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

Weifeng Zhu[1,2], Yan Deng[2,3], Jiucun Wang[4,5,6], Xinjian Guo[2], Weifeng Ding[2,7], Jiashuo Chao[8], Dan Lin[9], Yuqin Wang[9] & Xiaodong Zhou[2]

Behçet's disease (BD) is a multi-systemic inflammatory disease. Previous reports indicated that MICA*009 confers susceptibility to BD. MICA*049 differs from MICA*009:01, a major MICA*009 subtype, only at codon 335 in exon 6. However, the potential association of MICA*049 with BD has not been addressed. In this study, we differentiated association among MICA*049, 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, and ARMS-PCR for HLA-B*51. The phenotype frequency of MICA*049 (41.5% versus 8.1%, OR = 8.01, $P = 1.91 \times 10^{-8}$) and HLA-B*51 (46.3% versus 15.7%, OR = 4.62, $P = 1.21 \times 10^{-5}$) were significantly higher in BD patients than those in controls, whereas MICA*009 showed no significant difference between the two groups (17.1% versus 13.2%, OR = 1.35, P = 0.51). After stratification for the effect of HLA-B*51, MICA*049 was still associated with BD in HLA-B*51 negative patients (OR = 40.61, P = 0.02). Our results indicate that MICA*049, not MICA*009, is a risk factor to BD, and that is independent from HLA-B*51 in the Han Chinese cohort.

Behçet's disease (BD) is a multi-system inflammatory disease characterized by recurrent oral and genital ulcers, uveitis, and skin lesions. Although the etiology and pathogenesis of BD are still uncertain, multiple genetic factors have been linked to BD[1,2]. Among them, HLA-B*51 appears to be the most strongly associated known genetic risk to BD in different ethnic groups[2].

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[2]. The MICA transmembrane (TM) A6 allele and the MICA*009 allele were associated with BD in multiple previous reports[3–11]. According to updated IMGT/HLA database, there are 107 MICA alleles identified. The MICA*009 can be further subtyped into MICA*00901, MICA*0090201 and MICA*0090202. The only difference between the MICA*00901 and the MICA*049 is at codon 335 in exon 6 (https://www.ebi.ac.uk/ipd/imgt/hla/align.html). In the previous studies[5,8–11], the ambiguity between the MICA*009 allele and the MICA*049 allele was not addressed, because exon 6 was not studied. Therefore, the MICA*009 allele maybe mixed with the MICA*049 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*049 allele.

[1]Department of Biochemistry and Molecular Biology, College of Basic Medical Sciences, Nanchang University, Nanchang, China. [2]Department of Internal Medicine, McGovern Medical School, University of Texas Health Science Center at Houston, Houston, Texas, USA. [3]Department of Ophthalmology of Children, The Second Affiliated Hospital of Nanchang University, Nanchang, China. [4]State Key Laboratory of Genetic Engineering, Collaborative Innovation Center for Genetics and Development, School of Life Sciences, Fudan University, Shanghai, China. [5]Institute of Rheumatology, Immunology and Allergy, Fudan University, Shanghai, China. [6]Human Phenome Institute, Fudan University, Shanghai, China. [7]Department of Laboratory Medicine, Affiliated Hospital of Nantong University, Nantong, China. [8]The First Clinic Medical College, School of Medicine, Nanchang University, Nanchang, China. [9]Department of Uveitis, The Eye Hospital, Wenzhou Medical University, Wenzhou, China. Correspondence and requests for materials should be addressed to Y.W. (email: yqwang57@163.com) or X.Z. (email: Xiaodong.Zhou@uth.tmc.edu)

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MICA Allele	Patients n = 82 (%)	Controls n = 394 (%)	χ^2	P value	OR (95% CI)
002:01	8 (9.8)	65 (16.5)			
004	0 (0.0)	3 (0.8)			
007:01	0 (0.0)	7 (1.8)			
008 (:01 or :04)	13 (15.9)	91 (23.1)			
008:02	0 (0.0)	4 (1.0)			
009:01	7 (8.5)	24 (6.1)	0.39	0.53	1.32 (0.55–3.16)
009:02	0 (0.0)	2 (0.5)			
010:01	22 (26.8)	89 (22.6)			
012:01	3 (3.7)	24 (6.1)			
017	0 (0.0)	5 (1.3)			
018:01	0 (0.0)	1 (0.3)			
019	8 (9.8)	22 (5.6)			
027	0 (0.0)	24 (6.1)			
033	0 (0.0)	1 (0.3)			
045	1 (1.2)	15 (3.8)			
049	20 (24.4)	17 (4.3)	38.16	6.52×10^{-10}	7.15 (3.55–14.41)

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

MICA Allele	Patients n = 41 (%)	Controls n = 197 (%)	χ^2	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^{-8}	8.01 (3.58–17.91)

Table 2. Phenotype frequencies of *MICA* alleles in BD patients and controls.

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, $P = 6.52 \times 10^{-10}$). 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, $P = 1.91 \times 10^{-8}$). 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, $P = 1.18 \times 10^{-6}$). The result of phenotype frequency was consistent with that of allele frequency (53.7% versus 21.8%, OR = 4.15, $P = 3.16 \times 10^{-5}$).

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

	Patients n = 41 (%)	Controls n = 197 (%)	χ^2	P value	OR (95% CI)
Presence	19 (46.3)	31 (15.7)	19.16	1.21×10^{-5}	4.62 (2.24–9.54)
Absence	22 (53.7)	166 (84.3)			

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
χ^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
χ^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[5,8–11], 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 *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[12]. 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[13]. 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

Primer	Sequence (5′ to 3′)	Concentration (µM)
FO	GATGGGAGGGAACTGGTAGGGGCT	0.4
FI	GGTCCTGGATCAACACCCAGTTGGTAC	0.8
RI	GGCATCCCTGTGGTCACGCA	1.4
RO	AGGCACCAAGAGGGAAAGTGCTCG	0.4

Table 6. Primers for the T-ARMS-PCR to distinguish *MICA**009:01 from *MICA**049. FO: Forward outer primer, FI: Forward inner primer, RI: Reverse inner primer, RO: Reverse outer primer.

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 inform consent was obtained from all participants.

Genomic DNA extraction. Genomic DNA was extracted from peripheral blood cells of all subjects using Bioteke 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 secore 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/*049, Sanger sequencing was used to distinguished the two alleles. Two primers (Forward primer: 5′-AGAGAAAGGGCGAATCTGGT-3′, Reverse primer: 5′-AAGAGGGAAA-GTGCTCGTGA-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/*049, T-ARMS-PCR was used to differentiate *MICA**009:01 from *MICA**049. 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 outer primer and reverse outer primer. The PCR was performed in a final volume of 10 µl containing 5 µl of 2 × Hot-start Taq Red Master Mix (PHENIX, CA, USA), 0.4–1.4 µM of each primer (IDT, Skokie, USA) and 10 ng of genomic DNA. The PCR program on the Veriti Thermal cycler was as follow: 95 °C for 10 min; 35 cycles of 20 s at 94 °C, 30 s at 62 °C and 25 s at 72 °C, followed by a final extension of 5 min at 72 °C. The results of T-ARMS-PCR were verified by DNA sequencing. Direct sequencing was done with the two outer primers. The PCR products were purified using DNA clean & concentrator kit (Irvine, CA, USA), then the purified PCR products were sent to company for sequencing (GENEWIZ, NJ, USA). The sequencing data were analyzed using chromas software.

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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