Genetic Variations of NLR family genes in Behcet’s Disease

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This study aimed to investigate whether single nucleotide polymorphisms (SNPs) of five NLR family genes (NOD1, NOD2, NLRP1, NLRP3 and CIITA) are associated with Behcet’s disease (BD) in a Chinese Han population. The study was carried out in 950 BD patients and 1440 controls for 19 SNPs in the selected NLR genes. In the first-stage study, significantly decreased frequencies of the CIITA/rs12932187 C allele ($P_c = 1.668 \times 10^{-2}$) and NOD1/rs2075818 G allele ($P_c = 4.694 \times 10^{-2}$) were found in BD patients as compared to controls. After performing a second stage validation study and combination of data we confirmed the association of CIITA/rs12932187 and NOD1/rs2075818 with BD. In CIITA/rs12932187, the frequencies of the CC genotype and C allele were significantly lower in BD than in controls ($P_c = 3.331 \times 10^{-6}$; $P_c = 6.004 \times 10^{-7}$, respectively). In NOD1/rs2075818, the GG genotype and G allele showed significantly decreased frequencies in BD patients when compared to controls ($P_c = 1.022 \times 10^{-2}$; $P_c = 6.811 \times 10^{-5}$, respectively). Functional experiments showed that carriers with the CC genotype in CIITA/rs12932187 had a lower CIITA mRNA expression level and an enhanced IL-10 secretion as compared to GG and CG carriers. This study provides evidence that the CIITA and NOD1 gene are involved in the susceptibility to Behcet’s disease.

Behçet’s disease (BD) is a multifactorial disease which presents with oral aphthae, genital ulcerations, ocular inflammation, skin lesions and a pathognomonic pathergy test. The etiology of BD is largely unknown, but cumulative evidence suggests that an excessive T-cell mediated inflammatory response is associated with disease activity. Previous studies revealed that a number of genetic factors are involved in disease susceptibility, such as STAT3, STAT4, TLR2, miR-182, FAS and CD40 genes.

BD is associated with important morbidity of which the intraocular inflammation may lead to serious visual handicap. The disease is currently being treated with corticosteroids and a variety of immunosuppressive agents. Further knowledge of the inflammatory pathways involved in the disease process may lead to the development of new drugs to target these disorders. One of the approaches currently used includes the analysis of the association of these diseases with gene polymorphisms of proteins involved in the immune or inflammatory response. Since BD may be triggered by an infectious process we focused on gene polymorphisms associated with the microbial immune response.

Nucleotide-binding domain and leucine-rich repeat containing (NLRs) including at least 22 known proteins, exist in the cytosol and play an important role in the recognition of microbial products. They are characterized by three structural domains: a NACHT-domain for oligomerization and activation of the NLRs, an LRR domain at the C-terminus which is responsible for recognition of microbial patterns, and a protein–protein interaction domain at the N-terminus that could be formed of a pyrin (PYD)-, caspase (CARD) or a baculo-virus inhibitor of apoptosis repeat (BIR) domain, triggering the signal transduction cascade.

Few NLRs have been well characterized thus far, however, more recent studies demonstrate that variation in NLRs genes are associated with autoimmune or inflammatory disease. NOD2 was the first identified CD (Crohn’s disease) susceptibility gene and variations of NOD1 have been shown to confer risk to inflammatory bowel diseases (IBD) and CD. NLRP3 variations have also been found to be associated with several autoimmune diseases including neonatal–onset multi-system inflammatory disease (NOMID), Muckle-Wells syndrome (MWS) and familial cold urticaria (FCU). NLRP1 has been found to confer risk to autoimmune rheumatoid arthritis (RA), Addison's disease, type I diabetes and vitiligo. Variation in CIITA was also found to be related to a number of autoimmune diseases such as RA, myocardial infarction and multiple sclerosis (MS).

