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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

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

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Abstract
Background The prevalence of nonalcoholic fatty liver disease (NAFLD) has been increasing. This study aimed to assess the recent prevalence of NAFLD and to predict the prevalence of nonalcoholic steatohepatitis (NASH) with liver fibrosis using established scoring systems in the general population.
Methods A cross-sectional study was conducted among 8352 subjects who received health checkups from 2009 to 2010 in three health centers in Japan. Subjects with an intake over 20 g of alcohol/day or with other chronic liver diseases were excluded. Fatty liver was detected by ultrasonography. The probability of NASH with advanced fibrosis was calculated according to the body mass index, age, ALT, and triglyceride (BAAT) and FIB-4 (based on age, aspartate aminotransferase and alanine aminotransferase levels, and platelet counts) indices.
Results A total of 5075 subjects were enrolled. The overall prevalence of NAFLD was 29.7%. There was a significant threefold difference in the mean prevalence between males (41.0%) and females (17.7%). This prevalence showed a linear increase with body mass index, triglycerides, and low-density lipoprotein cholesterol regardless of threshold values, even without obesity. The estimated prevalence of NASH according to the BAAT index ≥ 3 was 2.7%, and according to the FIB-4 index it was 1.9%.
Conclusions The prevalence of NAFLD has increased in the general population, especially in males. There is a linear relationship between the prevalence of NAFLD and various metabolic parameters, even in nonobese subjects. The prevalence of NASH with advanced fibrosis is estimated to be considerably high in subjects with NAFLD.

Keywords Abdominal obesity · Central obesity · Metabolic syndrome

Abbreviations

Ht	Body height
BW	Body weight
BMI	Body mass index
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
AAR	AST/ALT ratio
ALP	Alkaline phosphatase
GGT	Gamma-glutamyl transferase
ChE	Cholinesterase
FPG	Fasting plasma glucose

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Hb	Hemoglobin
PLT	Platelet
TC	Total cholesterol
TG	Triglyceride
HDL-C	High-density lipoprotein cholesterol
LDL-C	Low-density lipoprotein cholesterol

Introduction

Obesity and life-related diseases due to obesity are rising at an alarming rate in Japan, many Western countries, and worldwide. Nonalcoholic fatty liver disease (NAFLD), a hepatic manifestation of metabolic syndrome, is associated with an increased risk for development of life-related disease including type 2 diabetes, cardiovascular disease, and cerebral vessel disease. NAFLD covers a spectrum of liver diseases that range from benign simple steatosis to hepatic inflammation and fibrosis of nonalcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma [1, 2].

NAFLD is rapidly becoming the most common liver disorder worldwide [3–6]. Currently, NAFLD is present in 20–40% the general population of industrialized countries [7, 8]. Among all subjects with NAFLD, features of NASH are observed in 10–20%. Recent studies reported that the prevalence of NASH in Western countries is approximately 2–12% [7–9].

The degree of fatty infiltration in NAFLD is graded according to the percentage of hepatocytes with fat deposits: mild NAFLD involves less than 30% hepatocytes, moderate NAFLD up to 60%, and severe NAFLD more than 60%. The degree of liver fibrosis must be estimated to determine the surveillance, prognosis, and optimal treatment for NAFLD, similar to the situation for other liver diseases such as chronic hepatitis C [10, 11]. Liver biopsy is recommended as the gold standard method for the diagnosis and staging of NAFLD/NASH, but it is invasive and is associated with a high risk of complications [1, 12]. In fact, it is impossible to recommend a liver biopsy to all NAFLD patients, because the number of NAFLD patients has reached 80–100 million in the USA and an estimated 10 million in Japan. Previous studies proposed novel scoring systems to estimate NASH with advanced liver fibrosis, because it was just not realistic to conduct a liver biopsy in a large number of subjects with fatty liver. The scoring system consisting of body mass index (BMI), age, serum alanine aminotransferase (ALT), and triglyceride (BAAT score) and the novel index proposed by Sterling et al. based on age, serum aspartate aminotransferase (AST), ALT level, and platelets (FIB-4 index) are simple and useful to predict NASH with advanced liver fibrosis. It might therefore be possible to estimate the approximate prevalence of NASH with advanced liver fibrosis in the Japanese general population by using these predictive formulae [13–15].

It is well known that there are age and gender differences in both the prevalence and severity of NAFLD. These age and gender differences are caused by differences in the prevalence of obesity and lifestyle-related diseases. According to annual health check findings in Japan, the prevalence of NAFLD in men is approximately 27% for all ages above 30 years. In contrast, in women, it gradually increases from 7% in their 30s to 23% above 60 years of age [16, 17]. However, this information was reported from studies conducted at the end of 1990–2000. According to the worldwide systemic analysis of health examination surveys and epidemiological studies, the prevalence of obesity is increasing year-on-year and varies substantially between nations. It is predicted that the prevalence of NAFLD in the general population is increasing and there might be a difference between each country [18].

Because of the dramatic increase in obesity in Japan and many other industrialized countries, it is plausible that there also has been a dramatic increase in the prevalence of NAFLD and NASH. However, the most recent prevalence of NAFLD has not been well established in Japan. Therefore, the aim of this study was to investigate the prevalence of NAFLD/NASH using the latest database of a large proportion of the general population who underwent an annual health checkup from 2009 to 2010 in Japan and to estimate the prevalence of NASH with liver fibrosis using established scoring systems.

