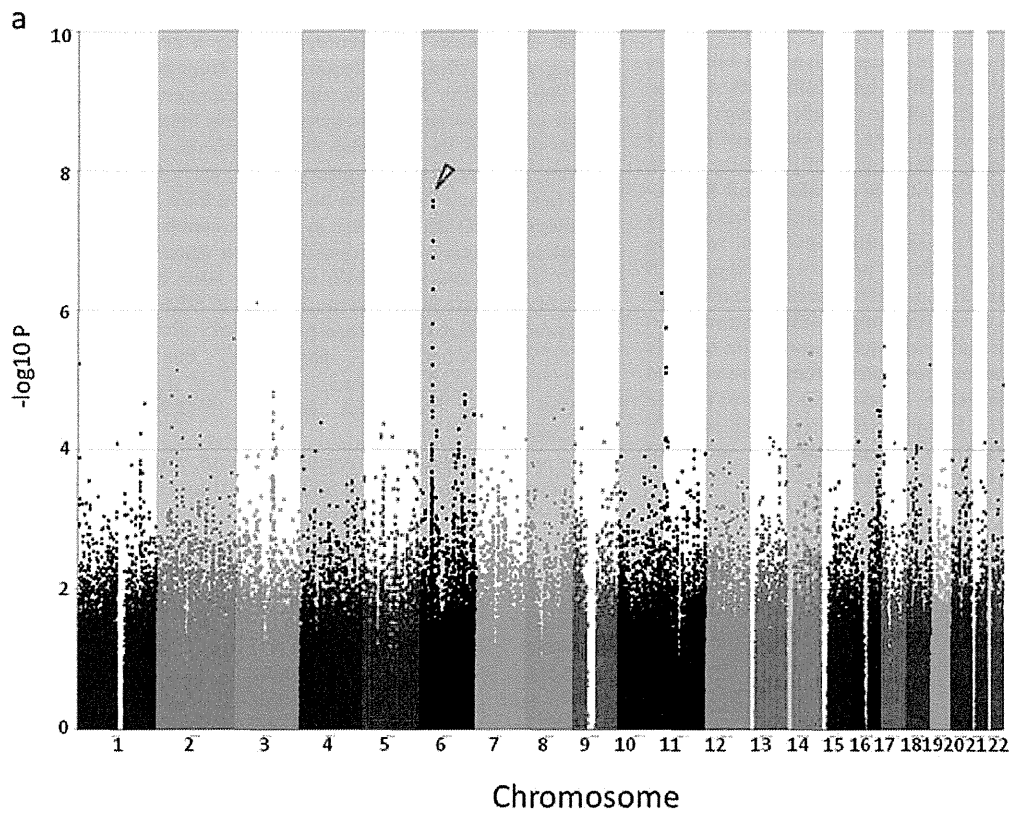
a
-log10 P
Chromosome

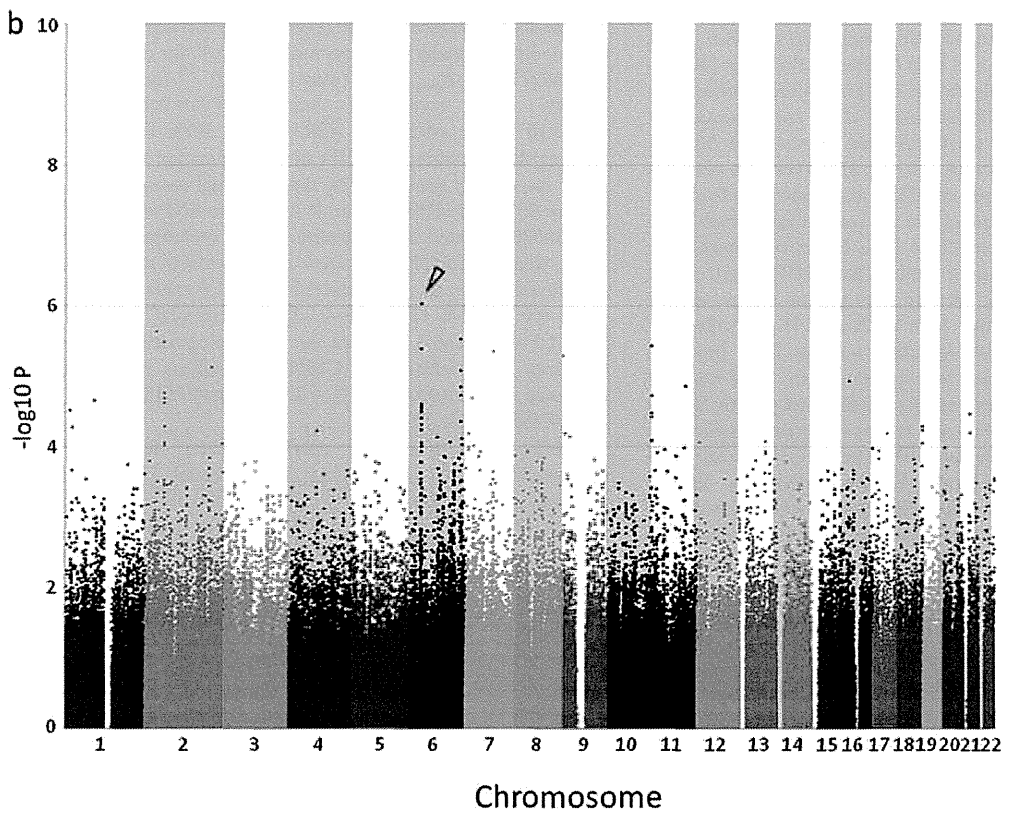
b
-log10 P
Chromosome

Figure 1. Results of genome-wide association studies. a) HBV carriers and healthy controls, and b) HBV carriers and HBV-resolved individuals were compared. *P* values were calculated by chi-squared test for allele frequencies. Dots with arrows on chromosome 6 show strong associations with protective effects against persistent HB infection and with HBV clearance.
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Clearance of Hepatitis B virus in Japanese and Korean Individuals

We also conducted a GWAS to identify the host genetic factors related to clearance of HBV in the above 181 Japanese HBV carriers and 185 Japanese HBV-resolved individuals using a genome-wide SNP typing array (Affymetrix Genome-Wide Human SNP Array 6.0 for 900 K SNPs). The same two SNPs (rs3077 and rs9277542) showed strong associations in the allele frequency model ($P = 9.24\times10^{-7}$ and $P = 3.15\times10^{-5}$) with clearance of HBV (Figure 1b).

The above 32 SNPs, including the two associated SNPs (rs3077 and rs9277542), were selected for a replication study in two independent sets of HBV carriers and HBV resolved individuals (replication-1:256 Japanese HBV carriers and 150 Japanese HBV resolved individuals; and replication-2:344 Korean HBV carriers and 106 Korean HBV resolved individuals; Table 1). All 32 SNPs were genotyped using the DigiTag2 assay and 29 of 32 SNPs were successfully genotyped (Table S3). The associations of the original SNPs were replicated in both replication sets [replication-1 (Japanese): rs3077, $P = 3.32\times10^{-2}$, OR = 0.72 and rs9277542, $P = 1.25\times10^{-2}$, OR = 0.68; replication-2 (Korean): rs3077, $P = 2.35\times10^{-7}$, OR = 0.41 and rs9277542, $P = 4.97\times10^{-6}$, OR = 0.46; Table 3]. Meta-analysis using random effects model showed $P_{meta} = 1.56\times10^{-4}$ for rs3077 (OR = 0.51, 95% CI = 0.36–0.72), and 5.91×10^{-7} for rs9277542 (OR = 0.55, 95% CI = 0.43–0.69). While there was evidence of heterogeneity between these studies for rs3077 ($P_{het} = 0.03$) and no evidence for rs9277542 ($P_{het} = 0.19$), significant associations with HBV clearance were observed with Mantel-Haenszel $P_{meta} = 3.28\times10^{-12}$ for rs3077 and 1.42×10^{-10} for rs9277542, when using CMH fixed-effects model. Among the remaining 27 SNPs in the replication study, two SNPs (rs9276431 and rs7768538), located in a genetic region including *HLA-DQ* gene, were marginally replicated in the two sets of HBV carriers and HBV resolved individuals with Mantel-Haenszel *P* values of 2.10×10^{-5} (OR = 0.59) and 1.10×10^{-5} (OR = 0.56), respectively (Table S3), when using CMH fixed-effect model. Due to the existing heterogeneity among three groups (GWAS, Replication-1 and Replication-2) ($P_{het} = 0.03$ for rs9276431 and 0.04 for rs7768538), weak associations were observed with $P_{meta} = 0.03$ for rs9276431 and 0.02 for rs7768538 by the random effects model meta-analysis.

