	7/12/10	44/141/142	1.5×10^{-9}	1.61(1.38–1.87)	6.5×10^{-5}
		(0.479)	(0.596)	(0.505)	(0.500)	(0.552)	(0.650)			
rs926633	G/A*	247/474/211	93/237/199	21/56/23	22/29/22	7/12/10	43/140/144	7.5×10^{-10}	1.62(1.39–1.89)	5.8×10^{-5}
		(0.481)	(0.600)	(0.510)	(0.500)	(0.552)	(0.654)			
rs3810622	T*/C	330/445/157	263/208/58	40/48/12	28/29/16	14/12/3	181/119/27	1.0×10^{-7}	0.64(0.55–0.75)	0.0017
		(0.407)	(0.306)	(0.360)	(0.418)	(0.310)	(0.265)			

Reference (A1) and non-reference (A2) alleles refer to NCBI Reference Sequence Build 36.3 with the effective allele marked by an asterisk. Genotyping results are shown by genotype count of A1A1/A1A2/A2A2 with allele frequency of A2 in parenthesis.
†*P*-values are calculated by exact trend test with odds ratios (OR) calculated for A2 with 95% confidence interval (CI).
‡*P*-values are calculated by Jonckheere-Terpstra test in NAFLD patients for Matteoni type and additive model of genotype. SNPs are ordered by chromosomal location.
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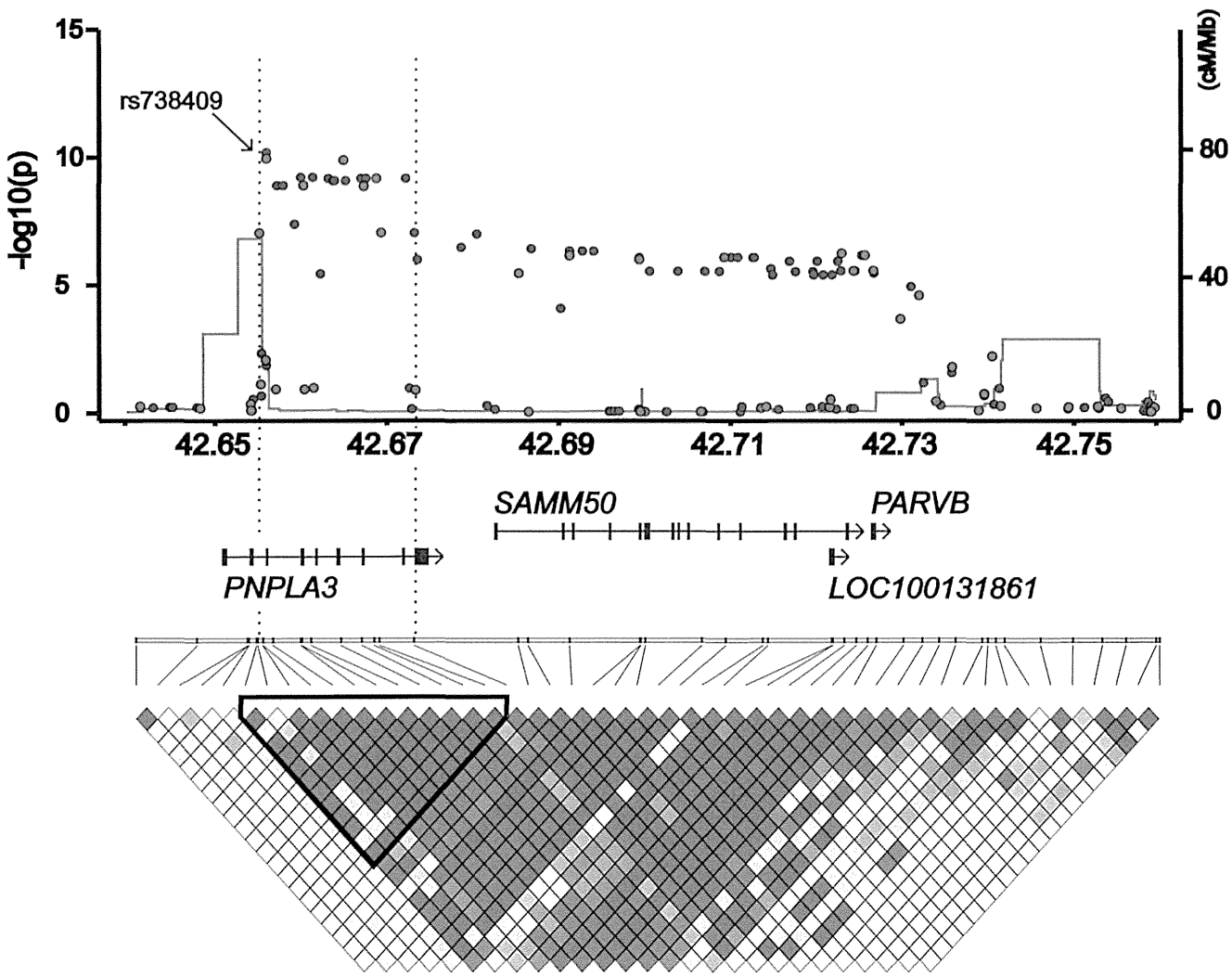

Figure 2. A schematic organization of the human *PNPLA3* locus at 22q13.31 with the genome scan results. *P*-values calculated by the exact trend test were plotted in $-\log_{10}$ scale. Red and blue dots indicate the *p*-values of genotyped and imputed SNPs, respectively. Local recombination rate obtained from HAPMAP release 22 is indicated by a red line plotted in cm/Mb scale. The structure and orientation of four genes in the region are shown below the plots with their transcriptional orientations according to NCBI Reference Sequence Build 36.3. LD blocks were generated according to pairwise LD estimates of the SNPs located within the region using the genome scan results. The LD block showing the strongest association is highlighted with the triangle, and the corresponding chromosomal region is represented by the dotted lines.
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chromosomes in the Japanese result of the International HapMap Project).

Discussion

NASH is a type of hepatic steatosis in NAFLD with poor prognosis accompanying liver fibrosis, and subsequent liver cirrhosis and hepatocellular carcinoma [18]. Despite the extensive biochemical and histological investigation of NAFLD, whether or not NASH forms a distinct disease entity in NAFLD still remains unclear. The principle aim of this study was to identify the genetic factors related to the pathogenic status of NAFLD by collecting DNA samples of Japanese NAFLD patients with critically diagnosed disease status by liver biopsy. To our knowledge, this is the first GWA study of NAFLD using patients with known histology-based Matteoni type. In the initial association study using pooled genotyping results of all the cases, we found a significant association of the *PNPLA3* gene at chromosome 22q13.31 with NAFLD in the Japanese. Rs738409 which showed the strongest association with NAFLD in the GWA study of Caucasians was also genotyped and its strongest association with NAFLD was confirmed. These results were in agreement with the former GWA analyses in populations of European descent and in Hispanics, giving strong evidence of the involvement of *PNPLA3* in NAFLD beyond ethnicities. Rs738409 is located in exon3 of the *PNPLA3* gene which is expressed in the liver and adipose tissue. This SNP introduces an amino acid substitution from isoleucine to methionine (I148M), and biological studies demonstrated that its risk allele (G) abolishes the triglyceride hydrolysis activity of *PNPLA3* [19]. These observations strongly suggest rs738409 to be a causative genetic variation for NAFLD. However, future genomic analyses by fine mapping or extensive sequencing may identify additional genetic determinants within the *PNPLA3* locus.

