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Association Studies Article

ASSOCIATION STUDIES ARTICLE

Genome-wide association studies identify *PRKCB* as a novel genetic susceptibility locus for primary biliary cholangitis in the Japanese population

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Abstract

A previous genome-wide association study (GWAS) performed in 963 Japanese individuals (487 primary biliary cholangitis [PBC] cases and 476 healthy controls) identified *TNFSF15* (rs4979462) and *POU2AF1* (rs4938534) as strong susceptibility loci for PBC. In this study, we performed GWAS in additional 1,923 Japanese individuals (894 PBC cases and 1,029 healthy controls),

and combined the results with the previous data. This GWAS, together with a subsequent replication study in an independent set of 7,024 Japanese individuals (512 PBC cases and 6,512 healthy controls), identified *PRKCB* (rs7404928) as a novel susceptibility locus for PBC (odds ratio [OR] = 1.26, $P = 4.13 \times 10^{-9}$). Furthermore, a primary functional variant of *PRKCB* (rs35015313) was identified by genotype imputation using a phased panel of 1,070 Japanese individuals from a prospective, general population cohort study and subsequent *in vitro* functional analyses. These results may lead to improved understanding of the disease pathways involved in PBC, forming a basis for prevention of PBC and development of novel therapeutics.

Introduction

Primary biliary cholangitis (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 high concordance rate in monozygotic twins compared to dizygotic twins and familial clustering of PBC patients indicates the involvement of strong genetic factors in the development of PBC (2). Previous genome-wide association studies (GWAS), immunochip analyses, and subsequent meta-analyses in populations of European descent (3–9) identified *HLA* and 25 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) - *SOCS1* (MIM603597), *NFKB1* (MIM 164012) - *MANBA* (MIM 609489), *RAD51L1* (MIM 602948), *MAP3K7IP1* (MIM 602615), *PLCL2* (MIM 614276), *RPS6KA4* (MIM 603606), *TNFAIP2* (MIM 603300), *ELMO1* (MIM 606420), *IRF8* (MIM 601565), *TNFSF11* (MIM 602642), *SH2B3* (MIM 605093), *CRHR1* (MIM 122561), and *TYK2* (MIM 176941)] for PBC. Among these susceptibility genes, *IL12A* and *IL12RB2* exhibited the strongest association with PBC, highlighting the importance of T-cell differentiation into Th1/Th17 cells via IL12/IL12R signalling in the development of PBC. Furthermore, an international genome-wide meta-analysis on European descents, which was recently performed in a total of 2,764 PBC cases and 10,475 controls followed by a validation study using 3,716 PBC cases and 4,261 controls, identified six new risk loci for PBC on chromosome 2q12.1, 2q36.3, 4p16.3, 5q21.1, 5q33.3, and 6q23.3 (10). These results indicate the involvement of formation and function of mucosal lymphoid tissues (via CCL20 and CCR6) and reinforce the importance of IL12 and JAK-STAT signalling in the development of PBC in European descents (10).

On the other hand, the first GWAS performed in the Japanese population (11) identified two susceptibility loci for PBC, *TNFSF15* (MIM 604052) and *POU2AF1* (MIM 601206), which were not identified in European populations. A subsequent replication study in Japanese subjects also identified five susceptibility loci previously identified in people of European descent: *IL7R*, *IKZF3*, and *CD80* at a genome-wide significance level (P-value $\leqq 5.0 \times 10^{-8}$) and *STAT4* and *NFKB1* at a sub-genome-wide significance level ($5.0 \times 10^{-8} < P \leqq 5.0 \times 10^{-6}$). By contrast, *IL12A* and *IL12RB2*, the most significant susceptibility genes outside the *HLA* region in European cohorts, were not replicated in the Japanese population. Thus, although there are ethnic differences in genetic susceptibility loci to PBC, common disease pathways are shared in both European descent and Japanese population: Th1/Th17 differentiation of T cells (via CD80, IL12A, IL12RB2, STAT4, TNFSF15, TYK2, and SOCS1) and B-cell differentiation into plasma cells (via IL7R, CXCR5, POU2AF1, SPIB, and IKZF3) (12). In a recent replication study of PBC susceptibility genes in the Han Chinese population, *TNFSF15* and *CD80* were replicated at a genome-wide significance level, but *POU2AF1*, *IL12A*, and *IL7R* were not replicated, indicating that Han Chinese patients share common genetic susceptibility genes with Japanese and European populations but still exhibit a distinct genetic susceptibility profile (13).