Meta-analysis across 6 independent studies, including 5 additional published data, showed $P_{meta} = 1.48\times10^{-9}$, OR = 0.60 for rs3077, $P_{meta} = 1.08\times10^{-17}$, OR = 0.66 for rs9277535 and $P_{meta} = 5.14\times10^{-5}$, OR = 0.55 for rs9277542 (Table S4). As shown in Table S4, the OR for the rs9277535 and rs9277542 were similar among the 6 independent studies, and heterogeneity was negligible ($P_{het} = 0.03$ for rs9277535 and 0.14 for rs9277542). However, significant level of heterogeneity for rs3077 was observed with $P_{het} = 9.57\times10^{-6}$ across 5 independent studies, including our study.

Table 1. Number of study samples.

		GWAS	Replication-1	Replication-2
population		**Japanese**	**Japanese**	**Korean**
HBV carriers	Total	**181**	**256**	**344**
	IC	20	94	–
	CH	67	101	177
	LC	3	10	–
	HCC	91	51	167
Healthy controls		**184**	**236**	**151**
Resolved individuals		**185**	**150**	**106**

Abbreviation: IC, Inactive Carrier; CH, Chronic Hepatitis; LC, Liver Cirrhosis; HCC, Hepatocellular Carcinoma.
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URLs

The results of the present GWAS are registered at a public database: https://gwas.lifesciencedb.jp/cgi-bin/gwasdb/gwas_top.cgi.

Discussion

The recent genome-wide association study showed that the SNPs located in a genetic region including *HLA-DPA1* and *HLA-DPB1* genes were associated with chronic HBV infection in the Japanese and Thai population [10,11]. In this study, we confirmed a significant association between SNPs (rs3077 and rs9277542) located in the same genetic region as *HLA-DPA1* and *HLA-DPB1* and protective effects against CHB in Korean and Japanese individuals. Mata-analysis using the random effects model across 6 independent studies including our study suggested that, widely in East Asian populations, variants in antigen binding sites of *HLA-DP* contribute to protective effects against persistent HBV infection (Table S2).

On GWAS and replication analysis with Japanese and Korean individuals, we identified associations between the same SNPs (rs3077 and rs9277542) in the *HLA-DPA1* and *HLA-DPB1* genes and HBV clearance; however, no new candidate SNPs from the GWAS were detected on replication analysis (Table S3). When the data of reference#18 was excluded from the meta-analysis across 6 independent studies, heterogeneity among 4 studies was estimated to be $P_{het} = 0.15$ and significant association of rs3077 with HBV clearance was observed with $P_{meta} = 5.88\times10^{-24}$, OR = 0.56 (Table S4). In our study, a negligible level of heterogeneity for rs3077 was also observed ($P_{het} = 0.03$) on meta-analysis by adding replication-1 (Table 3). Despite the heterogeneity in replication-1, a marginal association was observed for rs3077 with the same downward trend in the odds ratio ($P = 3.32\times10^{-2}$, OR = 0.72). Moreover, meta-analysis using GWAS and replication-2 showed significant association of $P_{meta} = 1.89\times10^{-12}$, OR = 0.43 for rs3077 with no evidence of heterogeneity ($P_{het} = 0.75$). Although the reason why heterogeneity was observed in replication-1 is unclear, one possible reason is the clinical heterogeneity due to different kits being used for antibody testing. The associations of *HLA-DPA1/−DPB1* with CHB and HBV clearance showed the same level of significance in the comparison of HBV patients with HBV resolved individuals (OR = 0.43 for rs3077 and 0.49 for rs9277542) as the one with healthy controls (OR = 0.46 for rs3077 and 0.50 for rs9277542), when the replication-1 was excluded in the analysis (Table 2 and Table 3). The results of meta-analysis across 6 independent studies including our study also showed the same or slightly weaker associations in the

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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Soluble MICA and a *MICA* Variation as Possible Prognostic Biomarkers for HBV-Induced Hepatocellular Carcinoma

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Abstract

MHC class I polypeptide-related chain A (MICA) molecule is induced in response to viral infection and various types of stress. We recently reported that a single nucleotide polymorphism (SNP) rs2596542 located in the *MICA* promoter region was significantly associated with the risk for hepatitis C virus (HCV)-induced hepatocellular carcinoma (HCC) and also with serum levels of soluble MICA (sMICA). In this study, we focused on the possible involvement of MICA in liver carcinogenesis related to hepatitis B virus (HBV) infection and examined correlation between the *MICA* polymorphism and the serum sMICA levels in HBV-induced HCC patients. The genetic association analysis revealed a nominal association with an SNP rs2596542; a G allele was considered to increase the risk of HBV-induced HCC ($P = 0.029$ with odds ratio of 1.19). We also found a significant elevation of sMICA in HBV-induced HCC cases. Moreover, a G allele of SNP rs2596542 was significantly associated with increased sMICA levels ($P = 0.009$). Interestingly, HCC patients with the high serum level of sMICA (>5 pg/ml) exhibited poorer prognosis than those with the low serum level of sMICA (≤5 pg/ml) ($P = 0.008$). Thus, our results highlight the importance of *MICA* genetic variations and the significance of sMICA as a predictive biomarker for HBV-induced HCC.

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Introduction

Hepatocellular carcinoma (HCC) reveals a very high mortality rate that is ranked the third among all cancers in the world [1]. HCC is known to develop in a multistep process which has been related to various risk factors such as genetic factors, environment toxins, alcohol and drug abuse, autoimmune disorders, elevated hepatic iron levels, obesity, and hepatotropic viral infections [2]. Among them, chronic infection with hepatitis B virus (HBV) is one of the major etiological factors for developing HCC with considerable regional variations ranging from 20% of HCC cases in Japan to 65% in China [3].

Interestingly, clinical outcome after the exposure to HBV considerably varies between individuals. The great majority of individuals infected with HBV spontaneously eliminate the viruses, but a subset of patients show the persistent chronic hepatitis B infection (CHB), and then progresses to liver cirrhosis and HCC through a complex interplay between multiple genetic and environmental factors [4]. In this regard, genome wide association studies (GWAS) using single nucleotide polymorphisms (SNPs) have highlighted the importance of genetic factors in the pathogenesis of various diseases including CHB as well as HBV-induced HCC [5,6,7,8,9,10,11,12,13]. Recently, we identified a genetic variant located at 4.7 kb upstream of the *MHC class I polypeptide-related chain A* (*MICA*) gene to be strongly associated with hepatitis C virus (HCV) -induced HCC development [14].

MICA is highly expressed on viral-infected cells or cancer cells, and acts as ligand for NKG2D to activate antitumor effects of Natural killer (NK) cells and $CD8^+$ T cells [15,16]. Our previous results indicated that a G allele of SNP rs2596542 was significantly associated with the lower cancer risk and the higher level of soluble MICA (sMICA) in the serum of HCV-induced HCC patients, demonstrating the possible role of MICA as a tumor suppressor. However, elevation of serum sMICA was shown to be associated with poor prognosis in various cancer patients [17,18,19,20].

Matrix metalloproteinases (MMPs) can cleave MICA at a transmembrane domain [21] and release sMICA proteins from cells. Since sMICA was shown to inhibit the antitumor effects of NK cells and $CD8^+$ T cells by reduction of their affinity to binding to target cells [22,23], the effect of MICA in cancer cells would be modulated by the expression of MMPs. To elucidate the role of MICA in HBV-induced hepatocellular carcinogenesis, we here report analysis of the *MICA* polymorphism and serum sMICA level in HBV-induced HCC cases.