Patients and methods

Study population

We studied 8352 subjects (51.8% males) aged 21–86 years (mean 50.0 years), who received a health checkup from 2009 to 2010 in three health centers, namely Eguchi Hospital Health Center in Saga prefecture, Kawamura Clinic Heath Center in Hiroshima prefecture, and Kochi Medical School Hospital in Kochi prefecture in Japan. Subjects were included if they fulfilled the following criteria: (1) absence of markers of hepatitis B virus infection (hepatitis B surface antigen and anti-hepatitis B core antibody) and hepatitis C virus infection (anti-hepatitis C virus antibodies); (2) no alcoholic liver disease (more than 20 g of alcohol per day); and (3) no use of insulin-sensitizing medication. Finally, 5075 subjects who met the inclusion criteria were enrolled. All subjects provided written informed consent to the use of their data for an epidemiological study under anonymity. The study design

was approved by each institutional review board (Saga Medical School, "2011-06-04" as Eguchi Hospital; Hiroshima University, "Eki-241" as Kawamura Clinic Health Center; Kochi Medical School, "23-74"). The study was conducted in accordance with the Declaration of Helsinki.

Physical examination and serum biochemistry

Body weight and height were obtained for both sets of subjects, and BMI was calculated. Waist circumference was measured at the umbilical level. Venous blood samples were taken from all subjects at 0900 hours following a 12-h overnight fast and AST, ALT, gamma-glutamyl transpeptidase (GGT), total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides, and fasting plasma glucose (FPG) levels were measured using standard techniques in the subjects who received a health checkup.

Abdominal ultrasound protocol and definition of fatty liver

All subjects received abdominal ultrasonography to determine fatty liver. First, examination of all visible liver parenchyma was performed with a conventional convex array transducer. Liver parenchyma was examined with sagittal as well as longitudinal guidance of a probe and completed by lateral and intercostals views. Use of tissue harmonic imaging with both transducers was encouraged. The presence of steatosis was recognized as a marked increase in hepatic echogenicity, poor penetration of the posterior segment of the right lobe of the liver, and poor or no visualization of the hepatic vessels and diaphragm. The severity of hepatic steatosis present by imaging was not graded with careful consideration of the error due to the difference of ultrasonography equipment and examiner. The liver was considered normal if the hepatic parenchyma was homogeneous with no acoustic attenuation, the portal veins were visible, the diaphragm was well visualized, and the echogenicity was similar or slightly higher than the echogenicity of the renal cortex.

Ultrasonography was performed with the following units: LOGIQ 7 with a 4-MHz convex array transducer (GE Health Care) at Eguchi Hospital; Pro Sound Alpha-10 with 3.5 MHz with a convex array transducer (Hitachi Aloka Medical) at Kawamura Clinic Health Center; and Xario with a 3.5-MHz convex array transducer (Toshiba Medical Systems) at Kochi Medical School. Experienced sonographers, who were trained by gastroenterologists with more than 5 years' experience, performed examinations over 5 years. Technical parameters were adjusted for each subject using the standard protocol for ultrasonography. Each certificated gastroenterologist independently reviewed the images and evaluated the liver for the presence of steatosis.

Algorithms for prediction of NASH

In this study, two representative algorithms based on the BAAT score and the recently proposed FIB-4 index [13, 14] were employed to predict the prevalence of subjects with NASH with advanced liver fibrosis. BAAT scores consist of the sum of the following categorical variables: BMI ($\geq$28 = 1, <28 = 0), age ($\geq$50 years = 1, <50 = 0), ALT [$\geq$2 UNL (males, ALT $\geq$60 IU/L; females, ALT $\geq$40 IU/L) = 1, <2 UNL = 0) and serum triglycerides [1.7 mmol/L (=150 mg/dL) = 1, <1.7 = 0], thus ranging from 0 to 4, and a cutoff value to predict NASH with advanced liver fibrosis was defined as BAAT score $\geq$3 in this study [13]. The FIB-4 index was calculated as [age (years) $\times$ AST (U/L)]/[platelets (10^9) $\times$ root ALT (U/L)]. The subjects were classified into three groups on the basis of the following values: FIB-4 index $\geq$2.67 and <1.30, because previous studies reported that a FIB-4 index $\geq$2.67 had an 80% positive predictive value and a FIB-4 index <1.30 had a 90% negative predictive value to predict NASH with advanced liver fibrosis [14, 15].

Statistical analysis

Descriptive statistics (means and standard deviations) were calculated for all continuous variables. Differences between the two groups were compared by the Mann–Whitney U test. Differences were considered significant at $p < 0.05$. All analyses were carried out using IBM SPSS Statistics Ver. 19.

Results

Clinical and biochemical characteristics and the prevalence of NAFLD in enrolled subjects

A total of 5075 subjects were enrolled from July 2009 to June 2010. The clinical and biochemical characteristics of these subjects are summarized in Tables 1 and 2. The subjects were predominantly middle-aged (50.0 $\pm$ 9.5 years; range 21–86 years) and 48.2% were female. The mean BMI of the whole cohort was 23.0 $\pm$ 3.3 kg/m^2 with 23.6% of the subjects meeting the criteria for obesity (BMI $\geq$25). The mean age was not significantly different between subjects with or without NAFLD (51.1 $\pm$ 8.9 vs. 49.5 $\pm$ 6.7 years). A total of 1509 subjects (29.7%) had evidence of NAFLD on ultrasonography. There was a significant threefold difference in the mean prevalence of NAFLD between males (41.0%) and females (17.7%). The

