Identification of susceptibility SNPs in *IL10* and *IL23R-IL12RB2* for Behçet's disease in Han Chinese

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Background: Although previous genome-wide association studies in various cohorts have identified several susceptibility loci underlying Behçet's disease (BD), this has not yet led to a breakthrough in the management of BD.

Objective: This study aimed to further investigate the association of 26 candidate single nucleotide polymorphisms with previous genome-wide association studies–identified nearly positive *P* values ($5.0 \times 10^{-8} < P < 1.0 \times 10^{-5}$) in Chinese Han patients with BD.

Methods: A case-control association study was performed in 1206 patients with BD and 2475 healthy controls. Genotyping was performed using iPLEX Gold genotyping assay. Gene expression and cytokine production was quantified by real-time PCR and ELISA.

Results: The results showed that significantly higher frequencies of the *IL23R-IL12RB2*/rs924080 TT genotype ($P = 2.03 \times 10^{-8}$; odds ratio [OR] = 1.50), *IL23R-IL12RB2*/rs12141431 CC genotype ($P = 2.18 \times 10^{-8}$; OR = 1.53), *IL10*/rs1800871 TT genotype ($P = 5.88 \times 10^{-8}$; OR = 1.47), and *IL10*/rs3024490 TT genotype ($P = 2.80 \times 10^{-5}$; OR = 1.34) were found in BD. Functional experiments showed an increased *IL23R* expression and IL-17 production in rs12141431/CC genotype carriers compared with GG genotype carriers. A decreased *IL10* expression and IL-10 production was observed in rs3024490/TT genotype carriers as compared with GG genotype carriers.

Conclusions: Our findings not only confirmed the association of *IL10*/rs1800871 and *IL23R-IL12RB2*/rs924080 with BD but also identified 2 susceptibility single nucleotide polymorphisms in *IL10* and *IL23R-IL12RB2* (rs3024490 and rs12141431) with BD in Han Chinese. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: IL23R, IL12RB2, IL10, *BD, susceptibility*

Uveitis is an inflammation of the uvea, consisting of iris, ciliary body, and choroid, and is a leading cause of visual impairment and blindness.[1] Behçet's disease (BD) is one of the most commonly seen uveitis entities in China,[2] and is an autoinflammatory disease characterized by various symptoms including recurrent uveitis, oral aphthae, genital ulcerations, arthritis, and skin lesions.[3] Although accumulating evidence showed that a large number of genetic factors, such as *HLA-B51*, *IL-23R*, *miR-146a*, *miR-182*, *IL12B*, *DHCR7*, *FAS*, *ERAP1*, and *ATG5*, are involved in the susceptibility to BD,[4-12] the exact etiology of BD remains unclear.

Recently, genome-wide association studies (GWAS) in a Japanese cohort identified 2 suggestive associations on chromosome 1p31.3 (rs12119179 in *IL23R-IL12RB2*) and 1q32.1 (rs1554286 in *IL10*).[13] Two other loci (rs1495965 in *IL23R-IL12RB2* and rs1800871 in *IL10*) have also been found to be significantly associated with BD after meta-analyzing the former 2 loci with results from additional Turkish and Korean cohorts.[13] In addition, a GWAS in a Turkish cohort has confirmed the known association of BD with *HLA-B51* and identified a new susceptibility locus (rs1518111 in *IL10*) with BD.[14] Besides this, another GWAS has identified new susceptibility loci for BD and epistasis between *HLA-B51* and *ERAP1*.[15] Furthermore, a GWAS in a Chinese BD cohort performed by our group identified rs897200 around *STAT4* as a novel locus for this disease.[16] It is obvious that the genetic background of BD may vary between different ethnic populations. A stringent *P* value below 5.0×10^{-8} is generally considered as the threshold for genome-wide significance, but may miss loci showing weaker associations that are also involved in the development of BD and there is therefore a need to further investigate these loci in various ethnic groups.

Hence, with the aim of investigating the possible role of the so-called GWAS-identified nearly positive associations ($5.0 \times 10^{-8} < P < 1.0 \times 10^{-5}$), we conducted a GWAS replication study in a Chinese Han population. Our results not only confirmed the association of *IL10*/rs1800871 and *IL23R-IL12RB2*/rs924080 with BD but also identified 2 susceptibility single nucleotide polymorphisms (SNPs) in *IL10* and *IL23R-IL12RB2* (rs3024490 and rs12141431).

METHODS

Case-control cohorts

One thousand two hundred six patients with BD were recruited from the Department of Ophthalmology in the First Affiliated Hospital of Chongqing

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Abbreviations used
BD: Behçet's disease
GWAS: Genome-wide association studies
LD: Linkage disequilibrium
OR: Odds ratio
SNP: Single nucleotide polymorphism

Medical University (Chongqing, China) between May 2008 and July 2015. The diagnosis of BD was made strictly according to the International Workshop criteria.[17] All patients with BD enrolled in this study had uveitis. In parallel, a total of 2475 healthy individuals representing the same ethnical population and geographic area with the patients with BD were enrolled in this study to serve as the controls. These controls were matched for sex and age with the patients with BD. The study was conducted in accordance with the tenets of the Declaration of Helsinki and received approval from the Ethics Research Committee at the Chongqing Medical University (permit no. 2009-201008). Before blood collection, all patients and controls were informed of this study and provided their written informed consent.