Materials and Methods

Study participants

The demographic details of study participants are summarized in Table 1. A total of 181 HCC cases, 597 CHB patients, and 4,549 non-HBV controls were obtained from BioBank Japan that was initiated in 2003 with the funding from the Ministry of Education, Culture, Sports, Science and Technology, Japan [24]. In the Biobank Japan Project, DNA and serum of patients with 47 diseases were collected through collaborating network of 66 hospitals throughout Japan. List of participating hospitals is shown in the following website (http://biobankjp.org/plan/member_hospital.html). A total of 226 HCC cases, 102 CHB patients, and 174 healthy controls were additionally obtained from the University of Tokyo. The diagnosis of chronic hepatitis B was conducted on the basis of HBsAg-seropositivity and elevated serum aminotransferase levels for more than six months according to the guideline for diagnosis and treatment of chronic hepatitis (The Japan Society of Hepatology, http://www.jsh.or.jp/medical/gudelines/index.html). Control Japanese DNA samples (n = 934) were obtained from Osaka-Midosuji Rotary Club, Osaka, Japan. All HCC patients were histopathologically diagnosed. Overall survival was defined as the time from blood sampling for sMICA test to the date of death due to HCC. Patients who were alive on the date of last follow-up were censored on that date. All participants provided written informed consent. This research project was approved by the ethics committee of the University of Tokyo and the ethics committee of RIKEN. All clinical assessments and specimen collections were conducted according to Declaration of Helsinki principles.

SNP genotyping

Genotyping platforms used in this study were shown in Table 1. We genotyped 181 HCC cases and 5,483 non-HBV control samples using either Illumina Human Hap610-Quad or Human Hap550v3. The other samples were genotyped at SNP rs2596542 by the Invader assay system (Third Wave Technologies, Madison, WI).

MICA variable number tandem repeat (VNTR) locus genotyping

Genotyping of the *MICA* VNTR locus in 176 HBV-induced HCC samples was performed using the primers reported previously by the method recommended by Applied Biosystems (Foster City, CA) [14]. Briefly, the 5′ end of forward primer was labeled with 6-FAM, and reverse primer was modified with GTGTCTT non-random sequence at the 5′ end to promote Plus A addition. The PCR products were mixed with Hi-Di Formamide and GeneScan-600 LIZ size standard, and separated by GeneScan system on a 3730×l DNA analyzer (Applied Biosystems, Foster City, CA). GeneMapper software (Applied Biosystems, Foster City, CA) was employed to assign the repeat fragment size (Figure S1).

Quantification of soluble MICA

We obtained serum samples of 111 HBV-positive HCC samples, 129 HCV-positive HCC samples, and 60 non-HBV controls from Biobank Japan. Soluble MICA levels were measured by sandwich enzyme-linked immunosorbent assay, as described in the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Statistical analysis

The association between an SNP rs2596542 and HBV-induced HCC was tested by Cochran-Armitage trend test. The Odds ratios were calculated by considering a major allele as a reference. Statistical comparisons between genotypes and sMICA levels were performed by Kruskal-Wallis test (if more than two classes for comparison) or Wilcoxon rank test using R. Overall survival rate of the patients was analyzed by Kaplan-Meier method in combination with log-rank test with SPSS 20 software. The period for the survival analysis was calculated from the date of blood sampling to the recorded date of death or the last follow-up date. Differences with a P value of <0.05 were considered statistically significant.

Results

Association of SNP rs2596542 with HBV-induced HCC

In order to examine the effect of rs2596542 genotypes on the susceptibility to HBV-induced HCC, a total of 407 HCC cases and 5,657 healthy controls were genotyped. The Cochran Armitage trend test of the data revealed a nominal association

Table 1. Demographic details of subjects analyzed.

Subjects	Source	Genotyping platform	Number of Sample	Female (%)	Age (mean+/−sd)
Liver Cancer	BioBank Japan	Illumina Human Hap610-Quad	181	17.9	62.94±9.42
	University of Tokyo	Invader assay	226		
Control	BioBank Japan	Illumina Human Hap550v3	4549	47.95	55.19±12.5
	Osaka**	Illumina Human Hap550v3	934		
	University of Tokyo	Invader assay	174		
Chronic hepatitis B*	BioBank Japan	Invader assay	597	45.66	61.31±12.6
	University of Tokyo	Invader assay	102		

*Chronic hepatitis B patients without liver cirrhosis and liver cancer during enrollment.
**Healthy volunteers from Osaka Midosuji Rotary Club, Osaka, Japan.
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between HBV-induced HCC and rs2596542 in which a risk allele G was more frequent among HBV-induced HCC cases than an A allele (P = 0.029, OR = 1.19, 95% CI: 1.02–1.4; Table 2). To further investigate the effect of rs2596542 on the progression from CHB to HBV-induced HCC, we genotyped a total of 699 CHB cases without HCC. Although the progression risk from CHB to HBV-induced HCC was not statistically significant with rs2596542 (P = 0.197 by the Cochran Armitage trend test with an allelic OR = 1.3 (0.94–1.36); Table 2), we found a similar trend of association in which the frequency of a risk-allele G was higher among HBV-induced HCC patients than that of CHB subjects. Since we previously revealed that an A allele was associated with a higher risk of HCV-induced HCC with OR of 1.36 [14], the s2596542 alleles that increased the risk of HCC were opposite in HBV-induced HCC and HCV-induced HCC.

Soluble MICA levels are associated with SNP rs2596542

We subsequently performed measurement of soluble MICA (sMICA) in serum samples using the ELISA method in 176 HBV-positive HCC cases and 60 non-HBV controls. Nearly 30% of the HBV-induced HCC cases revealed the serum sMICA level of >5 pg/ml (defined as high) while the all control individuals except one showed that of ≤5 pg/ml (defined as low) ($P = 4.5 \times 10^{-6}$; Figure 1A). Then, we examined correlation between SNP rs2596542 genotypes and serum sMICA levels in HBV-positive HCC cases. Interestingly, rs2596542 genotypes were significantly associated with serum sMICA levels (P = 0.009; Figure 1B); 39% of individuals with the GG genotype and 20% of those with the AG genotype were classified as high for serum sMICA, but only 11% of those with the AA genotype were classified as high (AA+AG vs GG; P = 0.003) (Figure 1B). These findings were similar with our previous reports in which a G allele was associated with higher serum sMICA levels in HCV-induced HCC patients [14].

Negative association of variable number of tandem repeat (VNTR) with sMICA level

The *MICA* gene harbors a VNTR locus in exon 5 that consists of 4, 5, 6, or 9 repeats of GCT as well as a G nucleotide insertion into a five-repeat allele (referred as A4, A5, A6, A9, and A5.1, respectively). The insertion of G (A5.1) causes a premature translation termination and results in loss of a transmembrane domain, which may produce the shorter form of the MICA protein that is likely be secreted into serum [25]. However, the association of this VNTR locus with serum sMICA level was controversial among studies [14,26,27,28]. Therefore, we examined the association between the VNTR locus and sMICA level in HBV-induced HCC patients, and found no significant association (Figure S1 and S2), concordant with our previous report for HCV-induced HCC patients [14].

Soluble MICA levels are associated with survival of HCC patients

In order to evaluate the prognostic significance of serum sMICA levels in HCC patients, we performed survival analysis of HCC patients. A total of 111 HBV-infected HCC patients and 129 HCV-infected HCC patients were included in this analysis. The mean survival period for HBV- and HCV-infected patients with less than 5 pg/ml of serum sMICA were 67.1 months (95% CI: 61.1–73.1, n = 83), and 58.2 months (95% CI: 51.4–65.0, n = 85), respectively. On the other hand, for patients with more than 5 pg/ml of serum sMICA, the mean survival periods were 47.8 months (95% CI: 34.8–30.9, n = 28) for HBV-induced HCC patients and 59.5 months (95% CI: 51.9–67.1, n = 44) for HCV-induced HCC patients. The Kaplan-Maier analysis and log-rank test indicated that among HBV-induced HCC subjects, the patients in the high serum sMICA group showed a significantly shorter survival than those in the low serum sMICA (P = 0.008; Figure 2). In addition, we performed multi-variate analysis to test whether sMICA is an independent prognostic factor by including age and gender as covariates. The results revealed significant association of sMICA levels with overall survival (P = 0.017) but not with age and gender (Table S1).However, we found no association between the serum sMICA level and the overall survival in the HCV-induced HCC subjects (P = 0.414; Figure S3). Taken together, our findings imply the distinct roles of the *MICA* variation and sMICA between HBV- and HCV-induced hepatocellular carcinogenesis.