Table 1 Characteristics of all patients

	All (n = 5075)	Non-NAFLD (n = 3566)	NAFLD (n = 1509)	p value
Gender (M/F)	2627/2448	1551/2015	1076/433	<0.0001
Age (years)	50.0 ± 9.5	49.5 ± 6.7	51.1 ± 8.9	<0.0001
Ht (m)	1.631 ± 0.086	1.62 ± 0.08	1.65 ± 0.08	<0.0001
BW (kg)	61.4 ± 11.7	57.7 ± 9.6	70.2 ± 11.3	<0.0001
BMI (kg/m^2)	23.0 ± 3.3	21.8 ± 2.6	25.6 ± 3.3	<0.0001
AST (IU/L)	21.5 ± 9.1	20.1 ± 6.7	24.7 ± 12.4	<0.0001
ALT (IU/L)	22.6 ± 16.6	18.3 ± 9.9	32.7 ± 23.5	<0.0001
AAR	1.11 ± 0.37	1.21 ± 0.35	0.87 ± 0.29	<0.0001
ALP (IU/L)	210.6 ± 65.7	205.3 ± 65.3	223.3 ± 64.7	<0.0001
GGT (IU/L)	34.4 ± 36.1	28.4 ± 27.0	48.5 ± 48.6	<0.0001
ChE (IU/L)	293.6 ± 126.0	277.0 ± 122.0	33.4 ± 126.3	<0.0001
Albumin (g/dL)	4.5 ± 0.2	4.5 ± 0.2	4.6 ± 0.2	<0.0001
FPG (mg/dL)	99.6 ± 17.7	96.3 ± 13.3	107.3 ± 23.3	<0.0001
TC (mg/dL)	207.1 ± 34.1	205.6 ± 33.9	210.5 ± 34.3	<0.0001
TG (mg/dL)	111.8 ± 76.9	93.0 ± 52.6	155.6 ± 102.3	<0.0001
HDL-C (mg/dL)	60.6 ± 16.2	64.4 ± 16.2	51.5 ± 12.1	<0.0001
LDL-C (mg/dL)	121.6 ± 32.1	118.3 ± 32.2	129.4 ± 30.6	<0.0001

Values are expressed as mean ± SD. Statistical analysis was conducted using Mann–Whitney U test

Ht body height, *BW* body weight, *BMI* body mass index, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *AAR* AST/ALT ratio, *ALP* alkaline phosphatase, *GGT* gamma-glutamyl transferase, *ChE* cholinesterase, *FPG* fasting plasma glucose, *Hb* hemoglobin, *PLT* platelet, *TC* total cholesterol, *TG* triglyceride, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol

prevalence of NAFLD in men was greater than 30% in all ages above the third decade. This prevalence was higher in males than that in females for all ages, and it gradually increased from only 3.3% in the second decade to 31.3% above the sixth decade in females (Fig. 1). Furthermore, there was a significant difference in most of the clinical factors except BMI and LDL cholesterol between males and females (Table 2). By multivariate logistic regression in each male and female with NAFLD, there was a difference in the independent variables significantly associated with NAFLD (Table 3).

Relationship between anthropometric and biochemical features and the presence of NAFLD

BMI in subjects with NAFLD was significantly higher than that in those without NAFLD ($p < 0.01$). The prevalence of NAFLD showed a linear increase with the increase of BMI (BMI <23 kg/m^2, 10.5%; BMI ≥23 kg/m^2 and <25 kg/m^2, 37.9%; BMI ≥25 kg/m^2 and <28 kg/m^2, 58.4%; BMI ≥28 kg/m^2, 84.2%; a 7.4–11.4% increase per 1 kg/m^2 between 20 and 30) (Fig. 2), and it was 18.4% in nonobese subjects (BMI <25 kg/m^2) with NAFLD, 63.4% in obese subjects (BMI >25 kg/m^2 but <30 kg/m^2) with NAFLD, and 89.1% in morbid obese (BMI >30 kg/m^2) subjects with NAFLD. Serum levels of LDL cholesterol, triglycerides (TG), FPG, and liver enzymes including AST and ALT were significantly higher in subjects with NAFLD than those in subjects without NAFLD ($p < 0.01$). Serum levels of HDL cholesterol were significantly lower in subjects with NAFLD than those without NAFLD ($p < 0.01$, Tables 1, 2). The prevalence of NAFLD showed a linear increase with the increase of serum triglycerides and LDL cholesterol levels (Fig. 3a, d), and a linear decrease with the increase of serum HDL cholesterol levels (Fig. 3c). The prevalence of NAFLD was 22.8% in subjects with normal triglyceride levels (triglycerides <150 mg/dL) and 59.5% in subjects with hypertriglyceridemia (triglycerides >150 mg/dL). The prevalence of NAFLD was 27.3% in subjects with normal HDL cholesterol levels (HDL cholesterol >40 mg/dL) and 61.7% in subjects with hypo-HDL cholesteremia (HDL cholesterol <40 mg/dL). The prevalence of NAFLD was 26.4% in subjects with normal LDL cholesterol levels (LDL cholesterol <140 mg/dL) and 38.5% in subjects with hyper-LDL cholesteremia (LDL cholesterol >140 mg/dL). The prevalence of NAFLD showed a linear increase with FPG levels (<120 mg/dL) and this prevalence was approximately 60% and reached a plateau with FPG ≥120 mg/dL, especially in males (Fig. 3e). The prevalence of NAFLD was 25.6% in subjects with a normal fasting glucose, 56.2% in subjects with impaired FPG (FPG >110 mg/dL

