Table 2. Results of replication study for protective effects against CHB.

	Position		MAF[a]	Allele	Stage	HBV carriers			Healthy controls				OR[b]		
dbSNP rsID	Chr	Buld 36.3 Nearest Gene	(allele)	(1/2)	(population)	11	12	22	11	12	22	HWEp	95% CI	*P*-value[c]	P_{het}[d]
rs3077	6	33141000 HLA-DPA1	0.44	T/C	GWAS	13	51	117	28	88	67	0.919	0.42	1.14×10^{-7}	
			(T)		(Japanese)	(7.2)	(28.2)	(64.6)	(15.3)	(48.1)	(36.6)		(0.30–0.58)		
					Replication-1	26	95	134	46	125	65	0.309	0.48	2.70×10^{-8}	
					(Japanese)	(10.2)	(37.3)	(52.5)	(19.5)	(53.0)	(27.5)		(0.37–0.62)		
					Replication-2	23	81	111	31	74	40	0.767	0.47	2.08×10^{-6}	
					(Korean)	(10.7)	(37.7)	(51.6)	(21.4)	(51.0)	(27.6)		(0.35–0.65)		
					Meta-analysis[e]								0.46	4.40×10^{-19}	0.80
													(0.39–0.54)		
rs9277542	6	33163225 HLA-DPB1	0.45	T/C	GWAS	18	53	110	29	102	52	0.073	0.42	5.32×10^{-8}	
			(T)		(Japanese)	(9.9)	(29.3)	(60.8)	(15.8)	(55.7)	(28.4)		(0.31–0.58)		
					Replication-1	30	106	118	54	114	67	0.681	0.54	3.33×10^{-6}	
					(Japanese)	(11.8)	(41.7)	(46.5)	(23.0)	(48.5)	(28.5)		(0.42–0.70)		
					Replication-2	30	87	94	35	72	36	0.933	0.54	8.29×10^{-5}	
					(Korean)	(14.2)	(41.2)	(44.5)	(24.5)	(50.3)	(25.2)		(0.40–0.74)		
					Meta-analysis[e]								0.50	1.28×10^{-15}	0.40
													(0.43–0.60)		

[a]Minor allele frequency and minor allele in 198 healthy Japanese (ref#19).
[b]Odds ratio of minor allele from two-by-two allele frequency table.
[c]P value of Pearson's chi-square test for allelic model.
[d]Heterogeneity was tested using general variance-based method.
[e]Meta-analysis was tested using the random effects model.
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comparison of HBV patients with HBV resolved individuals (OR = 0.56 for rs3077, 0.66 for rs9277535 and 0.55 for rs9277542) than in the one with healthy controls (OR = 0.55 for rs3077, 0.61 for rs9277535 and 0.51 for rs9277542), which was the opposite result as we expected (Table S2 and Table S4). These results may suggest that other unknown immune system(s) exist to eliminate the HBV in the HBV resolved individuals.

Among the HLA class II loci (*HLA-DPA1*, *HLA-DPB1* and *HLA-DQB2*), which were associated with CHB and HBV clearance, a weak linkage disequilibrium ($r^2<0.1$) was observed between *HLA-DQB2* locus and *HLA-DPA1/−DPB1* loci in Japanese and Korean populations (Figure S2). We also found that similar linkage disequilibrium blocks (r^2) were observed among three subgroups (HBV carriers, HBV resolved individuals and Healthy controls). Moreover, logistic regression analysis of *HLA-DP* (rs3077 and rs92775542) with use of *HLA-DQ* (rs9276431 and rs768538) as covariates showed that the same level of significant associations of *HLA-DP* with CHB and HBV clearance as shown in the single-point association analysis, while no associations of *HLA-DQ* with $P_{log} > 0.05$ were detected both in Japanese and in Korean (Table S5). These results show that *HLA-DP* is the main genetic factor for susceptibility to CHB and HBV clearance, and the associations of *HLA-DQB2* would result from linkage disequilibrium of *HLA-DP*A1/−DPB1.

In this study, we confirmed the significant associations between *HLA-DPA1* and *HLA-DPB1*, and protective effects against CHB and HBV clearance in Japanese and Korean individuals. These results suggest that the associations between the *HLA-DP* locus, CHB and HBV clearance are widely replicated in East Asian populations, including Chinese, Thai, Japanese and Korean individuals; however, there have been no similar GWAS performed in Caucasian and African populations. Moreover, there were no significant SNPs associated with HCC development in this study, thus suggesting that it is necessary to increase the sample size. To clarify the pathogenesis of CHB or the mechanisms of HBV clearance, further studies are necessary, including a functional study of the *HLA-DP* molecule, identification of novel host genetic factors other than *HLA-DP*, and variation analysis of HBV.

Materials and Methods

Ethics Statement

All study protocols conform to the relevant ethical guidelines, as reflected in the *a priori* approval by the ethics committees of all participating universities and hospitals. The written informed consent was obtained from each patient who participated in this study and all samples were anonymized.

Genomic DNA Samples and Clinical Data

All of the 1,793 Japanese and Korean samples, including individuals with CHB, healthy controls and HBV-resolved individuals (HBsAg-negative and anti-HBc-positive), were collected at 20 multi-center hospitals (liver units with hepatologists) throughout Japan and Korea. The 19 hospitals in Japan were grouped into the following 8 areas: Hokkaido area (Hokkaido University Hospital, Teine Keijinkai Hospital), Tohoku area (Iwate Medical University Hospital), Kanto area (Musashino Red Cross Hospital, Saitama Medical University, Kitasato University Hospital, University of Tokyo), Koshin area (Shinshu University Hospital, Kanazawa University Hospital), Tokai area (Nagoya City University Hospital, Nagoya Daini Red Cross Hospital), Kinki area (Kyoto Prefectural University of Medicine Hospital, National Hospital Organization Osaka National Hospital, Osaka

Table 3. Results of replication study for clearance of hepatitis B virus.