Vascular invasion in HBV-related HCC patients is associated with soluble MICA levels

Since sMICA levels were associated with the overall survival of HBV-related HCC patients, we tested whether sMICA levels affect survival through modulating invasive properties of tumors or size of the tumors. We tested the association between sMICA levels and vascular invasion in 35 HBV-related HCC cases, among whom 7 cases were positive and 21 cases were negative for vascular invasion. We found significant association between sMICA levels and vascular invasion (Figure 3; P = 0.014) in which 7 cases with positive vascular invasion showed high levels of sMICA (mean = 54 pg/ml) than 21 cases without vascular invasion (mean = 7.51 pg/ml). However, we found no association between tumor size and sMICA levels (P = 0.56; data not shown). These results suggest that sMICA may reduce the survival of HBV-related HCC patients by affecting the invasive properties of tumors.

Discussion

Several mechanisms such as HBV-genome integration into host chromosomal DNA [29] and effects of viral proteins including HBx [30] are shown to contribute to development and progression of HCC, while the immune cells such as NK and T cells function as key antiviral and antitumor effectors. MICA protein has been

Table 2. Association between HCC and rs2596542.

SNP	Comparison	Chr	Locus	Case MAF	Control MAF	P*	OR*	95% CI
rs2596542	HCC vs. Healthy control	6	*MICA*	0.294	0.332	0.029	1.19	1.02–1.4
rs2596542	HCC vs. CHB	6	*MICA*	0.294	0.320	0.197	1.13	0.94–1.36

Note: 407 HCC cases, 699 CHB subjects and 5,657 non-HBV controls were used in the analysis.
Chr.,chromosome; MAF, minor allele frequency; OR, odds ratio for minor allele; CI, confidence interval.
*Obtained by Armitage trend test.
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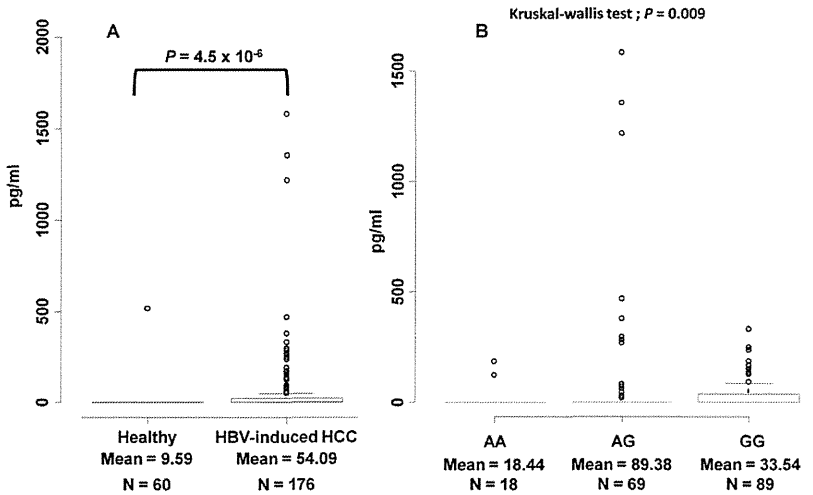

Figure 1. Soluble MICA levels are associated with HBV-related HCC. (A) Correlation between soluble MICA levels and HBV-induced HCC subjects. The y-axis displays the concentration of soluble MICA in pg/ml. The number of independent samples tested in each group is shown in the x-axis. Each group is shown as a box plot and the mean values are shown in the x-axis. The difference between two groups is tested by Wilcoxon rank test. The box plots are plotted using default settings in R. (B) Correlation between soluble MICA levels and rs2596542 genotype in HBV-positive HCC subjects. The x-axis shows the genotypes at rs2596542 and y-axis display the concentration of soluble MICA in pg/ml. Each group is shown as a box plot. $P = 0.027$ and 0.013 for AA vs. GG and AA vs. AG, respectively. The association between genotypes and sMICA levels was tested by Kruskal-wallis test, whereas the difference in the sMICA levels between AA and GG is tested by Wilcoxon rank test. The box plots are plotted using default settings in R.
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considered as a stress marker of gastrointestinal epithelial cells because of its induced expression by several external stimuli such as heat, DNA damage, and viral infections [31,32,33,34]. Here, we examined the association of rs2596542 and serum sMICA levels with HBV-induced HCC. Like in HCV-induced HCC [14], our results from ELISA revealed a significantly higher proportion

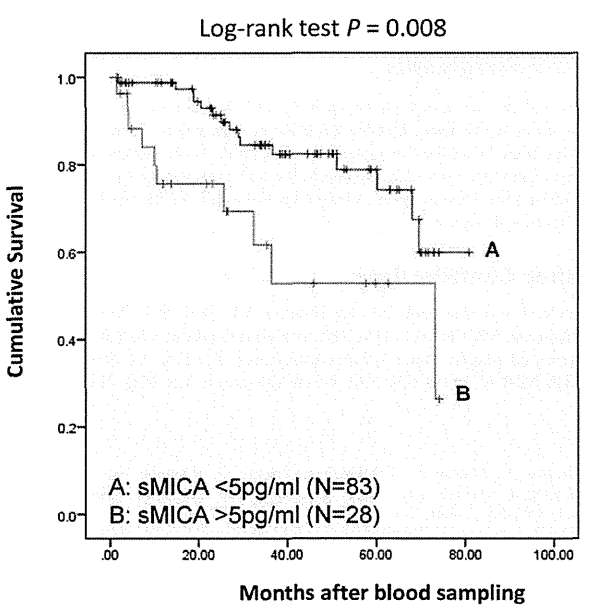

Figure 2. Kaplan-Meier curves of the patients with HBV-induced HCC. The patients were divided into two groups according to their sMICA concentration (high: >5 pg/ml and low: =<5 pg/ml). Statistical difference was analyzed by log-rank test. The y-axis shows the cumulative survival probability and x-axis display the months of the patients survival after blood sampling.
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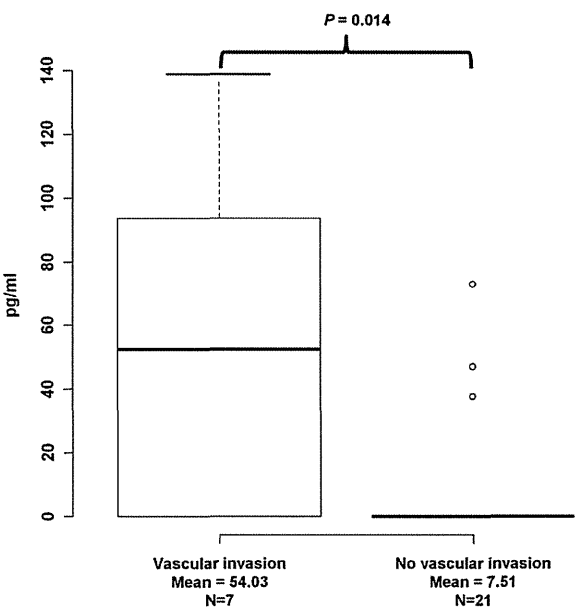

Figure 3. Correlation between soluble MICA levels and vascular invasion in HBV-induced HCC subjects. The y-axis displays the concentration of soluble MICA in pg/ml. The number of independent samples tested in each group is shown in the x-axis. Each group is shown as a box plot and the mean values are shown in the x-axis. The difference between two groups is tested by Wilcoxon rank test. The box plots are plotted using default settings in R.
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of high serum sMICA cases (nearly 30%) in the HBV-induced HCC group, compared to non-HBV individuals (1.7%). Moreover, the serum sMICA level was significantly associated with rs2596542, but not with the copy number differences of the VNTR locus, as concordant with our previous report [14].

Several studies have already indicated the roles of sMICA as prognostic markers for different types of malignant diseases [17,18,19,20]. Therefore, it is of medical importance to test whether serum sMICA levels can be used as a prognostic marker for patients with HCC. To our best knowledge, this is the first study to demonstrate the prognostic potential of sMICA for HBV-positive HCC patients; we found 19.3 months of improvement in survival among patients carrying less than 5 pg/ml of serum sMICA, compared to those having more than 5 pg/ml.