To further dissect the genetic architecture of PBC in the Japanese population, we conducted a GWAS in additional 1,923 Japanese individuals (894 PBC cases and 1,029 healthy controls) and combined the data with those of the previous GWAS which was performed using the same genotyping array of Affymetrix Axiom Genome-Wide ASI in 963 Japanese individuals (487 PBC cases and 476 healthy controls) (11). This GWAS, together with a subsequent replication study in an independent set of 7,024 Japanese individuals (512 PBC cases and 6,512 healthy controls) identified PRKCB (MIM 176970) as a novel susceptibility locus for PBC at a genome-wide significance level. In addition, a primary variant of *PRKCB* (rs35015313) was identified by genotype imputation using a phased panel of 1,070 Japanese individuals from a prospective, general population cohort study (14) and subsequent *in vitro* functional analyses. This study not only expands our knowledge of disease pathways involved in PBC, but may also facilitate the development of preventive measures or therapies for PBC in the future.

Results

GWAS

We genotyped 1,929 samples (896 PBC cases and 1,033 healthy controls) using the Affymetrix Axiom Genome-Wide ASI 1 Array. No samples were excluded by Dish QC (< 0.82) or overall call rate for a total of 600K SNPs (< 0.97), which were calculated using the Genotyping Console™ Software v4.1. All samples passed a heterozygosity check. The average overall call rate was 0.9939 (0.9710–0.9981) in PBC cases and 0.9951 (0.9710–0.9986) in healthy controls. Next, we added the genotyping data of the previous GWAS which was performed using Affymetrix Axiom Genome-Wide ASI 1 Array (487 PBC cases and 476 healthy controls) (11) to that of the current GWAS (896 PBC cases and 1,033 healthy controls). One related sample was identified in identity-by-descent testing and removed from further analysis. The demographics of PBC cases and controls used for the present association analysis are shown in Supplementary Material, Table S1.

Principal component analysis was performed using 1,383 PBC cases, 1,508 healthy controls, and HapMap samples (43 JPT, 40 CHB, 91 YRI, and 91 CEU samples). Two samples (one PBC case and one control) were located in the CHB cluster, which was drawn using the first and second components. After excluding these two samples, 1,382 PBC cases and 1,507 healthy controls formed the same cluster, along with the HapMap-JPT samples (Supplementary Material, Fig. S1A and B), indicating that the effect of population stratification (*i.e.* mainland Honshu vs. neighbouring islands Kyushu and Shikoku) was negligible in the subjects studied (15).

During data cleaning, we applied the following thresholds for SNP quality control: SNP call rate ≥ 95% and minor allele

frequency (MAF) ≥ 5% in both PBC cases and healthy controls, and Hardy-Weinberg equilibrium (HWE) P-value ≥ 0.001 in healthy controls (16). Of the SNPs on autosomal chromosomes, 425,290 SNPs passed the thresholds for SNP quality control and were used for the association analysis (Supplementary Material, Table S2). At this stage, three samples (one PBC case and two healthy controls) showed call rates less than 97% for a total of 425,290 SNPs. Thus, 1,381 PBC cases and 1,505 healthy controls were ultimately used for the association study (Supplementary Material, Table S1).

A quantile–quantile plot of the distribution of test statistics for the comparison of allele frequencies in PBC cases and healthy controls showed that the inflation factor lambda was 1.079 for all tested SNPs including those in the *HLA* region (from *HLA-F* to *KIFC1*; chr.6: 29,645,000–33,365,000; 3.72Mb [hg19]) (Supplementary Material, Fig. S2A), and 1.062 when SNPs in the *HLA* region were excluded (Supplementary Material, Fig. S2B). LD score regression intercept (17) was 1.021 (SE 0.0124) for the dataset with all tested SNPs.