	Position		MAF[a]	Allele	Stage	HBV carriers			Resolved individuals			OR[b]		
dbSNP rsID	Chr	Buld 36.3 Nearest Gene	(allele)	(1/2)	(population)	11	12	22	11	12	22	95% CI	*P*-value[c]	P_{het}[d]
rs3077	6	33141000 HLA-DPA1	0.44	T/C	GWAS	13	51	117	29	82	74	0.44	9.24×10^{-7}	
			(T)		(Japanese)	(7.2)	(28.2)	(64.6)	(15.7)	(44.3)	(40.0)	(0.32–0.61)		
					Replication-1	26	95	134	20	64	60	0.72	3.32×10^{-2}	
					(Japanese)	(10.2)	(37.3)	(52.5)	(13.9)	(44.4)	(41.7)	(0.53–0.97)		
					Replication-2	23	81	111	29	48	28	0.41	2.35×10^{-7}	
					(Korean)	(10.7)	(37.7)	(51.6)	(27.6)	(45.7)	(26.7)	(0.29–0.58)		
					Meta-analysis[e]							0.51	1.56×10^{-4}	0.03
												(0.36–0.72)		
					Meta-analysis[e]							0.43	1.89×10^{-12}	0.75
					(GWAS+replication-2)							(0.34–0.54)		
rs9277542	6	33163225 HLA-DPB1	0.45	T/C	GWAS	18	53	110	28	88	69	0.51	3.15×10^{-5}	
			(T)		(Japanese)	(9.9)	(29.3)	(60.8)	(15.1)	(47.6)	(37.3)	(0.37–0.70)		
					Replication-1	30	106	118	28	62	52	0.68	1.25×10^{-2}	
					(Japanese)	(11.8)	(41.7)	(46.5)	(19.7)	(43.7)	(36.6)	(0.51–0.92)		
					Replication-2	30	87	94	30	53	22	0.46	4.97×10^{-6}	
					(Korean)	(14.2)	(41.2)	(44.5)	(28.6)	(50.5)	(21.0)	(0.33–0.64)		
					Meta-analysis[e]							0.55	5.91×10^{-7}	0.19
												(0.43–0.69)		
					Meta-analysis[e]							0.49	9.69×10^{-10}	0.65
					(GWAS+replication-2)							(0.39–0.61)		

[a]Minor allele frequency and minor allele in 198 healthy Japanese (ref#19).
[b]Odds ratio of minor allele from two-by-two allele frequency table.
[c]P value of Pearson's chi-square test for allelic model.
[d]Heterogeneity was tested using general variance-based method.
[e]Meta-analysis was tested using the random effects model.
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City University), Chugoku/Shikoku area (Tottori University Hospital, Ehime University Hospital, Yamaguchi University Hospital, Kawasaki Medical College Hospital) and Kyushu area (Kurume University Hospital). Korean samples were collected at Yonsei University College of Medicine.

HBV status was measured based on serological results for HBsAg and anti-HBc with a fully automated chemiluminescent enzyme immunoassay system (Abbott ARCHITECT; Abbott Japan, Tokyo, Japan, or LUMIPULSE f or G1200; Fujirebio, Inc., Tokyo, Japan). For clinical staging, inactive carrier (IC) state was defined by the presence of HBsAg with normal ALT levels over 1 year (examined at least four times at 3-month intervals) and without evidence of portal hypertension. Chronic hepatitis (CH) was defined by elevated ALT levels (>1.5 times the upper limit of normal [35 IU/L]) persisting over 6 months (at least by 3 bimonthly tests). Liver cirrhosis (LC) was diagnosed principally by ultrasonography (coarse liver architecture, nodular liver surface, blunt liver edges and hypersplenism), platelet counts <100,000/cm^3, or a combination thereof. Histological confirmation by fine-needle biopsy of the liver was performed as required. Hepatocellular carcinoma (HCC) was diagnosed by ultrasonography, computerized tomography, magnetic resonance imaging, angiography, tumor biopsy or a combination thereof.

The Japanese control samples from HBV-resolved subjects (HBsAg-negative and anti-HBc-positive) at Nagoya City University-affiliated healthcare center were used by comprehensive agreement (anonymization in an unlinkable manner) in this study. Some of the unrelated Japanese healthy controls were obtained from the Japan Health Science Research Resources Bank (Osaka, Japan). One microgram of purified genomic DNA was dissolved in 100 μl of TE buffer (pH 8.0) (Wako, Osaka, Japan), followed by storage at −20°C until use.

SNP Genotyping and Data Cleaning

For GWAS, we genotyped a total of 550 individuals, including 181 Japanese HBV carriers, 184 Japanese healthy controls and 185 spontaneously HBV-resolved Japanese individuals (HBsAg-negative and anti-HBc-positive), using the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Inc., Santa Clara, CA), in accordance with the manufacturer's instructions. The average QC call rate for 550 samples reached 98.47% (95.00–99.92%), which had an average sample call rate of 98.91% (93.55–99.74%) by determining the genotype calls of over 900 K SNPs using the Genotyping Console v4.1 software (with Birdseed v1 algorithm) provided by the manufacturer [19]. We then applied the following thresholds for SNP quality control in data cleaning: SNP call rate ≥95% and MAF ≥1% for three groups (HBV carriers, healthy controls and HBV-resolved individuals), and HWE *P*-value ≥0.001 for healthy controls [20]. Here, SNP call rate is defined for each SNP as the number of successfully genotyped samples divided by the number of total samples genotyped. A total of 597,789 SNPs and 590,278 SNPs on autosomal chromosomes

passed the quality control filters in the genome-wide association analysis using HBV carriers and healthy controls, and using HBV carriers and HBV-resolved individuals, respectively (Figure 1). All cluster plots for the SNPs showing $P<0.0001$ on association analyses in the allele frequency model were confirmed by visual inspection, and SNPs with ambiguous cluster plots were excluded.

