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Identification of Novel Risk Loci for Behçet's Disease–Related Uveitis in a Chinese Population in a Genome-Wide Association Study

Guannan Su,[1] Zhenyu Zhong,[1] Qingyun Zhou,[1] Liping Du,[2] Zi Ye,[1] Fuzhen Li,[2] Wenjuan Zhuang,[3] Chaokui Wang,[1] Liang Liang,[1] Yan Ji,[1] Qingfeng Cao,[1] Qingfeng Wang,[1] Rui Chang,[1] Handan Tan,[1] Shenglan Yi,[1] Yujing Li,[1] Xiaojie Feng,[1] Weiting Liao,[1] Wanyun Zhang,[1] Jia Shu,[1] Shiyao Tan,[1] Jing Xu,[1] Su Pan,[1] Hongxi Li,[1] Jing Shi,[1] Zhijun Chen,[1] Ying Zhu,[1] Xingsheng Ye,[1] Xiao Tan,[1] Jun Zhang,[1] Zhangluxi Liu,[1] Fanfan Huang,[1] Gangxiang Yuan,[1] Tingting Pang,[2] Yizong Liu,[2] Jiadong Ding,[2] Yingnan Gao,[2] Meifen Zhang,[4] Wei Chi,[5] Xiaoli Liu,[6] Yuqin Wang,[7] Ling Chen,[8] Akira Meguro,[9] Masaki Takeuchi,[9] Nobuhisa Mizuki,[9] Shigeaki Ohno,[10] Xianbo Zuo,[11] Aize Kijlstra,[12] and Peizeng Yang[1]

Objective. To explore susceptibility loci associated with uveitis in Behçet's disease (BD).

Methods. We conducted a 2-stage study, consisting of a genome-wide association study (GWAS) stage and a replication stage, in a Chinese population. The GWAS stage included 978 cases with BD-related uveitis and 4,388 controls, and the replication stage included 953 cases with BD-related uveitis and 2,129 controls. Luciferase reporter analysis and chromatin immunoprecipitation assay were performed to explore the functional role of susceptibility genetic variants near ZMIZ1.

Results. Three independent HLA alleles (HLA–B51 [3.75×10^{-190}], HLA–A26 [1.50×10^{-18}], and HLA–C0704 [3.44×10^{-16}]) were identified as having a genome-wide association with BD-related uveitis. In the non-HLA region, in addition to confirming 7 previously reported loci, we identified 22 novel susceptibility variants located in 16 loci. Meta-analysis of the Chinese cohort consisting of 1,931 cases and 6,517 controls and a published Japanese cohort of 611 cases and 737 controls showed genome-wide significant associations with ZMIZ1, RPS6KA4, IL10RA, SIPA1-FIBP-FOSL1, and VAMP1. Functional experiments demonstrated that genetic variants of ZMIZ1 were associated with enhanced transcription activity and increased expression of ZMIZ1.

Conclusion. This GWAS study identified a novel set of genetic variants that are associated with susceptibility to uveitis in BD. These findings enrich our understanding of the contribution of genetic factors to the disease.

INTRODUCTION

Behçet's disease (BD) is a systemic inflammatory disease that typically manifests with recurrent oral and genital ulcers, uveitis, skin lesions, and even inflammation in the nervous and gastrointestinal systems (1). BD has an estimated prevalence of 14.0 per 100,000 persons in China, 13.5 per 100,000 persons in Japan, and 80–420 per 100,000 persons in Turkey. The disease has a much lower prevalence in Europe and the US, estimated to be 0.27–5.2 per 100,000 persons (1–3). Although the etiology of BD is far from being fully understood, it is currently recognized that environmental factors combined with a susceptible genetic background are responsible for its development (4).

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[1]Guannan Su, PhD, Zhenyu Zhong, MD, Qingyun Zhou, MD, Zi Ye, MD, Chaokui Wang, MD, Liang Liang, MD, Yan Ji, MD, Qingfeng Cao, MSc, Qingfeng Wang, MD, Rui Chang, MD, Handan Tan, MD, Shenglan Yi, MD, Yujing Li, MD, Xiaojie Feng, MD, Weiting Liao, MD, Wanyun Zhang, MD, Jia Shu, MD, Shiyao Tan, MD, Jing Xu, MD, Su Pan, MD, Hongxi Li, MD, Jing Shi, MD, Zhijun Chen, MD, Ying Zhu, MD, Xingsheng Ye, MD, Xiao Tan, MD, Jun Zhang, MD, Zhangluxi Liu, MD, Fanfan Huang, MD, Gangxiang Yuan, PhD, Peizeng Yang, MD, PhD: The First Affiliated Hospital of Chongqing Medical University and Chongqing Branch of National Clinical Research Center for Ocular Diseases, Chongqing, China; [2]Liping Du, MD, PhD, Fuzhen Li, MD, PhD, Tingting Pang, MD, Yizong Liu, MD, Jiadong Ding, MD, Yingnan Gao, MD: The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China; [3]Wenjuan Zhuang, MD: People's Hospital of Ningxia Hui Autonomous Region, Yinchuan, China; [4]Meifen Zhang, MD, PhD: Peking Union

Studies of the immunogenetic association with BD started with the identification of HLA–B*51 (originally named HL–A5) in 1973 (5). Recent genome-wide association studies (GWAS) and ImmunoChip studies revealed numerous loci located in both HLA and non-HLA regions, including HLA–A*03, HLA–B*49, HLA–B*15, ERAP1, IL23R, IL12RB2, IL10, and GIMAP (6–11). Nevertheless, the susceptibility loci identified could only explain a limited proportion of variance in the disease risk, and current understanding of the genetic background of BD, especially in the Chinese population, is still far from complete.

We previously reported the results of a GWAS study in Han Chinese patients with BD, which included 149 patients and 951 healthy controls in the discovery stage and 554 patients and 1,159 controls in the replication stage, in which we identified STAT4 as a susceptibility locus (12). That study was, however, limited by the small sample size and therefore had insufficient statistical power (12). Here we performed a larger GWAS, recruiting 1,015 patients and 4,502 controls (978 cases and 4,388 controls passed quality control and were included in subsequent analysis), followed by a replication stage with 953 patients and 2,129 controls. Besides confirming a number of previously identified loci, we identified a set of novel susceptibility genes.

PATIENTS AND METHODS

Recruitment of patients and healthy controls. We conducted a 2-stage study comprising a GWAS stage and a validation stage. The GWAS stage included 1,015 BD patients with uveitis and 4,502 controls, and the validation stage included a separate set of 953 BD patients and 2,129 controls. Patients were diagnosed as having BD based on the criteria of the International Study Group for BD (13). All BD patients had uveitis. Participants were recruited from the First Affiliated Hospital of Chongqing Medical University, the First Affiliated Hospital of Zhengzhou University, Peking Union Medical College Hospital, the First Affiliated Hospital of Anhui Medical University, and the Zhongshan Ophthalmic Center of Sun Yat-sen University between April 2008 and October 2019. Participant enrollment in the Japanese cohort of 611 cases and 737 controls has been described previously (7).