Supplementary Material, Table S3 shows the 271 SNPs with $P < 0.0001$ in the GWAS in which the *HLA* region was excluded. Regional Manhattan plots including 14,017 SNPs on chromosome 6p, 4,007 SNPs in the *HLA* region, and 1,177 SNPs in the surrounding region of the top-hit SNP (chr.6: 32,062,000–32,735,000; 673 kb [hg19]) are illustrated in Supplementary Material, Fig. S3A,B, and C, respectively. All cluster plots for the SNPs with $P < 0.0001$ in a chi-square test of the allele frequency model were checked by visual inspection, and SNPs with ambiguous genotype calls were excluded.

Figure 1 shows a genome-wide view of the single-point association data based on allele frequencies. Consistent with the previous study (11), rs3129887 (odds ratio [OR] = 3.13, 95% confidence interval [CI] = 2.54–3.85, $P = 2.04 \times 10^{-29}$), which is located in intron 2 of *HLA-DRA* (MIM 142860) and is also 79 kb downstream of *HLA-DRB1* (MIM 142857), showed the strongest association with PBC. The loci *TNFSF15*, *POU2AF1*, *IL7R*, and *IKZF3* also showed significant associations with PBC, as reported in the previous GWAS performed in the Japanese population (*TNFSF15* rs4979462: OR = 1.60, 95% CI = 1.44–1.78, $P = 7.98 \times 10^{-19}$; *POU2AF1* rs4938534: OR = 1.35, 95% CI = 1.22 –1.50, $P = 1.37 \times 10^{-8}$; *IL7R* rs6897932: OR = 1.52, 95% CI = 1.32–1.75, $P = 5.72 \times 10^{-9}$; *IKZF3* rs9303277: OR = 1.43, 95% CI = 1.29–1.60, $P = 8.37 \times 10^{-11}$) (Supplementary Material, Table S3). In addition to these regions, loci *PRKCB* and *ETS1* showed the evidence of association with PBC (*PRKCB* rs3785396: OR = 1.35, 95% CI = 1.21–1.50, $P = 7.10 \times 10^{-8}$; *ETS1* rs12574073: OR = 1.33, 95% CI = 1.20–1.48, $P = 1.09 \times 10^{-7}$) (Supplementary Material, Table S3). The SNPs which showed the lowest P-value in *IL12A and STAT4* loci were rs644587 ($P = 1.49 \times 10^{-6}$) and rs7574865 ($P = 2.75 \times 10^{-5}$), respectively, in the Japanese population (Supplementary Material, Table S3). In addition, the SNPs which showed the lowest P-value in *CD80* (annotated as *C3orf1* in the present study) *and NFKB1* loci was *rs2293370* ($P = 1.40 \times 10^{-6}$) and *rs230534* ($P = 1.50 \times 10^{-7}$), respectively, in the Japanese population (Supplementary Material, Table S3). A genome-wide set of summary association statistics will be available at the National Bioscience Database Center (NBDC): http://humandbs.biosciencedbc.jp/en/; date last accessed December 4, 2016.

Replication study for candidate SNPs

Fine mapping of the surrounding regions of *PRKCB* rs3785396 and *ETS1* rs12574073 was first performed by DigiTag2 method (11) using 58 and 96 Tag SNPs, respectively (data not shown). This mapping revealed that *PRKCB* rs7404928 (linkage disequilibrium [LD] with rs3785396: $r^2 = 0.87$) and *ETS1* rs12575600 (LD with rs12574073: $r^2 = 0.99$) were the most significant SNPs for susceptibility to PBC (data not shown). Therefore, two new candidates (*PRKCB* rs7404928 and *ETS1* rs12575600) were subjected to a replication study using an independent set of 7,024 Japanese individuals (512 PBC cases and 6,512 controls). The