In the current study we did not find other genetic loci showing genome-wide significance ($p<1.0\times10^{-7}$). However, two additional chromosomal loci with *p*-values being smaller than 1×10^{-5} were

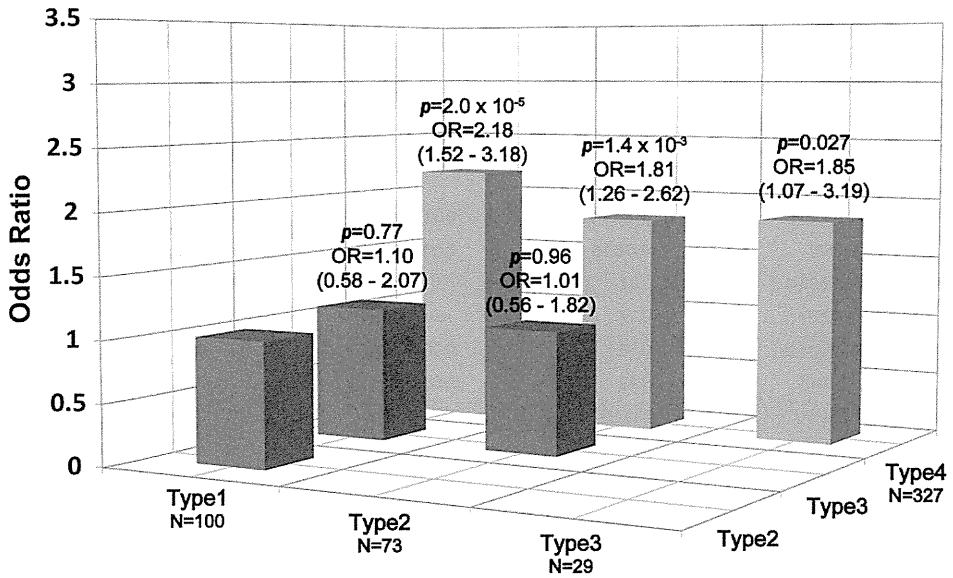

Figure 3. Histogram of odds ratios for genotype distribution of rs738409 between Matteoni types. Each box denotes the odds ratio (OR) comparing the corresponding Matteoni types on the horizontal axes. N represents the number of samples. Odds ratios and *p*-values are calculated for the higher Matteoni type per risk allele (G) on additive model by multivariable logistic regression adjusted for age, sex and BMI, and are shown with 95% CI above each box.
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identified on chromosome 1p (rs11206226) and chromosome 4p (rs1390096) neither of which has been reported as being associated with NAFLD in Caucasians (Table S1). Statistical calculation by taking their allele frequencies and effect sizes into account showed that approximately three times as many case and control samples are required to obtain sufficient statistical power (>0.8) for genome wide significance. Hence, further confirmation is required using a larger collection of patients and controls although they may be potential candidates of low-penetrance genes for susceptibility to NAFLD in Japanese.

Subsequent analyses through comparison of genotype distribution among four subgroups of NAFLD (type1 to type4) categorized by Matteoni's classification revealed that the seven NAFLD-associated SNPs in the *PNPLA3* gene were also significantly associated with the pathogenic status of NAFLD. There were also marked differences in genotype distribution of rs738409 between type4 subgroup and the other three groups ($p = 4.8 \times 10^{-6}$, OR = 1.96, 95%CI: 1.47–2.62 between type4 and pooled genotypes of type1 to type3). Moreover, a case/control analysis of rs738409 between Matteoni type4 and controls returned a surprisingly strong association ($p = 1.7 \times 10^{-16}$) which was much stronger than the initial analysis using all NAFLD cases ($p = 1.4 \times 10^{-10}$), whereas the analysis using Matteoni type1 to type3 as cases didn't show significance ($p = 0.41$). There were differences in the score of HOMA-IR and hs-CRP, indicators of insulin resistance and inflammation, respectively, between Matteoni type1 to type3 and type4 subgroups (Table 1). Our results provide compelling evidence that NASH corresponding to Matteoni type4 is both a clinically and genetically different disease subset from other spectrums of NAFLD. Previous studies showed association between *PNPLA3* and fatty liver, inflammation, fibrosis grade and NASH [13]. In our result, strong association between rs738409 and fatty liver was not observed by comparing control and Matteoni type1. In addition, strong association between rs738409 and lobular inflammation was not observed by comparing Matteoni Type1 and Type2. In contrast, a strong association between rs738409 and NASH was observed. Although we could not observe the strong association between rs738409 and fibrosis stage, strong association between rs738409 and Hyaluronic acid suggests that an association exists between *PNPLA3* and fibrosis.

We have also undertaken association analyses of rs738409 and clinical traits in the patients. The multivariable regression analysis adjusted for age, sex, BMI and Matteoni type followed by the correction for multiple testing revealed hyaluronic acid and HbA1c as being significantly associated with rs738409. Hyaluronic acid is one of the principle components of the extracellular matrix and its involvement in fibrosis has been previously suggested [20]. This may indicate another possible functional involvement of *PNPLA3* in the progression of liver fibrosis by influencing the circulating hyaluronic acid levels. A weak association of rs738409 and HbA1c levels was observed in our study population. However, there are no reports to date indicating such an association, and confirmation with different sample sets is needed for definitive conclusion. Also, the association between rs738409 and iron deposition was demonstrated by an ordinal logistic regression analysis. Since the association still remained after the results were adjusted with Matteoni type, rs738409 may play a functional role in the oxidative stress through iron absorption in the liver.

Recently, a genetic analysis of Japanese NAFLD patients was reported demonstrating a significant association in the increase of AST, ALT, ferritin levels and fibrosis stage (Brunt stage) and in the decrease of serum triglyceride with the risk allele (G) of rs738409 [12]. In our study, the association of rs738409 with AST ($p = 1.2 \times 10^{-4}$) and ALT ($p = 0.0016$) was reproduced and that of AST still remained after the results were adjusted for Matteoni type ($p = 0.038$). No association was observed for ferritin level. Brunt stage was available for Matteoni type4 patients only in our study. Although the odds ratio was slightly high (OR = 1.28, 95%CI: 0.95–1.72), it was not possible to examine the association. In addition, the inverse association of the risk allele of rs738409 with decrease of serum triglyceride was confirmed in our study ($p = 0.013$ after being adjusted for Matteoni type). For all of these

Table 3. Association of rs738409 with clinical traits.

