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

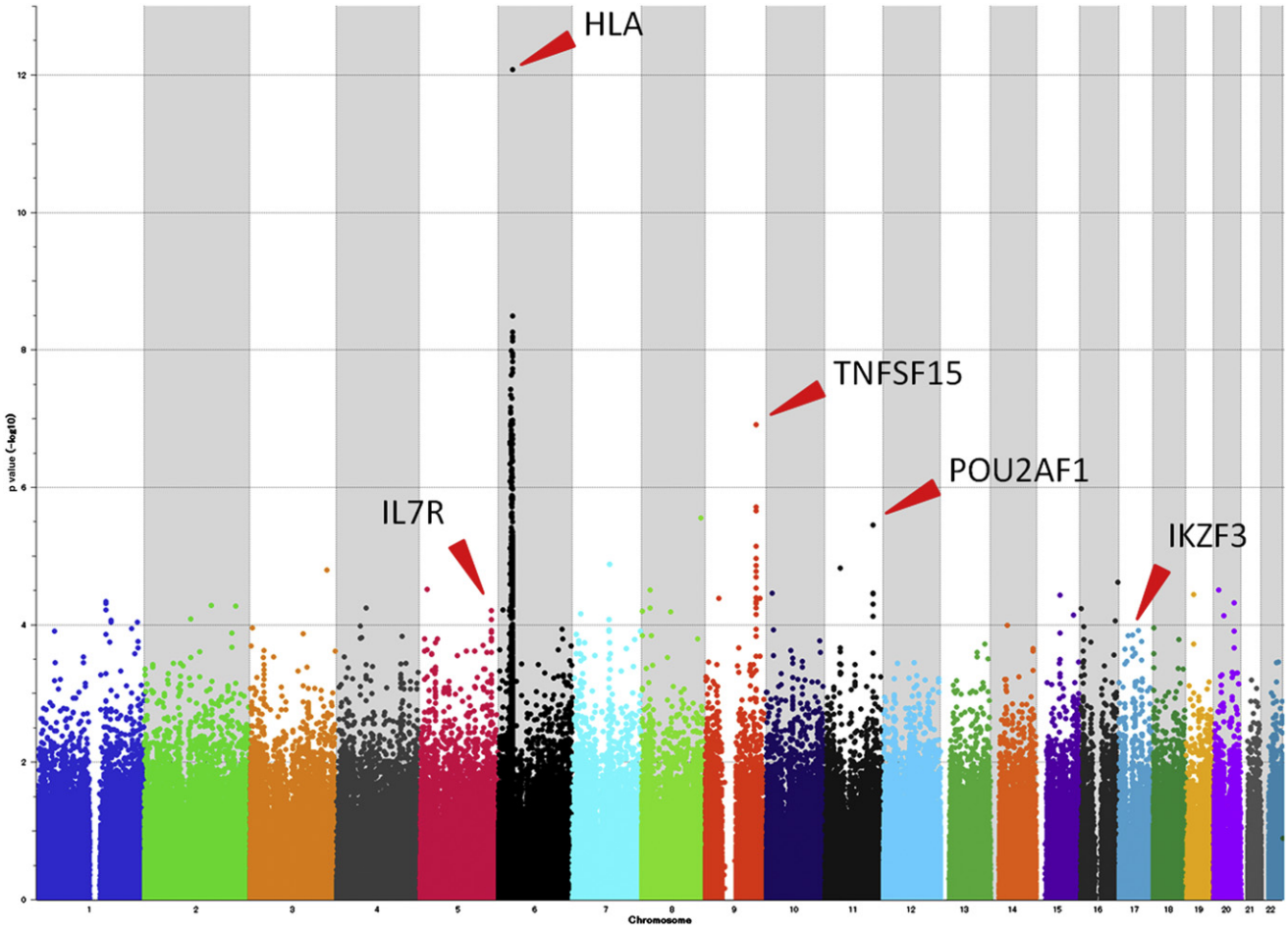

Figure 1. GWAS Results
From 963 samples (487 Japanese PBC cases and 476 Japanese healthy controls), p values were calculated with a chi-square test for allele frequencies among 420,928 SNPs.