Table 2 Characteristics of the patients according to gender

	Male			Female			
	Non-NAFLD (n = 1551)	NAFLD (n = 1076)	p value*	Non-NAFLD (n = 2015)	NAFLD (n = 433)	p value*	p value**
Age (years)	49.8 ± 10.2	49.9 ± 8.8	0.651	49.2 ± 9.3	54.0 ± 8.3	<0.0001	<0.0001
Ht (m)	1.69 ± 0.58	1.694 ± 0.059	0.886	1.567 ± 0.055	1.552 ± 0.055	<0.0001	<0.0001
BW (kg)	64.8 ± 7.9	73.6 ± 10.3	<0.0001	52.2 ± 6.8	61.8 ± 9.2	<0.0001	<0.0001
BMI (kg/m^2)	22.6 ± 2.4	25.6 ± 3.1	<0.0001	21.2 ± 2.6	25.7 ± 3.7	<0.0001	0.5978
AST (IU/L)	20.9 ± 7.8	25.2 ± 11.7	<0.0001	19.5 ± 5.7	23.5 ± 14.0	<0.0001	<0.0001
ALT (IU/L)	21.1 ± 11.7	35.3 ± 24.0	<0.0001	16.1 ± 7.5	26.2 ± 20.6	<0.0001	<0.0001
AAR	1.09 ± 0.34	0.81 ± 0.26	<0.0001	1.30 ± 0.32	1.01 ± 0.30	<0.0001	<0.0001
ALP (IU/l)	214.3 ± 63.9	218.6 ± 58.5	<0.01	198.1 ± 65.6	235.4 ± 77.3	<0.0001	<0.01
GGT(IU/L)	36.5 ± 31.5	53.8 ± 48.7	<0.0001	22.1 ± 20.9	35.4 ± 45.9	<0.0001	<0.0001
ChE (IU/L)	289.2 ± 120.5	333.2 ± 130.0	<0.0001	267.2 ± 122.3	333.8 ± 116.9	<0.0001	<0.05
Albumin (g/dL)	4.5 ± 0.2	4.6 ± 0.2	<0.0001	4.4 ± 0.2	4.5 ± 0.2	<0.0001	<0.0001
FPG (mg/dL)	99.7 ± 15.2	108.7 ± 23.8	<0.0001	93.7 ± 11.0	103.9 ± 21.5	<0.0001	<0.0001
TC (mg/dL)	200.7 ± 32.1	208.0 ± 33.9	<0.0001	209.4 ± 34.7	216.6 ± 34.7	<0.0001	<0.0001
TG (mg/dL)	109.6 ± 59.1	167.2 ± 106.7	<0.0001	80.2 ± 42.9	127.0 ± 84.1	<0.0001	<0.0001
HDL-C (mg/dL)	57.5 ± 14.5	48.6 ± 10.4	<0.0001	69.8 ± 15.4	58.6 ± 13.0	<0.0001	<0.0001
LDL-C (mg/dL)	118.5 ± 31.8	128.6 ± 30.5	<0.0001	118.1 ± 32.5	131.3 ± 30.8	<0.0001	0.1288

Values are expressed as mean ± SD. Statistical analysis was conducted using Mann–Whitney U test. Abbreviations are the same as those in Table 1

*p value for comparison between non-NAFLD and NAFLD in each gender group

**p value for comparison between male and female with NAFLD

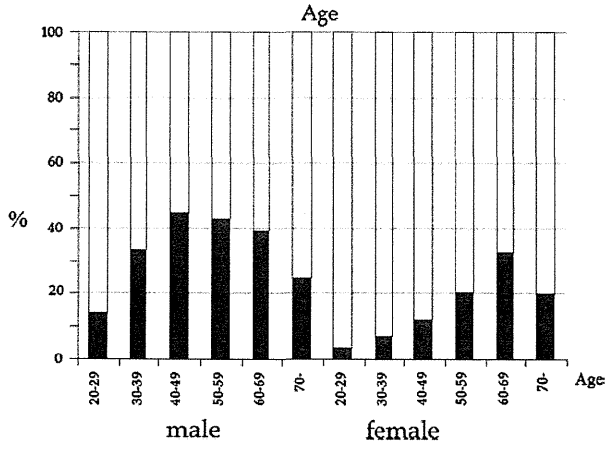

Fig. 1 Prevalence of NAFLD in patients according to age. The prevalence of NAFLD is higher in males than that in females at all ages, and it gradually increases with age in females

but <126 mg/dL), and 68.0% in subjects with FPG greater than 126 mg/dL, respectively. The prevalence of NAFLD gradually increased with an elevation of ALT. The prevalence of NAFLD was 70.6 and 35.8% in subjects with abnormal ALT levels in males (ALT ≥30) and females (ALT ≥20); the prevalence of NAFLD was 29.5 and 10.7% in subjects with normal ALT levels in males (ALT <30) and females (ALT <20), respectively (Fig. 3f).

Prevalence of NASH in the general population and subjects with fatty liver predicted by established scoring systems

In this study, the prevalence of NASH was estimated by BAAT score and FIB-4 index. Tables 4 and 5 show the distribution of subjects estimated by BAAT score and FIB-4 index, respectively. The estimated prevalence of NASH according to the BAAT index was 16.7% (BAAT score ≥2) and 2.7% (BAAT score ≥3) in the whole cohort, whereas it was 36.1% (BAAT score ≥2) and 8.3% (BAAT score ≥3) in subjects with NAFLD.

Mean FIB-4 indices in the whole cohort, in subjects without NAFLD, and in those with NAFLD were 1.15 ± 0.60, 1.17 ± 0.62, and 1.10 ± 0.55, respectively. The estimated prevalence of NASH according to the FIB-4 index was 1.9% (cutoff ≥2.67) in the whole cohort and it was 2.7% in subjects with NAFLD. In contrast, the estimated prevalence of NAFLD without advanced fibrosis was 74.0% (cutoff <1.30) in subjects with NAFLD.

