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ORIGINAL INVESTIGATION

TNFAIP3 gene polymorphisms confer risk for Behcet's disease in a Chinese Han population

Hong Li · Qing Liu · Shengping Hou · Liping Du · Qingyun Zhou · Yan Zhou · Aize Kijlstra · Zheng Li · Peizeng Yang

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Abstract The tumor necrosis factor alpha-inducible protein 3 (*TNFAIP3*) gene polymorphisms have recently been reported to be associated with the susceptibility to several immune-related diseases. This study was performed to evaluate the potential association of *TNFAIP3* polymorphisms with Behcet's disease (BD) in a Chinese Han population. Five single-nucleotide polymorphisms (SNPs), rs10499194, rs610604, rs7753873, rs5029928, and rs9494885 of *TNFAIP3* were genotyped in 722 BD patients and 1,415 healthy controls using a PCR-restriction fragment length polymorphism assay. Allele and genotype frequencies were compared between patients and controls using the χ^2 test. The results showed a significantly increased prevalence of the rs9494885 TC genotype and C allele in BD patients compared with controls (Bonferroni corrected p (p_c) = 1.83×10^{-10}, odds ratio (OR) [95 % CI] 2.03 [1.65–2.49]; $p_c = 8.35 \times 10^{-10}$, OR [95 % CI] 1.81 [1.51–2.18], respectively).The frequency of the TT genotype and T allele of rs9494885 was markedly lower in BD patients than that in controls ($p_c = 1.23 \times 10^{-10}$, OR [95 % CI] 0.50 [0.40–0.61]; $p_c = 8.35 \times 10^{-10}$, OR [95 % CI] 0.55 [0.46–0.66], respectively). For rs10499194, a higher frequency of the CC genotype ($p_c = 0.015$, OR [95 % CI] 1.96 [1.30–2.97]) and C allele ($p_c = 0.005$, OR [95 % CI] 1.92 [1.28–2.90]), and a lower frequency of the TC genotype ($p_c = 0.015$, OR [95 % CI] 0.51 [0.34–0.77]) and T allele ($p_c = 0.005$, OR [95 % CI] 0.52 [0.35–2.97]) were found in BD patients. Concerning rs7753873, a higher frequency of the AC genotype ($p_c = 0.015$, OR [95 % CI] 1.49 [1.17–1.91]) and C allele ($p_c = 0.025$, OR [95 % CI] 1.39 [1.11–1.76]), and a lower frequency of the AA genotype ($p_c = 0.03$, OR [95 % CI] 0.68 [0.53–0.87]) and A allele ($p_c = 0.025$, OR [95 % CI] 0.72 [0.57–0.91]) were observed in BD patients. This study identified one strong risk SNP rs9494885 and two weak risk SNPs rs10499194 and rs7753873 of *TNFAIP3* in Chinese Han BD patients.

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H. Li · S. Hou · L. Du · Q. Zhou · Y. Zhou · P. Yang (✉)
The First Affiliated Hospital of Chongqing Medical University, Chongqing Key Laboratory of Ophthalmology, and Chongqing Eye Institute, Chongqing 400016, People's Republic of China
e-mail: peizengycmu@126.com

Q. Liu (✉)
The First Affiliated Hospital of Chongqing Medical University, The Clinical Research Center, Chongqing 400016, People's Republic of China
e-mail: qingliu5566@yahoo.cn

A. Kijlstra
University Eye Clinic Maastricht, Maastricht, The Netherlands

Z. Li
Ocular Genomics, Singapore Eye Research Institute, Singapore, Singapore

Introduction

Behçet's disease (BD) is an idiopathic, multisystem, recurrent chronic inflammatory disease, clinically characterized as recurrent uveitis, recurrent oral ulceration, genital ulceration, and erythema nodosum. It is quite common along the ancient 'Silk Road' countries extending from China to the Mediterranean area (Keino and Okada 2007; Yang et al. 2008). Although the precise etiology of BD remains unknown, extensive studies suggest that the environment and genetic factors are all involved in its pathogenesis. It has been shown that polymorphisms of several genes such as human leukocyte antigen-B51 (HLA-B51) (Cohen et al. 2002), intercellular