In the following replication stage, we selected a set of 32 SNPs with $P<0.0001$ in the GWAS using HBV carriers and HBV-resolved individuals. SNP genotyping in two independent sets of 256 Japanese HBV carriers, 236 Japanese healthy controls and 150 Japanese HBV-resolved individuals (Table 1, replication-1), and 344 Korean HBV carriers, 151 Korean healthy controls and 106 Korean HBV-resolved individuals (Table 1, replication-2) was completed for the selected 32 SNPs using the DigiTag2 assay [21,22] and custom TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) on the LightCycler 480 Real-Time PCR System (Roche, Mannheim, Germany).

Statistical Analysis

The observed associations between SNPs and the protective effects on chronic hepatitis B or clearance of hepatitis virus B were assessed by chi-squared test with a two-by-two contingency table in allele frequency model. SNPs on chromosome X were removed because gender was not matched among HBV carriers, healthy controls and HBV-resolved individuals. A total of 597,789 SNPs and 590,278 SNPs passed the quality control filters in the GWAS stage; therefore, significance levels after Bonferroni correction for multiple testing were $P=8.36\times10^{-8}$ (0.05/597,789) and $P=8.47\times10^{-8}$ (0.05/590,278), respectively. For the replication study, 29 of 32 SNPs were successfully genotyped; therefore, we applied $P=0.0017$ (0.05/29) as a significance level, and none of the 29 markers genotyped in the replication stage showed deviations from the Hardy-Weinberg equilibrium in healthy controls ($P>0.01$).

The genetic inflation factor λ was estimated by applying the Cochrane-Armitage test on all SNPs and was found to be 1.056 and 1.030 in the GWAS using HBV carriers and healthy controls, and using HBV carriers and HBV-resolved individuals, respectively (Figure S3). These results suggest that the population substructure should not have any substantial effect on statistical analysis. In addition, the principal component analysis in a total of 550 individuals in the GWAS stage together with the HapMap samples also revealed that the effect of population stratification was negligible (Figure S4).

Based on the genotype data of a total of 1,793 samples including 1,192 Japanese samples and 601 Korean samples in both GWAS and replication stages, haplotype blocks were estimated using the Gabriel's algorithm using the Haploview software (v4.2) (Figure S2). In the logistic regression analysis, two SNPs (rs9276431 and rs7768538) within the HLA-DQ locus were individually involved as a covariate (Table S5). Statistical analyses were performed using the SNP & Variation Suite 7 software (Golden Helix, MT, USA).

Supporting Information

Figure S1 GWAS using samples from HBV carriers with LC or HCC, and HBV carriers without LC and HCC. *P* values were calculated using chi-squared test for allele frequencies.
(PPTX)

Figure S2 Estimation of linkage disequilibrium blocks in HBV patients, HBV resolved individuals and healthy controls in Japanese and Korean. The LD blocks (r^2) were analyzed using the Gabriel's algorithm.
(PPTX)

Figure S3 Quantile-quantile plot for test statistics (allele-based chi-squared tests) for GWAS results. Dots represent P values of each SNP that passed the quality control filters. Inflation factor λ was estimated to be: a) 1.056 in the analysis with HBV carriers and healthy controls; and b) 1.030 with HBV carriers and HBV-resolved individuals.
(PPTX)

Figure S4 Principal component analysis on a total of 550 individuals in GWAS, together with HapMap samples (CEU, YRI and JPT).
(PPTX)

Table S1 Results for 29 SNPs selected in replication study using samples of HBV carriers and healthy controls. [a]*P* values by chi-squared test for allelic model. [b]Odds ratio of minor allele from two-by-two allele frequency table. [c]Meta-analysis was tested using additive, two-tailed CMH fixed-effects model.
(XLSX)

Table S2 Results of meta-analysis for protective effects against persistent HB infection across 6 independent studies, including this study. [a]Minor allele frequency and minor allele in 198 healthy Japanese (ref#19). [b]Odds ratio of minor allele from two-by-two allele frequency table. [c]*P* value of Pearson's chi-squared test for allele model. [d]Heterogeneity was tested using general variance-based method. [e]Meta-analysis was tested using the random effects model.
(XLSX)

Table S3 Results for 29 SNPs selected in replication study using samples from HBV carriers and HBV-resolved individuals. [a]*P* values by chi-squared test for allelic model. [b]Odds ratio of minor allele from two-by-two allele frequency table. [c]Meta-analysis was tested using additive, two-tailed CMH fixed-effects model.
(XLSX)

Table S4 Results of meta-analysis for clearance of HBV across 6 independent studies, including this study. [a]Minor allele frequency and minor allele in 198 healthy Japanese (ref#19). [b]Odds ratio of minor allele from two-by-two allele frequency table. [c]*P* value of Pearson's chi-squared test for allele model. [d]Heterogeneity was tested using general variance-based method. [e]Meta-analysis was tested using the random effects model.
(XLSX)

Table S5 Logistic regression analysis of *HLA-DP* (rs3077 and rs9277542) and *HLA-DQ* (rs9276431 and rs7768538) with susceptibility to CHB and HBV clearance using the *HLA-DQ* genotypes individually as a covariate.
(XLSX)

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Author Contributions

Conceived and designed the experiments: NN HS YT. Performed the experiments: HS Y. Mawatari M. Sageshima YO. Analyzed the data: NN MK AK. Contributed reagents/materials/analysis tools: KM M. Sugiyama SHA JYP SH JHK KS M. Kurosaki YA SM MW ET MH SK EO YI EM AT Y. Murawaki YH IS M. Korenaga KH TI NI KHH YT MM. Wrote the paper: NN M. Kawashima YT KT MM.