On the contrary, we found no significant correlation between sMICA levels and the prognosis of HCV-induced HCC cases. These opposite effects of *MICA* variation could be explained by the following mechanism. The individuals who carry the G allele would express high levels of membrane-bound MICA upon HCV infection and thus lead to the activation of immune cells against virus infected cells. On one hand, HBV infection results in increased expression of membrane-bound MICA as well as MMPs through viral protein HBx [35], which would result in the elevation of sMICA and the reduction of membrane-bound MICA. Since sMICA could block CD8+T cells, NK-CTL, and NK cells, higher sMICA would cause the inactivation of immune surveillance system against HBV infected cells. In other words, HBV may use this strategy to evade immune response and hence, higher levels of sMICA could be associated with lower survival rate among HBV-associated HCC. On the other hand, since HCV is not known to induce the cleavage of membrane bound MICA, individuals with low level membrane bound MICA expression (carriers of rs2596542-allele A) could be inherently susceptible for HCV-induced HCC. Thus, HBx-medicated induction of MMPs could partially explain the intriguing contradictory effect of MICA between HBV-induced HCC and HCV-induced HCC. Since we observed significant correlation of sMICA levels with vascular invasion, it may be the case that high levels of sMICA cause poor prognosis of HBV-related HCC cases by making tumors more aggressive and invasive. However it is important in future to determine the ratio of membrane-bound MICA to sMICA in case of HCV- and HBV-related HCC.

Interestingly, the immune therapy against melanoma patients induced the production of auto-antibodies against MICA [36]. Anti-MICA antibodies would exert antitumor effects through antibody-dependent cellular cytotoxicity against cells expressing membrane-bound MICA and/or activation of NK cells by inhibiting the sMICA-NKG2D interaction. However, further studies are necessary, using well-defined HBV-related HCC cohort, to investigate whether sMICA levels could be included as an additional factor to predict the survival rate among HBV-related HCC subjects. Taken together, our results indicate the potential of *MICA* variant and sMICA as prognostic biomarkers. Thus, MICA could be a useful therapeutic target for HBV-induced HCC.

Supporting Information

Figure S1 MICA repeat genotyping using capillary-based method. The alleles are annotated using GeneMapper software based on the size of the PCR product (185 bp = A4 allele, 188 bp = A5, 189 bp = A5.1, 191 bp = A6 and 200 bp = A9). The inset at the base of each peak shows the size of the PCR product with corresponding allele call by the software. The figure display all observed heterozygotes at A5.1 allele.
(TIF)

Figure S2 MICA VNTR alleles are not associated with soluble MICA levels. Each group is shown as a box plot. The difference in the sMICA values among each group is tested by Wilcoxon rank test. The box plots are plotted using default settings in R.
(TIF)

Figure S3 Kaplan-Meier curves of the patients with HCV-induced HCC. The patients were divided into two groups according to their sMICA concentration (<5 pg/ml or >5 pg/ml). Statistical difference was analyzed by log-rank test. The y-axis shows the cumulative survival probability and x-axis display the months of the patients survival after blood sampling.
(TIF)

Table S1 Clinical parameters of HBV-related HCC patients available for prognostic analyses.
(XLS)

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Author Contributions

Conceived and designed the experiments: VK KM YN. Performed the experiments: VK PHL YU HM ZD. Analyzed the data: VK PHL CT RM. Contributed reagents/materials/analysis tools: YN NK AT MK HS KT YT MS MM RT MO KK NK. Wrote the paper: VK PHL KM YN.

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Genome-wide Association Study Identifies *TNFSF15* and *POU2AF1* as Susceptibility Loci for Primary Biliary Cirrhosis in the Japanese Population

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For the identification of susceptibility loci for primary biliary cirrhosis (PBC), a genome-wide association study (GWAS) was performed in 963 Japanese individuals (487 PBC cases and 476 healthy controls) and in a subsequent replication study that included 1,402 other Japanese individuals (787 cases and 615 controls). In addition to the most significant susceptibility region, human leukocyte antigen (HLA), we identified two significant susceptibility loci, *TNFSF15* (rs4979462) and *POU2AF1* (rs4938534) (combined odds ratio [OR] = 1.56, $p = 2.84 \times 10^{-14}$ for rs4979462, and combined OR = 1.39, $p = 2.38 \times 10^{-8}$ for rs4938534). Among 21 non-HLA susceptibility loci for PBC identified in GWASs of individuals of European descent, three loci (*IL7R*, *IKZF3*, and *CD80*) showed significant associations (combined $p = 3.66 \times 10^{-8}$, 3.66×10^{-9}, and 3.04×10^{-9}, respectively) and *STAT4* and *NFKB1* loci showed suggestive association with PBC

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(combined $p = 1.11 \times 10^{-6}$ and 1.42×10^{-7}, respectively) in the Japanese population. These observations indicated the existence of ethnic differences in genetic susceptibility loci to PBC and the importance of TNF signaling and B cell differentiation for the development of PBC in individuals of European descent and Japanese individuals.

Primary biliary cirrhosis (PBC, MIM 109720) is a chronic and progressive cholestatic liver disease, presumably caused by autoimmune reactions against biliary epithelial cells, leading to liver cirrhosis and hepatic failure.[1] The incidence and prevalence of PBC range from 0.33 to 5.8 and from 2 to 40 per 100,000 inhabitants, respectively, in different geographical areas.[2] This may indicate the contribution of environmental or genetic factors in the development of PBC, whereas the clinical profiles of PBC are thought to be similar between different ethnicities and/or different geographical areas, including European-descent and eastern Asian populations. The high concordance rate in monozygotic twins compared to dizygotic twins[3] and familial clustering of individuals with PBC indicate the involvement of strong genetic factors in the development of PBC; however, the pathogenesis of PBC is still poorly understood. Previous genome-wide association studies (GWASs) and subsequent meta-analyses have identified *HLA* and 21 non-HLA susceptibility loci (*IL12A* [MIM 161560], *IL12RB2* [MIM 601642], *STAT4* [MIM 600558], *IRF5* [MIM 607218], *IKZF3* [MIM 606221], *MMEL1* [MIM 120520], *SPIB* [MIM 606802], *DENND1B* [MIM 613292], *CD80* [MIM 112203], *IL7R* [MIM 146661], *CXCR5* [MIM 601613], *TNFRSF1A* [MIM 191190], *CLEC16A* [MIM 611303], *NFKB* [MIM 164012], *RAD51L1* [MIM 602948], *MAP3K7IP1* [MIM 602615], *PLCL2* [MIM 614276], *RPS6KA4* [MIM 603606], *TNFAIP2* [MIM 603300], 7p14, and 16q24) to PBC in individuals of European descent,[4–7] indicating the important role of several autoimmune pathways (i.e., IL12A signaling, TNF/TLR-NF-κB signaling, and B cell differentiation) in the development of PBC. However, GWASs for PBC have never been reported for ethnicities other than European descent, limiting our knowledge of the genetic architecture of PBC. Here, we conducted a GWAS for PBC in the Japanese population to identify host genetic factors related to PBC, which would not only expand our knowledge of pathogenic pathways in PBC but also lead to the development of rationale for therapies in the future.

Samples from 2,395 individuals (1,295 cases with PBC and 1,100 healthy volunteers working at the National Hospital Organization (NHO) in Japan as a medical staff who declared having no apparent diseases, including chronic liver diseases and autoimmune diseases [healthy controls]) were collected by members of the Japan PBC-GWAS Consortium, which consists of 31 hospitals participating in the NHO Study Group for Liver Disease in Japan (NHOSLJ) and 24 university hospitals participating in the gp210 Working Group in Intractable Liver Disease Research Project Team of the Ministry of Health and Welfare in Japan. Most of the case and control samples were collected from the mainland and the neighboring islands of Japan (Honshu, Kyushu, and Shikoku). Previous studies have shown that there is little genetic heterogeneity in resident populations in these areas.[8] In fact, the genetic inflation factor was close to 1.00, and only a small portion of the samples were identified as outliers in the principal component analysis. The cases were diagnosed with PBC if they met at least two of the following internationally accepted criteria:[9] biochemical evidence of cholestasis based mainly on alkaline phosphatase elevation, presence of serum anti-mitochondrial antibodies, histological evidence of non-suppurative destructive cholangitis, and destruction of interlobular bile ducts. The demographic details of PBC cases are summarized in Table S1, available online. Of the 487 PBC cases in the GWAS, 57 were male and 430 were female, ages ranged from 33 to 90 years, the median age was 66 years, 320 cases had early-stage PBC (a stage without any signs indicating portal hypertension or liver cirrhosis), 110 had late-stage PBC without jaundice (a stage with signs of portal hypertension or liver cirrhosis but without persistent jaundice), and 57 were at the late stage with jaundice (persistent presence of jaundice [total bilirubin >2 mg/dl]). Of the 476 healthy controls in the GWAS, 170 were male and 306 were female, ages ranged from 25 to 87 years, and the median age was 40. Of the 808 PBC cases in the replication set, 120 were male and 688 were female, ages ranged from 24 to 85 years, the median age was 61 years, 646 had early-stage PBC, 121 had late-stage PBC without jaundice, and 39 were at the late stage with jaundice. Of the 624 healthy controls in the replication set, 271 were male and 353 were female, ages ranged from 24 to 74 years, and the median age was 33 years. Concomitant autoimmune diseases are also shown in Table S1. As for inflammatory bowel diseases such as Crohn disease (CD, MIM 266600) and ulcerative colitis (UC, MIM 266600), only one out of 1,274 PBC cases had UC, but none had CD. DNA was extracted from whole peripheral blood with the QIAamp DNA Blood Midi Kit (QIAGEN, Tokyo).