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 ≥95%, minor allele frequency ≥5% in both PBC cases and healthy controls, and Hardy-Weinberg Equilibrium (HWE) p value ≥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

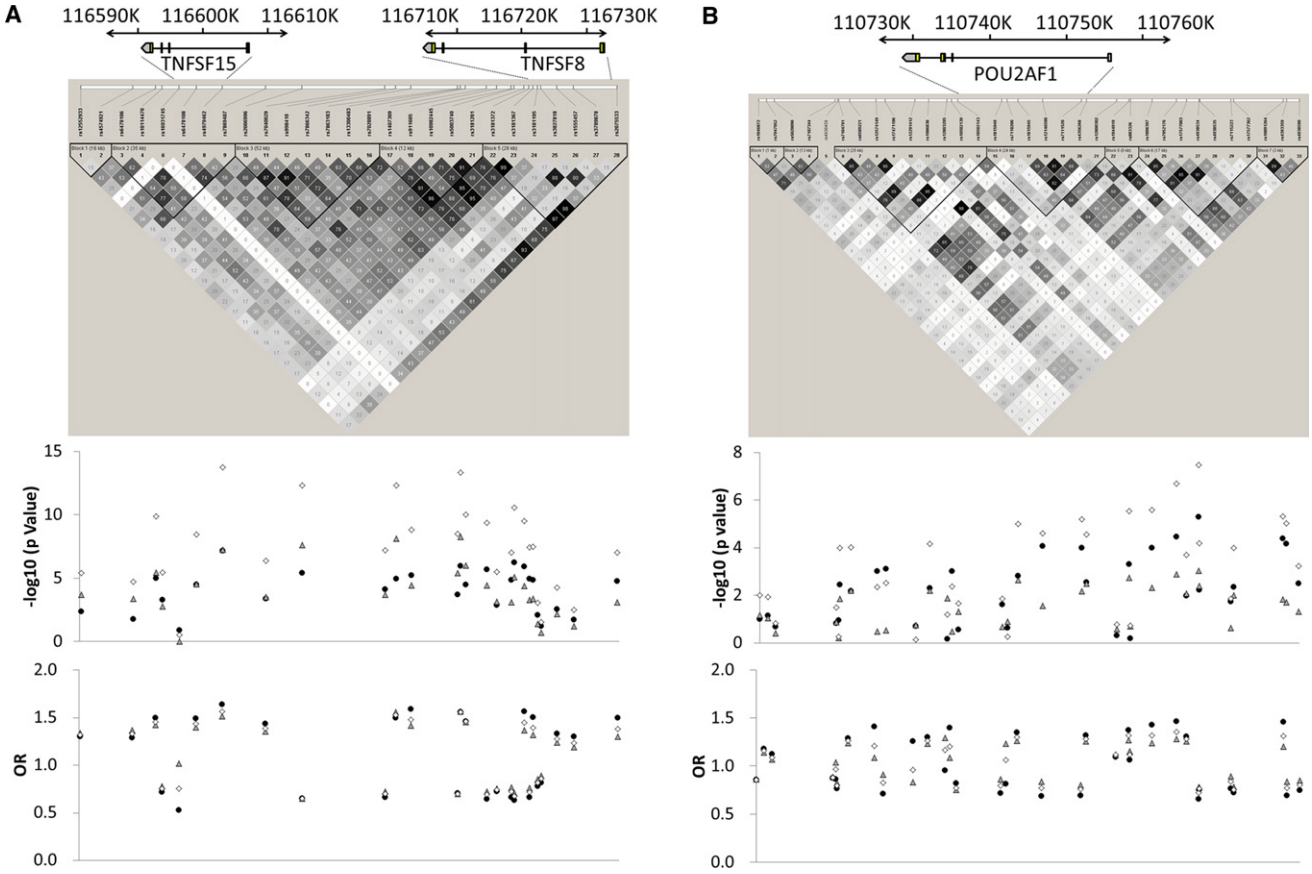

Figure 2. LD Structure, p Values, and OR Plots in the Association Analysis
LD maps (A) around *TNFSF15* (chr9: nucleotide position: 116561403–116733452; build 36.3) and (B) around *POU2AF1* (chr11: nucleotide position: 110684600–110802128; build 36.3). The middle panels show estimates of pairwise r^2 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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