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RESEARCH ARTICLE Open Access

No association for Chinese HBV-related hepatocellular carcinoma susceptibility SNP in other East Asian populations

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Abstract

Background: A recent genome-wide association study (GWAS) using chronic HBV (hepatitis B virus) carriers with and without hepatocellular carcinoma (HCC) in five independent Chinese populations found that one SNP (rs17401966) in *KIF1B* was associated with susceptibility to HCC. In the present study, a total of 580 HBV-derived HCC cases and 1351 individuals with chronic hepatitis B (CHB) or asymptomatic carrier (ASC) were used for replication studies in order to evaluate the reported association with HBV-derived HCC in other East Asian populations.

Results: We did not detect any associations between rs17401966 and HCC in the Japanese cohorts (replication 1: OR = 1.09, 95 % CI = 0.82-1.43; replication 2: OR = 0.79, 95 % CI = 0.54-1.15), in the Korean cohort (replication 3: OR = 0.95, 95 % CI = 0.66-1.36), or in the Hong Kong Chinese cohort (replication 4: OR = 1.17, 95 % CI = 0.79-1.75). Meta-analysis using these cohorts also did not show any associations with P = 0.97.

Conclusions: None of the replication cohorts showed associations between rs17401966 and HBV-derived HCC. This may be due to differences in the genetic diversity among the Japanese, Korean and Chinese populations. Other reasons could be the high complexity of multivariate interactions between the genomic information and the phenotype that is manifesting. A much wider range of investigations is needed in order to elucidate the differences in HCC susceptibility among these Asian populations.

Keywords: Hepatitis B, hepatocellular carcinoma, candidate SNP, replication study, genome-wide association study

Background

Hepatitis B (HB) is a potentially life-threatening liver infection caused by the hepatitis B virus (HBV), and approximately 360 million people worldwide are thought to be chronically infected with HBV. The clinical course of HBV infection is variable, including acute self-limiting infection, fulminant hepatic failure, inactive carrier state and chronic hepatitis with progression to cirrhosis and hepatocellular carcinoma (HCC). Although some HBV carriers spontaneously eliminate the virus, 2-10 % of individuals with chronic HB (CHB) develop liver cirrhosis every year, and a subset of these individuals suffer from liver failure or HCC. Around 600,000 new HCC cases are diagnosed annually worldwide, with HCC being relatively common in Asia-Pacific countries and sub-Saharan Africa; more than 70 % of HCC patients are diagnosed in Asia (with 55 % in China) [1]. However, HCC is relatively uncommon in the USA, Europe and Australia [1,2]. The majority of HCC develops in patients with cirrhosis, which is most often attributable

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to chronic HBV infection followed by chronic HCV in the Asia-Pacific region [3].

A recent genome-wide association study (GWAS) using Japanese CHB cases and controls confirmed that 11 SNPs in a region including *HLA-DPA1* and *-DPB1* were associated with CHB [4]. Moreover, a GWAS using chronic HBV carriers with and without HCC in five independent Chinese populations reported that one SNP (rs17401966) in *KIF1B* was associated with HCC susceptibility [5]. In the present study, we performed replication studies using Japanese, Korean and Hong Kong Chinese cases and controls in order to evaluate the reported association with HBV-derived HCC in other East Asian populations.

Results

We performed SNP genotyping of rs17401966 located in the *KIF1B* gene for the purpose of replication analysis of the previous GWAS report [5]. Four distinct cohorts were used for these replication analyses (Table 1). We first examined two independent Japanese case–control samples including 179 cases and 769 controls from Biobank Japan (replication 1), and 142 cases and 251 controls from various hospitals (replication 2). We did not detect any associations between rs17401966 and HCC in the Japanese cohorts (replication 1: OR = 1.09; 95 % CI = 0.82-1.43, replication 2: OR = 0.79; 95 % CI = 0.54-1.15). We further examined Korean case–control samples comprising 164 cases and 144 controls (replication 3) and Hongkongese 94 HCC cases and 187 CHB controls (replication 4), but again did not detect any association (replication 3: OR = 0.95; 95 % CI = 0.66-1.36, replication 4: OR = 1.17; 95 % CI = 0.79-1.75). Logistic regression analysis adjusted for age and gender also did not show any association (P_{log} = 0.65, 0.27, 0.11, 0.56 for each replication panel). Moreover, we conducted meta-analysis to combine these studies, also not detect any association (P_{meta} = 0.97).

Discussion and conclusions

Zhang et al. [5] reported that SNP rs17401966 was significantly associated with HBV-related HCC (joint OR = 0.61). They conducted a GWAS using 348 cases and 359 controls in a population in Guangxi in southern China, and selected 45 SNPs for the replication study based on the results ($P < 10^{-4}$). In the first replication study, they used 276 cases and 266 controls from Beijing in northern China, and 5 SNPs showed the same direction of association as in the GWAS ($P < 0.05$). They performed a further replication study (of 507 cases and 215 controls) in Jiangsu in eastern China and only one SNP showed the same trend ($P = 3.9 \times 10^{-5}$). Guangdong and Shanghai samples from southern and eastern China were used for further replication studies. The association yielded a p-value of 1.7×10^{-18} on meta-analysis.

We performed four replication analyses using Japanese, Korean and Hong Kong Chinese samples (Table 1). Although sample size of each cohort is smaller than that of the previous GWAS, we conducted mete-analysis of all our study. The result did not show any association between rs17401966 and HBV-derived HCC (P_{meta} = 0.97).