adhesion molecule-1(ICAM-1) (Verity et al. 2000), small ubiquitin-like modifier4 (SUMO4) (Hou et al. 2008), interleukin-23 receptor (IL-23R)-IL12RB2 (Jiang et al. 2010; Mizuki et al. 2010; Remmers et al. 2010), IL10 (Mizuki et al. 2010; Remmers et al. 2010), monocyte chemoattractant protein-1 (MCP)-1 (Hou et al. 2010), signal transducers and activators of transcription 4(STAT4) (Hou et al. 2012c; Hu et al. 2010), CCR1/CCR3 (Hou et al. 2012b), UBAC2 (Hou et al. 2012a), and Fc receptor-like 3 gene (FCRL3) (Li et al. 2008) are established or suggested to be associated with susceptibility to BD. However, the identified risk genes such as HLA-B51 only account for approximately 20 % of the genetic-risk effect in siblings of affected individuals (Gul et al. 2001). Therefore, there are many non-HLA risk genes which need to be investigated.

TNFAIP3 encodes the ubiquitin-modifying enzyme A20, a key regulator of the NF-κB signaling pathway of tumor necrosis factor alpha (TNFα), toll-like receptor (TLR), interleukin 1 receptor (IL1R), and nucleotide-binding oligomerization domain containing 2 (NOD2) (Boone et al. 2004; Hitotsumatsu et al. 2008; Jaattela et al. 1996; Lee et al. 2000). Several studies have suggested a role for *TNFAIP3* polymorphisms in the susceptibility to complex genetic autoimmune disorders, including rheumatoid arthritis (RA) (Plenge et al. 2007; Thomson et al. 2007), systemic sclerosis (SSc) (Dieude et al. 2010),systemic lupus erythematosus (SLE) (Adrianto et al. 2011; Graham et al. 2008; Musone et al. 2008), psoriasis (Musone et al. 2011; Nair et al. 2009), Sjögren's syndrome (SS) (Musone et al. 2011), Crohn's disease (Musone et al. 2011; WTCCC 2007), celiac disease (Dubois et al. 2010; Trynka et al. 2009; Zhernakova et al. 2011), diabetes (Fung et al. 2009), and ulcerative colitis (Wang et al. 2010a). These findings suggest that *TNFAIP3* may be a common risk gene for a number of immune-related disorders. Moreover, these genetic findings described in human patients linking *TNFAIP3* with autoimmune inflammatory pathology such as RA, SLE, psoriasis, and CD were experimentally confirmed in mice (Chu et al. 2011; Hammer et al. 2011; Kool et al. 2011; Matmati et al. 2011; Tavares et al. 2010; Vereecke et al. 2010). Based on this evidence, we postulated that *TNFAIP3* might also be a risk gene for BD and therefore examined whether the *TNFAIP3* gene was associated with the susceptibility to BD in a Chinese Han population. In this study we identified a strong association between rs9494885 of this gene with BD and two weak associations were observed with rs7753873 and rs10499194.

Methods

Study population

Seven hundred and twenty-two BD patients who all belonged to the Chinese Han population were included in this study. The control population consisted of 1,415 unrelated healthy individuals who were selected from the same geographical regions as the BD patients. There were no differences in age, sex, and ethnicity between patients and controls. The blood samples were obtained from the First Affiliated Hospital, Chongqing Medical University (Chongqing, China) or the Uveitis Study Center of the Sun Yat-sen University (Guangzhou, China). The diagnosis of BD was based on the criteria of the International Study Group (1990). The clinical characteristics of the BD patients were assessed at the time of diagnosis and are summarized in Table 1. All study participants gave written informed consent and the local ethical committee of both hospitals approved the study.

Table 1 Clinical features of the investigated BD patients

Clinical features	Total (n = 722)	%
Age at onset (years ± SD)	34.5 ± 6.7	
Male	594	82.3
Female	128	17.7
Uveitis	722	100
Oral ulcer	722	100
Genital ulcer	314	43.5
Hypopyon	186	25.8
Skin lesions	356	49.3
Positive pathergy test	258	35.7
Arthritis	205	28.4

BD Behcet's disease

SNP selection and genotyping

We studied 5 SNPs rs10499194, rs610604, rs7753873, rs5029928 and rs9494885 in the *TNFAIP3* region on 6q23, which were demonstrated earlier by other groups to be associated with certain immune-related diseases. SNPs rs10499194, rs7753873, and rs9494885 are located in the intergenic region of *TNFAIP3*. SNPs rs610604, rs5029928 are located in an intron of *TNFAIP3*. Genomic DNA was isolated from blood leukocytes using the commercial kit Qiagen DNA Blood Mini kit (Qiagen, Valencia, CA). The extracted DNA was stored at −20 °C until use. Amplification of the target DNA was performed by PCR. The primers used in this study are shown in Table 2. These five SNPs were genotyped by restriction fragment length polymorphism analysis. The amplification was performed using initial denaturation at 95 °C for 5 min, 95 °C for 30 s, 58–62 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min followed by 37 cycles. The PCR products were incubated with restriction enzymes (Table 2) for at least 4 h. Digestion products were visualized on a 4.0 % agarose gel and stained with Goodview (SBS Genetech, Beijing, China). Direct sequencing was performed by the Invitrogen

