**MICA*049, not MICA*009, is associated with Behçet’s disease in a Chinese population**

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Behçet’s disease (BD) is a multi-system inflammatory disease. Previous reports indicated that MICA*009 confers susceptibility to BD. MICA*009 differs from MICA*009:01, a major MICA*009 subtype, only at codon 335 in exon 6. However, the potential association of MICA*009 with BD has not been addressed. In this study, we differentiated association among MICA*009, MICA*009 and HLA-B*51 with BD. A Han Chinese cohort consisting of 41 BD patients and 197 ethnically matched controls were examined with sequencing and T-ARMS-PCR for genotyping of MICA*00901 allele and MICA*009 allele. Here, we examined the association between MICA*009 and BD in a Han Chinese cohort. According to updated IMGT/HLA database, there are 107 HLA-B*51 alleles identified. The MICA*009 can be further subtyped into MICA*009:01, a major histocompatibility complex class I chain related gene A), located only 46 kb centromeric of HLA-B, is a highly polymorphic gene. It normally expresses on the cell membrane, and functions in immune activation under cellular stress conditions, such as infections, tissue injury, pro-inflammatory signals, and malignant transformation. The MICA transmembrane (TM) A6 allele and the MICA*009 allele were associated with BD in multiple previous reports. Among them, HLA-B*51 appears to be the most strongly associated known genetic risk to BD in different ethnic groups. MICA (major histocompatibility complex class I chain related gene A), located only 46 kb centromeric of HLA-B, is a highly polymorphic gene. It normally expresses on the cell membrane, and functions in immune activation under cellular stress conditions, such as infections, tissue injury, pro-inflammatory signals, and malignant transformation. The MICA transmembrane (TM) A6 allele and the MICA*009 allele were associated with BD in multiple previous reports. HLA-B*51 appears to be the most strongly associated known genetic risk to BD in different ethnic groups. MICA (major histocompatibility complex class I chain related gene A), located only 46 kb centromeric of HLA-B, is a highly polymorphic gene. It normally expresses on the cell membrane, and functions in immune activation under cellular stress conditions, such as infections, tissue injury, pro-inflammatory signals, and malignant transformation. The MICA transmembrane (TM) A6 allele and the MICA*009 allele were associated with BD in multiple previous reports. Among them, MICA*00901 allele and MICA*0090201 and MICA*0090202. The only difference between the MICA*00901 and the MICA*009 is at codon 335 in exon 6 (https://www.ebi.ac.uk/ipd/imgt/hla/align.html). In the previous studies, the ambiguity between the MICA*009 allele and the MICA*009 allele was not addressed, because exon 6 was not studied. Therefore, the MICA*009 allele maybe mixed with the MICA*009 allele. Here, we examined the association between MICA and BD in a Han Chinese cohort with MICA sequencing approach, along with a simple tetra-primer amplification refractory mutation system-polymerase chain reaction (T-ARMS-PCR) method to discriminate between the MICA*00901 allele and the MICA*009 allele.

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Results

The frequencies of MICA alleles in the 41 BD patients and 197 healthy controls were shown in Table 1. There were 8 different MICA alleles in patients and 16 in controls. The frequency of MICA*049 was significantly higher in the patient group (24.4% in BD versus 4.3% in control, OR = 38.16, P = 6.52 × 10^{-10}). However, the frequency of MICA*009 (including MICA*009:01 and MICA*009:02) was similar between the two groups (8.5% versus 6.6%, OR = 1.32, P = 0.53).

The genotype frequencies of MICA*008(:01 or :04)/MICA*049, MICA*010:01/MICA*049 and MICA*049/ MICA*049 were significantly higher in the patients (see Supplementary Table S1).

The MICA allele phenotype frequencies in BD patients and controls were shown in Table 2. The MICA*049 was significantly increased in BD patients compared to that in controls (41.5% versus 8.1%, OR = 8.01, P = 1.91 × 10^{-8}). The difference of the MICA*009 frequency between patients and controls was not significant (17.1% in BD versus 13.2% in control, OR = 1.35, P = 0.51). The allele frequency of the MICA*A6 was significantly higher in BD patients than that in controls (32.9% versus 11.7%, OR = 3.71, P = 1.18 × 10^{-4}). The result of phenotype frequency was consistent with that of allele frequency (53.7% versus 21.8%, OR = 4.15, P = 3.16 × 10^{-5}).

The presence of HLA-B*51 in BD patients and controls were 46.3% and 15.7% (OR = 4.62, P = 1.21 × 10^{-5}), respectively (Table 3).

