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TNFSF15 (rs3810936) in Behçet's disease

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Behçet's disease is a chronic multi-systemic disorder of relapsing mucocutaneous, ocular, vascular and central nervous system manifestation with vasculitis as a main pathological finding [1]. The affected individuals are mainly between the ages of 20 to 40 [2]. Prognosis depends on the clinical involvement and the disease may result in considerable morbidity and mortality. Loss of visual acuity and neurological disease are major causes of morbidity and disability [1]. The exact aetiopathology is unknown with both autoimmune and auto-inflammatory mechanisms are suggested [3]. HLA association, infectious agents, environmental factors as well as different gene polymorphism have been investigated [4,5]. *TNFSF15* (Tumour Necrosis Factor Superfamily Member 15), lies on the long arm of chromosome 9, and codes for TNF-like ligand 1A (TL1A), a cytokine that belongs to TNF superfamily. TL1A affects the major pro-inflammatory pathways (Th1, Th2, Th17, and Treg cells) [6]. Abnormalities in the expression of TL1A and/or *TNFSF15* polymorphisms have been addressed in different autoimmune diseases (e.g. ulcerative colitis), and various cancers [7,8]. We hypothesized altered *TNFSF15* (rs3810936) polymorphism distribution, as well as different peripheral blood *TNFSF15* expression in Behçet's disease.

The current cross-sectional, case-control study was conducted at Zagazig University Hospitals/Sharkia Governorate-Egypt from December 2015 to August 2019. The approval of the Zagazig University Institutional Review Board was obtained before starting the present study. Sample size was calculated using the Epi Info program (Atlanta, Georgia, USA). We recruited 70 Behçet's disease patients and 140 age and sex-matched healthy controls. The diagnosis of Behçet's disease was according to international criteria [9]. None of the control group individuals nor their first degree relatives had a history or showed the manifestation of Behçet's disease. Patients were subjected to full clinical evaluation, including complete medical history regarding: (a) onset, course and duration of the disease, (b) symptoms suggesting ocular, oral or genital affection, (c) joint pain and swelling, (d) vasculitic lesion in the form of ulceration, gangrene or tender finger nodules and (e) symptoms suggesting weakness of lower limbs, upper limbs and sensory affection. Clinical examinations were dermatological (pathergy test, erythema nodosum, thrombophlebitis, pseudofolliculitis, papulopustular lesions, and acneiform nodules), vascular, neurological (motor and sensory) and ophthalmological (uveitis, conjunctivitis and retinal vasculitis). Patients were assessed for disease activity according to Behçet disease current activity form [10].

From each subject, a minimum of 2 ml of venous blood was collected by venipuncture on sterile EDTA vacutainer. Genomic DNA was extracted from EDTA whole blood using a spin column method per the manufacturer's protocol (QIAamp Blood Kit; Qiagen GmbH, Hilden, Germany). The TNFSF15 gene polymorphism was detected by PCR RFLP analysis method described elsewhere [7]. The used primers were: forward 5′-GGAACCAGTTGCTACCTACTTCGTA-3′ and reverse 3′-CCGTGGGATGACCTCTGAGTG-5′. PCRs were performed in a 25 µl volume containing 100 ng of DNA template, 12.5 µl one-step PCR mixture containing 1 unit Taq polymerase, 10 mM KCl, 10 mM (NH4)2SO4, 20 mM Tris-HCl (pH 8.75), 0.1% Triton X-100, 0.1 mg/ml BSA and 200 µM dNTPs (Bio-Basic Inc., Ontario, Canada), and 0.2 µM of each primer (Biosynthesis Inc., Lewisville, Tx, USA). After initial denaturation, the reaction was performed for 35 cycles under the following conditions 95 °C for 30 seconds, 58 °C for 30 seconds, 72 °C for 30 seconds, and 72 °C for 5 minutes. The PCR product was digested with restriction endonuclease RasI (Thermo Fisher Scientific, Vilnius, Lithuania) then visualized under UV trans-illuminator on 3% agarose gels with ethidium bromide staining.