All participants provided written informed consent, and the study was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (permit number 2009-201008) and local research ethics committees. The study was conducted in accordance with the Declaration of Helsinki.

Genomic DNA was obtained from peripheral blood samples using a QIAamp DNA Blood Mini kit (Qiagen) and Magnetic Bead DNA extraction kit (Bio-Base) and was stored at −80°C until used.

GWAS genotyping and analysis. The discovery GWAS stage genotyping was performed using a Human Omni ZhongHua-8 Bead Chip (Illumina). Single-nucleotide polymorphisms (SNPs) were excluded if they had 1) a call rate of <98% in cases or controls; 2) a minor allele frequency (MAF) of <1%; or 3) significant deviation from Hardy-Weinberg equilibrium in healthy controls, with $P < 10^{-4}$. Samples were removed if they 1) had an overall call rate of <98%, as assessed by GenomeStudio Software Modules V2.0; 2) were duplicates or showed familial relationships based on pairwise identity by state (PI_HAT >0.25 for second-degree relatives; the sample with a lower call rate was excluded); or 3) showed genetic gender inconsistent with clinical data. We did not analyze variants on the X chromosome.

Principal components analysis (PCA) was performed using EIGENSTRAT software, and linkage disequilibrium pruning was performed with the parameters $R^2 = 0.25$ and a window size of 200 kb. SNPs (on chromosomes 1–22) that passed quality control were included in the calculation of genomic inflation factor (λ_{GC}). The λ_{GC} value was calculated using R programming language using the formula: λ_{GC} = round(median((qnorm (p_value/2))^2, na.rm = TRUE)/0.454, 3). Ungenotyped SNPs were imputed using the IMPUTE program (v2.0; https://mathgen.stats.ox.ac.uk/impute/impute.html) according to the Han Chinese in Beijing, China (CHB) and Japanese in Tokyo, Japan data from the 1000 Genomes Project integrated phase 3 release (http://www.internationalgenome.org/).

Analyses of HLA amino acid variation were performed as previously described (14). Briefly, HLA typing was based on an independent analysis pipeline that used all currently known HLA gene sequences in the IMGT/HLA database (database release 3.13.1). We thus substituted the called genotypes for sites located in the exonic regions of the 29 HLA genes with the HLA typing results. Imputation of the HLA region was performed using SNP2HLA software (http://software.broadinstitute.org/mpg/snp2hla/). The reference panels were provided by HIBAG

Medical College Hospital, Beijing, China; [5]Wei Chi, MD, PhD: Sun Yat-sen University, Guangzhou, China; [6]Xiaoli Liu, MD, PhD: Jilin University, Changchun, China; [7]Yuqin Wang, MD, PhD: The Eye Hospital of Wenzhou Medical University, Wenzhou, China; [8]Ling Chen, MD, PhD: The Eye and ENT Hospital of Fudan University, Shanghai, China; [9]Akira Meguro, PhD, Masaki Takeuchi, MD, PhD, Nobuhisa Mizuki, MD, PhD: Yokohama City University School of Medicine, Yokohama, Japan; [10]Shigeaki Ohno, MD, PhD: Hokkaido University, Sapporo, Japan; [11]Xianbo Zuo, PhD: China-Japan Friendship Hospital, Beijing, China, and No. 1 Hospital, Anhui Medical University, Anhui, China; [12]Aize Kijlstra, PhD: University Eye Clinic Maastricht, Maastricht, The Netherlands.

Drs. Su, Zhong, Zhou, and Du contributed equally to this work.

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Address correspondence to Peizeng Yang, MD, PhD, Peizeng No. 1 Friendship Road, Yuanjiagang, Yuzhong District Chongqing, China 400016 (email: peizengycmu@126.com); or to Xianbo Zuo, PhD, Yinghuadongjie No. 2, Chaoyang District, Beijing, China, 100029 (email: zuoxianbo@qq.com).

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(https://github.com/zhengxwen/HIBAG) (the 4-digit resolution of multiple GlaxoSmithKline clinical trials of Asian ancestry, east and south Asia), HapMap Phase 3, and a Han Chinese major histocompatibility complex database (14). Imputed SNPs were excluded if they had 1) a call rate of <95% in cases or controls; 2) a MAF of <5%; or 3) a significant deviation from Hardy-Weinberg equilibrium in healthy controls, with $P < 10^{-4}$.

SNP selection and genotyping in the replication stage. Non-HLA region SNPs with valid genotype scatter plots and $P < 10^{-4}$ were selected for validation using a Sequenom MassARRAY system (https://support.agenabio.com/s/online-tools). Genotyped SNPs were excluded if they had 1) a call rate of <90% in cases or controls; 2) a significant deviation from Hardy–Weinberg equilibrium in healthy controls, with $P < 10^{-4}$; or 3) a significant deviation from the frequency of CHB.

Genome-wide pathway association analysis. The pathways related to susceptibility genes were analyzed using the DAVID functional annotation tool (v6.7) (https://david.ncifcrf.gov/tools.jsp). The associated genes found in our study as well as previously reported genes were all included in this analysis.

Expression quantitative trait locus (eQTL) analyses. Expression data for specific tissues were obtained from the GTEx Portal (http://www.gtexportal.org/home/) and filtered using a false discovery rate of ≤0.05.

Real-time polymerase chain reaction (PCR). Total RNA was extracted from peripheral blood mononuclear cells (PBMCs) using TRIzol (Invitrogen), followed by reverse transcription and amplification with SYBR Green Real-time PCR Master Mix (Bio-Rad). β-actin was used as an internal normalization control. The assays were performed on an ABI 7500 real-time PCR instrument. Comparative quantification was calculated using the $2^{-\Delta\Delta Ct}$ method.

Luciferase reporter analysis. The construction of vectors was performed by Wuhan GeneCreate Biological Engineering. Haplotypes (2,569 bp length) and 201-bp–long DNA sequences harboring the wild-type or risk allele of candidate SNPs were cloned into pGL3 plasmid vectors. These constructed vectors and pRL-TK were cotransfected into HEK 293T cells. Cells were harvested 48 hours after transfection and luciferase activities were analyzed using a Dual-Luciferase Reporter Assay system (Promega).

Chromatin immunoprecipitation (ChIP) assay. PBMCs (>2 × 10^7cells) were crosslinked with 1% formaldehyde for 10 minutes at room temperature, and protein/DNA crosslinking was stopped by incubating in 0.125*M* glycine for 5 minutes. Cells were lysed in ChIP lysis buffer for 10 minutes. After centrifugation, the pellet was lysed in lysis buffer and subjected to sonication. Sheared chromatin was immunoprecipitated overnight at 4°C with specific antibodies bound to the Pierce TM Protein A/G Agarose Beads (ThermoFisher Scientific), followed by elution and reverse crosslinking at 65°C overnight. DNA was isolated and purified subsequently. Quantitative reverse transcriptase–PCR was performed to calculate the percentage of input. The following antibodies were used: ELF4 (catalog no. ab13581; Abcam), IRF1 (catalog no. ab186384; Abcam), GABPA (catalog no. sc-28312; Santa Cruz Biotechnology), ELF3 (catalog no. sc-376055; Santa Cruz Biotechnology), IRF7 (catalog no. sc-74472; Santa Cruz Biotechnology), ETV3 (catalog no. ab176717; Abcam), PAX6 (catalog no. ab5790; Abcam), TEAD1 (catalog no. ab133533; Abcam), H3K27ac (catalog no. ab4729; Abcam), and H3K4me1 (catalog no. ab176877; Abcam).