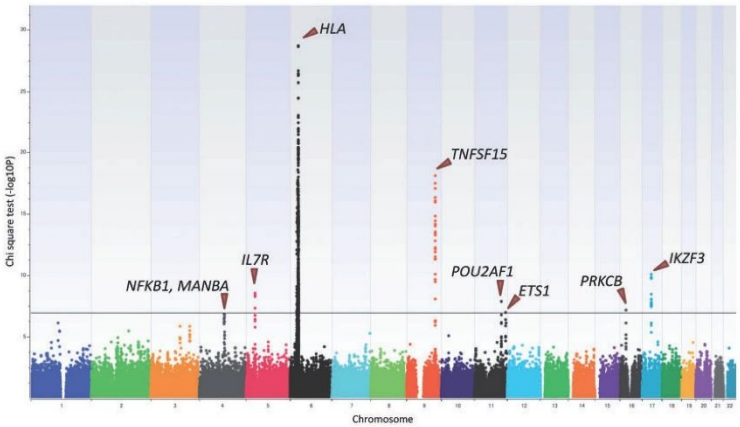

Figure 1. Genome-wide Manhattan plot. P-values were calculated for each SNP from data of 2,886 samples (1,381 PBC cases and 1,505 healthy controls) with a chi-square test for allele frequencies among 425,290 SNPs that passed a quality control. A threshold line is shown at 6.93 ($-\log_{10} P$ [0.05/425,290]).

Table 1. rs7404928 associated with susceptibility to PBC

dbSNP rsID	Nearest Gene	Risk Allele	Allele (1/2)	Stage	PBC cases					Healthy Controls					OR[a]	
					11	12	22	Total	RAF	11	12	22	Total	RAF	95% CI	P-value[b]
rs7404928	*PRKCB*	T	T/C	GWAS	648	559	160	1367	0.678	574	685	243	1502	0.610	1.35	8.10×10^{-8}
					0.474	0.409	0.117			0.382	0.456	0.162			(1.21–1.50)	
				Replication	236	223	51	510	0.681	2589	3050	871	6510	0.632	1.25	1.78×10^{-3}
					0.463	0.437	0.100			0.398	0.469	0.134			(1.09–1.43)	
				Combined	884	782	211	1877	0.679	3163	3735	1114	8012	0.628	1.26	4.13×10^{-9}
					0.471	0.417	0.112			0.395	0.466	0.139			(1.16–1.35)	

Parenthetical numbers indicate the percentage of genotype 11, 12, or 22 among all genotypes in PBC cases or healthy controls. The following abbreviations are used: PBC, primary biliary cholangitis; RAF, risk allele frequency; and GWAS, genome-wide association study.

[a]Odds ratio (OR) of minor allele from two-by-two allele frequency table.

[b]P-value of Pearson's chi-square test for allelic model.

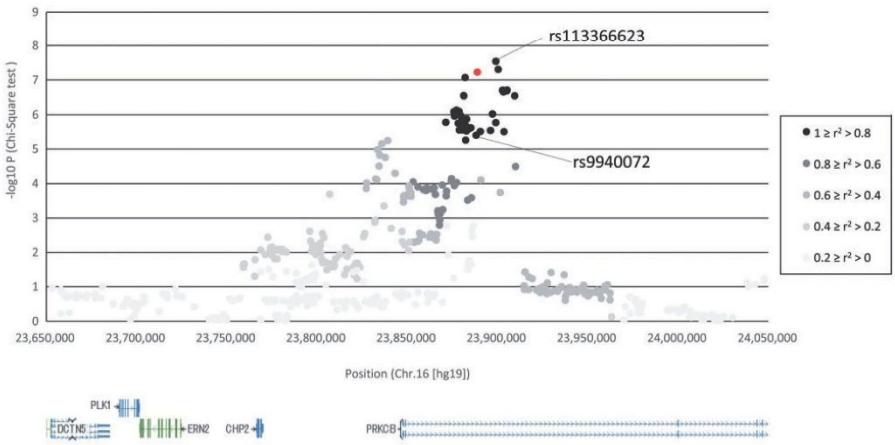

Figure 2. SNP imputation analyses for the surrounding region of *PRKCB* rs7404928 (Chr.16: 23,650,000–24,050,000 [hg19]). High-density association mapping was performed by genotype imputation using a phased reference panel of 1,070 Japanese individuals (14). The differences in allele frequencies were evaluated using Pearson's chi-square test (2 × 2 contingency tables). Each dot shows the *P*-value of each SNP. The dot colour (black to light gray) indicates the strength of LD with rs7404928 (red dot). Pairwise LD is reported as *r*-squared.

combined *P*-values of the GWAS data set and the replication study data set were 4.13×10^{-9} (OR = 1.26, 95% CI = 1.16–1.35) for *PRKCB* rs7404928 which surpassed the genome-wide significance level (Table 1). The combined *P*-value for *ETS1* rs12575600 became less significant, $P = 1.02 \times 10^{-4}$ (OR = 1.15, 95% CI = 1.07–1.24).