Table 2 Primers and restriction enzymes used for RFLP analysis of the *TNFAIP3* gene

rs number	Primers	Tm (°C)	Restriction enzyme
rs10499194	5′CCACCTTGAATTTCTTAGCTCTG 3′	62	MseI/TRUII
rs610604	5′TCCCCTGCTCGCTGTTTT 3′	60	SacI
	5′GCGCCTTTGAGTGTGTCTGC 3′		
rs7753873	5′ATGCCTCATTTATTCACTCAAC 3′	60	TSP509I
	5′CCAAAGGGATGCTCTGTC 3′		
rs5029928	5′GGGAGAAGAGTTTGAGTAAC 3′	60	XapI (ApoI)
	5′GCAGCTAAGGCAATGGAG 3′		
rs9494885	5′TACCAGCCACATAGCAAGCA 3‘	58	hinfI
	5′CAGGGCATATGTGGGAGAAA 3′		

RFLP restriction fragment length polymorphism

Biotechnology Company (Guangzhou, Guangdong province, China) using randomly selected subjects (20 % of all samples) to validate the method used in this study.

Real-time PCR

Peripheral blood mononuclear cells (PBMCs) were prepared from peripheral blood by Ficoll-Hypaque density-gradient centrifugation. Total RNA was extracted from PBMCs using TRIzol (Invitrogen), followed by reverse transcription using a transcriptase kit (Applied Biosysterms). The relative expression level of mRNA was normalized to that of the internal control β-actin by using the $2^{-\Delta\Delta Ct}$ cycle threshold method.

Statistical analysis

Distribution of genotypes and alleles between patients and normal controls was analyzed using SPSS version 17.0 (SPSS, Inc., Chicago, IL). The Chi square test was used to compare allele and genotype distributions. In case a genotype had a frequency less than 10, the Fisher's exact test was applied. Bonferroni correction was applied for multiple testing. We also performed a power analysis using Quanto software to verify the association between BD and the tested SNPs. The result showed a power value of 0.84 for SNP rs10499194, 0.26 for SNP rs610604, 0.05 for SNP rs5029928, 0.48 for SNP rs7753873, and 0.96 for SNP rs9494885 using a prevalence of Behcet's disease in our population of 0.01 % (Wang et al. 2010b) (Supplementary Table 1).

Results

There were no differences in age and sex between patients and controls. The results showed that the five SNPS rs10499194, rs610604, rs7753873. and rs5029928 rs9494885of *TNFAIP3* were in Hardy–Weinberg equilibrium in the cases and controls ($P > 0.01$). The distribution of both genotype frequencies and allele frequencies of the five tested *TNFAIP3* polymorphisms are shown in Table 3. The call rate for the examined five SNPs is 100 %. There were no statistically significant differences in the proportions of missing genotype data between cases and controls ($P > 0.05$). The results showed that there were significant differences between BD patients and controls concerning the frequencies of rs9494885, rs10499194, and rs7753873. A significantly increased prevalence of the rs9494885 TC genotype and C allele was found in BD patients compared with controls ($p_c = 1.83 \times 10^{-10}$, OR 2.02, 95 % CI 1.65–2.49; $p_c = 8.35 \times 10^{-10}$, OR 1.81, 95 % CI 1.51–2.18, respectively). The frequencies of the TT genotype and T allele were significantly lower in BD patients than that in controls ($p_c = 1.23 \times 10^{-10}$, OR 0.50, 95 % CI 0.40–0.61; $p_c = 8.35 \times 10^{-10}$, OR 0.55, 95 % CI 0.46–0.66, respectively). The frequency of the rs10499194 CC genotype ($p_c = 0.015$, OR 1.96, 95 % CI 1.30–2.97) and C allele ($p_c = 0.005$, OR 1.924,95 % CI 1.28–2.90) were higher in BD patients than that in controls. The frequency of the rs10499194 TC genotype ($p_c = 0.015$, OR 0.51, 95 % CI 0.34–0.77) and T allele ($p_c = 0.005$, OR 0.52, 95 % CI 0.35–0.78) were lower in BD patients than that in controls. For rs7753873, a higher frequency of the AC genotype ($p_c = 0.015$, OR 1.49, 95 % CI 1.17–1.91) and C allele ($p_c = 0.025$, OR 1.39, 95 % CI 1.11–1.76), and a lower frequency of the AA genotype ($p_c = 0.03$, OR 0.68, 95 % CI 0.53–0.87) and A allele ($p_c = 0.025$, OR 0.72, 95 % CI 0.57–0.91) were found in BD patients compared with controls. There was no association of BD with rs610604 or with rs5029928. The haplotype block was constructed using haploview software 4.0. The results showed that the five examined SNPs in this study did not exibit strong linkage disequilibrium to each other ($r^2 < 0.8$). There results suggest that the association with SNPs rs10499194 and rs7753873 may be independent from SNP rs9494885.