Statistical analysis. The association of each SNP with BD in the GWAS, replication, and meta-analysis stages was analyzed using an additive model in logistic regression using PLINK v1.07 (http://zzz.bwh.harvard.edu/plink/) and SNPTEST v2.5.4-beta3 (http://mathgen.stats.ox.ac.uk/genetics_software/snptest/snptest.html) (15). Odds ratios (ORs) and 95% confidence intervals (95% CIs) were adjusted for the top 10 eigenvectors in the logistic regression analysis. PCA implemented in the EIGENSOFT package (http://genetics.med.harvard.edu/reich/Reich_Lab/Software.html) was used to evaluate ancestry and population stratification. Regional plots were generated using LocusZoom31 (http://csg.sph.umich.edu/locuszoom/). R was used to create quantile–quantile plots to evaluate the overall significance of the GWAS results (http://www.r-project.org/). Heterogeneity was examined using Cochran's Q and I^2 statistics. A fixed-effects (Mantel–Haenszel) model was applied if I^2 ≤30%; a random-effects model was adopted if I^2 >30%.

RESULTS

We performed a GWAS study in 1,015 cases with BD-related uveitis and 4,502 controls in a Chinese population. After sample and SNP quality control, 753,745 SNPs were genotyped in 978 cases with BD-related uveitis and 4,388 controls (Supplementary Table 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41998). PCA results showed that patients and healthy controls were well matched (Supplementary Figure 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41998). Quantile–quantile plot analysis showed that the genomic inflation factor value (λ) without the HLA region was 1.04 (Supplementary Figure 2, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41998), demonstrating no substantial population stratification effects.

The strongest association was found within the HLA region (Figure 1). We further performed an imputation analysis within the HLA region (chromosome 6; 29–34 Mb), and then analyzed HLA alleles, amino acids, and SNPs for their association with BD (Supplementary Tables 2 and 3, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41998). HLA–B51 was the most strongly associated allele ($P = 3.75 \times 10^{-190}$; OR 5.86) (Table 1 and Supplementary Figure 3, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41998). An HLA allele previously reported to be associated with BD, HLA–A26 (16), was also found to be of independent genome-wide significance in our study, and, for the first time, we identified a genome-wide significant association of HLA–C0704 with BD-related uveitis (Table 1).

A stepwise conditional logistic regression analysis was next performed on the amino acids and SNPs, respectively. We identified 6 independent amino acid variations (AA_B_67_31432515_F, AA_A_-15_30018338_L, AA_C_1_31347624_C, AA_C_156_31346909_D, AA_B_80_31432476_I, and AA_A_76_30018738_A) and 8 independent SNPs (rs41546114, rs9277724, rs2245961, rs17195089, rs2395031, rs6920323, rs9261403, and rs2022539) in genome-wide association with BD (Table 1). These independent amino acids and SNPs were next conditioned on the effect of HLA–B51, HLA–A26, and HLA–C0704, respectively (Table 1). The variant rs6920323, located between HLA–B and MICA, lost significance after conditioning on HLA–B51. In the non-HLA region, we confirmed 7 previously reported loci, including IL23R-IL12RB2, IL10, STAT4, ERAP1, IFNGR1, LACC1, and CEBPB-PTPN1, showing a consistent direction of effect on risk (Table 2).

A total of 98 unreported SNPs with suggestive evidence of an association ($P < 0.0001$) were selected for a further replication study in an additional set of 953 cases and 2,129 controls. Data

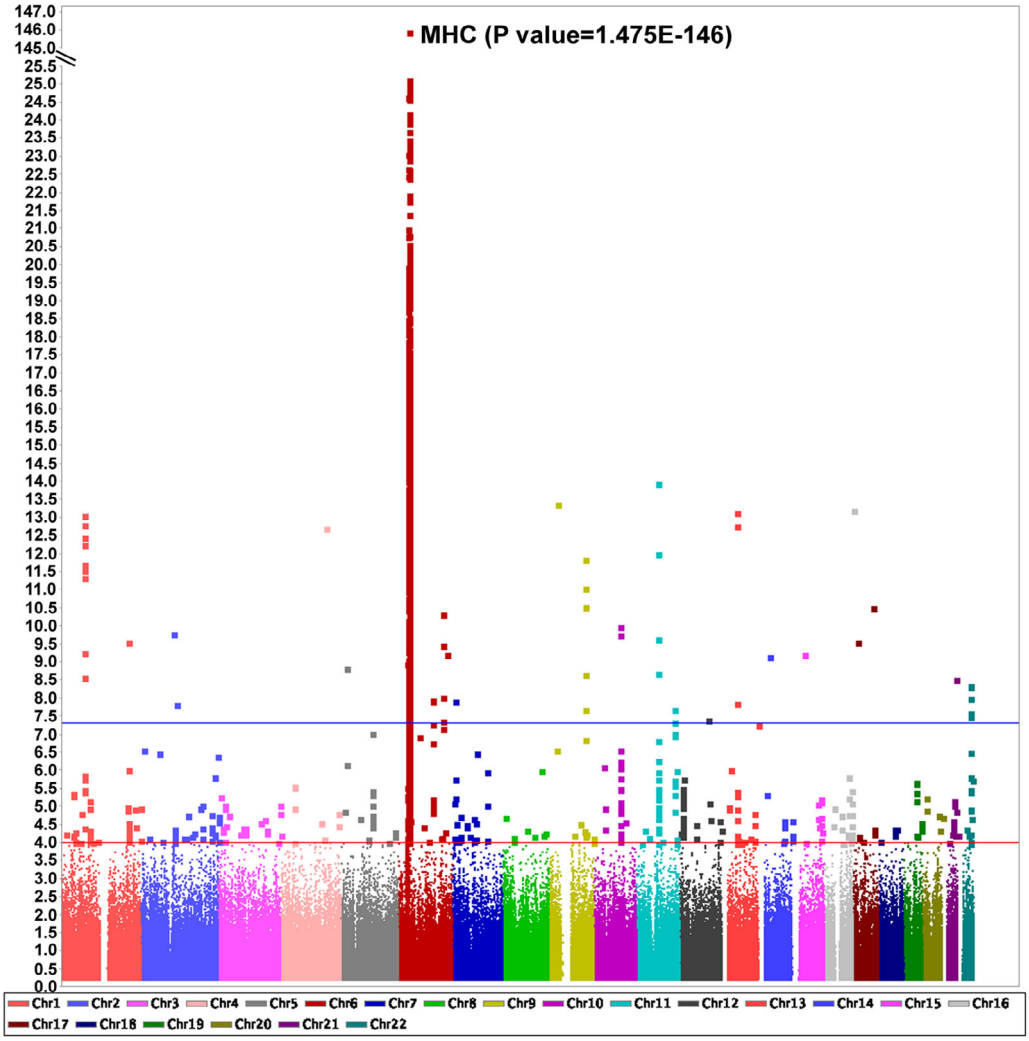

Figure 1. Manhattan plot of *P* values on a $-\log_{10}$ scale for 753,745 genotyped single-nucleotide polymorphisms (SNPs) in a genome-wide association study consisting of 978 cases with Behçet's disease and 4,388 healthy controls. The red line represents $P = 1 \times 10^{-4}$ and the blue line represents $P = 5 \times 10^{-8}$. SNP locations are from build 37/hg19. MHC = major histocompatibility complex.