Table 1. Comparison of MICA alleles between BD patients and controls.

| MICA Allele | Patients n = 41 (%) | Controls n = 197 (%) | χ² | P value | OR (95% CI) |
|-------------|---------------------|----------------------|----|---------|-------------|
| 002:01      | 8 (19.5)            | 60 (30.5)            |    |         |             |
| 004         | 0 (0.0)             | 3 (1.5)              |    |         |             |
| 007:01      | 0 (0.0)             | 5 (2.5)              |    |         |             |
| 008(01or:04)| 11 (26.8)           | 78 (39.6)            |    |         |             |
| 008:02      | 0 (0.0)             | 4 (2.0)              |    |         |             |
| 009:01      | 7 (17.1)            | 24 (12.2)            | 0.43| 0.51    | 1.35 (0.54–3.37) |
| 009:02      | 0 (0.0)             | 2 (1.0)              |    |         |             |
| 010:01      | 20 (48.8)           | 79 (40.1)            |    |         |             |
| 012:01      | 3 (7.3)             | 23 (11.7)            |    |         |             |
| 017         | 0 (0.0)             | 4 (2.0)              |    |         |             |
| 018:01      | 0 (0.0)             | 1 (0.5)              |    |         |             |
| 019         | 8 (19.5)            | 21 (10.7)            |    |         |             |
| 027         | 0 (0.0)             | 23 (11.7)            |    |         |             |
| 033         | 0 (0.0)             | 1 (0.5)              |    |         |             |
| 045         | 1 (2.4)             | 14 (7.1)             |    |         |             |
| 049         | 17 (41.5)           | 16 (8.1)             | 31.59| 1.91 × 10^{-4} | 8.01 (3.58–17.91) |

Table 2. Phenotype frequencies of MICA alleles in BD patients and controls.
Table 3. Association of BD with HLA-B*51.

| MICA*049 | Absence of HLA-B*51 | Presence of HLA-B*51 |
|----------|---------------------|---------------------|
|          | Patients            | Controls            | Patients            | Controls            |
| Presence | 2                   | 0                   | 15                  | 16                  |
| Absence  | 20                  | 166                 | 4                   | 15                  |
| $\chi^2$ | 15.25               | 3.74                |                     |                     |
| P value  | 0.02                | 0.07                |                     |                     |
| OR (95% CI) | 40.61 (1.88–875.58) | 3.52 (0.95–13.01)  |                     |                     |

Table 4. Association of MICA*049 with BD stratified for the effect of HLA-B*51.

| HLA-B*51 | Absence of MICA*049 | Presence of MICA*049 |
|----------|---------------------|---------------------|
|          | Patients            | Controls            | Patients            | Controls            |
| Presence | 4                   | 15                  | 15                  | 16                  |
| Absence  | 20                  | 166                 | 2                   | 0                   |
| $\chi^2$ | 1.77                | 2.00                |                     |                     |
| P value  | 0.25                | 0.29                |                     |                     |
| OR (95% CI) | 2.21 (0.67–7.32)   | 0.19 (0.01–4.23)   |                     |                     |

Table 5. Association of HLA-B*51 with BD stratified for the effect of MICA*049.

To examine whether the observed BD association of MICA*049 and HLA-B*51 are independent from each other, we performed subclonal analysis in HLA-B*51 negative subjects for MICA*049, and in MICA*049 negative subjects for HLA-B*51. As shown in Table 4, the MICA*049 remained significantly associated with BD (OR = 40.61, P = 0.02) in HLA-B*51 negative BD patients, but the association of HLA-B*51 with BD appeared lost in MICA*049 negative patients (Table 5).

Discussion

Previously, MICA*009 and MICA*A6 were suggested as susceptibility alleles for BD. The MICA*A6 is a polymorphism with 6 tendent repeats of GCT in exon 5 of MICA gene. This polymorphism is included in the MICA*009, and shared by MICA*049 and a number of other MICA alleles. In the previous studies, the MICA alleles were identified by PCR-SSP or PCR-SBT based on sequences of exon 2 to exon 5. However, the MICA*00901 and the MICA*049 differ by only one nucleotide at codon 335 of exon 6. Therefore, the ambiguity between these two alleles could not be addressed, and the MICA*009 allele reported in the previous studies may be mixed with MICA*049. According to allelic functional analysis using SIFT program (http://sift.bii.a-star.edu.sg/), the change at codon 335 may impact MICA function.

In the present study, we developed a rapid and cost-efficient T-ARMS-PCR to discriminate the MICA*009 from the MICA*049. Comparison analysis between BD patients and controls showed that the MICA*049, not MICA*009, was strongly associated with BD. As we expected, the MICA*A6 showed a consistent BD association with previous reports as it is within the MICA*049 polymorphism. It is worth noting that the allele frequency of the MICA*009 and *049 in controls were consistent with the previous report of MICA alleles in a Chinese population. Considering MICA and HLA-B genes are located next to each other, and strong linkage disequilibrium (LD) exists between alleles of these two genes, it is necessary to determine whether the observed association is due to LD effect from HLA-B*51. According to the clonal analysis, the MICA*049 was independently associated with BD in the Chinese cohort.