For the GWAS, we genotyped 1,015 samples (515 Japanese PBC cases and 500 Japanese healthy controls) using the Affymetrix Axiom Genome-Wide ASI 1 Array, according to the manufacturer's instructions. After excluding three PBC samples with a Dish QC of less than 0.82, we recalled the remaining 1,012 samples (512 cases and 500 controls) using the Genotyping Console v4.1 software. Here, Dish QC represents the recommended sample quality control (QC) metric for the Axiom arrays.[10] Of the 600,000 SNPs embedded in the array, samples with an overall call rate of less than 97% were also excluded. As a result, 508 cases and 484 controls were subjected to further analysis. All samples used for GWAS passed a heterozygosity check, and no duplicated and related samples were identified in identity by descent testing. Moreover, principal component analysis found 29 outliers to be excluded via the Smirnov-Grubbs test

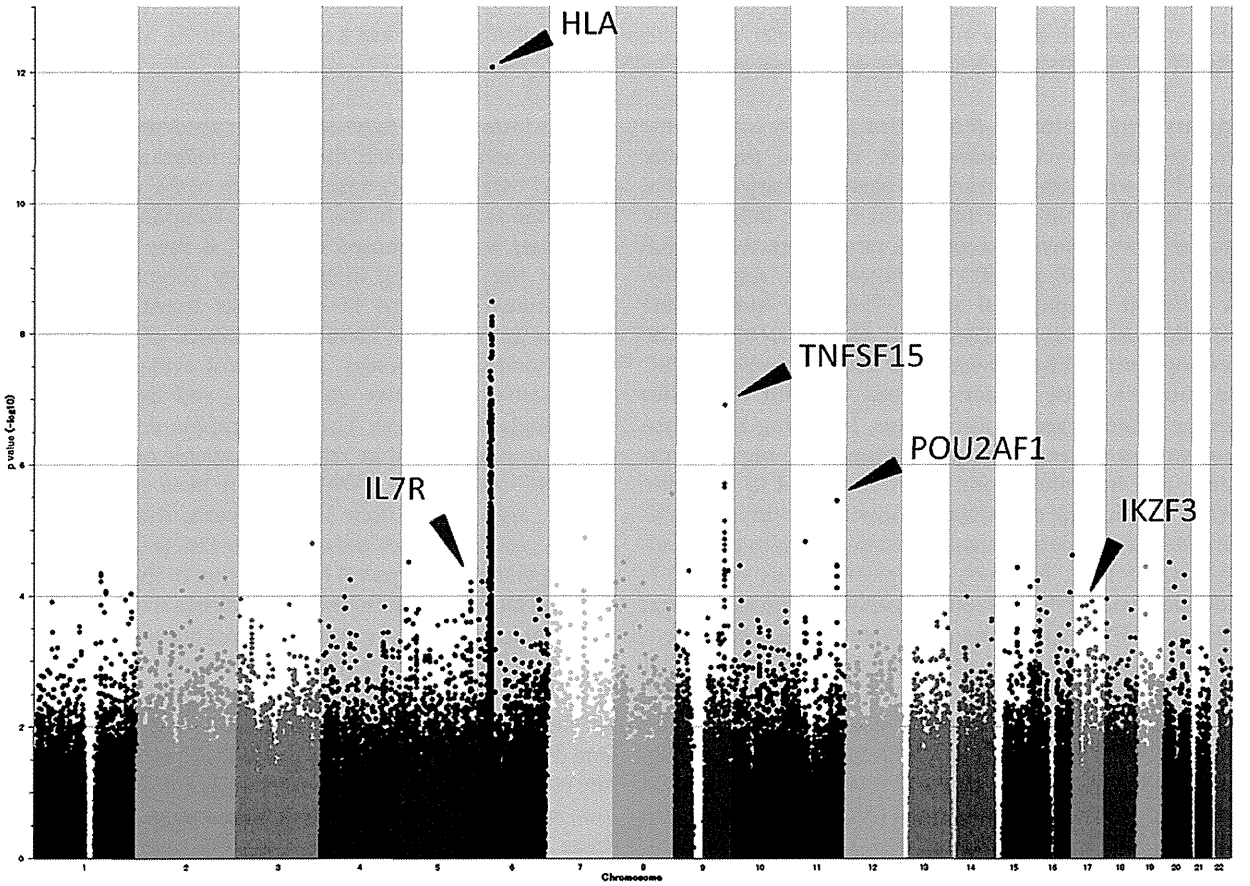

Figure 1. GWAS Results
From 963 samples (487 Japanese PBC cases and 476 Japanese healthy controls), p values were calculated with a chi-square test for allele frequencies among 420,928 SNPs.

and finally showed that all PBC cases (n = 487) and healthy controls (n = 476) formed a single cluster together with the HapMap JPT (Japanese in Tokyo from the CEPH collection), but not with CHB (Han Chinese in Beijing) samples (Figure S1, Table S2). These results indicate that the effect of population stratification was negligible. The average overall call rates of the remaining 487 PBC cases and 476 healthy controls were 99.38% (97.15–99.80) and 99.27% (97.01–99.81), respectively.[11] We then applied the following thresholds for SNP quality control during the data cleaning: SNP call rate $\geq$95%, minor allele frequency $\geq$5% in both PBC cases and healthy controls, and Hardy-Weinberg Equilibrium (HWE) p value $\geq$0.001 in healthy controls.[12] Of the SNPs on autosomal chromosomes and in the pseudoautosomal regions on the X chromosome, 420,928 and 317 passed the quality control filters and were used for the association analysis, respectively (Table S3). A quantile-quantile plot of the distribution of test statistics for the comparison of genotype frequencies in PBC cases and healthy controls showed that the inflation factor lambda was 1.039 for all the tested SNPs, including those in the HLA region, and was 1.026 when SNPs in the HLA region were excluded (Figures S2A and S2B). Table S4 shows the 298 SNPs with $p < 0.0001$ in the GWAS. All cluster plots for the SNPs with a $p < 0.0001$ from a chi-square test of the allele frequency model were checked by visual inspection, and SNPs with ambiguous genotype calls were excluded. For the GWAS and replication study, a chi-square test was applied to a two-by-two contingency table in an allele frequency model.

Figure 1 shows a genome-wide view of the single-point association data, which are based on allele frequencies. We found that the *HLA-DQB1* locus (MIM 604305) had the strongest association with susceptibility to PBC (rs9275175, odds ratio [OR] = 1.94; 95% confidence interval [CI] = 1.62–2.33, $p = 8.30 \times 10^{-13}$) (Figure 1 and Table S4); this finding was consistent with findings from previous studies.[4–7] In addition to the HLA class II region, loci *TNFSF15* and *POU2AF1* showed evidence indicative of association with PBC (rs4979462, OR = 1.63; 95% CI = 1.36–1.95, $p = 1.21 \times 10^{-7}$ for *TNFSF15*; rs4938534, OR = 1.53; 95% CI = 1.28–1.83, $p = 3.51 \times 10^{-6}$ for *POU2AF1*).