Table 1. Association of HLA variants with BD*

	A1/A2	MAF in cases	MAF in controls	*P*	OR (95% CI)	*P*, conditioned on HLA–B51	*P*, conditioned on HLA–A26	*P*, conditioned on HLA–C0704	*P*, stepwise conditional analysis
Independent allele									
HLA–B51	P/A	0.2964	0.06711	3.75×10^{-190}	5.856 (5.151–6.657)	NA	NA	–	–
HLA–A26	P/A	0.07231	0.03099	9.77×10^{-18}	2.437 (1.976–3.005)	1.50×10^{-18}	NA	–	–
HLA–C0704	P/A	0.03282	0.008888	6.07×10^{-17}	3.784 (2.709–5.286)	1.01×10^{-15}	3.44×10^{-16}†	–	–
Independent amino acid									
AA_B_67_31432515_F	P/A	0.3451	0.1158	1.346×10^{-139}	4.025 (3.592–4.511)	0.004282	1.419×10^{-108}	3.202×10^{-112}	NA
AA_A_-15_30018338_L	P/A	0.3564	0.4905	6.6×10^{-27}	0.5751 (0.5195–0.6367)	4.7×10^{-21}	9.947×10^{-20}	2.191×10^{-24}	4.10×10^{-22}
AA_C_1_31347624_C	A/P	0.1933	0.3018	5.898×10^{-22}	0.5543 (0.491–0.6258)	0.0000846	2.427×10^{-22}	1.813×10^{-19}	1.66×10^{-16}
AA_C_156_31346909_D	P/A	0.03282	0.008888	6.071×10^{-17}	3.784 (2.709–5.286)	1.01×10^{-15}	1.529×10^{-14}	NA	6.11×10^{-13}
AA_B_80_31432476_I	P/A	0.359	0.1745	1.084×10^{-73}	2.65 (2.379–2.951)	0.03048	5.338×10^{-68}	9.106×10^{-70}	4.84×10^{-13}
AA_A_76_30018738_A	P/A	0.121	0.07065	1.122×10^{-13}	1.811 (1.545–2.123)	2.17×10^{-16}	0.01997	4.958×10^{-14}	1.22×10^{-12}
Independent SNP									
rs41546114	A/G	0.1948	0.03583	1.475×10^{-146}	6.509 (5.552–7.63)	0.0002327	8.24×10^{-102}	1.027×10^{-103}	NA
rs9277724	A/G	0.1874	0.3374	6.055×10^{-38}	0.4531 (0.4007–0.5122)	5.04×10^{-28}	6.114×10^{-34}	5.271×10^{-33}	3.392×10^{-30}
rs2245961	C/A	0.2933	0.4445	1.75×10^{-34}	0.5187 (0.4665–0.5769)	5.75×10^{-31}	8.271×10^{-27}	8.857×10^{-32}	8.17×10^{-25}
rs17195089	A/G	0.1022	0.2043	3.668×10^{-20}	0.4434 (0.3713–0.5294)	4.38×10^{-21}	5.816×10^{-17}	1.431×10^{-19}	6.03×10^{-21}
rs2395031	A/G	0.03846	0.008776	1.115×10^{-23}	4.518 (3.275–6.233)	1.8×10^{-18}	8.993×10^{-19}	6.83×10^{-09}	3.09×10^{-18}
rs6920323	G/A	0.3686	0.1787	1.928×10^{-76}	2.682 (2.41–2.985)	0.3677	1.527×10^{-69}	1.573×10^{-71}	1.10×10^{-13}
rs9261403	T/G	0.05385	0.01571	2.1×10^{-24}	3.566 (2.75–4.625)	1.96×10^{-19}	0.007938	5.314×10^{-22}	1.25×10^{-11}
rs2022539	C/A	0.4722	0.3816	2.921×10^{-13}	1.449 (1.311–1.602)	9.81×10^{-14}	5.932×10^{-13}	2.623×10^{-11}	1.53×10^{-09}

* $P < 5 \times 10^{-8}$ was considered to indicate an independent association in the conditional regression analysis. BD = Behçet's disease; A1 = minor allele; A2 = major allele; MAF = minor allele frequency; OR = odds ratio; 95% CI = 95% confidence interval; NA = not applicable.
† *P* conditioned on both HLA–B51 and HLA–A26.

Table 2. Results for loci previously reported as having a genome-wide significant or suggestive association with BD in the non-HLA region*

Chr.	SNP	BP	Gene	A1	MAF		A2	*P*	OR (95%CI)†
					Cases	Controls			
1	rs11209032	67740092	IL23R-IL12RB2	G	0.4179	0.5104	A	1.50×10^{-13}	0.6886 (0.6235–0.7605)
1	rs34426521‡	67745768	IL23R-IL12RB2	A	0.4169	0.5081	G	3.24×10^{-13}	0.6922 (0.6268–0.7645)
1	rs12119179	67747415	IL23R-IL12RB2	A	0.4174	0.5056	C	1.83×10^{-12}	0.7004 (0.6342–0.7736)
1	rs1495965	67753508	IL23R-IL12RB2	A	0.4056	0.4959	G	5.36×10^{-13}	0.6938 (0.628–0.7665)
1	rs3021094	206944952	IL10	C	0.5215	0.471	A	5.40×10^{-5}	1.224 (1.109–1.35)
2	rs7572482	192015072	STAT4	G	0.3901	0.4452	A	8.90×10^{-6}	0.7972 (0.7212–0.8811)
5	rs1065407§	96112083	ERAP1	C	0.09151	0.05864	A	8.93×10^{-8}	1.617 (1.354–1.932)
5	rs10050860	96122210	ERAP1	A	0.07495	0.05117	G	3.38×10^{-5}	1.502 (1.238–1.823)
5	rs2287987¶	96129535	ERAP1	G	0.07566	0.051	A	1.66×10^{-5}	1.523 (1.256–1.847)
5	rs2013717¶	96134175	ERAP1	C	0.07566	0.05136	A	2.29×10^{-5}	1.512 (1.247–1.833)
6	rs9376268	137532751	IFNGR1	A	0.3456	0.4267	G	4.41×10^{-11}	0.7095 (0.6405–0.786)
13	rs3764147	44457925	LACC1	G	0.2336	0.3199	A	6.71×10^{-14}	0.6481 (0.5782–0.7264)
13	rs1373904	44475398	LACC1	G	0.2359	0.321	A	1.60×10^{-13}	0.653 (0.5828–0.7317)
20	rs913678	48955424	CEBPB-PTPN1	A	0.2669	0.3165	G	1.72×10^{-5}	0.7863 (0.7045–0.8775)