Discussion

Using the latest large database in Japan, our study showed that the prevalence of NAFLD was high in the general

Table 3 Clinical factors associated with NAFLD in each male and female group using multivariate logistic regression analysis

	Male				Female			
	Coefficient	*p*	Odds ratio	95% confidence interval	Coefficient	*p*	Odds ratio	95% confidence interval
BMI >25	1.34	0.00	3.81	3.11–4.67	1.98	0.00	7.23	5.50–9.50
Age >50	–	–	–	–	0.59	0.00	1.80	1.37–2.37
ALT (M >30; F >20)	0.93	0.00	2.54	2.00–3.23	0.50	0.00	1.65	1.20–2.67
AAR <1	1.08	0.00	2.53	2.02–3.15	1.07	0.00	2.56	1.87–3.48
FPG >110	0.851	0.00	2.34	1.83–2.99	0.96	0.00	2.61	1.76–3.87
TG >150	0.792	0.00	2.21	1.78–2.74	0.95	0.00	2.58	1.83–3.63
GGT >35	0.360	0.00	1.43	1.17–1.75	–	–	–	–
HDL <40	0.306	<0.05	1.36	1.00–1.84	–	–	–	–
LDL >140	0.264	<0.05	1.30	1.05–1.60				

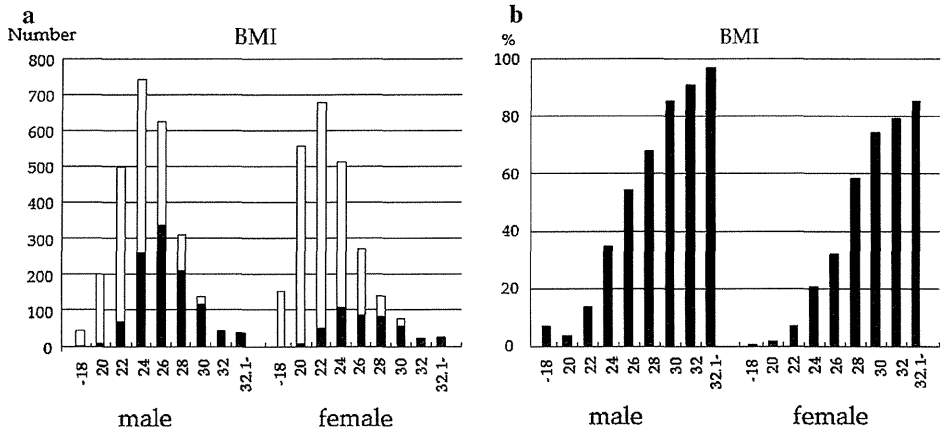

Fig. 2 **a** Distribution of subjects with NAFLD (*black columns*) and without NAFLD (*white columns*) according to body mass index (BMI). **b** Relative percentage of NAFLD according to BMI. The prevalence of NAFLD shows a tendency to increase linearly with BMI in males and females

population, especially in males, even though subjects were not obese. Our study suggested that the prevalence of NAFLD is still increasing in Japan. The present study showed the most recent frequency of NAFLD and a 10% increase from a previous Japanese study conducted in subjects who received a health checkup from 1989 to 2000 [17]. A recent study reported that mean BMI has globally increased in adults 20 years and older in 199 countries and territories between 1980 and 2008 [18]. In 2008, an estimated 1.46 billion adults worldwide had a BMI of 25 kg/m^2 or greater, and of these, 205 million men and 297 million women were obese.

Our study found that there was a linear relationship between the prevalence of NAFLD and an increase in BMI, serum triglycerides, and cholesterol, whereas the increase of prevalence showed a plateau at 120 mg/dL for FPG levels, especially in males. It is well known that NAFLD and NASH are strongly associated with the presence of obesity and lifestyle-related diseases, especially type 2 diabetes mellitus [6–8]. According to annual health check findings in Japan and Asian countries, the prevalence of NAFLD increases with BMI; it has been reported to be 10–20% in nonobese subjects, approximately 50% in those with a BMI ranging from more than 25 kg/m^2 to less than 30 kg/m^2, and approximately 80% in those with a BMI over 30 kg/m^2 [19].

A previous study reported that the crude prevalence of NAFLD increased with deterioration of glucose homeostasis, from 27% in patients with normal fasting glucose, 43% in patients with impaired fasting glucose, and 62% in patients with newly diagnosed and thus untreated diabetes [20]. This study revealed that there were a certain number of NAFLD subjects with normal range in various parameters. It is unclear whether NAFLD causes metabolic dysfunction or whether metabolic dysfunction is responsible for hepatic fat accumulation, or both. As shown in our study, there was a close relationship between the pathogenesis of NAFLD and that of glucose and lipid metabolism abnormalities.

Our study confirmed previous findings that various traditional metabolic parameters and aminotransferases may be normal in an appreciable proportion of patients with NAFLD, and therefore, are not sensitive enough for the diagnosis of NAFLD [7, 21–23]. It is widely accepted that