Biochemical traits	Statistical calculation1		Statistical calculation 2	
Phenotype	**Coef. (S.E.)**	***p*-value**	**Coef. (S.E.)**	***p*-value**
Biological traits				
AST (IU/L)	0.22 (0.056)	**1.2×10^{-4}**	0.11 (0.052)	0.038
ALT (IU/L)	0.19 (0.058)	**0.0016**	0.093 (0.056)	0.098
GGT (IU/L)	−0.056 (0.061)	0.37	−0.088 (0.062)	0.16
Albumin (g/dL) *	0.015 (0.051)	0.77	−0.012 (0.052)	0.81
Total bilirubin (mg/dL)	−0.011 (0.063)	0.86	0.0059 (0.064)	0.93
Cholinesterase (unit) *	0.062 (0.040)	0.12	0.069 (0.041)	0.092
Type IV collagen 7S (ng/dL) *	−0.19 (0.064)	0.0025	−0.11 (0.062)	0.069
Hyaluronic acid (ng/dL)	0.30 (0.065)	**4.9×10^{-6}**	0.22 (0.063)	**4.6×10^{-4}**
Triglycerides (mg/dL)	−0.10 (0.058)	0.072	−0.15 (0.059)	0.013
Total cholesterol (mg/dL)	−0.066 (0.060)	0.27	−0.057 (0.061)	0.34
HbA1c (%)	−0.17 (0.053)	**0.0012**	−0.18 (0.054)	**0.0011**
IRI (μg/dL)	0.16 (0.063)	0.012	0.086 (0.061)	0.16
FPG (mg/dL)	−0.14 (0.049)	0.0047	−0.15 (0.05)	0.0035
HOMA-IR	0.084 (0.064)	0.19	0.0092 (0.062)	0.88
Hs-CRP (mg/dL)	−0.013 (0.048)	0.79	−0.031 (0.049)	0.52
Adiponectin (μg/mL)	0.048 (0.066)	0.47	0.12 (0.066)	0.072
Leptin (ng/mL)	0.11 (0.068)	0.11	0.10 (0.069)	0.15
Ferritin (ng/mL)	0.031 (0.047)	0.51	−0.0042 (0.048)	0.93
Uric acid (mg/dL)	−0.097 (0.061)	0.11	−0.11 (0.062)	0.067
PLT ($x10^4$/μL)	−0.056 (0.020)	0.0052	−0.045 (0.020)	0.028
Immunological/histological traits				
ANA (0/1/2/3/4)	0.92 (0.70–1.21)	0.56	0.86 (0.65–1.15)	0.31
Brunt grade (1/2/3)	1.42 (1.06–1.92)	0.021	1.38 (1.02–1.87)	0.036
Brunt stage (1/2/3/4)	1.28 (0.95–1.72)	0.11		
Fat deposition (1/2/3/4)	1.44 (1.15–1.81)	0.0019	1.24 (0.98–1.56)	0.76
Iron deposition (0/1/2/3/4)	0.61 (0.47–0.80)	**3.0×10^{-4}**	0.62 (0.47–0.81)	**5.6×10^{-4}**

Associations between distribution of rs738409 genotypes and clinical traits are calculated by multivariable regression. Statistical calculation1 is adjusted for age, sex and BMI, while the Matteoni types are additionally included as covariate in statistical calculation 2. Statistics are calculated by multivariable linear regression for biochemical traits and by multivariable ordinal logistic regression for immunological and histological traits.

Coefficients and odds ratios are calculated for the increase of each trait per risk allele (G). The *p*-values showing significance after Bonferroni's correction for multiple testing ($p = 0.0021$) was shown in bold.

*Reciprocal numbers are used for normalization and a negative coefficient implicates an increase in value according to the increase of the risk allele.

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biomarkers, however, the significance was lost after the correction for multiple testing.

A replication analysis of other genetic loci that had been reported for their association with NAFLD in East coast white Americans [14] was performed in our sample collection. We confirmed the association of rs780094 in *GCKR* with NAFLD in a case/control analysis but at a much weaker level ($p = 0.011$, OR = 0.82, 95%CI: 0.70–0.95) than that shown for the populations of European-descent. No associations were found for *LYPLAL1* and *NCAN* loci in our study. There are several reasons to explain such differences, such as the insufficient statistical power with a limited number of study subjects in our study due to the difficulty in the collection of a larger number of histologically diagnosed NAFLD patients. The difference in genetic background between the Japanese and Europeans is also conceivable. Indeed, the risk allele frequency of rs12137855 in *LYPLAL1* was 0.944 in our control subjects but approximately 0.79 in the European populations [14]. Similarly, there was a difference in the risk allele frequency of rs2228603 in *NCAN* (0.049 in Japanese and 0.08 in Europeans). Rs4240624 in *PPP1R3B* was not polymorphic in the Japanese while its risk allele frequency was 0.91 in Europeans.

Materials and Methods

Ethics Statement

In compliance with the Declaration of Helsinki, ethical approval for this study was given by the respective Institutional Review Board and subject written informed consent were obtained for all subjects (Ethical committee of Nara City Hospital; Ethical committee of Saiseikai Suita Hospital; Medical Ethics Committee of Kanazawa University; Ethics committee of Kyoto Prefectural University of Medicine; Ethical Committee of Aichi Cancer Center; Ethical Committee of Kochi Medical School, Kochi University; Ethics Committee of Tokyo Women's Medical University; Ethical Committee on Kawasaki Medical School and Kawasaki Medical School Hospital; Ethical Committee of

Juntendo University; Ethics Committee of Yamagata University School of Medicine; Ethical Committee of the Ikeda Municipal Hospital; Institutional Review Board and Ethics Committee of Kyoto University School of Medicine).

Study Population

A total of 543 patients histologically diagnosed for NAFLD in 2007–2009 were recruited through the Japan study of Nonalcoholic Fatty Liver Disease. Biopsy specimens were stained with H&E and Masson's trichrome for morphological review and assessment of fibrosis. Perl's Prussian blue was performed to evaluate iron load. Biopsy specimens were reviewed by a hepatopathologist (T.O). NAFLD patients were classified into four categories by liver histology according to the classification by Matteoni *et al* [2] as follows; type1: fatty liver alone, type2: fat accumulation and lobular inflammation, type3: fat accumulation and ballooning degeneration, type4: fat accumulation, ballooning degeneration, and either Mallory-Denk body or fibrosis. With these criteria, the 543 patients were classified as type1; 102, type2; 75, type3; 31 and type4; 335. The histological grade and fibrosis stage were also evaluated by the classification of Brunt *et al* [21] for advanced NAFLD cases (type3 and type4) as follows; grade 1: steatosis involving up to 66% of biopsy, occasional ballooned zone 3 hepatocytes and absence or mild portal chronic inflammation, grade2: steatosis, ballooning hepatocytes mild to moderate chronic inflammation, grade3: panacinar steatosis, ballooning and disarray obvious and mild or portal mild to moderate inflammation, stage1: perivenular and/or perisinusoidal fibrosis in zone3, stage2: combined pericellular portal fibrosis, stage3: septal/bridging fibrosis, stage4: cirrhosis. The degree of fat deposition was evaluated by amount of fat droplets as observed under the microscope as follows; 0: <5%, 1: 5–<10%, 2: 10–<34%, 3: 34–<67%, 4: >67%. The degree of iron deposition was categorized by the presence of granules of free iron observed under the microscope as follows; 0: absence by x400, 1: easily identifiable by x400 and rarely identifiable by x250, 2: identifiable by x100, 3: identifiable by x25, 4: identifiable at lower than x25.

Inclusion criteria for NAFLD patients were as follows; (i) no history of alcoholism, (ii) no history for HBV/HCV/HIV infection, (iii) diagnosed by liver biopsy, (iv) information regarding age and BMI available. The sex of two samples was unknown, and was imputed from the results of the genome scan. As general Japanese population controls, the genome scan results of 942 healthy Japanese volunteers from Aichi Cancer Center Hospital and Research Institute were used [22].

Anthropometric and Laboratory Evaluation

We employed conventional methods for the measurement of anthropometry (height, weight, amount of visceral fat and abdominal circumscript). BMI was calculated from the measurements. The following biochemical/hematological/immunological traits were also measured by conventional methods; aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (GGT), albumin, total bilirubin, cholinesterase, type IV collagen 7S, hyaluronic acid, triglyceride, total cholesterol, hemoglobin A1c (HbA1c), fasting immunoreactive insulin (IRI), fasting plasma glucose (FBS), high sensitive CRP (hs-CRP), adiponectin, leptin, ferritin, uric acid, and platelet (PLT) count. Anti nuclear antibody (ANA) was measured by ELISA and categorized by the detection limit in a serial dilution as follows; 0: <40x, 1: 40–80x, 2: 81–160x, 3: 160x, 4: >320x. Homeostasis model assessment-insulin resistance (HOMA-IR) was calculated from the measurements. Patients were assigned a diagnosis of diabetes mellitus (DM) when they had documented use of oral hypoglycemic medication, a random glucose level >200 mg/dl, or FPG >126 mg/dl. Hyperlipidemia was diagnosed with the cholesterol level being >200 mg/dl and/or triglyceride level being >160 mg/dl. Hypertension was diagnosed when the patient was taking antihypertensive medication and/or had a resting recumbent blood pressure ≥140/90 mmHg on at least two occasions.