On the basis of these previous studies, we conducted this research to investigate whether polymorphisms of NLR family genes including NOD1, NOD2, NLRP1, NLRP3 and CIITA gene were associated with BD.
CIITA in the BD patients (Pc = 0.629–0.799, respectively). In NOD1//rs2075818, the frequencies of the GG genotype and G allele were also decreased in BD patients compared to controls (Table 2). The other seventeen SNPs did not show a significant association with BD (Supplementary Table S1). In the second stage, we tested another set of 566 BD patients and 870 healthy controls to confirm the result of the first stage study. After combination of the two stages, we found that the CC genotype and C allele of CIITA//rs12932187 had a lower mRNA expression of CIITA compared with the GG or CG genotype carriers (P = 0.004, Fig. 1). anti-CD3/anti-CD28 stimulation did not affect CIITA expression (Supplementary Fig. S2) and no effect on NOD1 mRNA expression was observed for the various rs2075818 genotypes by either normal or stimulated PBMCs (Supplementary Fig. S4 and Supplementary Fig. S5).

Because of the significant association of CIITA//rs12932187 and NOD1//rs2075818 with BD, we tested the expression of NOD1 and CIITA in PBMCs obtained from healthy individuals with known genotypes of the two SNPs. Real-time PCR did not show a detectable association between the various genotypes and the expression of NOD1 and CIITA when testing unstimulated PBMCs (Supplementary Fig. S1 and Supplementary Fig. S3). Following stimulation by LPS, carriers with the CC genotype of the CIITA//rs12932187 had a lower secretion level of IL-10 as compared to GG and CG carriers (P = 0.017, Fig. 2).

Since the different genotypes of CIITA//rs12932187 had an effect on CIITA mRNA expression, we decided to investigate whether the different genotypes influenced the cytokine response of PBMCs following LPS stimulation. We measured the PBMC expression levels of IL-6, IL-8, IL-10, IL-1β, TNF-α and MCP-1 by ELISA. These cytokines have all been shown to play a role in the development of BD as shown by earlier studies22,23. Carriers of the CC genotype had a higher secretion level of IL-10 as compared to GG and CG carriers (P = 0.017, Fig. 2). No significant effect on secretion levels of IL-6, IL-8, IL-1β, TNF-α and MCP-1 was found (Supplementary Fig. S6–S10).

Discussion
In the present study, we investigated the associations of 19 SNPs in NOD1, NOD2, NLRP1, NLRP3 and CIITA with BD in a Chinese Han population. Two SNPs, rs12932187 of CIITA and rs2075818 of NOD1 contributed to the genetic susceptibility of BD. Functional studies showed that carriers of the CC genotype of CIITA//rs12932187 had a lower CIITA mRNA expression level and an increased IL-10 secretion by PBMCs as compared to GG and CG carriers.

CIITA acts as a transcriptional coactivator and has been associated with various inflammatory and autoimmune diseases24,25. CIITA mediates activated immune responses and its deficiency has been shown to cause Type II Bare lymphocyte syndrome (BLS)19. Variability in the CIITA gene has also been reported to be associated with

| Clinical Features | Total | % |
|-------------------|-------|---|
| Ocular BD Patients | 950   |   |
| Age at onset, year ± SD | 33.1 ± 8.4 |   |
| Female | 950 | 19.6 |
| Male | 755 | 80.4 |
| Uveitis | 950 | 100 |
| Oral ulcer | 950 | 100 |
| Genital ulcer | 466 | 49.1 |
| Skin lesions | 570 | 60.0 |
| Pathergy reaction | 231 | 24.3 |
| Hypopyon | 202 | 21.3 |
| Arthritis | 151 | 15.9 |
| Controls | 1440 |   |
| Age at onset, year ± SD | 35.9 ± 11.2 |   |
| Female | 321 | 22.3 |
| Male | 1119 | 77.7 |

Table 1. Clinical Features, Age and Gender Distribution in Controls as well as Patients with Ocular BD. SD = standard deviation. BD = Behcet disease.
IL-10 is considered an immune regulatory cytokine which controls innate and adaptive immune responses. CIITA negatively regulates the expression of IL-10 by DCs, which supports the findings in humans as previously reported.

CIITA has been shown to function not only as a transcriptional regulator of MHC genes, but is also a transcriptional regulator of over 60 immunologically important genes, including IL-4, IL-10 and a number of thyroid-specific genes. A study in CIITA-deficient (CIITA\(^{-/-}\)) mice showed low IL-10 serum levels in Asian patients with BD\(^{33}\). The functional tests we performed showed that carriers with the CC genotype had a lower CIITA mRNA expression level and an enhanced IL-10 secretion as compared to GG and CG carriers. The protective effect of the C allele and CC genotype concerning BD development could thus be explained by the fact that these individuals produce more anti-inflammatory IL-10 in response to a microbial stimulus than carriers of the G allele. Further studies are needed to support this hypothesis.