* BP = base position; MAF = minor allele frequency; A2 = major allele.
† Odds ratio (OR) and 95% confidence interval (95% CI) for the minor allele (A1).
‡ The single-nucleotide polymorphism (SNP) rs34426521 in IL23R-IL12RB2 is in strong linkage disequilibrium ($r^2 = 0.99$, $D' = 0.99$) with the SNP rs11209032, which was previously reported to have an association with Behçet's disease (BD) in a Japanese genome-wide association study (7).
§ The SNP rs1065407 was previously reported to have an association with BD in our ERAP1 gene polymorphisms study (34).
¶ The SNPs rs2287987 and rs2013717 in ERAP1 are in strong linkage disequilibrium with the SNP rs10050860 ($r^2 = 0.85$, $D' = 0.92$).

from both stages were included in a meta-analysis, and 22 novel variants achieved (or nearly achieved) the genome-wide significance threshold ($P < 5 \times 10^{-8}$). These variants were located within the following loci: RHOH, PRDM1, MTHFD1L, KLF4, ZMIZ1, RPS6KA4-PRDX5, SIPA1-FIBP-FOSL1, IL10RA, VAMP1, AGBL1 ($P = 1.17 \times 10^{-7}$), CMIP, CDH15-ZNF778, TCF4, MRPL39-JAM2, GART, and MIS18A (Table 3 and Supplementary Figure 4, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41998). For further validation, we analyzed these 16 novel loci by direct genotyping using a GeneChip Human Mapping 500K array set (Affymetrix) or imputation (imputation $R^2 > 0.85$) in a published Japanese cohort involving 611 cases and 737 controls (7). In a meta-analysis of the Chinese and Japanese populations, the loci within ZMIZ1, RPS6KA4, IL10RA, VAMP1, and SIPA1-FIBP-FOSL1 exceeded genome-wide significance (Table 4).

We performed pathway enrichment analysis using all newly identified susceptibility genes from this study along with earlier reported associated genes and found that genes in these loci contribute to the pathways involved in JAK/STAT signaling, cytokine receptor activity, and immune response (Supplementary Table 4, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41998).

The aforementioned result identified a strong association of rs1250569 of ZMIZ1with BD. To analyze the functional significance of this SNP, we measured the expression of ZMIZ1 in PBMCs derived from 36 healthy individuals with a known rs1250569 genotype. The mean expression of ZMIZ1 in GG carriers was 2.69-fold higher and 1.59-fold higher than that in AA carriers ($P = 0.001$) and GA carriers ($P = 0.028$), respectively (Figure 2A).

Furthermore, we explored whether the BD susceptibility SNPs within ZMIZ1 had potential regulatory functions. The SNP rs1250569 is located in the intron 8 region of ZMIZ1. There are 2 SNPs, rs1250568 and rs2802372, in strong linkage disequilibrium ($r^2 = 0.82$, $D' = 1$; and $r^2 = 0.8$, $D' = 1$, respectively) with rs1250569 in Asian (ASN) populations as shown in the 1000 Genomes Project using HaploReg v4.1. Interestingly, rs2802372 in ZMIZ1 was also identified in our GWAS-stage data as having a strong association with BD ($P_{GWAS} = 1.67 \times 10^{-10}$) (second only to rs1250569 [$P_{GWAS} = 9.77 \times 10^{-11}$]), although the rs2802372 association lost significance in the analysis combining data from both stages. Therefore, markers located within approximately ±2 Mb of these 2 SNPs (rs1250569 and rs2802372) were selected for imputation (https://mathgen.stats.ox.ac.uk/impute/impute.html). Imputation identified another 3 SNPs (rs1250568, rs1250564, and rs1250565) that were in high linkage disequilibrium with rs1250569 or rs2802372 ($r^2 \geq 0.8$), and showed genome-wide significant associations ($P < 5 \times 10^{-8}$) (Supplementary Table 5, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41998). To validate whether these 5 SNPs lead to an allele-specific functional effect on transcription, we cloned haplotypes comprising the 5 SNPs and 201-bp–long DNA fragments harboring non-risk and risk alleles of the 5 SNPs (Supplementary Table 6, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41998), respectively, into pGL3 vectors and performed a luciferase reporter assay. We observed significant differences in transcription activity between the haplotypes and between the risk and non-risk alleles of rs1250569, rs1250568, and rs1250565 (Figure 2B).

Transcription factors are considered to be the major mediator of sequence-dependent regulation of gene expression (17,18).

Table 3. Newly identified loci in 978 cases with BD and 4,388 controls in a Han Chinese population*