DNA Preparation

Genomic DNA was extracted from peripheral blood mononuclear cells by standard phenol-chloroform extraction and resuspended in TE buffer. DNA concentration and purity were measured with Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The samples were stored at −20°C until use.

Genome-wide Genotyping and Quality Control

Genome scan was conducted for 543 patients with NAFLD and 942 healthy subjects using Illumina Human 610-Quad Bead Chip on a Bead Station 500G Genotyping System (Illumina, Inc., San Diego, CA, USA) and subjected to the following quality controls. Initially, ten patients and six control subjects were removed due to low call rates (<0.99). Regarding the SNP markers, 85,472 SNPs with minor allele frequency of smaller than 0.01 in either case or control group, 6,479 SNPs with lower success rates (<0.98) and 35 SNPs with distorted Hardy-Weinberg equilibrium ($p<10^{-7}$) were removed, resulting in 484,751 SNP markers being used for analysis. Principal component analysis by EIGENSOFT [17] including phase II HapMap (http://hapmap.ncbi.nlm.nih.gov/) samples identified no samples that were deviated from the Japanese population. Subsequently, the degree of kinship between individuals was examined by pi-hat in PLINK 1.07 (http://pngu.mgh.harvard.edu/purcell/plink/) [23]. Of the eight pairs of samples (four case pairs and four control pairs) showing high degrees of kinship (PI-HAT>0.4), the sample with the lower call rate in each pair was removed. After these steps, 529 case and 932 controls were used for the analysis.

Statistical Analysis

A case/control association analysis was performed by exact trend test between NAFLD patients and control subjects [24]. The correction of obtained *p*-values for population stratification was performed using EIGENSTRAT [17]. In addition, an association between Matteoni classification (type1 to type4) and additive model of genotype for each SNP was examined using Jonckheere-Terpstra test for NAFLD patients. Assessment of population stratification of inflation of *p*-value was carried out by the genomic control method for asymptotic trend test [25]. Association between each quantitative trait and the genotype of significant SNPs in NAFLD patients were calculated by multivariable linear regression or multivariable ordinal regression adjusted for age, sex and BMI. Each quantitative trait was transformed as follows; natural log for ALT, AST, HOMA-IR, HbA1c, IRI, triglyceride, total bilirubin, adiponectin, hs-CRP, hyaluronic acid, leptin, reciprocal number for albumin, cholinesterase, type IV collagen 7S and square root for uric acid and ferritin. The values of FPG, PLT, total cholesterol, amount of visceral fat, and abdominal circumscript were not transformed. For each trait, values that were within only 4 S.D. were included for analysis. LD indices were calculated by default setting of Haploview [26] and the LD block was defined manually.

Table 4. Replication study of previously reported SNPs.

			Genotyping Result and Allele Frequency of A2					Statistics		
			NAFLD					NAFLD vs. Control		Matteoni
dbSNPID	A1/A2	Gene	Control	Type 1	Type 2	Type 3	Type 4	*p*-value†	OR (95%CI)	*p*-value‡
rs12137855	C*/T	*LYPLAL1*	828/102/2	90/10/0	67/6/0	24/5/0	294/33/0	0.55	0.89	0.98
			(0.056)	(0.050)	(0.041)	(0.086)	(0.050)		(0.64–1.25)	
rs780094	T*/C	*GCKR*	321/433/178	34/54/12	28/34/11	17/11/1	133/139/55	0.011	0.82	0.92
			(0.423)	(0.390)	(0.383)	(0.224)	(0.381)		(0.70–0.95)	
rs4240624	G/A	*PPP1R3B*	–	–	–	–	–	–	–	–
rs2228603	C/T*	*NCAN*	842/88/2	93/7/0	65/8/0	28/1/0	292/31/4	0.80	1.05	0.58
			(0.049)	(0.035)	(0.054)	(0.017)	(0.059)		(0.75–1.48)	

Reference (A1) and non-reference (A2) alleles refer to NCBI Reference Sequence Build 36.3 with the effective allele marked by an asterisk. Genotyping results are shown by genotype count of A1A1/A1A2/A2A2 with allele frequency of A2 in parenthesis. †*P*-values are calculated by exact trend test with odds ratios (OR) calculated for A2 with 95% confidence interval (CI). ‡*P*-values are calculated by Jonckheere-Terpstra test in NAFLD patients for Matteoni type and additive model of genotype.
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Supporting Information

Figure S1 QQ plot of the GWA study comparing distribution of the observed and expected *p*-values. Upper box is expressed in antilog scale and the lower box is expressed in $-\log_{10}$ scale. The X- and Y-axis correspond to expected and observed *p*-values. Blue and red dots denote before and after correction by genomic control method ($\lambda = 1.04$), respectively.
(DOC)

Table S1 List of the SNPs showing $p<1.0\times10^{-5}$ in the GWA study. Reference (A1) and non-reference (A2) alleles refer to NCBI Reference Sequence Build 36.3 with the effective allele marked by an asterisk. Genotyping results are shown by genotype count of A1A1/A1A2/A2A2 with allele frequency of A2 in parenthesis. †*P*-values are calculated by exact trend test with odds ratios (OR) calculated for A2 with 95% confidence interval (CI). ‡*P*-values are calculated by Jonckheere-Terpstra test in NAFLD patients for Matteoni type and additive model of genotype. SNPs are ordered by chromosomal location.
(DOC)

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Author Contributions

Conceived and designed the experiments: FM TO. Performed the experiments: MT M. Kokubo. Analyzed the data: TK RY FM. Contributed reagents/materials/analysis tools: TK YS AU KM MT TT KY T. Saibara EH M. Kokubo SW SK YI M. Kawanaka T. Shima HP HT KT RY. Wrote the paper: TK MT RY FM TO.

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Original Article

Hepatocellular carcinoma based on cryptogenic liver disease: The most common non-viral hepatocellular carcinoma in patients aged over 80 years

Katsutoshi Tokushige,[1] Etsuko Hashimoto,[1] Yoshinori Horie,[2] Makiko Taniai[1] and Susumu Higuchi[3]

[1]*Department of Internal Medicine and Gastroenterology, Tokyo Women's Medical University,* [2]*Department of Internal Medicine, Sanno Hospital, International University of Health and Welfare, Tokyo, and* [3]*National Hospital Organization Kurihama Alcoholism Center, Kanagawa, Japan*

Aim: To clarify the clinical features of patients with hepatocellular carcinoma (HCC) with cryptogenic liver diseases, we analyzed the data from a nationwide survey in Japan.

Methods: The survey was conducted in 2009. The factors examined included age and underlying liver diseases: alcoholic liver disease (ALD; $n = 991$), non-alcoholic fatty liver disease ($n = 292$), modest alcohol intake (intake between 20 and 70 g/day, $n = 214$) and cryptogenic liver diseases ($n = 316$). We compared the clinical features of cryptogenic HCC among patient-age subgroups.

Results: HCC with ALD etiology was most common among the non-viral HCC patients under 80 years old; for those aged 80 years or older, cryptogenic HCC was the most common etiology. Among the cryptogenic HCC patients, the body mass index values and the prevalences of liver cirrhosis (LC) and diabetes mellitus (DM) were significantly lower in the 80 years or older group versus the 50–79 years group. In the 80 years or older group, 28% of the patients developed HCC without cirrhosis, obesity and DM.