In a subsequent replication analysis, 27 SNPs with $p < 0.0001$ in the initial GWAS were also studied, in addition to SNPs at the *TNFSF15* and *POU2AF1* loci. Tagging SNPs were selected from the regions surrounding *TNFSF15* and

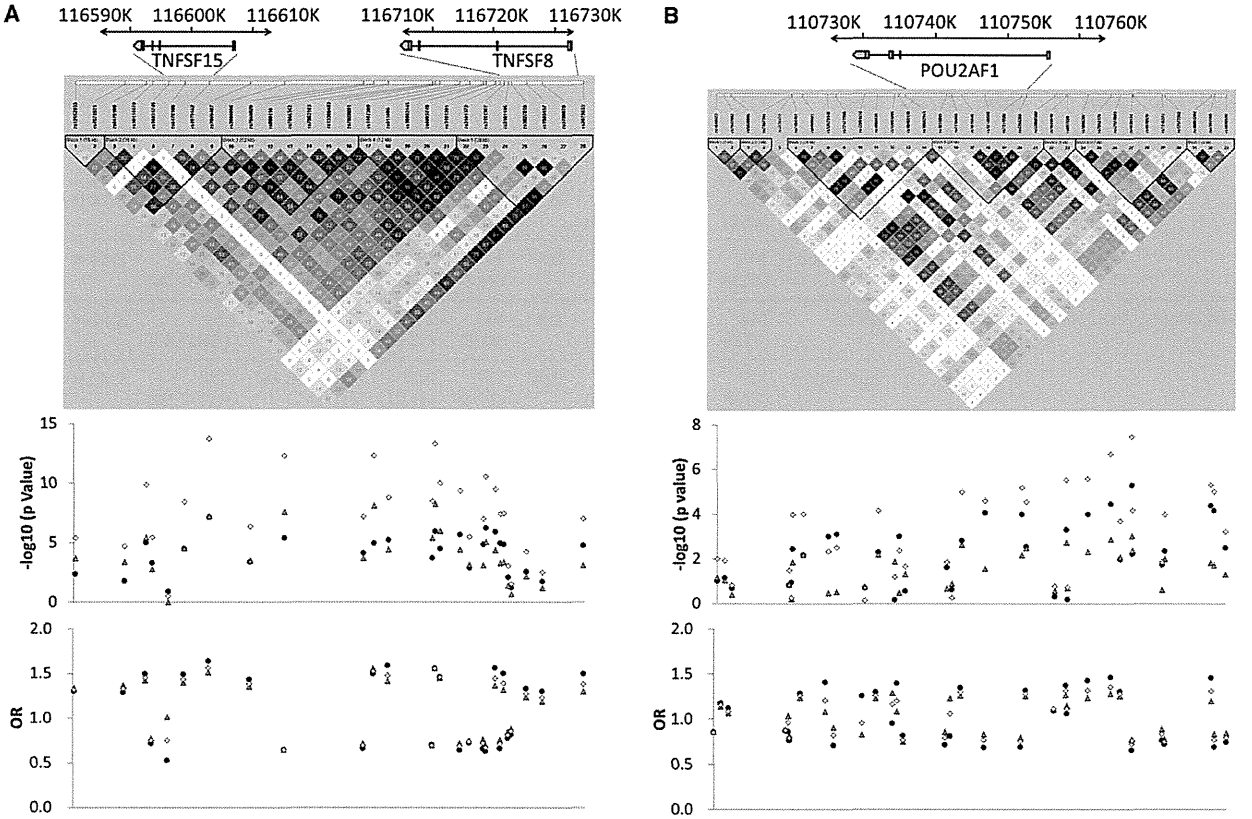

Figure 2. LD Structure, p Values, and OR Plots in the Association Analysis
LD maps (A) around *TNFSF15* (chr9: nucleotide position: 116561403–116733452; build 36.3) and (B) around *POU2AF1* (chr11: nucleotide position: 110684600–110802128; build 36.3). The middle panels show estimates of pairwise r^2 for (A) 28 SNPs and (B) 33 SNPs in the high-density mapping with a total of 2,365 samples used. The bottom panels show p values and OR-based chi-square tests for the allelic model for the left panels of 963 samples in the GWAS (●), the right panels of 1,402 samples in the replication study (▲), and the combined analysis (◇).

POU2AF1 (28 and 33, respectively) for high-density association mapping (Table S5, Figures 2A and 2B). For this follow-up replication analysis, an independent set of 1,402 samples (787 Japanese PBC cases and 615 Japanese healthy controls) and the original set of 963 samples (487 PBC cases and 476 healthy controls) were genotyped with the DigiTag2[13] and custom TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA, USA) on the LightCycler 480 Real-Time PCR System (Roche, Mannheim, Germany). The strongest associations identified in the initial GWAS were replicated in the independent set of 1,402 samples (OR = 1.52, $p = 5.79 \times 10^{-8}$ for rs4979462; OR = 1.29, $p = 9.32 \times 10^{-4}$ for rs4938534, Table 1). The combined p values were 2.84×10^{-14} (OR = 1.56; 95% CI = 1.39–1.76) for rs4979462 and 2.38×10^{-8} (OR = 1.39; 95% CI = 1.24–1.56) for rs4938534 (Table 1), both of which reached the genome-wide significance level of $p < 5 \times 10^{-8}$. In contrast, the other 27 weakly associated SNPs identified in the initial GWAS (p values <0.0001) were not found to have significant associations with PBC (Table S5). Moreover, no strongly associated SNPs were observed when comparing PBC cases between the early and late stages (Table S5).

A haplotype analysis of the *TNFSF15* and *POU2AF1* regions was conducted with the use of the genotype data from all 2,365 samples (1,274 PBC cases and 1,091 healthy controls). Linkage disequilibrium (LD) blocks were analyzed with Gabriel's algorithm,[14] and five blocks were observed in the *TNFSF15* region and seven blocks in the *POU2AF1* region (Figures 2A and 2B). There were no differences in the LD blocks between PBC cases and healthy controls. The risk haplotypes in each region showed a lower level of association than did the individual SNPs ($p = 8.26 \times 10^{-14}$ for *TNFSF15* and $p = 1.00 \times 10^{-4}$ for *POU2AF1*) (Tables S6 and S7).

Next, we focused on data from our initial GWAS in 21 loci that are reportedly associated with susceptibility to PBC in populations of European descent.[4–7] We found that three such loci (*IL7R*, *IKZF3*, and *STAT4*) had p values of less than 0.001 and eight other such loci (*RAD51L1*, *CXCR5*, *PLCL2*, *IL12RB2*, *NFKB1*, *CD80*, *DENND1B*, and 7p14) showed evidence of marginal associations ($p < 0.05$) in the initial GWAS in 487 Japanese PBC cases and 476 Japanese healthy controls (data not shown). We genotyped three SNPs (rs6890503 for *IL7R*, rs9303277 for *IKZF3*, and rs7574865 for *STAT4*) in an independent set

Table 1. ***TNFSF15*** **SNP rs4979462 and** ***POU2AF1*** **SNP rs4938534 Associated with Susceptibility to PBC**

dbSNP rsID	Nearest Gene	Risk Allele	Allele (1/2)	Stage	PBC Cases				Healthy Controls				OR[a]	
					11	12	22	RAF	11	12	22	RAF	95% CI	p Value[b]
rs4979462	*TNFSF15*	T	T/C	GWAS	154 (31.8)	244 (50.4)	86 (17.8)	0.57	98 (20.7)	230 (48.5)	146 (30.8)	0.45	1.63 (1.36–1.95)	1.21×10^{-7}
				Replication	253 (32.3)	390 (49.7)	141 (18.0)	0.57	131 (21.6)	305 (50.3)	170 (28.1)	0.47	1.52 (1.30–1.76)	5.79×10^{-8}
				Combined	407 (32.1)	634 (50.0)	227 (17.9)	0.57	229 (21.2)	535 (49.5)	316 (29.3)	0.46	1.56 (1.39–1.76)	2.84×10^{-14}
rs4938534	*POU2AF1*	A	G/A	GWAS	114 (23.6)	229 (47.3)	141 (29.1)	0.53	151 (31.8)	247 (52.0)	77 (16.2)	0.42	1.53 (1.28–1.83)	3.51×10^{-6}
				Replication	179 (22.8)	391 (49.8)	215 (27.4)	0.52	179 (29.4)	299 (49.2)	130 (21.4)	0.46	1.29 (1.11–1.50)	9.32×10^{-4}
				Combined	293 (23.1)	620 (48.9)	356 (28.1)	0.52	330 (30.5)	546 (50.4)	207 (19.1)	0.44	1.39 (1.24–1.56)	2.38×10^{-8}

Parenthetical numbers indicate the percentage of allele 11, 12, or 22 among total alleles in PBC cases or healthy controls. The following abbreviations are used: PBC, primary biliary cirrhosis; RAF, risk allele frequency; and GWAS, genome-wide association study.
[a]Odds ratio (OR) of minor allele from the two-by-two allele frequency table.
[b]p value of Pearson's chi-square test for the allelic model.

of 1,402 samples (787 Japanese PBC cases and 615 Japanese healthy controls) and the original set of 963 samples (487 PBC cases and 476 healthy controls) using the DigiTag2[13] and custom TaqMan SNP genotyping assays. Two SNPs, rs6890853 and rs9303277 located in loci *IL7R* and *IKZF3*, respectively, showed significant associations and the *STAT4* locus (rs7574865) showed suggestive association with PBC in 2,365 Japanese samples (1,274 PBC cases and 1,091 healthy controls) (rs6890853, combined p value = 3.66×10^{-8}, OR = 1.47 for *IL7R*; rs9303277, combined p value = 3.66×10^{-9}, OR = 1.44 for *IKZF3*; rs7574865, combined p value = 1.11×10^{-6}, OR = 1.35 for *STAT4*) (Tables S5 and S8).