					GWAS				Replication study						
					MAF				MAF				Meta-analysis		
Chr.	SNP	Gene	A1	A2	Cases	Controls	*P*	OR (95% CI)	Cases	Controls	*P*	OR (95% CI)	*P*	OR (95% CI)	I^2, %
4	rs9683756	RHOH	G	A	0.29	0.35	2.81×10^{-6}	0.77 (0.70–0.86)	0.29	0.34	1.81×10^{-3}	0.78 (0.67–0.91)	1.93×10^{-8}	0.78 (0.72–0.85)	0
6	rs13437093	PRDM1	A	G	0.55	0.47	1.14×10^{-8}	1.33 (1.21–1.47)	0.56	0.47	7.23×10^{-6}	1.40 (1.21–1.63)	5.36×10^{-13}	1.35 (1.25–1.47)	0
6	rs12216229	MTHFD1L	G	A	0.07	0.12	5.99×10^{-10}	0.54 (0.44–0.66)	0.07	0.13	1.46×10^{-8}	0.51 (0.41–0.65)	1.43×10^{-16}	0.53 (0.46–0.62)	0
9	rs10733565	KLF4	G	A	0.50	0.41	8.29×10^{-12}	1.41 (1.28–1.55)	0.49	0.41	1.78×10^{-7}	1.40 (1.24–1.59)	9.92×10^{-18}	1.41 (1.30–1.52)	0
9	rs7018799	KLF4	G	A	0.51	0.43	2.76×10^{-11}	1.40 (1.27–1.54)	0.49	0.42	2.81×10^{-6}	1.29 (1.16–1.44)	7.33×10^{-16}	1.35 (1.25–1.44)	10.22
9	rs10979075	KLF4	A	G	0.48	0.40	1.36×10^{-12}	1.43 (1.29–1.57)	0.46	0.39	3.66×10^{-6}	1.30 (1.16–1.45)	1.69×10^{-11}†	1.37 (1.26–1.46)†	33.84
10	rs1250569	ZMIZ1	G	A	0.43	0.51	9.77×10^{-11}	0.72 (0.65–0.80)	0.43	0.51	3.47×10^{-6}	0.74 (0.65–0.84)	1.98×10^{-15}	0.73 (0.67–0.78)	0
11	rs6591843	RPS6KA4	G	A	0.38	0.30	9.66×10^{-13}	1.45 (1.31–1.60)	0.36	0.31	2.31×10^{-5}	1.29 (1.14–1.44)	1.23×10^{-7}†	1.37 (1.27–1.48)†	55.95
11	rs7130280	RPS6KA4	G	A	0.47	0.38	1.07×10^{-14}	1.47 (1.34–1.63)	0.45	0.38	3.93×10^{-8}	1.38 (1.23–1.54)	4.72×10^{-21}	1.43 (1.33–1.53)	0
11	rs2448490	SIPA1-FIBP-FOSL1	A	G	0.19	0.24	5.12×10^{-7}	0.73 (0.65–0.83)	0.18	0.24	2.93×10^{-5}	0.70 (0.60–0.83)	7.88×10^{-11}	0.72 (0.65–0.79)	0
11	rs568617	SIPA1-FIBP-FOSL1	A	G	0.38	0.44	1.10×10^{-5}	0.80 (0.72–0.88)	0.39	0.45	1.66×10^{-5}	0.78 (0.70–0.88)	8.11×10^{-10}	0.79 (0.74–0.86)	0
11	rs10791830	SIPA1-FIBP-FOSL1	A	G	0.36	0.41	1.44×10^{-5}	0.80 (0.72–0.88)	0.38	0.44	1.99×10^{-6}	0.76 (0.68–0.85)	1.59×10^{-10}	0.78 (0.75–0.87)	0
11	rs2228054	IL10RA	A	G	0.24	0.30	1.00×10^{-7}	0.73 (0.66–0.82)	0.20	0.27	3.46×10^{-6}	0.69 (0.59–0.81)	2.29×10^{-12}	0.72 (0.65–0.78)	0
11	rs2228055	IL10RA	G	A	0.24	0.30	8.81×10^{-8}	0.73 (0.65–0.82)	0.20	0.27	2.67×10^{-7}	0.66 (0.56–0.77)	2.52×10^{-13}	0.71 (0.64–0.77)	12.29
12	rs1034969	VAMP1	A	C	0.27	0.33	4.72×10^{-6}	0.78 (0.7–0.86)	0.29	0.35	2.60×10^{-5}	0.78 (0.70–0.88)	5.43×10^{-10}	0.78 (0.73–0.86)	0
15	rs896615‡	AGBL1	G	A	0.29	0.34	1.91×10^{-5}	0.79 (0.71–0.88)	0.27	0.32	1.69×10^{-3}	0.79 (0.68–0.92)	1.17×10^{-7}	0.79 (0.72–0.86)	0
16	rs9888833	CMIP	G	A	0.23	0.29	3.49×10^{-6}	0.76 (0.68–0.86)	0.21	0.27	4.70×10^{-5}	0.72 (0.62–0.84)	8.65×10^{-10}	0.75 (0.68–0.81)	0
16	rs79831785	CDH15	G	A	0.07	0.14	5.83×10^{-14}	0.47 (0.39–0.58)	0.07	0.12	6.80×10^{-7}	0.54 (0.42–0.69)	1.39×10^{-18}	0.50 (0.42–0.57)	0
18	rs7237392	RNA5SP459-TCF4	A	G	0.29	0.34	4.07×10^{-5}	0.80 (0.72–0.89)	0.27	0.33	9.96×10^{-6}	0.76 (0.67–0.86)	2.11×10^{-9}	0.78 (0.72–0.84)	0
21	rs2829839	MRPL39-JAM2	A	C	0.23	0.27	5.62×10^{-5}	0.79 (0.7–0.89)	0.20	0.26	7.93×10^{-5}	0.73 (0.62–0.85)	2.53×10^{-8}	0.77 (0.69–0.84)	0
21	rs6517178	GART	A	G	0.34	0.39	6.53×10^{-6}	0.79 (0.71–0.87)	0.30	0.36	3.95×10^{-4}	0.77 (0.67–0.89)	1.05×10^{-8}	0.78 (0.71–0.84)	0
21	rs4817467	MIS18A	G	A	0.29	0.35	4.04×10^{-8}	0.74 (0.66–0.82)	0.24	0.31	1.42×10^{-5}	0.72 (0.62–0.83)	3.08×10^{-12}	0.73 (0.66–0.79)	0

* Odds ratios (ORs) and 95% confidence intervals (95% CIs) are for the minor allele (A1). BD = Behçet's disease; GWAS = genome-wide association study; MAF = minor allele frequency; SNP = single-nucleotide polymorphism; A2 = major allele.
† In terms of heterogeneity, the OR and *P* value were estimated using a random-effects model.
‡ This variant lost genome-wide significance in the meta-analysis.

Table 4. Results of association tests and meta-analysis of new susceptibility loci for BD identified in this study*

SNP/population	BP	Gene	A1	A2	*P*	OR (95% CI)†	I^2, %	*P* for heterogeneity
rs1250569	81045207	ZMIZ1	G	A				
Chinese	–	–	–	–	8.75×10^{-16}	0.73 (0.67–0.78)	–	–
Japanese	–	–	–	–	0.0006	0.76 (0.6–0.89)	–	–
Chinese and Japanese	–	–	–	–	3.40×10^{-18}	0.73 (0.68–0.79)	0.0	0.585
rs6591843	64147083	RPS6KA4	G	A				
Chinese	–	–	–	–	1.76×10^{-16}	1.37 (1.27–1.48)	–	–
Japanese	–	–	–	–	0.0008	1.30 (1.12–1.52)	–	–
Chinese and Japanese	–	–	–		6.96×10^{-19}	1.36 (1.27–1.45)	0.0	0.543
rs7130280	64156585	RPS6KA4	G	A				
Chinese	–	–	–	–	1.66×10^{-21}	1.43 (1.32–1.53)	–	–
Japanese	–	–	–	—	0.0024	1.27 (1.09–1.48)	–	–
Chinese and Japanese	–	–	–		1.35×10^{-8}‡	1.37 (1.31–1.49)‡	46.2	0.173
rs2228055	117864846	IL10RA	G	A				
Chinese	–	–	–	–	3.22×10^{-14}	0.70 (0.64–0.77)	–	–
Japanese	–	–	–	–	0.0026	0.79 (0.67–0.92)	–	–
Chinese and Japanese	–	–	–	–	1.07×10^{-8}‡	0.73 (0.67–0.78)‡	35.67	0.213
rs2228054	117864113	IL10RA	A	G				
Chinese	–	–	–	–	1.41×10^{-13}	0.71 (0.65–0.78)	–	–
Japanese	–	–	–	–	0.0023	0.78 (0.67–0.92)	–	–
Chinese and Japanese	–	–		–	3.24×10^{-15}	0.73 (0.67–0.79)	16.76	0.273
rs10791830	65661291	SIPA1-FIBP-FOSL1	A	G				
Chinese	–	–	–	–	8.24×10^{-9}	0.80 (0.75–0.87)	–	–
Japanese	–	–	–	–	0.0223	0.83 (0.71–0.97)	–	–
Chinese and Japanese	–	–	–	–	5.85×10^{-10}	0.81 (0.76–0.86)	0.0	0.699
rs568617	65653242	SIPA1-FIBP-FOSL1	A	G				
Chinese	–	–	–	–	1.27×10^{-9}	0.80 (0.74–0.86)	–	–
Japanese	–	–	–	–	0.0212	0.83 (0.71–0.97)	–	–
Chinese and Japanese	–	–	–	–	1.04×10^{-10}	0.80 (0.75–0.86)	0.0	0.607
rs1034969	6573856	VAMP1	A	C				
Chinese	–	–	–	–	3.82×10^{-9}	0.79 (0.73–0.86)	–	–
Japanese	–	–	–	–	0.0141	0.82 (0.69–0.96)	–	–
Chinese and Japanese	–	–	–	–	2.12×10^{-10}	0.80 (0.74–0.85)	0.0	0.741