Imputation-based high-density association mapping

Imputation-based high-density association mapping was subsequently performed using a phased reference panel of 1,070 Japanese individuals (14) and IMPUTE2 (ver.2.3.1) (18) for the region surrounding *PRKCB* rs7404928, which showed the genome-wide significant association with PBC. The strongest associations were observed in intron 2 of *PRKCB* rs113366623 (Fig. 2).

Identification of the primary functional variants in the *PRKCB* locus by *in silico/in vitro* analysis

Among 11 SNPs that exhibited strong LD ($r^2 > 0.9$) with *PRKCB* rs113366623 in the 1,000 Genomes project Asian panel (1000 Genomes ASN) (Supplementary Material, Table S4), rs7404928 and rs35015313 are located in transcription regulatory elements (as reflected by DNase hypersensitivity site and H3K27Ac marks). The effects of these SNPs in 'promoter histone marks', 'enhancer histone marks', 'DNase hypersensitivity', or 'transcription factor motif change' which are shown in HaploReg v4.1 database (http://www.broadinstitute.org/mammals/haploreg/haploreg.php; date last accessed December 4, 2016.) also supported these evidences. Therefore, we presumed that binding affinities of transcription factors and transcription efficiency might differ between the major and minor alleles of these SNPs. To explore a set of important

causal configulations surrounding rs113366623, the fine-mapping was also performed using FINEMAP (19). In this analysis, rs7404928 but not rs35015313 was listed in the higher rank (Supplementary Material, Table S5).

To identify the primary functional variants in the *PRKCB* locus, we performed an electrophoretic mobility shift assay (EMSA) using the human T-cell line Jurkat or human liver cell line HepG2, in which the *PRKCB* protein product is abundantly expressed (Supplementary Material, Fig. S4). A difference in mobility shift between the major and the minor alleles of rs35015313, but not of rs7404928, was detected in both Jurkat (Supplementary Material, Fig. 3A) and HepG2 (Supplementary Material, Fig. S5). In order to further analyse the differences in transcription efficiency between the major and the minor alleles of rs35015313, luciferase assay was performed. The luciferase activities were significantly higher in Jurkat and HepG2 cells transfected with pGL4.23 vector containing PBC-susceptibility allele (*PRKCB* rs35015313-TTG) than in cells transfected with the minor allele (*PRKCB* rs35015313-del) ($P < 0.005$ and $P < 0.05$, respectively, Student's *t*-test; Fig. 3B and C, and Supplementary Material, Fig. S6). A similar tendency was observed in the human bile duct cell line, HuCCT1, after transfection with the same vectors (Supplementary Material, Fig. S6).

To assess the influence of rs35015313 on the endogenous *PRKCB* expression, *PRKCB* mRNA expression levels were compared among genotypes of *PRKCB* rs9940072 which showed relatively high r^2 value with rs35015313 ($r^2 = 0.94$ in the 1000 Genomes ASN) using the GTEx portal database (http://gtexportal.org/home/; date last accessed December 4, 2016.) (20). In accordance with the results of our *in vitro* functional assays, the endogenous expression level of *PRKCB* was significantly higher in whole-blood with the PBC susceptible genotype of rs9940072 than in those with other genotypes of rs9940072 ($P = 0.0049$, effect-size = 0.073) (Fig. 3D).

Pathway analysis

Pathway analysis (excluding HLA), which was performed using DEPICT (21), indicated the involvement of regulation for inflammatory response, defense response and autoimmune response in the development of PBC (Supplementary Material, Table S6).

Discussion

In the current GWAS conducted in the Japanese population, we identified a novel genetic susceptibility locus for PBC, *PRKCB* rs7404928, which was not identified in previous GWAS for PBC in the European and Japanese populations (3–11). We also identified a functional variant of *PRKCB* rs35015313 that alter its gene expression level. These results suggest a role for novel disease pathways that act via PRKCB in addition to the previously reported immune-related disease pathways such as T-cell differentiation into Th1/TH17 cells (via CD80, IL12A, IL12RB2, STAT4, NFKB1, TNFSF15, TYK2, SOCS1) and B-cell differentiation into plasma cells (via IL7R, NFKB1, CXCR5, IKZF3, SPIB1, POU2AF1), in the development of PBC (12,22,23).