We further investigated whether the *TNFAIP3* SNPs studied were associated with certain clinical findings of BD, such as genital ulceration, hypopyon, skin lesions, and arthritis. The analysis failed to find any association of these parameters with the tested *TNFAIP3* SNPs.

Table 3 Frequencies of alleles and genotypes of *TNFAIP3* polymorphisms in BD patients and controls

SNP	Allele Genoty	BD (%) ($n = 722$)	Controls (%) ($n = 1415$)	χ^2	*P* value	p_c value	OR (95 % CI)
rs10499194	C	1,414 (97.9)	2,719 (96.1)	10.199	0.001	0.005	1.92 (1.28–2.90)
	T	30 (2.1)	111 (3.9)	10.199	0.001	0.005	0.52 (0.35–0.78)
	CC	692 (95.8)	1,304 (92.2)	10.599	0.001	0.015	1.96 (1.30–2.97)
	TC	30 (4.2)	111 (7.8)	10.599	0.001	0.015	0.51 (0.34–0.77)
	TT	0 (0.0)	0 (0.0)				
rs610604	A	1,317 (91.2)	2,628 (92.9)	3.696	0.055	NS	0.80 (0.63–1.10)
	C	127 (8.8)	202 (7.1)	3.696	0.055	NS	1.26 (1.00–1.58)
	AA	597 (82.7)	1,214 (85.8)	3.572	0.059	NS	0.79 (0.62–1.10)
	AC	123 (17.0)	200 (14.1)	3.137	0.077	NS	1.25 (0.98–1.59)
	CC	2 (0.3)	1 (0.1)	1.294	0.265※	NS	3.93 (0.36–43.39)
rs5029928	C	1,342 (92.9)	2,633 (93.0)	0.015	0.901	NS	0.98 (0.77–1.26)
	T	102 (7.1)	197 (7.0)	0.015	0.901	NS	1.02 (0.79–1.30)
	CC	620 (85.9)	1,220 (86.2)	0.048	0.827	NS	0.97 (0.75–1.26)
	CT	102 (14.1)	193 (13.6)	0.096	0.757	NS	1.04 (0.80–1.35)
	TT	0 (0.0)	2 (0.1)	–	–	–	–
rs7753873	A	1,313 (90.9)	2,641 (93.3)	7.909	0.005	0.025	0.72 (0.57–0.91)
	C	131 (9.1)	189 (6.7)	7.909	0.005	0.025	1.39 (1.11–1.76)
	AA	593 (82.1)	1,232 (87.0)	9.335	0.002	0.03	0.68 (0.53–0.87)
	AC	127 (17.6)	177 (12.5)	10.116	0.001	0.015	1.49 (1.17–1.91)
	CC	2 (0.3)	6 (0.4)	0.277	0.725※	NS	0.65 (0.13–3.24)
rs9494885	C	243 (16.8)	284 (10.0)	40.814	1.67×10^{-10}	8.35×10^{-10}	1.81 (1.51–2.18)
	T	1,201 (83.2)	2,546 (90.0)	40.814	1.67×10^{-10}	8.35×10^{-10}	0.55 (0.46–0.66)
	CC	7 (1.0)	10 (0.7)	0.417	0.609※	NS	1.37 (0.52–3.61)
	TC	229 (31.7)	264 (18.7)	45.946	1.22×10^{-11}	1.83×10^{-10}	2.03 (1.65–2.49)
	TT	486 (67.3)	1,141 (80.6)	46.703	8.26×10^{-12}	1.23×10^{-10}	0.50 (0.40–0.61)