* BD = Behçet's disease; SNP = single-nucleotide polymorphism; BP = base position; A2 = major allele.
† Odds ratio (OR) and 95% confidence interval (95% CI) for the minor allele (A1).
‡ In terms of heterogeneity, the OR and *P* value were estimated using a random-effects model.

Therefore, we next predicted whether these 3 SNPs (rs1250569, rs1250568, and rs1250565) overlap with transcription factor binding sites using the JASPAR (2018) database (19) and found that 8 transcription factors (PAX6, ELF5, IRF1, GABPA, ELF3, IRF7, ETV3, and TEAD1) were predicted to bind to only 1 of the alleles with a score of >9 (Supplementary Table 7, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41998). To further validate the transcription factor binding prediction and explore whether risk alleles and non-risk alleles manifest with differential binding activities, we performed ChIP assays for these 8 transcription factors. We detected a significant recruitment of PAX6 to rs1250569 and ETV3 to rs1250568 (both with preferential recruitment to the non-risk allele G compared to the risk allele A) (Figures 2C and D).

Other transcription factors were not found to bind rs1250568, and no transcription factor was identified to bind rs1250565 (Supplementary Figure 5, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41998). The ENCODE database showed that rs1250565 was located in gene regions with high H3K27ac expression, and database annotations primarily showed that the highest H3K27ac Z-scores in this gene region were associated with the KMS-11 cell line, a human multiple myeloma cell line. This locus might also regulate other genes in *cis* configuration, such as PPIF, ZCCHC24, and RP11-342M3.5. Subsequently, we performed ChIP assays in PBMCs for H3K27ac and H3K4me1, 2 markers for enhancers, and detected significant enrichments in the region covering rs1250569-G or rs1250568-G (Figures 2E–H and Supplementary Table 8, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41998).

DISCUSSION

This study included 1,931 BD cases and 6,517 controls in a Chinese population and identified 22 novel disease susceptibility variants. In addition, meta-analysis of the Chinese cohort and a published Japanese cohort showed genome-wide significant associations with novel loci in ZMIZ1, RPS6KA4, IL10RA, SIPA1-FIBP-FOSL1, and VAMP1. Functional experiments indicated that the risk allele of variants within ZMIZ1 may affect the expression of ZMIZ1 by altering enhancer activities and transcription factor binding activities.