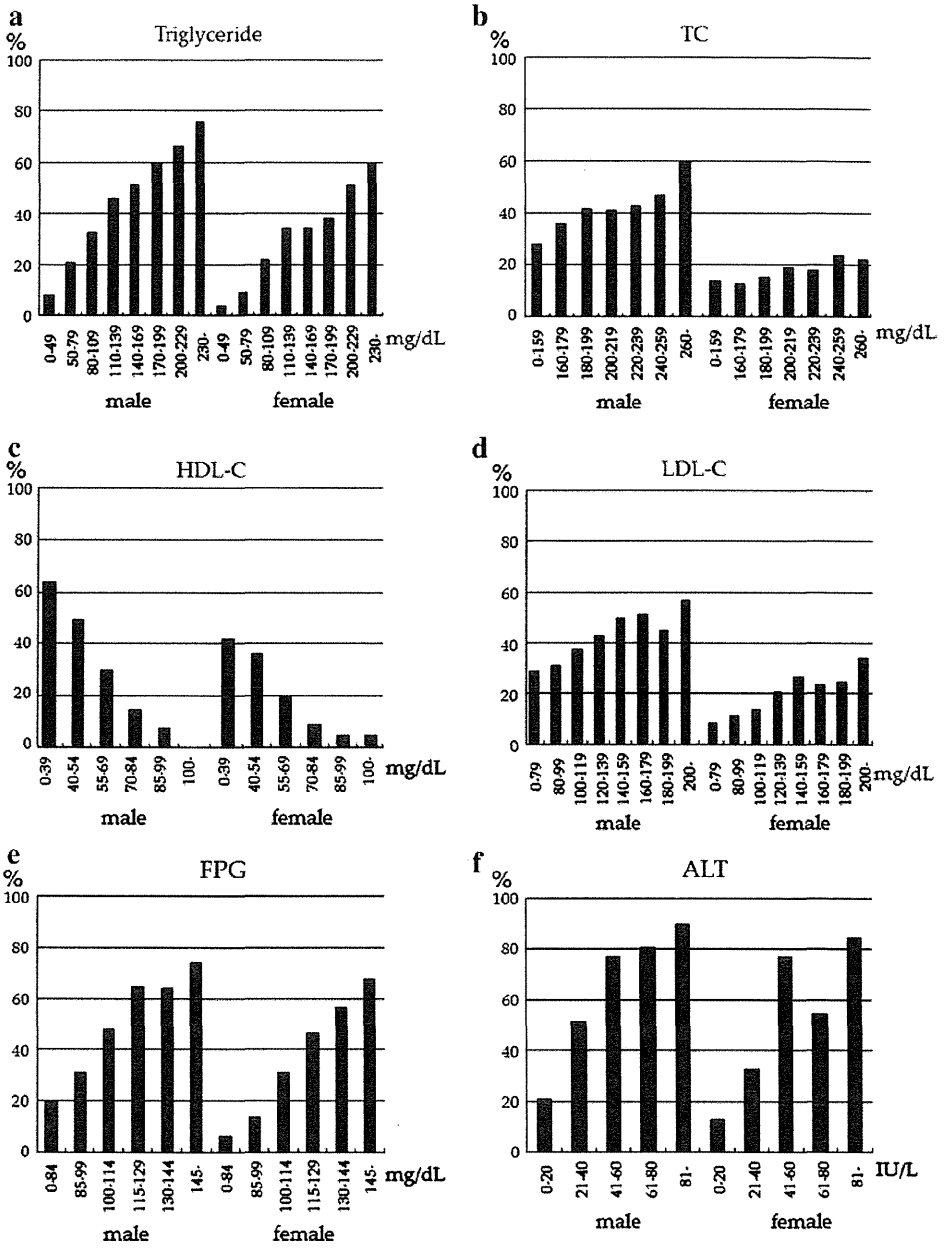

Fig. 3 Prevalence of NAFLD in various variables. The prevalence of NAFLD shows a linear increase with serum levels of triglycerides and low-density lipoprotein cholesterol (LDL-C) (**a**, **d**), whereas there is a linear decrease with high-density lipoprotein cholesterol (HDL-C) (**c**). The prevalence of NAFLD shows a linear increase with fasting plasma glucose (FPG) (<120 mg/dL), and this prevalence is approximately 60% and reaches a plateau with glucose ≥120 mg/dL, especially in males (**e**). The prevalence of NAFLD gradually increases with the elevation of alanine aminotransferase (ALT). There is an appreciable amount of NAFLD subjects with normal ALT levels (**f**)

Table 4 Distribution of subjects according to BAAT score

	BAAT score					Total
	0	1	2	3	4	
Non-NAFLD	1555	1708	290	13	0	3566
NAFLD	277	687	419	113	13	1509
Total	1832	2395	709	126	13	5075

serum aminotransferase levels are neither specific nor sensitive enough for diagnosis of NAFLD [2, 24, 25], which was consistent with the present study.

AAR was significantly lower in subjects with NAFLD compared with those without NAFLD. Similarly, lower values for AAR were found in NAFLD subjects compared

Table 5 Distribution of subjects according to FIB-4 index

	FIB-4 index			Total
	<1.3	≥1.3 but <2.67	≥2.67	
Non-NAFLD	2495	1011	60	3566
NAFLD	1117	352	40	1509
Total	3612	1363	100	5075

with those with alcoholic liver disease; thus, AAR can be used to differentiate between these conditions [26]. Another study demonstrated the significance of AAR, even within the spectrum of NAFLD, as a lower AAR was associated with a higher histopathological degree of hepatic steatosis in obese NAFLD subjects [27].

Although standard body weight is determined differently depending on ethnicity, obesity indicates excessive fat accumulation, and there is a relationship between the degree of obesity and the incidence of dyslipidemia, type 2 diabetes mellitus, and hypertension. For example, in Japan, a BMI of 22 is used to indicate the ideal body weight, because the incidence of obesity-related diseases is observed least frequently when the BMI is approximately 22.5 [28]. The incidence of obesity-related diseases is significantly increased in subjects with a BMI of more than 23 in Hong Kong [29]. In the current study, the prevalence of NAFLD showed a linear increase even though each variable was within the normal range. These results suggest that there is no threshold for the incidence of NAFLD and there are differences in the incidence of NAFLD among subjects.

In the present study, we focused on the gender difference for the relationship between the prevalence of NAFLD and metabolic abnormalities. It is well known that NAFLD and NASH exhibit age and sex differences in both prevalence and severity [30]. These age and gender differences are caused by differences in the prevalence of obesity and lifestyle-related diseases [31].

Computed tomography and magnetic resonance imaging are the most reliable procedures for measuring hepatic fat accumulation, but these procedures are not simple enough that they can be used for mass screening. Ultrasonography has many advantages for mass screening. Although ultrasonography is probably the least reliable of these three imaging methods for the quantitative assessment of the degree of hepatic steatosis, ultrasonography is simple and sensitive enough to evaluate hepatic fat accumulation when typical findings of hepatic steatosis are detected. A previous study indicated that the use of ultrasonography for diagnosing NAFLD had a sensitivity of 89% and specificity of 93% for the identification of fatty liver [32].