BD Behcet's disease, *OR* odds ratio, p_c Bonferroni corrected *P* value, *SNP* single-nucleotide polymorphism

※ Fisher's exact *P* value

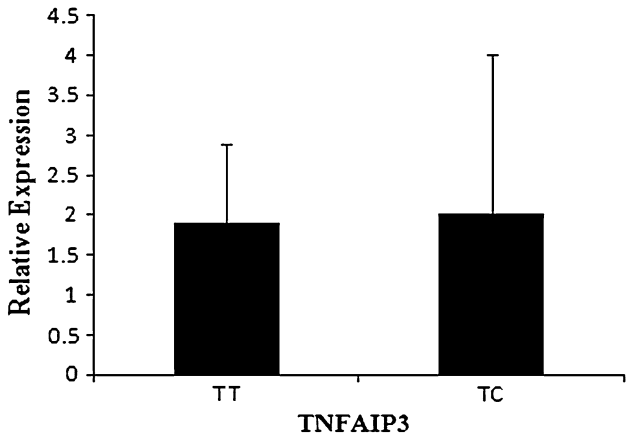

Fig. 1 *TNFAIP3* expression with two different genotypes of SNP rs9494885 in PBMCs. Real-time PCR analysis of mature *TNFAIP3* expression in PBMCs derived from healthy individuals of SNP rs9494885 TT, and TC genotypes ($n = 8$ per group)

The results stated above showed a strong association of rs9494885 in *TNFAIP3* with BD.A further study was performed on a set of PBMCs from 16 individuals (with 8 TT and 8 TC) derived from healthy individuals of known SNP (rs9494885) status to examine whether this SNP influences the expression. *TNFAIP3* mRNA levels were measured by the SYBGREEN assay (Takara, Dalian, China) with available RNA samples. The results showed that there was no difference in *TNFAIP3* expression between individuals with the TT or TC genotype (Fig. 1).

Discussion

This study identified a novel association between SNP rs9494885 in *TNFAIP3* and BD in a Chinese Han population. The results suggest that the rs9494885 TT genotype and T allele of *TNFAIP3* could provide strong protection against BD. The rs9494885 TC genotype and C allele were high-risk factors for this disease. Our study also showed that SNP rs10499194 and rs7753873 were weakly associated with susceptibility to BD. The CC genotype and C allele of rs10499194, and the AC genotype and C allele of rs7753873 were the risk factors for BD whereby the AA

genotype and A allele of rs7753873, and the TC genotype and T allele of rs10499194 provided protection.

In this study we chose *TNFAIP3* as a candidate gene to investigate its relationship with BD principally based on its involvement in several signaling pathways (Boone et al. 2004; Hitotsumatsu et al. 2008; Jaattela et al. 1996; Lee et al. 2000) and its association with various autoimmune diseases (Adrianto et al. 2011; Dieude et al. 2010; Graham et al. 2008; Musone et al. 2011; Nair et al. 2009; Plenge et al. 2007; Thomson et al. 2007; Wang et al. 2010a). The fact that TNF-α, an important cytokine involved in BD pathogenesis, triggers *TNFAIP3* gene expression (Arida et al. 2011; Song et al. 1996), also stimulated us to investigate the relationship between this gene and BD. As to the tested SNPs, we chose these five SNPs predominantly based on the results previously reported on one or more autoimmune diseases including psoriatic arthritis (Bowes et al. 2011), psoriasis (Nair et al. 2009; Tejasvi et al. 2012), RA (Plenge et al. 2007; Shimane et al. 2010), and SLE (Adrianto et al. 2011). Although other variants in this gene have also been shown to be associated with autoimmune disease (Bowes et al. 2010; Plenge et al. 2007), they were not included in our study due to the absence of polymorphisms in the Chinese Han population.

Numerous factors have been reported to influence the results of studies on the association of gene polymorphisms with complex diseases. Efforts were made to decrease the influence of confounding factors on the results. BD patients were strictly selected according to the criteria of the International Study Group. Unrelated healthy individuals acting as controls were selected from the same geographical regions as the BD patients. Furthermore, we selected age- and sex-matched normal individuals as controls. Finally, 20 % of the samples were randomly chosen and investigated by direct sequencing for the purpose of validating the method used in this study and the results showed no difference.