PRKCB is a member of the protein kinase C gene family encoded by the *PRKCB1* or *PRKCB2* gene, which consists of 18 exons spanning 375 kb on chromosome 16p (24). PRKCB is a calcium-activated, phospholipid- and diacylglycerol-dependent serine/threonine-protein kinase involved in various cellular processes such as regulation of the B-cell receptor (BCR) signalosome,

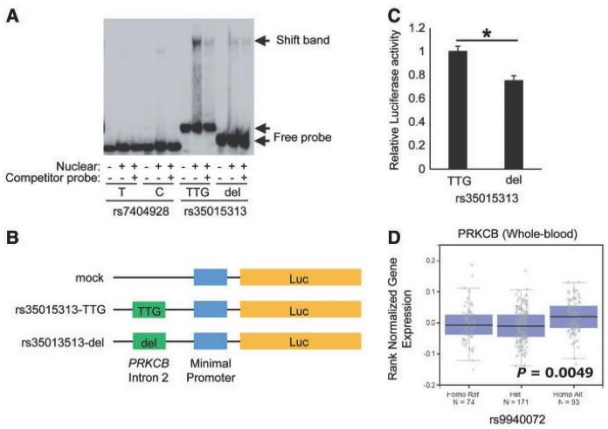

Figure 3. Identification of the primary functional variant in the *PRKCB* locus by *in vitro* functional analysis and e-QTL analysis. (**A**) An Electrophoretic Mobility Shift Assay (EMSA) of candidate primary functional variants using nuclear extract of human T-cell line Jurkat and biotin-labelled probes corresponding to each allele. Rs35015313 showed a difference in mobility shift between major allele and minor allele. An unlabelled probe was used as a competitor. (**B**) Plasmid constructs of fragments of the genomic DNA of the *PRKCB* locus used for transfection. Part of the 2nd intron containing rs35015313 were subcloned into the pGL4.23 vector. (**C**) The transcriptional enhancing activity of these plasmid constructs was measured by assay of luciferase (luc) activity of the transfected human T-cell line Jurkat 24 h after transfection. Cells transfected with the susceptible allele of rs35015313 showed enhanced luciferase activities compared to the minor allele. Values of relative luciferase activity are shown as means ± SD. *$P < 0.005$ (Student's *t*-test). (**D**) Difference in the endogenous expression level of *PRKCB* among people with *PRKCB* rs9940072 ($r^2 = 0.94$ with rs35015313 in 1000 Genomes ASN) genotypes in the whole-blood. The data of endogenous *PRKCB* expressions were extracted from GTEx portal database (20).

oxidative stress–induced apoptosis, androgen receptor–dependent transcription regulation, insulin signalling, and endothelial cell proliferation, autophagy, and energy metabolism (24–26). Genetic polymorphism of *PRKCB* is associated with various diseases or pathophysiological conditions, including end-stage renal disease in patients with type 2 diabetes (27), α2-adrenoreceptor–mediated vasoconstriction (28), lower levels of 25-hydroxyvitamin D and relapse in multiple sclerosis (29), depressive and anxiety symptoms in post-partum women (30), and systemic lupus erythematosus (SLE) (31). However, most of these associations were identified by a candidate gene approach, and their significance levels were not robust, necessitating further replication studies. Only one disease, SLE, is significantly associated with *PRKCB* rs16972959 ($P = 1.35 \times 10^{-9}$) at a genome-wide significance level in the Chinese Han population (31), implying that PRKCB might be involved in the development of SLE via the NFκB pathway.

PBC-susceptibility SNPs identified in this study (*i.e.* *PRKCB* rs35015313) are located in intron 2 of *PRKCB*, where the SLE-susceptibility SNP rs16972959 is also located. Although the pairwise LD between *PRKCB* rs16972959 and *PRKCB* rs35015313 is not strong ($r^2 = 0.46$) in the Japanese population, according to our genotyping data for 1,070 Japanese individuals (14), it is possible that rs35015313, a primary PBC-susceptibility SNP that influences the gene expression of *PRKCB*, is also involved in the development of SLE in Chinese Han via similar disease pathways with PBC in the Japanese population.