Conclusion: In the HCC patients aged 80 years and over, the etiology of most of the non-viral HCC cases was classified as cryptogenic. In light of our finding that the prevalences of obesity, DM and LC in the 80 years or older group of cryptogenic HCC patients were significantly lower those in the younger patients, it is apparent that analyses of HCC cases must take age differences into account.

Key words: cryptogenic liver disease, diabetes mellitus, hepatocellular carcinoma, liver cirrhosis, old age

INTRODUCTION

PRIMARY LIVER CANCER is the fifth most common cancer worldwide, and the third most common cause of cancer mortality.[1–3] According to the most recent nationwide Japanese registry data, primary liver cancer ranked fourth for men and sixth for women as a cause of death from malignancy.[4] Several recent Japanese surveys of hepatocellular carcinoma (HCC) studies have shown that the underlying liver diseases for HCC have changed; the incidence of hepatitis C virus (HCV)-related HCC has gradually decreased to approximately 60–70%, whereas the incidence of HCC associated with non-viral chronic liver disease has gradually increased to approximately 15–25%.[5–8] Among the cases of non-viral HCC, alcoholic liver disease (ALD)-HCC was found to account for 43–51% of cases, followed by unknown etiology liver disease HCC (18–35%) and non-alcoholic fatty liver disease (NAFLD)-HCC (13–28%).[6–8]

Non-alcoholic fatty liver disease is usually defined by a daily alcohol consumption of less than 20 g in women and less than 30 g in men, because ALD can occur above these thresholds.[9,10] However, there is no clear consensus regarding the threshold alcohol consumption for defining NAFLD and non-alcoholic steatohepatitis (NASH), and because the definitions are not clear, it is difficult to summarize the etiological analyses of liver disease underlying non-viral HCC.

To clarify the etiology of HCC in Japanese patients with non-viral liver disease, we performed a nationwide

Correspondence: Dr Etsuko Hashimoto, Department of Internal Medicine and Gastroenterology, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan. Email: drs-hashimoto@mti.biglobe.ne.jp

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survey of HCC patients in 2009.[8] We studied the clinical features of HCC patients with NAFLD, ALD (alcohol consumption, ≥70 g/day) and chronic liver disease of unknown etiology. We divided the cases of unknown etiology HCC into two subgroups: no alcohol intake group (alcohol consumption, <20 g/day) and modest alcohol intake group (alcohol consumption, 20–70 g/day).

We found that among the non-viral HCC cases, ALD-HCC was the most common etiology, and we observed that the patients in the ALD-HCC group were the youngest and showed the lowest percentage of females. The patients in the modest alcohol intake HCC group showed the same tendencies as the ALD-HCC patients regarding sex, body mass index (BMI), prevalence of lifestyle-related disease, and liver function. We reported that a modest intake of alcohol may have a more significant role in hepatic carcinogenesis than is presently thought.

In the present study, we focused on the clinical features and pathogenesis of HCC patients who reported consuming no alcohol and those who had cryptogenic HCC. In our experience, it is not rare that patients over 80 years old develop HCC in normal liver with no etiology (unpubl. data). To investigate the characteristics of cryptogenic HCC, we focused on age. First, we assessed the etiologies of non-viral HCC patients divided into 10-year age subgroups, and then we compared the clinical features of the cryptogenic HCC patients in the different age subgroups.

METHODS

IN 2009, WE conducted a nationwide survey of patients who received a diagnosis of HCC in Japan. We sent questionnaires to all of the hospitals in Japan that are approved by the Japanese Society of Gastroenterology, asking about the etiology of their HCC cases, and we sent case cards for ALD-HCC, NAFLD-HCC, modest alcohol intake HCC and cryptogenic HCC cases. We asked for data on all patients who were diagnosed with HCC between April 2006 and March 2009.

A total of 115 hospitals across the country responded to the questionnaire and provided case cards. These institutions are listed in Appendix I. The present retrospective study was conducted according to the Declaration of Helsinki (2000 version). We enrolled 14 530 patients with a clinical and/or histological diagnosis of HCC. Among these patients, 2299 (15.8%) were diagnosed as having non-hepatitis B virus (HBV), non-hepatitis C virus (HCV)-HCC and we analyzed their case cards.

All patients with non-viral HCC were shown to be negative for hepatitis B surface antigen and for anti-HCV antibody and/or HCV RNA by polymerase chain reaction analysis.

Among the 2299 non-viral HCC patients, we excluded the cases of 274 patients because their clinical data, such as the amount of alcohol intake and laboratory data, were not sufficient for the analysis (Fig. 1). We categorized each of the remaining 2025 non-viral HCC patients into one of five groups according to the etiology of their HCC: (i) ALD-HCC; (ii) NAFLD-HCC; (iii) modest alcohol intake HCC; (iv) cryptogenic HCC; and (v) miscellaneous disease.

Alcoholic liver disease (ALD-HCC group, $n = 991$) was diagnosed according to the modified criteria proposed by Takada *et al.*,[11] and the alcohol consumption in ALD was defined as habitual alcohol consumption over 70 g daily. The diagnosis of NAFLD ($n = 292$) was based on the following criteria: (i) detection of hepatic steatosis (or steatohepatitis) by liver biopsy or imaging; (ii) intake of less than 20–30 g of ethanol daily (as confirmed by the attending physician and family members in close contact with the patient); and (iii) the appropriate exclusion of other liver diseases.[8,12,13] "Modest intake of alcohol" ($n = 214$) was defined as unknown liver disease with alcohol consumption of 20–70 g/day. "Cryptogenic HCC" was defined as unknown liver disease without steatosis by imaging modalities or liver biopsy among patients

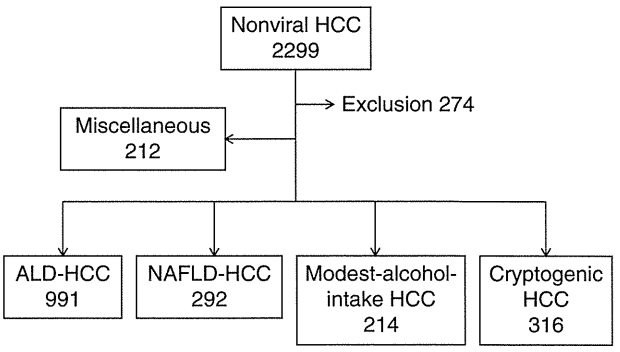

Figure 1 The cases of 2299 patients with non-viral HCC were collected by a national survey in Japan, and the cases of 274 patients were excluded because of incomplete clinical data. We analyzed the cases of the remaining 991 patients with ALD-HCC, 292 with NAFLD-HCC, 214 with modest-alcohol-intake HCC, and 316 with cryptogenic HCC; the underlying causes of the other 212 patients were classified as miscellaneous disease. ALD, alcoholic liver disease; HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease.

with alcohol consumption of less than 20 g/day. Many miscellaneous diseases were excluded, such as congestive disease, metabolic disease (e.g. Wilson's disease, hemochromatosis), primary biliary cirrhosis, autoimmune hepatitis and primary sclerosing cholangitis. The final diagnosis of HCC and the assessment of etiology were conducted at each participating institution.

Etiologies of non-viral HCC divided by 10-year age subgroups

To elucidate the etiological characteristics of the non-viral HCC cases, we investigated the deviations of the following four non-viral HCC groups: ALD-HCC, NAFLD-HCC, modest alcohol intake HCC and cryptogenic HCC. We divided these patients into five subgroups according to age in 10-year increments: less than 50, 50–59, 60–69, 70–79 and 80 years or older. We compared the number of patients and the percentages of these groups against the total population of non-viral HCC patients.