We found a significantly increased prevalence of the rs9494885 TC genotype and a lower frequency of the TT genotype in BD patients. This result is consistent with that reported in SLE in European-ancestry and Korean populations (Adrianto et al. 2011). We also found a higher frequency of the CC genotype and a lower frequency TC in rs10499194, in BD patients compared with controls. This result is consistent with that reported in RA and SLE in Japanese patients (Shimane et al. 2010). In addition, we observed a higher frequency of the AC genotype and a lower frequency of the AA genotype in rs7753873.This result is consistent with that reported in SLE in European-ancestry and Korean populations (Adrianto et al. 2011). These results seem to suggest that BD has, to a certain extent, similarity in genetic background with SLE and RA. The latter are considered autoimmune diseases, whereas BD is generally seen as an auto-inflammatory disease. We failed to find any association between rs610604 and rs5029928 with BD though both SNPs have been reported to be associated with a number of autoimmune diseases (Table 4).

Previous genome-wide association studies have identified multiple risk genes for Behcet's disease including IL23R-IL12RB2, IL10, UBAC2, and STAT4 (Fei et al. 2009; Hou et al. 2012c; Mizuki et al. 2010; Remmers et al. 2010). However, these studies did not find the association

Table 4 Relations between SNPs with autoimmune diseases reported earlier by others and in the present study

SNP	References	Disease	Cases	Controls	Minor alleles	p_c	OR	Ethnic
rs10499197	Shimane et al. (2010)	RA	3,411	2,299	T	8.4×10^{-4}	1.30	Japanese
		SLE	376	933		0.03	1.42	
	Present study	BD	772	1,415	T	0.005	0.52	Chinese Han
rs610604	Nair et al. (2009)	Psoriasis	6,407	6,451	G	9.0×10^{-12}	1.19	European ancestry
	Present study	BD	772	1,415	C	NS	1.26	Chinese Han
rs7753873	Adrianto et al. (2011)	SLE	853	978	C	2.7×10^{-6}	1.72	Korean
			3,936	3,491	C	2.9×10^{-5}	1.28	European ancestry
	Present study	BD	772	1,415	C	0.025	1.39	Chinese Han
rs5029928	Adrianto et al. (2011)	SLE	853	978	T	7.8×10^{-8}	1.95	Korean
			3,936	3,491	T	1.65×10^{-5}	1.29	European ancestry
	Present study	BD	772	1,415	T	NS	0.98	Chinese Han
rs9494885	Adrianto et al. (2011)	SLE	853	978	G	8.96×10^{-8}	1.94	Korean
			3,936	3,491	G	2.43×10^{-5}	1.28	European ancestry
	Present study	BD	772	1,415	C	8.35×10^{-10}	1.81	Chinese Han

RA rheumatoid arthritis, *SLE* systemic lupus erythematosus

of *TNFAIP3* with Behcet's disease. These results suggest that Behcet's disease displays genetic heterogeneity in different ethnic populations.

The above findings suggest that rs9494885 may be an important susceptibility factor for autoimmune disease. Functional studies of the *TNFAIP3* polymorphism will be an important advance to explain how polymorphisms of this gene affect the susceptibility to autoimmune disease. As yet little is known concerning its effect on the function or expression of this gene. We therefore evaluated the association of the rs9494885 genotypes and *TNFAIP3* expression. The results showed no differences in *TNFAIP3* expression between the individuals with the TT and those with the TC genotype of rs9494885. This study suggests that the diseases associated SNP rs9494885 may be involved in this disease through an unknown mechanisms rather than directly regulating *TNFAIP3* transcriptional regulation. In addition to mechanisms other than transcription, rs94948855 may be in linkage disequilibrium (LD) with the causal variant. Although other sites such as rs6920220, rs6933404, and rs6927172 in *TNFAIP3* have also been shown to be associated with rheumatoid arthritis (Elsby et al. 2010) and showed a functional role in disease, we did not study the association of these SNPs with Behcet's disease due to nonpolymorphism in Chinese population based on the database of HapMap.

It is worth mentioning that there are some limitations in the present study. BD is a systemic disease involving various organs and the patients enrolled in our study originated from an ophthalmology department and may, therefore, represent a subpopulation of this disease. The susceptible SNPs identified in this study are, therefore, associated only with uveitis in BD and more studies are needed to confirm the present results using BD patients from other medical departments.

As far as we are aware this is the first study to report an association between *TNFAIP3* SNPs rs10499194, rs7753873, and rs9494885 with BD in Chinese Han patients.

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Conflict of interest All authors do not have any conflict of interest to disclose.

Ethical standard This study was conducted with the approval of the Ethical Committee of Chongqing Medical University.

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