PRKCB is required for lupus development in SLE mice (32). PRKCB plays a key role in B-cell activation by regulating BCR-induced NFκB activation (33), and missense mutation in prkcb (S552P) causes selective deficits in T cell–independent antibody responses against polysaccharides in mice, leading to diminished serum IgM and IgG3 levels (34). PRKCB is also involved in energy homeostasis via regulation of mitochondrial function (26), and it is a key regulator of autophagy via the mitochondrial axis (25). These findings may indicate that PRKCB is involved not only in B-cell activation and differentiation, but also in energy homeostasis and autophagy via the mitochondria axis, in the development of PBC. Further studies are needed to clarify the role of PRKCB-mediated pathways in the development of PBC.

IL12A and *STAT4* constitute the most significant PBC-susceptibility genes in European descent. It is therefore very interesting that *IL12A* and *STAT4* are identified at the suggestive level of significance ($P = 1.49 \times 10^{-6}$ and $P = 2.75 \times 10^{-5}$, respectively) in the present study. Although further analysis is needed to confirm the significance of these genes in validation cohort in the Japanese population, these results may indicate that IL12A - STAT4 signalling pathway is also involved in the development of PBC in addition to TNFSF15-mediated pathways of T cell differentiation in the Japanese population. In addition, pathway analysis by DEPICT using the present GWAS-dataset indicated that the pathways of regulation for inflammatory-, defense- and autoimmune-responses are involved in the development of PBC. This may support the importance in PBC of non-specific immune- pathways which are shared with many other autoimmune diseases. Further studies are needed to identify pathways specific for PBC.

In conclusion, the current genome-wide association study of PBC in the Japanese population identified one novel PBC-susceptibility gene, *PRKCB*. Thus, *TNFSF15*, *POU2AF1* and *PRKCB* constitute a significant genetic structure for susceptibility to PBC, which is a distinctive feature in the Japanese population as compared to European descents. The ethnic differences in the disease-susceptibility genes between European and East Asian populations may provide an important opportunity for dissection of disease pathways that will be useful for the development of preventive methods and therapeutics for PBC in the future.

Materials and Methods

Samples for GWAS stage

DNA samples for GWAS were collected from 1,929 individuals (896 PBC cases and 1,033 healthy controls) in the PBC-GWAS Consortium, consisting of the National Hospital Organization (NHO) Study Group for Liver Disease in Japan (NHOSLJ) and the gp210 Working Group in Intractable Liver Disease Research Project Team of the Ministry of Health and Welfare in Japan, as previously described (11).

The cases were diagnosed with PBC if they met at least two of the following internationally accepted criteria (1): biochemical evidence of cholestasis, based mainly on alkaline phosphatase elevation; presence of serum anti-mitochondrial antibodies; or histological evidence of non-suppurative destructive cholangitis and destruction of interlobular bile ducts. Demographic details of all PBC cases enrolled in the previous ($n = 487$) and present ($n = 894$) GWAS are summarized in Supplementary Material, Table S1. Of the 1,381 PBC cases, 1,199 (86.8%) were female; age ranged from 23 to 93 years; median age was 58 years. Of the 1,505 healthy controls enrolled in the previous ($n = 476$) and present ($n = 1,029$) GWAS, 840 (55.8%) were female.

GWAS

Genomic DNA was extracted from the whole peripheral blood of cases and controls using the QIAamp DNA Blood Midi Kit (Qiagen, Tokyo, Japan). DNA samples from 1,929 Japanese individuals (896 PBC cases and 1033 healthy controls) were genotyped as previously described (11) using the Affymetrix Axiom Genome-Wide ASI 1 Array. SNP filtering for statistical analysis was performed as indicated in Supplementary Material, Table S2. Data shown in this manuscript are a combination of the data obtained in the GWAS performed for this study and those obtained in the previous GWAS (487 PBC cases and 476 healthy controls) (11).