This may be due to differences in genetic diversity among Japanese, Korean and Chinese populations. A maximum-likelihood tree of 126 populations based on 19,934 SNPs showed that Japanese and Korean populations form a monophyletic clade with a 100 % bootstrap value [6]. However, Chinese populations form a paraphyletic clade with two other populations. This indicates that Japanese and Korean populations are genetically closer to one another than the Chinese population.

Table 1 Association between rs17401966 and HBV-derived HCC

cohort	sample size	cases			controls				OR		
	(cases/controls)	GG	AG	AA	GG	AG	AA	HWE p	(95 % CI)	P [a]	P_{het} [b]
replication 1	179/769	13	61	105	45	261	463	0.599	1.09	0.578	
(Japan 1)		(7.2)	(34.1)	(58.7)	(5.9)	(33.9)	(60.2)		(0.82-1.43)		
replication 2	142/251	5	46	91	14	91	146	1	0.79	0.212	
(Japan 2)		(3.5)	(32.4)	(64.1)	(5.6)	(36.2)	(58.2)		(0.54-1.15)		
replication 3	164/144	17	59	88	15	55	74	0.616	0.95	0.790	
(Korea)		(10.4)	(36.0)	(53.6)	(10.4)	(38.2)	(51.4)		(0.66-1.36)		
replication 4	94/187	10	39	44	13	80	94	0.767	1.17	0.432	
(Hong Kong)		(10.6)	(41.5)	(46.8)	(6.9)	(42.8)	(50.3)		(0.79-1.75)		
Meta-analysis [c]									0.996	0.965	0.423
									(0.84-1.18)		

[a]P value of fisher's exact test for allele model.
[b]Result of Breslow-Day test.
[c]Results of meta-analysis were calculated by the Mantel-Haenzel method.

We did not find any association with Hong Kong Chinese cohort (P = 0.43). Moreover, a study using 357 HCC cases and 354 HBV-positive non-HCC controls in Hong Kong Chinese did not show any significant difference (P = 0.91) [7]. Previous population studies have revealed that various Han Chinese populations show varying degrees of admixture between a northern Altaic cluster and a southern cluster of Sino-Tibetan/Tai-Kadai populations in southern China and northern Thailand [6]. Although Hong Kong is located closed to the Guangdong (cohort 3 of Zhang et al study), there is great heterogeneity for rs17401966 between Hong Kong cohorts (our study and Chan's study [7]) and Guangdong cohort (our study versus Zhang's study: $P_{het} = 0.0066$; Chan's study versus Zhang's study: $P_{het} = 0.035$). This result suggests the existence of other confounding factors, which can differentiate the previous study in China and this study.

One of the possible reasons could be the high complexity of multivariate interactions between the genomic information and the phenotype that is manifesting. HCC development is a multiple process which links to causative factors such as age, gender, environmental toxins, alcohol and drug abuse, higher HBV DNA levels, and HBV genotype variations [8]. The eight HBV genotypes display distinct geographical and ethnic distributions. Genotypes B and C are prevalent in Asia. Specific variations in HBV have been associated with cirrhosis and HCC. These variations include in particular mutations in pre-core region (Pre-C), in basal core promoter (BCP) and in ORF encoding Pre-S1/Pre-S2/S and Pre-C/C. Because there is an overlap between Pre-C or BCP mutations and genotypes, these mutations appear to be more common in genotype C as compared to other genotypes [9].

Aflatoxins are a group of 20 related metabolites and Aflatoxin B1 is the most potent naturally occurring chemical liver carcinogen known. Aflatoxin exposures multiplicatively increase the risk of HCC in people chronically infected with HBV, which illustrates the deleterious impact that even low toxin levels in the diet can have on human health [10–12]. Liu and Wu estimated population risk for aflatoxin-induced HCC around the world [13]. Most cases occur in sub-Saharan Africa, Southeast Asia and China, where populations suffer from both high HBV prevalence and largely uncontrolled exposure to aflatoxin in food. But we could not obtain the information of these confounding factors from both of the previous GWAS study and this study. A much wider range of investigations is thus needed in order to elucidate the differences in HCC susceptibility among these Asian populations.

Methods

Samples

Case and control samples used in this study were collected from Japan, Korea and Hong Kong listed in supplementary Additional file 1: Table S1. A total of 179 cases and 769 control subjects were analyzed in the first replication study. DNA samples from both CHB controls and HBV-related HCC cases used in this study were obtained from the BioBank Japan at the Institute of Medical Science, the University of Tokyo [14]. Among the BioBank Japan samples, we selected HBsAg-seropositive CHB patients with elevated serum aminotransferase levels for more than six months, according to the guidelines for diagnosis and treatment of chronic hepatisis from The Japan Society of Hepatology (http://www.jsh.or.jp/medical/gudelines/index.html). The mean (and standard deviation; SD) age was 62.0 (9.4) years for the cases and 54.7 (13.5) years for the controls. The second Japanese replication sample sets for the cases (n = 142) and controls (n = 251) study were obtained from 16 hospitals. The case samples for the second replication included 142 HCC patients and the controls included 135 CHB patients and 116 asymptomatic carriers (ASC). The mean (SD) age was 61.3 (10.2) years for the cases and 56.2 (10.9) years for the controls. The Korean replication samples were collected from Yonsei University College of Medicine. The third replication set was composed of 165 HCC patients and 144 CHB patients. The mean (SD) age was 52.2 (8.9) and 37.3 (11.3) years for the cases and controls, respectively. The samples in Hong Kong were collected from the University of Hong Kong, Queen Mary Hospital. The fourth replication set was composed of 94 HCC patients and 187 CHB patients. The mean (SD) age was 58.0 (10.5) and 56.9 (8.3) years for the cases and controls, respectively. All participants provided written informed consent. This research project was approved by the Research Ethics Committees at the Institute of Medical Science and the Graduate School of Medicine, the University of Tokyo, Yonsei University College of Medicine, the University of Hong Kong, Nationa Center for Global Health and Medicine, Hokkaido University Graduate School of Medicine, Teine Keijinkai Hospital, Iwate Medical University, Saitama Medical University, Kitasato University School of Medicine, Musashino Red Cross Hospital, Kanazawa University Graduate School of Medicine, Shinshu University School of Medicine, Nagoya City University Graduate School of Medical Sciences, Kyoto Prefectural University of Medicine, National Hospital Organization Osaka National Hospital, Kawasaki Medical College, Tottori University, Ehime University Graduate School of Medicine, and Kurume University School of Medicine.