The prevalence of NASH in the general population is still not clearly documented. A recent study revealed that NASH was confirmed in 12.2% of a largely middle-aged population and 29.9% of patients with ultrasonographic fatty liver [9]. An autopsy study from the late 1980s found that the prevalence of NASH was 2.7% among lean subjects, rising to 18.5% among markedly obese patients [33]. More recently, three studies evaluating donor livers before transplantation found that the prevalence of NASH was 1.1–14% [34–36]. Since it is known that almost 10–20% of subjects with NAFLD have NASH, the prevalence of NASH is estimated to be 13% of the adult Japanese population, which is an extremely large number of potential patients [19]. However, no studies have estimated the prevalence of NASH in the Japanese general population.

In the present study, the prevalence of NAFLD with advanced fibrosis determined as a BAAT ≥3 was predicted as 8.3% in individuals with NAFLD and 2.8% in all subjects. There were 0.4% of subjects with a BAAT ≥3 in the cohort without NAFLD. Further analysis is required to clarify the characteristics of those subjects. The FIB-4 index was developed as a noninvasive panel to stage liver disease in subjects with human immunodeficiency virus and hepatitis C virus co-infection [14]. It has recently been demonstrated that its performance characteristics for the diagnosis of advanced fibrosis in NAFLD are better than those of other similar panels that do not require additional testing, and are comparable with several others that require additional tests [15]. In our study, the estimated prevalence of NASH according to the FIB-4 index was 1.9% in the whole cohort and it was 2.7% in subjects with NAFLD (cutoff ≥2.67). These results, which were predicted using representative scoring indices, suggest that there are potential patients with advanced NASH in the general population and the prevalence is similar to previous studies in Japan [31].

Recently, Sumida et al. [37] suggested a novel scoring system determined by serum ferritin, insulin, and type IV collagen 7S levels (NAFIC score) conducted with Japanese NAFLD patients. Although the scoring system is expected to accurately predict NASH with advanced liver fibrosis, we could not use the NAFIC score because of the lack of parameters.

Some limitations of this study should be noted. First, its cross-sectional design precluded any causal and temporal inferences about the relationships between the presence of NAFLD and various parameters. Second, the diagnosis of NAFLD was made by ultrasonography and exclusion of

other causes of chronic liver disease, but this was not confirmed by liver biopsy, and there were some limitations as mentioned above. Imaging modalities have several limitations in this respect. There might be some possible errors to examine due to the difference of ultrasonography equipment and examiners among each medical facility. The most important limitations of ultrasonography are that (1) it might detect only moderate to severe steatosis, which affects more than one-third of hepatocytes, and it cannot detect mild steatosis, (2) it is difficult to determine an accurate quantitative diagnosis, and (3) there might be differences in measurement deviations in each examiner in a multicenter study, even though a common ultrasonographic definition of NAFLD has been established [38]. Third, there was a lack of some important parameters required to evaluate the background of NAFLD such as waist circumference. We have previously demonstrated a relationship between visceral fat accumulation and development of insulin resistance in patients with NAFLD [39, 40]. Because the relationship between the pathogenesis of NAFLD and visceral fat accumulation is important, further studies are required to clarify the relationship between the prevalence of NAFLD and visceral fat accumulation and the prevalence of metabolic syndrome.

In conclusion, the present study showed that the prevalence of NAFLD is high in the general population in Japan and has increased compared with previous studies, especially in males, even though subjects are not obese. There is a linear relationship between the prevalence of NAFLD and various metabolic parameters, even in nonobese subjects. The prevalence of NASH with advanced fibrosis is estimated to be considerably high in subjects with NAFLD in Japan.

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Conflict of interest None.

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

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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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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.
doi:10.1371/journal.pone.0038322.t001

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,

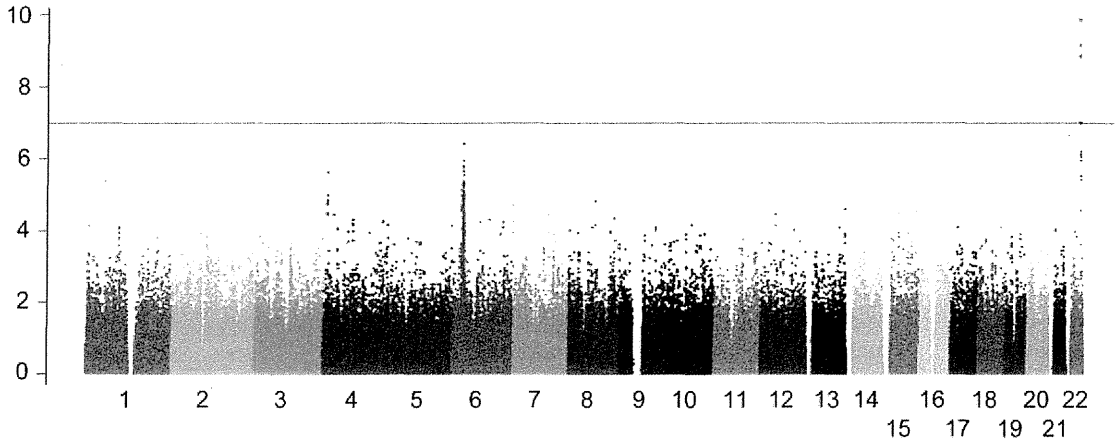

Figure 1. Manhattan plot of the GWA study. Association *p*-values are calculated by exact trend test and plotted along the chromosome in $-\log_{10}$ scale. The horizontal line indicates Bonferroni-adjusted significance threshold ($p = 1.03\times10^{-7}$).
doi:10.1371/journal.pone.0038322.g001

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.
doi:10.1371/journal.pone.0038322.t002