Moreover, we genotyped 16 additional associated SNPs, all of which were the same SNPs as identified in previous studies,[4–7] and revealed that six out of 16 SNPs (located on *CXCR5*, *NFKB1*, *CD80*, *DENND1B*, *MAP3K7IP1*, and *TNFAIP2*) were replicated ($p < 0.05$) in 2,365 Japanese samples (Table S8). The SNP rs2293370, located in the *CD80* locus, showed a significant association and the *NFKB1* locus (rs7665090) showed a suggestive association with PBC in the Japanese population (rs2293370, combined p value = 3.04×10^{-9}, OR = 1.48 for *CD80*; rs7665090, combined p value = 1.42×10^{-7}, OR = 1.35 for *NFKB1*). Although further study for determining the primary SNP at each locus is necessary, the remaining ten loci (*RAD51L1*, *PLCL2*, *IL12RB2*, *IRF5*, *SPIB*, *RPS6KA4*, *CLEC16A*, *TNFRSF1A*, *IL12A*, and *MMEL1*) did not show significant association ($p < 0.05$) with PBC in the Japanese population (Table S8).

In the current GWAS in the Japanese population, we identified two significant susceptibility loci for PBC, *TNFSF15* (rs4979462) and *POU2AF1* (rs4938534), which had not been identified in the previous GWAS in populations of European descent. In addition, of the 21 PBC susceptibility loci that have been identified in populations of European descent, three loci (*IL7R*, *IKZF3*, and *CD80*) showed significant associations and two loci (*STAT4* and *NFKB1*) showed suggestive associations with PBC in the Japanese population. Eight other loci (*RAD51L1*, *CXCR5*, *PLCL2*, *IL12RB2*, *DENND1B*, *MAP3K7IP1*, *TNFAIP2*, and 7p14) also showed marginal associations with PBC in the Japanese population. These results indicate the presence of additional important disease pathways (via TNFSF15 and POU2AF1)—differentiation to T helper 1 (Th1) cells (via IL7R and STAT4), B cell differentiation (via IL7R and IKZF3), T cell activation (via CD80), and NF-κB signaling—in addition to the previously reported disease pathways in the development of PBC in Japanese populations.

TNFSF15 is a newly described member of the TNF superfamily that interacts with death receptor 3 (*DR3* [MIM 603366], also known as *TNFRSF25*) not only to promote effector T cell expansion (i.e., Th1 and Th17 cells) and cytokine production (i.e., interferon-γ [IFN-γ, MIM 147570]) at the site of inflammation, but also to induce apoptosis in cells that overexpress DR3.[15] Interestingly, genetic polymorphisms in *TNFSF15* are associated with susceptibility to CD, UC, ankylosing spondylitis (AS, MIM 106300), and leprosy (MIM 609888)[16–20] (Table S8). Strong association of five SNPs (rs3810936, rs6478108, rs6478109, rs7848647, and rs7869487) in the *TNFSF15* region with CD was first reported for a Japanese population,[16] and the finding was replicated in an independent Japanese population and in European-descent and Korean populations.[21–25] Another SNP within *TNFSF15* (rs4263839) is also associated with susceptibility to CD in populations of European descent.[17,20,26] In addition, the risk alleles of the SNPs were significantly associated with *TNFSF15* mRNA expression in peripheral blood.[27,28] Given that there exists strong LD among SNPs in *TNFSF15*, including those in the promoter region (rs6478109 and

rs7848647) and introns (rs4263839 and rs4979462), it is very probable that the PBC susceptibility haplotype containing rs4979462 also influences *TNFSF15* mRNA expression. Additionally, TNFSF15 signaling via DR3 synergizes with interleukin-12 (IL-12) and IL-18 to promote IFN-γ production.[15] The IL-12 signaling pathway includes *IL12A* and *IL12RB* (MIM 601604), variants of which have been identified as PBC susceptibility loci in previous GWASs of peoples of European ancestry, and has been implicated as a key player in the pathogenesis of PBC.[4–7] STAT4 is essential for IL-12 signal transduction via the IL-12 receptor (IL12R) for IFN-γ production and Th1 polarization.[29] Thus, the evidence that *TNFSF15* and *STAT4* were identified and confirmed as PBC susceptibility loci in the present study might indicate that the IL-12 signaling pathway via IL12R is also operative in PBC pathogenesis in Japanese populations, as it is in populations of European descent.

POU2AF1 is a B cell-specific transcriptional factor that coactivates octamer-binding transcriptional factors *POU2F1* (MIM 164175) and *POU2F2* (MIM 164176) on B cell-specific promoters; thus, *POU2AF1* is essential for B cell maturation and germinal center formation.[30] The E-twenty six transcription factor *Spi-B* was recently identified as a direct target of the coactivator *POU2AF1*.[31] *Spi-B* is an important mediator of both B cell receptor signaling and early T cell lineage decisions.[32,33] *Spi-B* also induces IL7R-induced CD40 (MIM 109535, MIM 300386) expression.[34] Given that *Spi-B* has been identified as a PBC susceptibility gene in previous GWASs of peoples of European ancestry,[6,7,35] variation of *POU2AF1* might function along with *Spi-B* in this pathway of B cell signaling and differentiation. The lack of *POU2AF1* reportedly prevents the development of autoimmunity in *Aiolos* (also known as *IKZF3*) mutant mice, which have a systemic lupus erythematosus (MIM 152700)-like phenotype, and in MRL-lpr mice.[36,37] *IKZF3* and *IL7R* were both replicated and confirmed as PBC susceptibility loci in this study; *IKZF3* functions as a transcription factor that participates in the generation of high-affinity bone marrow plasma cells responsible for long-term immunity, and *IL7R* participates in pre-B cell expansion.[38,39] Collectively, these results strengthen the notion that the B cell signaling pathway is involved in the development of PBC.

In conclusion, *TNFSF15* and *POU2AF1* were identified as significant susceptibility loci for PBC in a Japanese population. Our results provide further evidence for the presence of (1) ethnic differences in genetic susceptibility loci (i.e., *TNFSF15*, *IL12A*, and *IL12RB2*), (2) a new autoimmune pathway (i.e., TNFSF15 signaling) shared with other autoimmune diseases (CD, UC, and AS), and (3) common pathogenic pathways such as B cell differentiation (i.e., *POU2AF1*, *IKZF3*, and *SPIB*), IL-12 signaling (i.e., *IL12A*, *IL12RB2*, and *STAT4*), and T cell activation (i.e., *CD80*) for the development of PBC in individuals of European descent and Japanese individuals (Table S8). Functional analysis of these genetic loci, as well as the identification of additional susceptibility loci associated with PBC in eastern Asian populations, should facilitate the analysis of the pathogenesis of PBC worldwide and aid the development of rationale for therapies in the future.

Supplemental Data

Supplemental Data include two figures, eight tables, and Supplemental Acknowledgments and can be found with this article online at http://www.cell.com/AJHG/.

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Web Resources

The URLs for data presented herein are as follows:

MEXT Integrated GWAS Database, https://gwas.biosciencedbc.jp/cgi-bin/gwasdb/gwas_top.cgi
Online Mendelian Inheritance in Man (OMIM), http://www.omim.org

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