SNP Genotyping

For the first replication samples, we genotyped rs17401966 using PCR-based Invader assay (Third Wave Technologies, Madison, WI) [15], and for the second, third and fourth replication samples, we used TaqMan genotyping assay (Applied Biosystems, Carlsbad, CA). In the TaqMan SNP

genotyping assay, PCR amplification was performed in a 5-μl reaction mixture containing 1 μl of genomic DNA, 2.5 μl of KAPA PROBE FAST qPCR Master Mix (Kapa Biosystems, Woburn, MA), and 40 x TaqMan SNP Genotyping Assay probe (ABI) for this SNP. QPCR thermal cycling was performed as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The SNP call rate of each replication panel was 100 %, 100 %, 99.7 % and 99.6 %.

Statistical analysis

We performed Hardy-Weinberg equilibrium test for the case and control samples in each replication study. Fisher's exact test was applied to two-by-two contingency tables for three different genetic models; allele frequency, dominant and recessive model. Odds ratios and confidence intervals were calculated using the major alleles as references. Meta-analysis was conducted using the Mantel-Haenszel method. Heterogeneity among studies was examined by using the Breslow-Day test. Genotype-phenotype association for the SNP rs17401966 was assessed using logistic regression analysis adjusted for age and gender in plink 1.07 (http://pngu.mgh.harvard.edu/~purcell/plink/index.shtml).

Additional file

Additional file 1: Table S1. Samples used in this study.

Abbreviations

HB: Hepatitis b; HBV: Hepatitis b virus; HCC: Hepatocellular carcinoma; CHB: Chronic hepatitis b; HCV: Hepatitis c virus; GWAS: Genome-wide association study; ASC: Asymptomatic carrier.

Competing interests

The authors declare that they have no competing interests.

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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,

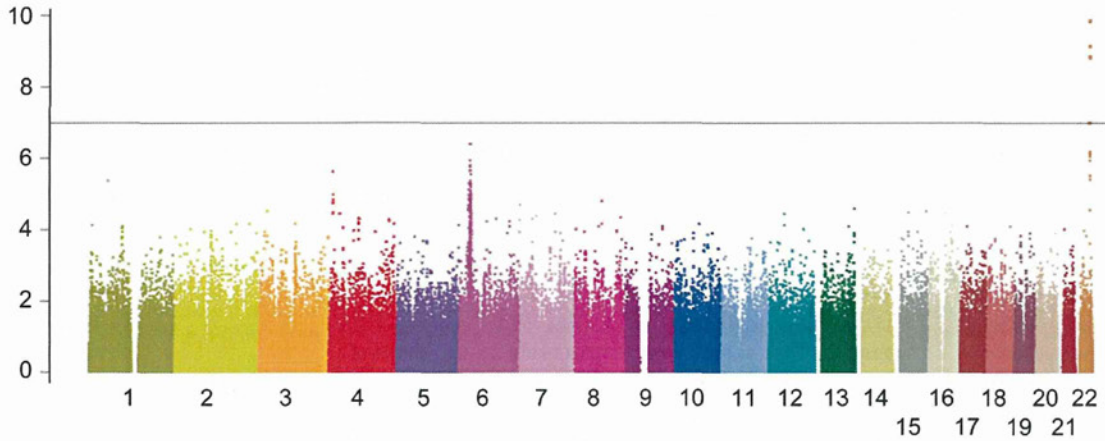

Figure 1. Manhattan plot of the GWA study. Association *p*-values are calculated by exact trend test and plotted along the chromosome in $-\log_{10}$ scale. The horizontal line indicates Bonferroni-adjusted significance threshold ($p = 1.03 \times 10^{-7}$).
doi:10.1371/journal.pone.0038322.g001