In conclusion, we investigated MICA polymorphisms in patients with BD of Chinese Han. It is the first report of MICA*049 in association with BD, and which appeared independent from HLA-B*51. Although the sample size is relatively small in the study, the association achieved significant P value with strong odd ratio. However, it still warrants further validation studies in a larger Chinese cohort and/or other ethnic populations. It may not rule out this observed association is ethnic specific for Chinese Han population.

Methods

Participants. A total of 41 Patients (34 male, 7 female) were enrolled between March 2010 and September 2017 from the Eye Hospital of Wenzhou Medical University. The diagnosis of BD was followed the criteria of the International Study Group of BD. The mean age of the patients was 37.8 years (range between 27–50 years) and the mean duration of the disease was 6.4 years (range between 1–18 years). A total of 197 unrelated healthy
of genomic DNA. The PCR program on the Veriti Thermal cycler was as follow: 95 °C for 10 min; 35 cycles of 20 s to 3′, Reverse primer: 5′-AGAGAAAGGGCGAATCTGGT-3′)

Table 6. Primers for the T-ARMS-PCR to distinguish MICA*009:01 from MICA*004. FO: Forward outer primer, FI: Forward inner primer, RI: Reverse inner primer, RO: Reverse outer primer.

| Primer | Sequence (5′ to 3′) | Concentration (μM) |
|--------|---------------------|--------------------|
| FO     | GATGGGAGGGGAATCCTGGGCT | 0.4 |
| FI     | GGTTCTGGATGCAACACCCAGTGATGAC | 0.8 |
| RI     | GGCATCCCAGTGCTACGGCA | 1.4 |
| RO     | AGGCACCAAGGAGGAAAGTGCTG | 0.4 |

individuals were recruited in the same geography. All of patients and controls were Chinese Han. The study was approved by the Ethics Committee of the Eye Hospital of Wenzhou Medical University and was conducted according to the Declaration of Helsinki Principles. Written informal consent was obtained from all participants.

### Genomic DNA extraction

Genomic DNA was extracted from peripheral blood cells of all subjects using Biotek DNA isolation kit (Beijing, China). After detecting DNA concentration by a Nanodrop 2000 spectrophotometer, a part of DNA of each subject was diluted to 10 ng/μl for genotyping assays.

### HLA-B*51 genotyping

For control samples, the HLA-B*51 genotyping was performed with sequence-based typing (SBT) method using secure kits (Life Technologies, USA)\(^{14}\). For patients, each sample was genotyped for HLA-B*51 positivity by ARMS PCR method\(^{15}\).

### MICA genotyping

MICA was genotyped by PCR sequencing exon 2–5 regions using bidirectional Sanger sequencing methods\(^{16}\). For samples in patient group which were discriminated as MICA*009:01/*004, Sanger sequencing was used to distinguished the two alleles. Two primers (Forward primer: 5′-AGAGAAAGGGCGAATCCTGGT-3′; Reverse primer: 5′-AAGAGGAAAA-GTGCTGGTGA-3′) were used to amplify 301 bp PCR products. The PCR was performed in a total volume of 20 μl containing 10 μl of 2 × Taq Master Mix (Jinan, Shanghai, China), 0.4 μM of each primer (Invitrogen, Shanghai, China) and 10 ng of genomic DNA. PCR was carried out on a Veriti Thermal cycler. The PCR thermal cycling condition was an initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 20 s, 60 °C for 20 s and 72 °C for 20 s, and a final extension at 72 °C for 5 min. For samples in control group which were detected as MICA*009:01/*004, T-ARMS-PCR was used to differentiate MICA*009:01 from MICA*004. The sequence of the four primers and concentration of each primer were listed in Table 6. Product sizes were 246 bp for T allele, 182 bp for C allele, and 382 bp for the forward primer were listed in Table 6. Product sizes were 246 bp for T allele, 182 bp for C allele, and 382 bp for the forward outer primer.

### Statistical analysis

HLA-B*51 and MICA allelic frequencies were calculated by direct counting. The significance of the distribution of alleles between the patient group and the control group was calculated by Chi-square or Fisher’s exact test using SPSS22.0 or Epi info software. If the cell frequency as zero, the odds ratio (OR) was calculated using MedCalc software (https://www.medcalc.org/calc/odds_ratio.php).

### Data Availability

The data generated and/or analyzed in the current study are available from the corresponding authors on reasonable request.

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Author Contributions

X.D.Z. and Y.Q.W. designed this study. J.C.W. and D.L. collected samples. W.F.Z., Y.D., J.C.W., X.J.G., W.F.D. and J.S.C. performed the experiments. W.F.Z. and X.D.Z. analyzed the data. W.F.Z. and Y.D. wrote the manuscript. X.D.Z. reviewed the manuscript. All authors read and approved the final manuscript.

Additional Information

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