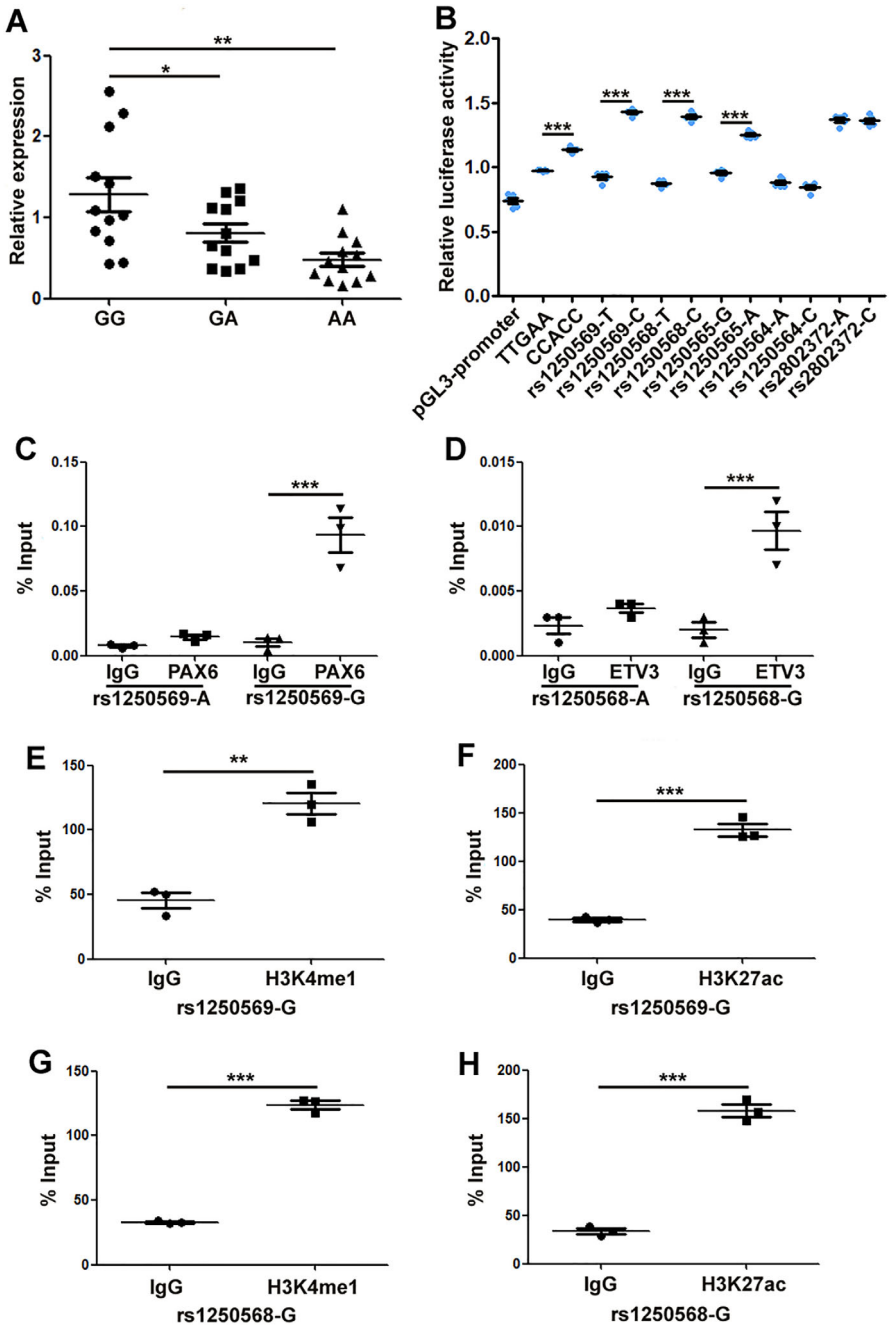

Figure 2. **A**, ZMIZ1 expression in peripheral blood mononuclear cells (PBMCs) from healthy controls with the GG, GA, and AA genotypes of the single-nucleotide polymorphism (SNP) rs1250569. The y-axis represents relative ZMIZ1 expression level, determined by real-time polymerase chain reaction. **B**, Transcription activity of the SNPs rs1250569, rs1250568, rs1250565, rs1250564, and rs2802372, determined by luciferase gene reporter assay. **C** and **D**, Differential allele transcription factor binding activities of PAX6 in the regions containing the A allele or G allele of rs1250569 (**C**) and of ETV3 in the regions containing the A allele or G allele of rs1250568 (**D**) in PBMCs from healthy controls, determined by chromatin immunoprecipitation (ChIP) assay. The relative abundance of PAX6 and ETV3 was normalized to the input controls. **E** and **F**, Enrichment of the enhancer-specific H3K4me1 (**E**) and H3K27ac (**F**) in the region containing the G allele of rs1250569, determined by ChIP assay. The relative abundance of H3K4me1 and H3K27ac was normalized to the input control. **G** and **H**, Enrichment of the enhancer-specific H3K4me1 (**G**) and H3K27ac (**H**) in the region containing the G allele of rs1250568, determined by ChIP assay. The relative abundance of H3K4me1 and H3K27ac was normalized to the input control. Symbols represent individual subjects; horizontal lines and error bars show the mean ± SD. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

Our study confirmed the significant association of BD with HLA–B51 and HLA–A26 and showed a novel independent association with HLA–C0704. We also identified a series of independent amino acid variations and SNPs in HLA regions associated with BD-related uveitis. Current understanding of these associations is largely limited, and further replications and

biologic studies are therefore needed to explore their underlying causal role in the development of BD.

Outside the HLA region, Chinese GWAS showed a strong association with genetic variants of ZMIZ1. ZMIZ1 encodes a member of the protein inhibitor of activated STAT family of proteins and has been found to be involved in thymocyte and T cell development (20). ZMIZ1 polymorphisms are associated with 46 traits or diseases recorded in the GWAS catalog database, including psoriasis, Crohn's disease, inflammatory bowel disease, and multiple sclerosis (21–23). These associations suggest that ZMIZ1 may play a role in inflammatory responses and that it might be a common target for a wide range of inflammatory diseases. The non-risk allele of the susceptibility locus within ZMIZ1 leads to an increased expression of ZMIZ1 in PBMCs. Luciferase reporter experiments and ChIP assays indicated that the non-risk allele G of the SNP within the ZMIZ1 gene could play a functional role with enhancer and transcription activities, thereby promoting the expression of ZMIZ1. These findings suggest that ZMIZ1 variants may have a causal association with BD.

Other susceptibility genes outside the HLA region that were found in our study are also involved in the regulation of the immune response and include KLF4, IL10RA, and IFNGR1. KLF4 is an evolutionarily conserved zinc finger–containing transcription factor involved in the regulation of diverse cellular processes such as cell growth, proliferation, and differentiation (24). It has been shown that KLF4 is implicated in T cell development and Th17 cell differentiation (25). IL10RA is a receptor for interleukin-10 (IL-10) that has been reported to mediate the immunosuppressive signal of IL-10, thus inhibiting the synthesis of proinflammatory cytokines (26). The newly identified susceptibility locus rs2228054 within IL10RA, which is in strong linkage disequilibrium with rs2228055 ($r^2 = 1$, $D' = 1$), was shown to be correlated with the expression of IL10RA in the eQTL analysis. Our study confirms the results of earlier GWAS analyses and supports the hypothesis that dysregulated IL-10 signaling is involved in the pathogenesis of BD (7,27,28).

We also identified susceptibility loci within IFNGR1, the gene encoding the ligand-binding chain (alpha) of the interferon-γ (IFNγ) receptor (29). This finding is consistent with a previous GWAS study that also identified another susceptibility locus (rs4896243) within IFNGR1 (30). Data from the eQTL database showed an association between the IFNGR1 susceptibility locus (rs9376268) and the expression of IFNGR1. Various studies have shown increased expression of IFNγ as well as enhanced IFNγ/T helper 1 cell immune response activity in BD (31–33). BD is thought to be caused by an aberrant immune reaction against certain triggers, and taken together, the newly discovered susceptibility genes we report here may provide a genetic basis to explain the dysregulated response in this disease.

Our GWAS data are consistent with previously reported susceptibility loci within IL23R-IL12RB2, IL10, ERAP1, IFNGR1, STAT4, LACC1, and CEBPB-PTPN1 (6,7,9,11,12). Pathway enrichment analyses indicated that these genes are involved in JAK/STAT signaling, cytokine receptor activity, and immune response. These findings extend our knowledge concerning the role of multiple genetic factors in the triggering of complex diseases, and may lead to the development of small-molecule drugs targeting these pathways in BD. Further replication in populations of other ethnicities as well as functional experiments are needed to evaluate the exact biologic role of these susceptibility genes as well as their relative contribution to the pathogenesis of BD.

One of the limitations of our study is that all Chinese BD patients included had uveitis and that new loci identified are to be considered risk loci for uveitis in BD and may not be generalized for all BD types. Further large sample validations in other BD subtypes and ethnic groups are therefore needed.

In conclusion, based on larger genomic data from an East Asian population, this GWAS identified a novel set of genetic variants that are associated with susceptibility to BD. Future research is needed to investigate whether some of these genes may function as a potential therapeutic target for BD.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Yang had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Su, Zhong, Zhou, Du, Q. Wang, Chang, H. Tan, Yi, Meguro, Takeuchi, Mizuki, Ohno, Zuo, Kijlstra, Yang.

Acquisition of data. Su, Zhong, Zhou, Du, Z. Ye, F. Li, Zhuang, C. Wang, Liang, Ji, Cao, Y. Li, Feng, Liao, W. Zhang, Shu, S. Tan, Xu, Pan, H. Li, Shi, Z. Chen, Zhu, X. Ye, X. Tan, J. Zhang, Z. Liu, Huang, Yuan, Pang, Y. Liu, Ding, Gao, M. Zhang, Chi, X. Liu, Y. Wang, L. Chen, Meguro, Takeuchi, Mizuki, Ohno, Zuo, Kijlstra, Yang.

Analysis and interpretation of data. Su, Zhong, Zhou, Du, Meguro, Takeuchi, Mizuki, Ohno, Zuo, Kijlstra, Yang.

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