Total RNA was extracted from EDTA peripheral blood samples using the Total RNA Purification kit (Jena Bioscience, Germany) per manufacturer's protocol. RNA was reverse-transcribed to the first-strand cDNA using

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the SCRIPT Reverse Transcriptase kit (Jena Bioscience, Germany). On ice 1.5 µl RNase-free water, 10 µl RNA template and 1 µl Oligo-(dT) primer were mixed together, then 4 µl SCRIPT RT buffer, 1 µl dNTP Mix, 1 µl RNase inhibitor, 1 µl Dithiothreitol stock solution and 0.5 µl SCRIPT reverse transcriptase were added and incubated at 30 °C for 10 min and 50 °C for 60 min. The cDNA was stored at −20 °C till analysis. Quantitative real-time RT-PCR for *TNFSF15* was performed on a Stratagene Mx3005P qPCR System (Agilent Technologies, Germany) using the qPCR GreenMaster (Jena Bioscience, Germany). PCR reaction was prepared by adding 10 µl qPCR Green Master, 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM), 5 µl template cDNA and 4 µl PCR grade water into real-time PCR wells. The final volume was 20 µl. The PCR reaction was performed under the following conditions: 95 °C for 10 min then 40 cycles (95 °C for 15 Sec, 58 °C for 1 min). GAPDH was used as a reference gene to normalize the transcription levels of target genes.

The normalized quantity of the target gene was obtained by subtracting the cycle threshold (CT) for RPS18 from the CT for the target gene (ΔCT sample). The same calculation was performed with controls (ΔCT control). Then ΔΔCT was calculated as the difference of these values (ΔΔCT = ΔCT sample − ΔCT control). Finally, the relative expression was expressed as fold change by 2 − ΔΔCT relative to control. The Hardy-Weinberg equilibrium (HWE) was evaluated. Comparisons between quantitative variables were done using the Kruskal–Wallis test and Mann Whitney U test. For comparing the categorical data chi-squared (χ2) test was performed. Genotype distributions were evaluated by (χ2) test and Fisher exact probability test. The odds ratios (OR) and their 95% confidence intervals (95% CI) were determined to describe the degree of association. The association between the polymorphism and disease severity was done using non-parametric Spearman's correlation coefficient method. Statistical analysis was carried out with SPSS® statistical software version 17.0 (SPSS Inc., Chicago, IL, USA). *p*-value < 0.05 was considered as significantly different.

The two groups were matched for age and sex: males/females were 54/16 and 112/28 for patients and control respectively ($p = 0.87$). Ages were median (range) of 36.5 (22–51) and 37.5 (20–51) for patients and control respectively ($p = 0.23$). Table 1 shows *TNFSF15* data in cases and controls. There was no difference in the frequency of the heterozygous C/T genotype, but patients with Behçet's disease were less likely to be homozygous for the T allele, and also less likely to have any T allele. There was no difference in the expression of *TNFSF15* between cases and controls. The effect of rs3810936 on relative *TNFSF15* messenger RNA expression in the patients revealed a significant difference between different genotypes (C/C 1.0 [0.64–1.16], C/T 1.1 [0.72–1.55], T/T 0.71 [0.51–1.36], $p < 0.001$). There was significant increase in the expression level of TNFSF15 in the C/T genotype when compared to the C/C genotype ($p = 0.027$) and when compared to the T/T genotype ($p < 0.001$), and a significance difference was detected when comparing C/C and T/T genotypes ($p = 0.019$). These findings suggest that T allele may influences the expression of TNFSF15. Results of the Behcet's disease activity index by genotype were C/C 10 (4–12), C/T 8 (3–12) and T/T 7.5 (2–10) ($p = 0.01$). The C/T and T/T genotypes difference was not significant ($p = 0.34$), whereas the C/C and C/T difference ($p = 0.049$) and the C/C and T/T difference ($p = 0.011$) were significant. Table 2 shows clinical data: the most prevalent features were ocular, genital, intestinal and dermal (all present in > 80%). The C allele was significantly more prevalent in those with ocular lesions, but less so in those with arthritis.