SNP selection and genotyping

Based on the potential susceptibility loci with a *P* value between 5.0×10^{-8} and 1.0×10^{-5} as identified in previous GWAS results,[13-16] 31 candidate SNPs (27 SNPs were in or near 22 genes, whereas 4 SNPs were in intergenic regions) were selected in this study (see Table I). Because the SNPs rs11209033 and rs12141431 were reported to be in strong linkage disequilibrium (LD) with each other,[13] we used only rs12141431 in our study. Similarly, SNPs rs1800871 and rs1554286 were also identified to be in a strong LD with each other,[18] and therefore only rs1800871 was used. Besides, no polymorphic data of the 3 SNPs (rs9819066, rs6549400, and rs11706279) were available in the Chinese Han database of single nucleotide polymorphism, and therefore we excluded them from this study. Genotyping analysis of the SNPs selected for validation was performed using iPLEX Gold Genotyping Assay and Sequenom MassArray (Sequenom, San Diego, Calif). Locus-specific PCR and detection primers were designed using the MassArray Assay Design 3.1 software (Sequenom) (see Table E1 in this article's Online Repository at www.jacionline.org). The complete experiment was carried out in strict accordance with standard procedures. For all SNPs, we examined the clustering patterns of genotypes and selected mass peaks and confirmed that the genotype calls were of good quality.

Cell isolation and culture

Ficoll-Hypaque density-gradient centrifugation was used to obtain PBMCs from venous blood of healthy controls. PBMCs were counted by microscope and then seeded into 24-well culture plates (1×10^6 cells per well). The cells were cultured in culture medium consisting of RPMI medium 1640, 10% FCS (Greiner, Wemmel, Belgium), 100 U/mL penicillin, and 100 μg/mL streptomycin. To investigate the effect of gene polymorphisms on the expression of *IL23R*, *IL12RB2*, and *IL10*, PBMCs were cultured with or without anti-CD3/CD28 antibodies (5:1) (Miltenyi Biotec, Palo Alto, Calif) at 37°C for 72 hours.[6]

Real-time PCR

Total RNA was extracted from unstimulated PBMCs and anti-CD3/CD28 antibodies-stimulated PBMCs using TRIzol Reagent (Invitrogen, Carlsbad, Calif), and the reverse transcription was performed using a transcriptase kit (Applied Biosysterms, ABI, Foster City, Calif). Real-time PCR was carried out using the Applied Biosystems 7500 System based on the SYBR-Green method. The expression of *IL23R*, *IL12RB2*, *IL10*, and β-*actin* (the internal reference) was detected using the primers as described in earlier studies (see Table E2 in this article's Online Repository at www.jacionline.org).[19-21] All tests were conducted in triplicate, and the relative expression levels of the candidate genes were quantified by using the $2^{-\Delta\Delta Ct}$ method.

Measurement of cytokines by ELISA

Supernatants of anti-CD3/CD28 antibodies-stimulated PBMCs were collected and stored at −80°C until laboratory testing. The production of IFN-γ, IL-10, and IL-17 was quantified using Duoset ELISA development kits (R&D Systems, Minneapolis, Minn) in accordance with the manufacturer instructions.

Statistical analysis

Hardy-Weinberg equilibrium was analyzed by the χ^2 test. Genotype and allele frequencies of candidate SNPs were compared between patients with BD and healthy controls by the χ^2 test using SPSS version 17.0 (SPSS, Inc, Chicago, Ill), which was also applied to calculate odds ratios (ORs) and 95% CI. For multiple comparisons, Bonferroni correction was applied in this study, and a *P* value of less than 4.90×10^{-4} (0.05/102) was considered to be statistically significant. The nonparametric Mann-Whitney *U* test or independent samples *t* test was used to compare *IL23R*, *IL12RB2*, and *IL10* expression levels as well as cytokine levels among different genotype groups.

RESULTS

Clinical manifestations of the enrolled patients with BD

The detailed demographic characteristics and clinical manifestations of patients with BD enrolled in this study are presented in Table II. The distribution of clinical symptoms in the patients with BD is as follows: 100% with oral ulcer, 51.1% with genital ulcer, 72.1% with skin lesions, 18.5% with arthritis, and 21.4% with a positive pathergy test.

Allele and genotype frequencies of tested SNPs in patients and controls in the study

Twenty-six SNPs (23 SNPs were in or near 22 genes, whereas 3 SNPs were in intergenic regions) were genotyped in 1206 patients with BD and 2475 healthy controls. Our results showed significantly increased frequencies of the *IL23R-IL12RB2*/rs924080 TT genotype ($P = 2.03 \times 10^{-8}$; OR = 1.50) and the *IL23R-IL12RB2*/rs12141431 CC genotype ($P = 2.18 \times 10^{-8}$; OR = 1.53) in BD (see Table III). In addition, the frequencies of the TT genotype for *IL10*/rs1800871 and the TT genotype for *IL10*/rs3024490 showed a significant increase in patients with BD ($P = 5.88 \times 10^{-8}$; OR = 1.47; $P = 2.80 \times 10^{-5}$; OR = 1.34) (see Table III). No significant differences in the other 22 investigated SNPs were detected between patients with BD and healthy individuals (see Table E3 in this article's Online Repository at www.jacionline.org).

Stratified analysis for rs924080, rs12141431, rs1800871, and rs3024490 with main clinical features of BD

A stratified analysis was performed to examine the association of rs924080, rs12141431, rs1800871, and rs3024490 with the main clinical features of BD. The main clinical manifestations of BD included genital ulcer, skin lesions, arthritis, and positive pathergy reaction. We could not demonstrate a significant association of the rs924080, rs12141431, rs1800871, and rs3024490 genotype frequency with any clinical manifestation of BD (see Tables E4-E7 in this article's Online Repository at www.jacionline.org).

The influence of rs924080 and rs12141431 on the expression of *IL23R* and *IL12RB2*

The aforementioned result revealed significant associations of SNPs rs924080 and rs12141431 of *IL23R-IL12RB2* with BD. To

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