Characteristic features of cryptogenic HCC: comparison of age-dependent groups

We also divided the group of 316 cryptogenic HCC patients into three broader age subgroups and compared their clinical data: (i) less than 50 years old ($n = 7$); (ii) 50–79 years old ($n = 216$); and (iii) 80 years and over ($n = 93$).

Obesity is defined by the Japanese Obesity Association criteria as a BMI of more than 25 kg/m^2.[14] For the present patient population, the diagnosis of type II diabetes mellitus (DM) was based on the World Health Organization (WHO) criteria.[15] Dyslipidemia was diagnosed if the patient was currently on lipid-lowering medications, or if the patient had shown elevated serum levels of total cholesterol (>220 mg/dL) and/or triglycerides (>150 mg/dL) on at least three occasions. Hypertension was diagnosed if the patient was receiving antihypertensive therapy or had a recorded blood pressure of more than 140/90 mmHg on at least three occasions.

Liver cirrhosis (LC) was diagnosed on the basis of histological biopsy findings, laparoscopy or abdominal imaging (left lobe hypertrophy with splenomegaly, nodular changes in the liver surface) and laboratory findings (lower platelet count, albumin level and/or prolonged prothrombin time) compatible with LC. Clinical findings of esophageal varices, ascites and/or hepatic encephalopathy were also taken into account.

The following laboratory parameters were measured: albumin, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (GGT), fasting blood sugar, hemoglobin Alc (HbAlc), platelet count, prothrombin time (PT), des-γ-carboxyprothrombin (DCP) and α-fetoprotein (AFP).

Statistical analysis

The statistical analyses were performed with SPSS version 13.0 J software (SPSS, Tokyo, Japan). Data are shown as the mean ± standard deviation (SD) or as percentages. The Mann–Whitney *U*-test or the χ^2-test were used to compare data between the 50–79 years and 80 years or older subgroups of the cryptogenic HCC patients. $P < 0.05$ was considered significant.

RESULTS

Etiologies of non-viral HCC in the 10-year age subgroups

THE DISTRIBUTION OF the patients with ALD-HCC, NAFLD-HCC, modest alcohol intake HCC and cryptogenic HCC divided by each 10-year age subgroup is shown in Figure 2(a). Among the patients under 70 years old, the number of ALD-HCC cases was markedly higher in each of the three under 70 years age groups compared to NAFLD-HCC, modest alcohol intake HCC and cryptogenic HCC. In contrast, among the patients aged 80 years or older, cryptogenic HCC was the most common etiology.

Among the patients with ALD-HCC, the age-grouped numbers of patients peaked at 60–69 years old, with a mean ± SD age of 67.1 ± 9.10 years, whereas in each of the groups of patients with NAFLD-HCC (71.6 ± 8.4 years), modest alcohol intake HCC (70.4 ± 9.0 years) and cryptogenic HCC (74.1 ± 10.2 years), the ages of the three groups peaked at 70–79 years old, respectively.

Figure 2(b) shows the percentages in the four non-viral HCC groups (ALD-HCC, NAFLD-HCC, modest alcohol intake HCC and cryptogenic HCC). Among the patients under 70 years old, ALD-HCC accounted for approximately 70% of the cases; among the patients 70 years old or older, this percentage was markedly decreased, and the percentage of NAFLD-HCC cases was slightly increased. Among the patients over 70 years old, the percentage of cryptogenic HCC cases was markedly increased.

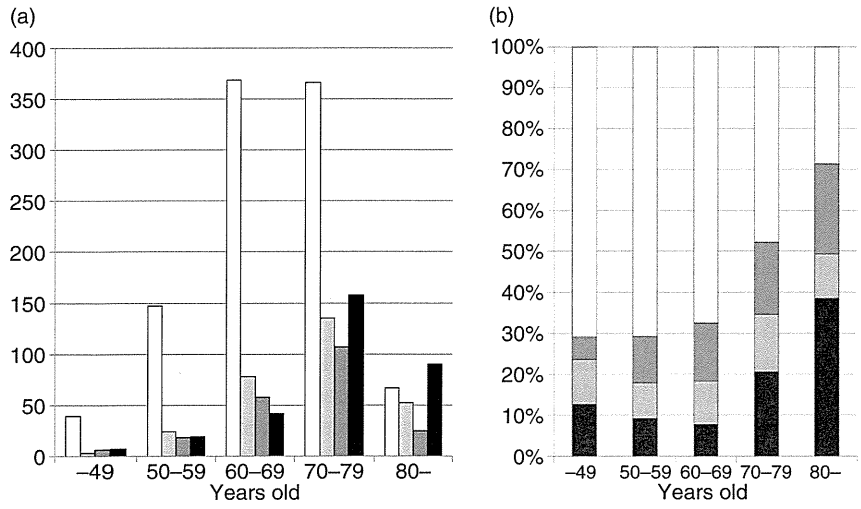

Figure 2 (a) Distribution of the patients in the four non-viral HCC groups (ALD-HCC, NAFLD-HCC, modest alcohol intake HCC and cryptogenic HCC). Among the patients under 70 years old, ALD-HCC was the most common etiology; among the patients aged 80 years or older, cryptogenic HCC was the most common etiology. (b) The percentages in the four non-viral HCC groups. □, ALD-HCC; ▨, NAFLD-HCC; ▨, modest alcohol intake HCC; ■, cryptogenic HCC. ALD, alcoholic liver disease; HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease.

Cryptogenic HCC cases classified by age

Table 1 shows the clinical characteristics and HCC features among the three age groups of less than 50 years, 50–79 years and 80 years or older. As there were only seven patients in the less than 50 years group, we performed the statistical analysis between the 50–79 years group ($n = 216$) and the 80 years or older group ($n = 93$).

Table 1 Characteristic features among age-dependent groups in cryptogenic HCC

	(1) <50 years old ($n = 7$)	(2) 50–79 years old ($n = 216$)	(3) ≥80 years old ($n = 93$)	*P*-value* (2 vs 3)
Age (year)	36.0 ± 11.9	71.2 ± 6.8	84.0 ± 3.4	
Sex (female)	49%	54%	63%	NS
Obesity (BMI, >25 kg/m^2)	0%	42%	33%	NS
BMI (kg/m^2)	19.2 ± 3.3	24.5 ± 4.64	23.2 ± 3.9	0.037
DM	14%	45%	33%	0.048
Hypertension	0%	44%	53%	NS
Dyslipidemia	0%	14%	19%	NS
Liver cirrhosis	0%	62%	49%	0.048
Albumin (g/dL)	4.1 ± 0.4	3.5 ± 0.7	3.6 ± 0.7	NS
Total bilirubin (mg/dL)	1.0 ± 0.5	1.2 ± 1.2	1.2 ± 2.0	NS
AST (IU/L)	38 ± 15.9	62 ± 68.0	61 ± 55.1	NS
ALT (IU/L)	48 ± 27.2	41 ± 48.1	38 ± 35.5	NS
GGT (IU/L)	95 ± 109.3	155 ± 215.5	107 ± 110.8	NS
FBS (mg/dL)	110 ± 50.2	124 ± 52.1	119 ± 49.1	NS
HbAlc (%)	5.3 ± 0.4	6.0 ± 1.4	5.7 ± 0.9	NS
Platelet count (×10^4/mm^3)	22.7 ± 6.6	15.6 ± 8.9	17.1 ± 9.4	NS
PT (%)	98 ± 17.5	79 ± 16.8	85 ± 16.8	0.008
AFP (ng/mL)	11 779 ± 22 849	8586 ± 57 379	6903 ± 30 775	NS
DCP (ng/mL)	1460 ± 3373	16 550 ± 87 884	69 666 ± 45 294	NS
HCC size (mm)	66.2 ± 50.5	47.4 ± 37.8	56.3 ± 67.4	0.076
No. of HCC	5.5 ± 4.9	2.9 ± 3.3	2.4 ± 2.9	0.066

Expressed as the mean ± standard deviation.
**P*-value, comparison between 50–79 years age group and ≥80 years group.
AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DCP, des-γ-carboxyprothrombin; DM, diabetes mellitus; FBS, fasting blood sugar; GGT, γ-glutamyltransferase; HbAlc, hemoglobin Alc; HCC, hepatocellular carcinoma; NS, not significant; PT, prothrombin time.