NOD1 has been characterized as a critical regulator of innate immunity. Various studies have reported the association between NOD1 gene variants and autoimmune disease\(^{10}\). The NOD1//rs2075818 G allele was found to decrease the risk of CD and rs2907748 AA and AG genotypes showed a decreased frequency in UC\(^{13}\). These findings are in agreement with our study and could be due to the fact that BD as well as these inflammatory bowel disease are considered as an autoinflammatory disease caused by an aberrant response to a microbial agent. We were not able to detect a functional explanation for the association with NOD1//rs2075818.

Table 2. Polymorphisms of NOD1//rs2075818 and CIITA//rs12932187 in Behcet’s Disease. NS = no significant difference; OR = odds ratio; \(P_c = P\) value with Bonferroni correction. SNP = single nucleotide polymorphism. First stage (stage 1), case-to-control ratio: 380:576; Replication stage (stage 2), case-to-control ratio: 939:1438.
NLRP1 and NLRP3 have been shown to play an important role in the processing of pivotal pro-inflammatory cytokines such as IL-1β and IL-18. Gene variants of NLRP3 have been shown to be associated with Psoriatic Juvenile Idiopathic Arthritis in a Caucasian population. Moreover, genetic variants of NLRP1 were observed to confer risk for the development of vitiligo. Nevertheless, our study did not find an association between NLRP1 and NLRP3 and BD in a Chinese Han population. NOD2, that was already identified as a CD-susceptibility gene, was not associated with BD.

Our study has some limitations. Since the subjects in our study were all Chinese Han, the conclusions of the study are only valid to the Chinese Han population and should be studied and replicated in other ethnic groups. Furthermore, all the BD patients in this study were recruited from ophthalmology departments and a selection bias in our patient population may be present. Whether our findings can be generalized to other uveitis entities is not known and deserves further study. We did test the SNPs described in this study on uveitis patients with Vogt Koyanagi Harada syndrome but did not observe statistically significant associations (data not shown).

In conclusion, this study for the first time reports an association of CIITA//rs12932187 and NOD1//rs2075818 with susceptibility to BD in a Chinese Han population. A functional variant of CIITA//rs12932187 was shown to regulate CIITA expression and IL-10 production.
Materials and Methods

Study population. In the first stage of this study, a total of 384 BD patients and 576 controls were enrolled to identify disease susceptibility loci in the family of NLR genes. In the second (confirmatory) stage, another set of 566 BD patients and 864 controls were added to replicate the susceptible SNPs found in the first stage study.

All blood samples were enrolled at the Zhongshan Ophthalmic Center (Guangzhou, China) and the First Affiliated Hospital of Chongqing Medical University (Chongqing, China) from November 2006 to February 2015. The diagnosis of BD patients is based on the criteria of the International Study Group for BD36. This study obtained the approval of the Local Ethics Research Council before the collection of blood, all the investigated individuals had signed the informed consent.

Ethical considerations. Before the collection of blood, all the investigated individuals had signed the informed consent. The investigation protocols obtained the approval of the Clinical Research Ethics Committee of the Zhongshan Ophthalmic Center of Sun Yat-sen University and the First Affiliated Hospital of Chongqing Medical University. All experiments were conducted in accordance with the approved guidelines and regulations. This study was conducted according to the tenets of the Declaration of Helsinki.

SNP selection. We selected nineteen SNPs of NLR family genes including NOD1 (rs2075818, rs2907749), NOD2 (rs8057431, rs3135499), NLRP1 (rs10754558, rs10925019, rs4925648, rs3806265, rs2027432) and CIITA (rs12932187, rs1107438, rs8048002, rs4774).
DNA extraction and genotyping. Genomic DNA was extracted from the blood of patients and healthy individuals using the QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA, USA), all the samples were stored at −80°C until used. All SNPs except NLRP3//rs10925019 were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). All the primers were designed using primer software 5.0 (Premier Biosoft International, Palo Alto, CA, USA). Primers and restriction enzymes are shown in Table 3. NLRP3//rs10925019 (TaqMan assay ID: C_26646027_10) genotyping was performed on the Applied Biosystems 7500 Real-Time PCR system using TaqMan® SNP assay (Applied Biosystems, CA, USA). The analysis was performed by TaqMan Genotyper Software. To verify the accuracy of genotyping, direct sequencing was carried out (Beijing Biomed Co. Ltd. China) using randomly selected samples (10% of all samples). The genotyping success rate was above 95%.