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

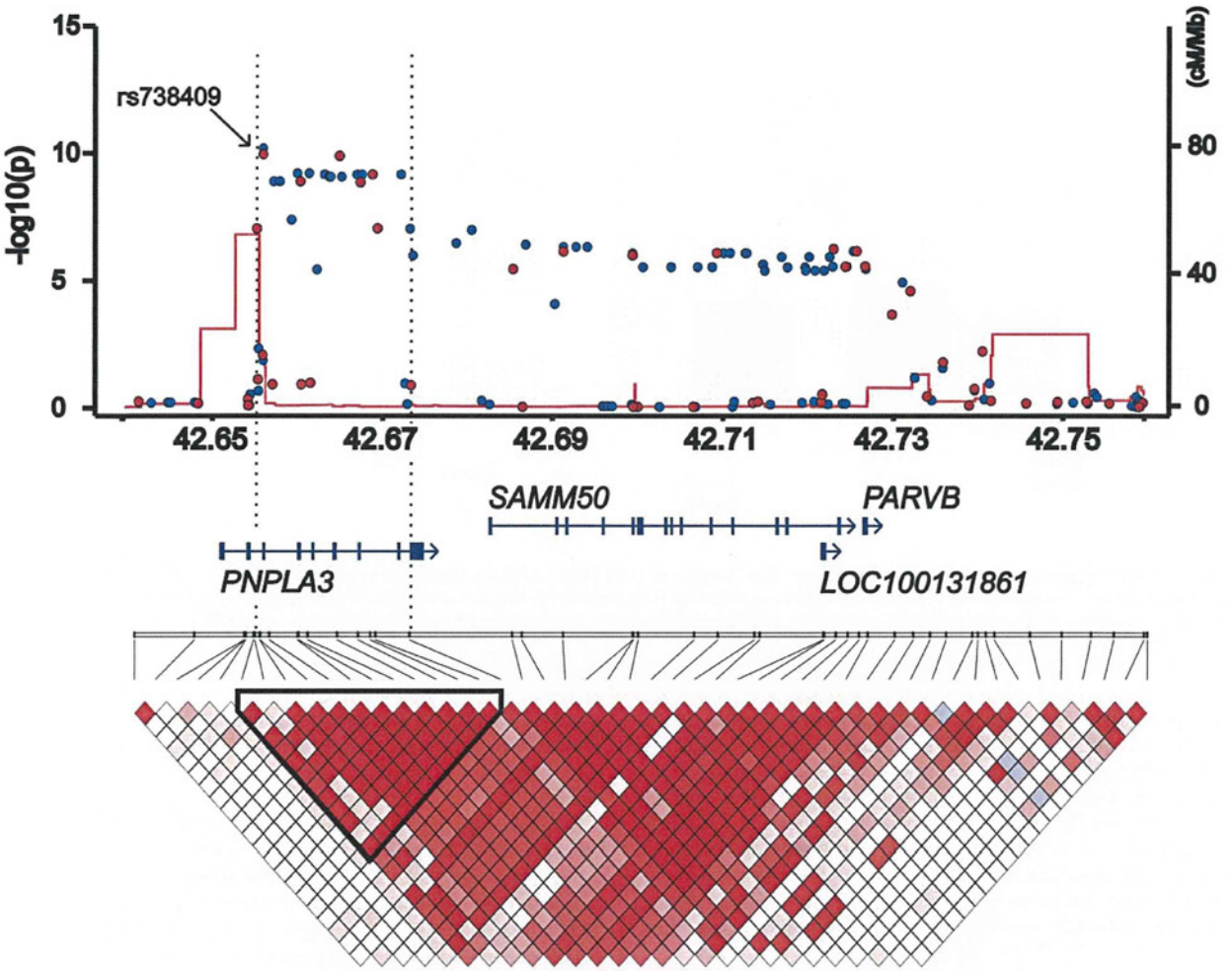

Figure 2. A schematic organization of the human *PNPLA3* locus at 22q13.31 with the genome scan results. *P*-values calculated by the exact trend test were plotted in $-\log_{10}$ scale. Red and blue dots indicate the *p*-values of genotyped and imputed SNPs, respectively. Local recombination rate obtained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

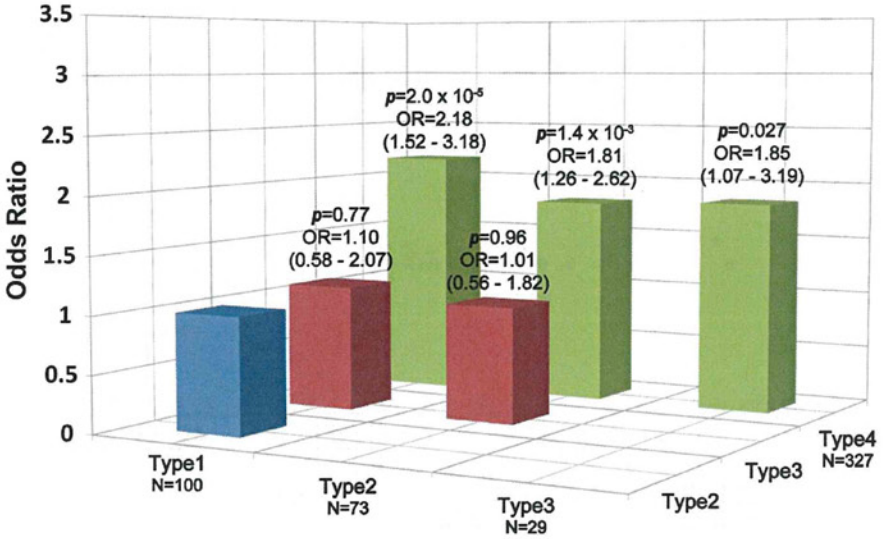

Figure 3. Histogram of odds ratios for genotype distribution of rs738409 between Matteoni types. Each box denotes the odds ratio (OR) comparing the corresponding Matteoni types on the horizontal axes. N represents the number of samples. Odds ratios and *p*-values are calculated for the higher Matteoni 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 ($x10^4/\mu L$)	−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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肝腫瘤の超音波診断基準

日本超音波医学会用語・診断基準委員会
委員長　貴田岡正史

「肝腫瘤の超音波診断基準（1988 / 11 / 30）の改訂」小委員会
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1. 目　的

腹部超音波検査の中で肝腫瘤性病変の存在診断および質的診断の占める重要性は高い．1988年11月30日に「日本超音波医学会医用超音波診断基準に関する委員会」より公示された「肝腫瘤の超音波診断基準」は一般に広く受け入れられ活用され，大きな役割を果たしてきた．しかし，最近の超音波診断装置の進歩および疾患概念の変化により，以前作成された肝腫瘤の超音波診断基準では対応ができなくなった点が多く見られるようになった．今回，これらを解決すべく肝腫瘤の超音波診断基準の改訂を試みた．