In terms of sex, the percentage of female patients was not significantly different between the two age groups. The BMI values of the 80 years or older group were significantly lower (23.2 ± 3.9 in the ≥80 years group vs 24.5 ± 4.6 in the 50–79 years group, $P = 0.037$). The prevalence of DM in the 80 years or older group was also significantly lower (33% in the ≥80 years group vs 45% in the 50–79 years group, $P = 0.048$).

The percentages of hypertension and dyslipidemia were not significantly different between the two age groups. The frequency of cirrhosis in the 80 years or older group was significantly lower (49% in the ≥80 years group vs 62% in the 50–79 years group, $P = 0.048$). In the 80 years or older group, 28% of the patients developed HCC without cirrhosis, obesity and DM.

The levels of serum albumin, total bilirubin, AST and ALT levels were similar between these two age groups. The serum GGT, fasting blood sugar and HbAlC levels were all slightly higher in the 50–79 years group, but the differences were not significant. The platelet count was slightly lower in the 50–79 years group. The percentage of prothrombin time in the 80 years or older group was significantly higher (mean PT%, 85% in the ≥80 years group vs 79% in the 50–79 years group, $P = 0.008$). The serum AFP and DCP levels were similar between the two groups. The maximum size of the HCC lesion in the 50–79 years group tended to be small, and the number of HCC tended to be larger.

We also investigated the clinical data of the patients with cryptogenic HCC as classified in the five age-dependent groups (<50, 50–59, 60–69, 70–79 and ≥80 years), shown in Table S1. The largest number of patients with cryptogenic HCC was in the 70–79 years group. The 50–59 years group of patients had clinical features similar to those of the less than 50 years group. The clinical features of the 60–69 years group were similar to those of the 70–79 years group. In the group of cryptogenic HCC patients aged 80 years or older, compared to the 70–79 years patients, the prevalences of LC and DM were significantly lower, the BMI values were significantly lower, and the PT values were significantly higher. There were no significant differences in clinical features between the 80 years or older group and the other age groups.

DISCUSSION

WE FOUND SEVERAL clinical characteristics of cryptogenic HCC that were related to age: (i) in the patients aged 80 years or older, cryptogenic HCC was the most common etiology among the non-viral HCC etiologies; and (ii) quite a few of the cases of HCC did not arise from obesity, DM or LC, especially in the 80 years or older group.

The etiology of cryptogenic HCC could include "burnt-out" NASH, occult HBV infection, HBV carriers with previous seroconversion to hepatitis B surface antigens and "burnt-out" autoimmune hepatitis. In the nationwide survey study used here, each hospital's gastrointestinal specialist conducted the final diagnosis of etiology. In the present study, we believe that the cases of cryptogenic HCC with obesity or DM did not have enough evidence of NASH, mild obesity or short history of DM. To exclude the possibility of including burnt-out NASH in the etiology of cryptogenic HCC, we compared the clinical features between the cryptogenic HCC patients with neither obesity nor DM and the cryptogenic HCC patients with obesity and/or DM. We found that except for the between-group differences in the prevalences of DM and hypertension and the difference in BMI, HbAlC and fasting blood sugar, no other clinical data were significantly different between these two groups (Table S2).

Both our and previous national surveys demonstrated that ALD is the most common disease among non-viral liver diseases in Japan.[5–7] However, according to the present study's detailed analysis, we found that the etiologies of HCC differed among the non-viral HCC patients by age: in the patients aged 80 years or older, cryptogenic HCC was the most common etiology of HCC.

It is well known that age and liver fibrosis are the most important risk factors for the development of HCC.[16,17] Obesity and DM also have been shown to be risk factors for HCC in both large cohort and experimental studies.[18,19] The increased risk of HCC associated with obesity and DM is probably due to two factors: the increased prevalence of NAFLD and the carcinogenic potential of obesity and DM. The most interesting finding of the present study was that the prevalences of obesity, DM and LC in the 80 years or older group of cryptogenic HCC patients were lower than those in the 50–79 years group. In this oldest group, 51% of the patients developed HCC without cirrhosis, and 28% developed HCC without cirrhosis, obesity and DM.

There were only seven patients in the cryptogenic HCC group under 50 years old. These patients had no risk factors for the development of HCC, such as LC and DM. Their HCC might have been associated with hepatoblastoma or genetic factors and occult HBV infection. Kato *et al.* reported that HBV genotype B may be associated with HCC in young (<50 years old)

Taiwanese.[20] However, in the present study's survey, we did not assess the prevalence of the hepatitis B core antibody due to the survey format. The group under 50 years old was a rather special group. Because a nationwide survey was used to query multiple institutions, we did not obtain further details of these patients; further investigation is needed to examine this group.

In summary, our data suggested that in the elderly, especially in those 80 years or older, there is a possibility of HCC arising even in the absence of risk factors for HCC. This phenomenon may be associated with elderly individuals' decreased immune defenses against cancer, DNA damage and gene mutations.[21,22] Our results may have significant implications for the future, when there is expected to be a very large increase in the elderly population in Japan and around the world.

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APPENDIX I

A TOTAL OF 115 hospitals across the country responded to the questionnaire and provided case cards in the present study: Hakodate City Hospital, Harada Hospital, Oji General Hospital, Hokkaido P.W.F.A.C Engaru-Kosei General Hospital, Dohkohkai Hospital, Hirosaki University School of Medicine and