Real-time PCR. In this study, peripheral blood mononuclear cells (PBMCs) were obtained from healthy controls by Ficoll-Hypaque density-gradient centrifugation; cells were stimulated with or without anti-CD3 (0.5ug/ml) and anti-CD28 antibodies (0.1ug/ml, ebSience, San Diego, CA, USA) to analog antigen presentation or lipopolysaccharide (LPS, 5ug/ml, Fluka, Buchs, Switzerland) to analog an inflammatory signal for 72 hours at a density of 1 × 10⁶ cells/ml. RNA was acquired from the cultured cells by TRIzol (Invitrogen), after reserve transcription (transcription kit, Takara Biotechnology Co. Ltd., Dalian, China.), mRNA expression of NOD1 gene (forward: 5′-TGACACCCCTGAGCTTGC3′, reserve: 5′-TCATTTTGGGTAGCCACAG3′) and CIITA gene (forward: 5′-TGAGGCTGTGCTCTGAG3′, reserve: 5′-ACACTGTAGCTGCTTGG3′) was measured by using real-time PCR equipment with a commercial dye kit (Applied Biosystems). β-Actin was selected as the internal reference gene and its expression was detected by the following primers: forward 5′-GGATGCGAAGGAGATCACCTG-3′ and reverse 5′-CGATCCACGGAGTAGCTTT-3′. Data were normalized to mRNA beta-actin and expression levels were calculated by the 2−ΔΔCT method.

Cytokine Measurements. The human Duoset enzyme-linked immunosorbent assay (ELISA) development kit (R&D System, Minneapolis, MN) was used to measure the concentration of IL-6, IL-8, IL-10, IL-1β, TNF-α and MCP-1 in cell culture supernatant in accordance with the manufacturers’ instructions.

Statistical analysis. Differences in alleles and genotypes of all SNP variations were evaluated by the Fisher’s exact test or X² test using SPSS (version 17.0; SPSS Inc, Chicago, IL). The p values were corrected with the Bonferroni correction method and a Pc < 0.05 was considered to be significant. The X² test was used to determine the Hardy-Weinberg equilibrium (HWE). The independent samples t test or nonparametric Mann-Whitney U test was used to compare CIITA, NOD1 and cytokine (IL-6, IL-8, IL-10, TNF-α, IL-1β and MCP-1) expression levels among three genotype groups.

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Corrigendum: Genetic Variations of NLR family genes in Behcet’s Disease

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This Article contains errors.

In Table 1, the number of Female ‘186’ and Male ‘764’ patients enrolled in this study is incorrectly given as ‘950’ and ‘755’ respectively.

In the Results section,

“The case group comprised 186 women and 764 men, and the average age of the BD patients was 33.0031 ± 8.4 years.”

should read:

“The case group comprised 186 women and 764 men, and the average age of the BD patients was 33.1 ± 8.4 years.”

In the Results section under subheading ‘Genotype Results’,

“In first stage study, the frequency of the CIITA/rs12932187 C allele (Pc = 1.668 × 10⁻², OR = 0.713, 95% CI = 0.591–0.861) and NOD1/rs2075818 G allele (Pc = 4.694E-02, OR = 0.698, 95% CI = 0.562–0.868) were decreased in BD patients compared to controls (Table 2).”

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“In NOD1/rs2075818, the frequencies of the GG genotype and G allele were also decreased in the BD patients (Pc = 1.022E-02, OR = 0.536, 95% CI = 0.386–0.745; Pc = 6.811 × 10⁻⁵, OR = 0.720, 95% CI = 0.629–0.824, respectively).”

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In Table 3, the Restriction enzyme ‘PvuII’ for gene NOD1 ’rs2907748’ is incorrectly given as ‘PvuIII’.

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