2. 対　象

腹部超音波検査の適応となる全ての人が対象となる．特に，肝細胞癌の高危険群（B型慢性肝炎，C型慢性肝炎，非B非C型肝硬変）と超高危険群（B型肝硬変，C型肝硬変）ではそれぞれ，6ヵ月に一度と3－4ヵ月に1度の腹部超音波検査を行う必要がある[1)]．

3. 存在診断

存在診断は，確診，疑診，判定保留の3つの段階に分けて記載する．

1）確診
　① 周囲肝組織との明らかなエコーレベルの相違もしくは明瞭な輪郭
　② 2方向以上での描出

2）疑診
　① 周囲肝組織との明らかなエコーレベルの相違もしくは明瞭な輪郭
　② 1方向のみでの描出

3）判定保留
　① 周囲肝組織とのわずかなエコーレベルの相違もしくは不明瞭な輪郭
　② 1方向以上での描出

注1）存在診断はあくまで肝腫瘤性病変に限ったものではなく，確診の中には限局性の脂肪浸潤の程度の異なる部位も含まれる．
注2）疑診では正常解剖を理解し円靱帯などを，腫瘤性病変と間違わないように注意する．また，所見，随伴疾患から必要に応じてCT（computed tomography）あるいはMRI（magnetic resonance imaging）などの他の画像検査を行い確認する．
注3）判定保留の場合には必要に応じて他の画像診断（CTあるいはMRI）もしくは超音波検査による経過観察を行い確認する．
注4）血管の圧排，途絶，腫瘍の凹凸，肝表面のhump sign，辺縁のedge signは，腫瘍の存在診断の重要な間接所見であるが，本邦に多い肝硬変の存在を考えた時，付加所見として記載する．
注5）肝内胆管の限局性の拡張所見は肝内胆管癌（胆管細胞癌）を強く示唆する所見なので，要精査とする．
注6）エコーレベルに差はないが，周囲肝組織と異なるエコーパターンを示す部分の認められる場合は，判定保留とする．
注7）2方向以上で描出という場合の2方向は，肋弓下走査と肋間走査のように互いに直角に近い2方向が望ましいが不可能な場合はこの限りでない．

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Table 1 Bモード所見

主分類	細分類	形状	境界・輪郭	腫瘍辺縁	腫瘍内部	後方エコー	付加所見
肝細胞癌	結節型（2 cm以下）	円形，類円形	やや不明瞭，整	辺縁低エコー帯（頻度少）	エコーレベルはさまざま（mosaic patternを認めることもある）	不変～時に増強	bright loop
	結節型（2 cmを越える）	円形，類円形	明瞭，整	薄い辺縁低エコー帯（ハロー）	mosaic pattern，nodule in nodule，（大きさや分化度により異なる）	増強	外側エコーの増強
	塊状型	不整形	不明瞭		エコーレベルはさまざま		門脈や肝静脈の腫瘍栓を有する場合がある
肝内胆管癌（胆管細胞癌）		不整形	不明瞭		エコーレベルはさまざま 血管が腫瘍を貫く		末梢胆管の拡張を認める場合がある．また末梢胆管の拡張のみで腫瘤が描出されない例もある
転移性肝腫瘍		不整形で，小さなものは円形	明瞭，時に不明瞭，不整（あらい凹凸）	厚い辺縁低エコー帯（bull's eye pattern，target pattern）	高エコー，低エコー，中心部に無エコー域，石灰化		cluster sign，strong echo，全肝で多数の結節が見られることが多い
肝細胞腺腫		円形，類円形	明瞭，整		エコーレベルはさまざま 隔壁を認めない		腫瘍内出血は低エコー，脂肪変性は高エコー
肝血管腫		円形，類円形	明瞭，不整（細かい凹凸）	辺縁に高エコー帯を認めることもある（marginal strong echo）	高エコー型，辺縁高エコー型，混在型，低エコー型に分けられる		chameleon sign，wax and wane sign，disappearing sign
限局性結節性過形成（FNH）		不整形	不明瞭		低～高エコーさまざま．中心部高エコー		

4. 存在部位診断

1) 小さな腫瘍ではCouinaud[2]の区域で記述する．また，2区域にまたがるような腫瘍の場合，多くの部分を占める区域を先に記載しその残りの区域を記載する（例：S_7－S_8にかけて腫瘤が存在しS_7が主体の場合には$S_{7,8}$としS_8が主体の場合には$S_{8,7}$とする）．
2) 大きな腫瘍ではHealey[3]の区域で記述する．
3) Healeyの区域間に存在する腫瘍では，肝静脈や門脈との立体的位置関係につき記述する．
4) Couinaudの上下区域の診断に迷う場合は，Healeyの区域門脈枝の何番目の枝によって支配されているかを記述する．門脈枝の分岐が複雑な場合は図示する．

5. 質的診断

腹部超音波検査には肝腫瘤診断の役割として存在診断に加えて質的診断がある．

質的診断には①Bモード所見，②ドプラ所見，③造影所見の3種類あり，それぞれの役割は異なる．

鑑別診断に必要な代表的な所見をそれぞれについて，主に肝細胞癌，肝内胆管癌（胆管細胞癌），転移性肝腫瘍，肝細胞腺腫，肝血管腫，限局性結節性過形成（FNH）の6疾患について以下に記載する．

5.1 Bモード所見

超音波検査所見の基本となる．**Table 1**に示すごとく，形状，境界・輪郭，腫瘍辺縁，腫瘍内部，後方エコー，付加所見から鑑別診断を行う．肝細胞癌においては結節型，塊状型の肝細胞癌が対象である[4]．

注1) いずれも典型的な所見を示した．転移性肝腫瘍（癌）は上皮性，非上皮性を区別していな