Hospital, Akita University Hospital, Yamagata University Hospital, Fukushima Medical University Hospital, Gunma University Hospital, Saitama Medical University Hospital, Saitama City Hospital, Saitama Shakai Hoken Hospital, Aikawa Naika Hospital, Koga Red Cross Hospital, Chiba University Hospital, Kohnodai Hospital, National Center for Global Health and Medicine, Kameda Medical Center, Showa University Hospital, Kashiwa City Hospital, Hachioji Syokaki Hospital, Tokyo Women's Medical University Medical Center East, Tokyo Hospital, Showa General Hospital, Tokyo Metropolitan Geriatric Hospital, Toshiba Hospital, JR Tokyo General Hospital, Kyorin University Hospital, National Cancer Center Hospital, Tokyo Kosei Nenkin Hospital, Nippon Medical School Hospital, Toho University Omori Medical Center, EIJU General Hospital, Tokyo Medical and Dental University Hospital Faculty of Medicine, Tamananbu Hospital, Yokohama City University Hospital, Teikyo University Mizonokuchi Hospital, National Hospital Organization Sagamihara National Hospital, Kanto Rosai Hospital, Saiseikai Yokohamashi Nanbu Hospital, St Marianna University School of Medicine, Nippon Medical University Muasi Kosugi Hospital, Yokohama General Hospital, Fujisawa Shounandai Hospital, Showa University Fujigaoka Hospital, Yokosuka Kyosai Hospital, Niigata Prefecture Yoshida Hospital, Niigata University Medical and Dental Hospital, Niigata Medical Center Hospital, Prefecture Nagano Kiso Hospital, Yodakubo Hospital, University of Yamanashi Hospital, Aichi Saiseikai Hospital, Tokoname Municipal Hospital, Mie University Hospital, Aichi Medical University Hospital, Hamamatsu University of School of Medicine, University Hospital, IUHW Atami Hospital, Kikugawa General Hospital, Kyoto Prefectural Yosanoumi Hospital, National Hospital Organization Kyoto Medical Center, Aiseikai Yamashina Hospital, Japan Post Kyoto Teishin Hospital, Mitsubishi Kyoto Hospital, Osaka University Hospital, Iseikai Hospital, Kinki University Hospital, Osaka Rosai Hospital, Osaka Police Hospital, Osaka City University Hospital, National Hospital Organization Osaka Minami Medical Center, Matsushita Memorial Hospital, Kansai Medical University Takii Hospital, Kobe Asahi Hospital, Kinki Central Hospital of Mutual Aid Association of Public School Teachers, Ono Municipal Hospital, The Hospital of Hyogo College of Medicine, Okayama Saiseikai General Hospital, Kurashiki Central Hospital, Kawasaki Medical School Hospital, The Sakakibara Heart Institute of Okayama, Hiroshima University Hospital, Tokushima University Hospital, Tottori University Hospital, Shimane University Hospital, Matsue Seikyo General Hospital, Ehime University Hospital, Kubo Hospital, Kochi Health Sciences Center, Fukuoka University Hospital, Kurume University Hospital, Japanese Red Cross Fukuoka Hospital, Shinkoga Hospital, Nagasaki University Hospital, Mitsubishi Nagasaki Hospital, Kamigoto Hospital, Nagasaki Municipal Medical Center, Saga University Hospital, Oita University Hospital, Arita GI Hospital, Kumamoto University Hospital, University of Miyazaki Hospital, Kagoshima University Medical and Dental University, Kimotsuki-gun Medical Associated Hospital, Kagoshima City Hospital, Kirishima Medical Center, Heart Life Hospital, Juntendo University Hospital, Japan Self Defense Forces Hanshin Hospital, Yokote Municipal Hospital, Kawasaki City Tama Hospital, Saiseikai Kawaguchi General Hospital, Tokyo Women's Medical University Hospital, Nihon University Itabashi Hospital, and Saitama Cooperative Hospital

SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article at the publisher's website:

Table S1 Characteristic features among the five age-dependent groups of patients with cryptogenic hepatocellular carcinoma (HCC).

Table S2 Comparison between cryptogenic hepatocellular carcinoma (HCC) without obesity and diabetes mellitus (DM) and those with obesity and/or DM.

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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).

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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.

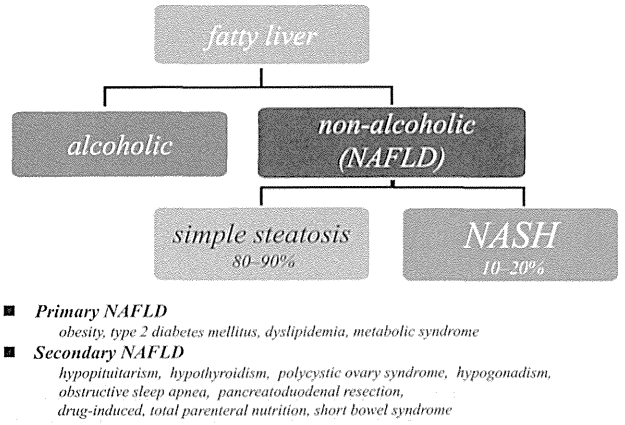


Figure 1 Fatty liver is classified as alcoholic or non-alcoholic. Then, non-alcoholic fatty liver disease (NAFLD) is divided into simple steatosis and non-alcoholic steatohepatitis (NASH) based on the histological features. NAFLD/NASH is classified into two categories depending on the causes: primary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

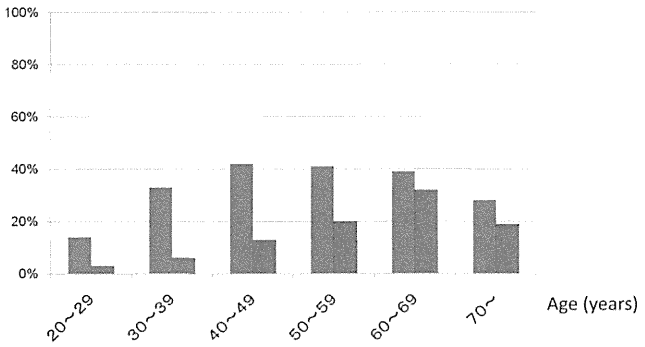


Figure 2 The prevalence of non-alcoholic fatty liver disease (NAFLD) by age and sex. Among men, the prevalence of NAFLD is around 40% in each age group. In women, the prevalence gradually increased with age.[9] A cross-sectional study: Japanese adults, $n = 5075$. (■) Male; (■) female (Source: Adapted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

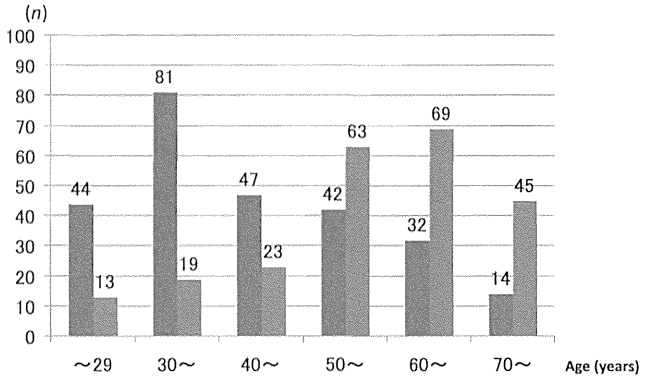


Figure 3 The age and sex distribution of the patients with biopsy-proven non-alcoholic steatohepatitis. Women were significantly more common above 50 years of age. (■) Male $n = 260$; (■) female $n = 232$ (Source: Adapted from Hashimoto *et al.*[11]).

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

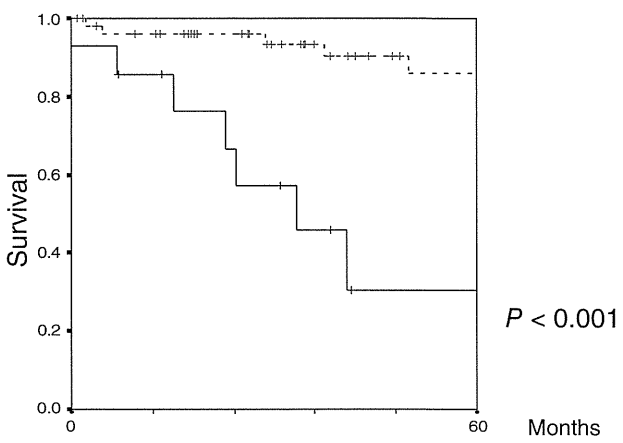
Author	Diagnosis	*n*	Average F/U (years)	Cirrhosis* prevalence *n* (%)	HCC* *n* <at baseline>	Death	
						Overall *n* (%)	Liver-related/ overall (%)
Adams *et al.*[17]	NAFLD**	420	7.6	21 (5%)	2 <0>	53 (12.6%)	13.2%
Ekstedt *et al.*[18]	NAFLD***	129	13.7	10 (7.8%)	3 <0>	26 (20.2%)	7.7%
Rafiq *et al.*[19]	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]).