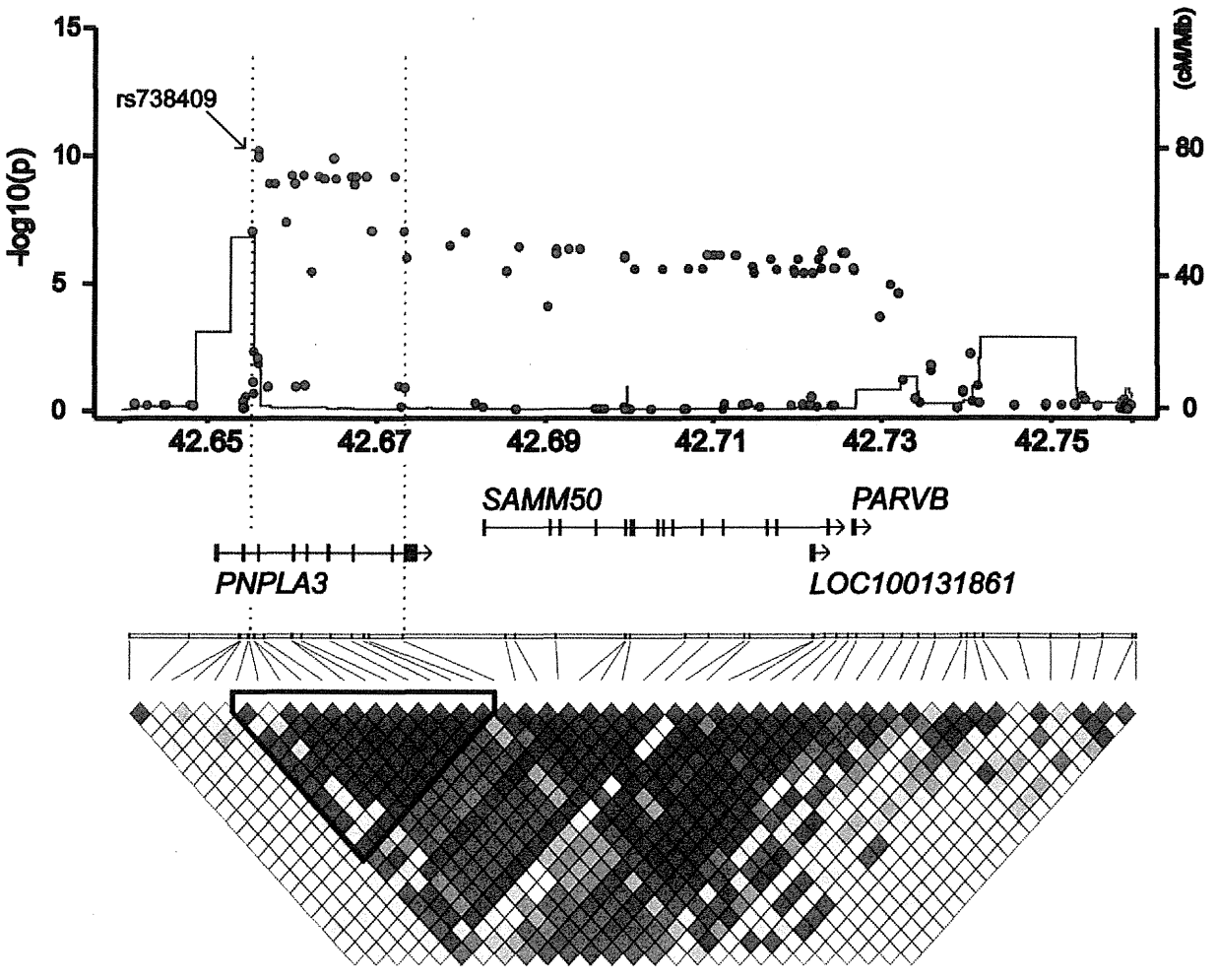

Figure 2. A schematic organization of the human *PNPLA3* locus at 22q13.31 with the genome scan results. *P*-values calculated by the exact trend test were plotted in $-\log_{10}$ scale. Red and blue dots indicate the *p*-values of genotyped and imputed SNPs, respectively. Local recombination rate obtained 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.
doi:10.1371/journal.pone.0038322.g002

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

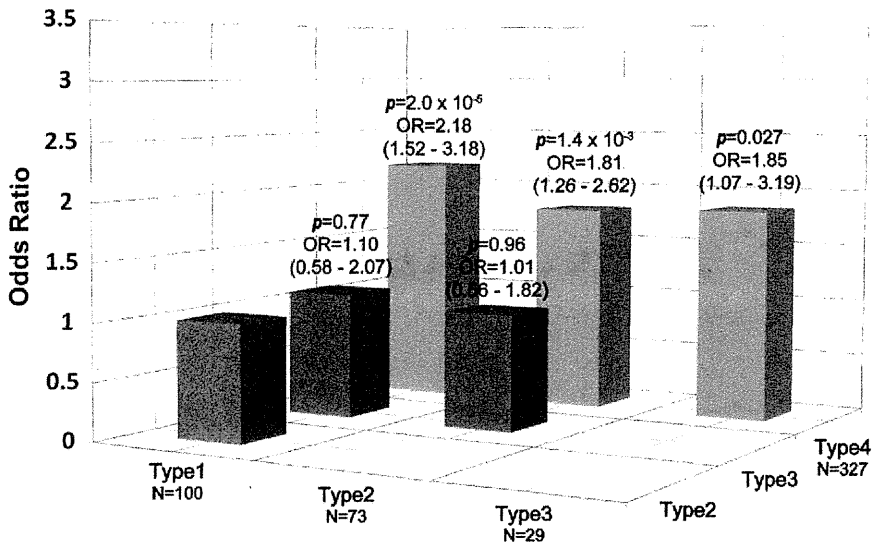

Figure 3. Histogram of odds ratios for genotype distribution of rs738409 between Matteoni types. Each box denotes the odds ratio (OR) comparing the corresponding Matteoni types on the horizontal axes. N represents the number of samples. Odds ratios and *p*-values are calculated for the higher Matteoni 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.
doi:10.1371/journal.pone.0038322.g003

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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