Replication study

A replication study was performed in an independent set of 512 PBC cases and 2,080 controls for two SNPs (*PRKCB* rs7404928 and *ETS1* rs12575600) newly identified in the GWAS stage ($P < 5.00 \times 10^{-7}$) using the DigiTag2 and custom TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA, USA) on a LightCycler 480 Real-Time PCR System (Roche, Mannheim, Germany) (11). Of the 512 PBC cases in the replication set, 439 (85.7%) were female; age ranged from 25 to 102 years; and median age was 62 years. In the replication set of 2,080 controls, 57.9% were female. The general population data of allele frequency for these two SNPs, which were obtained by genotyping with Illumina Human OmniExpressExome BeadChips in 4,432 Japanese individuals (female 66.5%) from the cohort study in the Tohoku Medical Megabank Organization (ToMMo), were also used as data from Japanese healthy controls. Thus, a total of 6,512 (63.8% female) Japanese individuals were included as controls in the replication set.

Genotype imputation

SNP genotypes of PBC cases ($n = 1,381$) and healthy controls ($n = 1,505$) that passed SNP filtering were phased with SHAPEIT2 (v2.r644) (35). The following options were used for SHAPEIT2: –burn 10, –prune 10, and –main 25. Genotype imputation was performed on the phased genotypes with IMPUTE2 (ver. 2.3.1) (18) using a phased reference panel of 1,070 Japanese individuals from a prospective, general population cohort study performed by the ToMMo (14). The panel contains 31,840,587 autosomal SNVs and short indel variants obtained as follows: sequencing data sets from HiSeq 2500 were aligned with Bowtie2 (version 2.1.0) (36), variant calling was performed on the aligned data with Bcftools (ver. 0.1.17-dev) (37), and called variants with *P*-value of HWE test $< 1.00 \times 10^{-4}$ were excluded. For IMPUTE2, the following options were used: -Ne 2000, -k_hap 1000, -k 120, -burnin 15, and -iter 50. In addition, fine-mapping was performed with FINEMAP v1.0 to explore causal variants (19).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were obtained from T-cell line Jurkat or human liver cell line HepG2 (Active Motif, Carlsbad, CA). The EMSA was performed by using a LightShift Chemiluminescent EMSA Kit (Thermo-Fisher Scientific, Waltham, MA), according to the manufacturer's instructions, using biotin-labelled double-stranded oligonucleotide probes corresponding to each major and minor allele. The sequences of the oligonucleotide probes are shown in Supplementary Material, Table S7. Nuclear extract (2.5 μg/ml) and the biotin-labelled probes (10 fmol/μl) were incubated for 30 min at 25°C.

Luciferase assay

Partial 2nd intron of *PRKCB* was amplified from human genomic DNA by using PCR with PrimeSTAR-GXL (TAKARA, Tokyo, Japan) and specific primers (Supplementary Material, Table S8). The PCR products of 2nd intron were subcloned into the reporter gene pGL4.23 (luc2/minP) vector (Promega, Madison, Wis). The pGL4.74 [hRluc/TK] vector was used to normalize for variations in transfection efficiency. These plasmids (pGL4.23 vector: 500 ng; pGL4.74 vector: 50 ng) were transfected into Jurkat T cells, HepG2, or HuCCT1 by using Lipofectamine 3000 reagents (Thermo-Fisher Scientific). Luciferase activities were determined by using the Dual-Luciferase Reporter Assay system (Promega) as described elsewhere (38).

eQTL

The data of the correlation between the *PRKCB* rs9940072 genotype and *PRKCB* expression was available at the GTEx portal database (http://gtexportal.org/home/; date last accessed December 4, 2016) (20).

Pathway analysis

Pathway analysis was performed by using DEPICT (21). SNPs that are with *P*-value $\leq 1 \times 10^{-5}$, included in DEPICT target SNP set, and not in the HLA region were extracted from the GWAS results, and applied to DEPICT after LD-based clumping by using Plink with the following option settings: –clump-p1 1e-5, –clump-r2 0.50, and –clump-kb 500.

Web resources

The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.omim.org; date last accessed December 4, 2016.

MEXT Integrated Database, https://gwas.lifesciencedb.jp/cgi-bin/gwasdb/gwas_top.cgi; date last accessed December 4, 2016.

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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