Table 1. Relative expression of TNFSF15 in Bechet's Disease patients compared to controls.

	Bechet's Disease (N = 70)		Control (N = 140)			
Genotype and allele	No.	%	No.	%	OR (95% CI)	*p*
C/C	25	35.7	40	28.6	1	
C/T	35	50	60	42.8	0.93 (0.49–1.78)	0.84
T/T	10	14.3	40	28.6	0.40 (0.17–0.94)	0.033
C	85	60.7	140	50	1	
T	55	39.3	140	50	0.64(0.42–0.97)	0.037
TNFSF15 Expression Median (range)	0.98 (0.51–1.55)		1 (0.8–1.15)		$p = 0.87$	

No.: Number of subjects, OR: odds ratio, CI: confidence interval.

TNFSF15 polymorphisms have been studied in different diseases [7,8,11], including one previous report in Behcet's disease [12], who found a link with *rs4246905* [12]. We add to the literature by focusing on *TNFSF15* SNP *rs3810936* and its relationship with different clinical features. We found no difference in the relative expression of TNFSF15 between the controls and patients, but report that the TT genotype and T allele were less often found in the patient group. In reciprocation, the CC genotype and C allele is over-represented in the patients. The most powerful clinical link was with ocular lesions. The association of the same SNP with acute

Table 2. The predictive role of T allele in clinical features of Bechet's Disease.

	C allele (N = 85)	T allele (N = 55)		
Clinical features	N (%)	N (%)	AOR (95% CI)	*p*
Ocular lesions	84 (98.8)	48 (87.3)	0.1 (0.01–0.63)	0.017
Genital ulceration	80 (94.1)	50 (90.0)	0.57 (0.15–2.17)	0.41
Oral ulcerations	78 (91.8)	52 (94.5)	1.48 (0.34–6.49)	0.60
Intestinal lesions	75 (88.2)	53 (96.4)	4.25 (0.83–21.7)	0.08
Erythema	70 (82.4)	48 (87.3)	1.8 (0.61–5.31)	0.29
Skin lesions	69 (81.2)	43 (87.2)	1.11 (0.42–2.93)	0.84
Arthralgia	67 (78.8)	45 (81.8)	0.75 (0.3–1.88)	0.54
Arthritis	66 (77.7)	52 (94.5)	6.95 (1.8–26.8)	0.005
Headache	59 (69.4)	39 (70.9)	1.28 (0.57–2.9)	0.55
Abdominal pain	54 (63.5)	28 (50.9)	1.75 (0.82–3.71)	0.15
Vascular lesions	50 (58.8)	36 (65.5)	1.03 (0.47–2.23)	0.95
CNS lesions	48 (56.5)	30 (54.5)	0.56 (0.23–1.39)	0.22
Diarrhoea	48 (56.5)	30 (54.5)	0.99 (0.48–2.05)	0.99

N: number of subjects; AOR: Adjusted odds ratio (to age, sex, and duration of disease); CI: confidence interval.

anterior uveitis (duration <3 months) has been reported [7]. However, exclusion criteria are not provided, so may include some patients with Behcet's disease. The study also examined the possible association between *rs3810936* and uveitis in patients with and without ankylosing spondylitis and found no association. Further links with ophthalmology include the report of a lower frequency of *rs3810936* in the ocular abnormalities of Grave's disease [11]. We are unable to speculate directly on a pathophysiological mechanism for links between molecular genetics and clinical features, but note that others [12] point to roles for *TNFSF15* genotypes and production of IL-6 and TNF-α by LPS-stimulated peripheral blood mononuclear cells.

We recognize limitations of our study. The sample size is modest, and the frequency of clinical features differ from those of a previous local Behcet's disease study [13]. Nevertheless, our is the first such study of this nature outside China [7,8,11,12]. As a single-centre study, further studies are recommended on a larger scale or a multicenter level to confirm these results.

This work represents an advance in biomedical science because it points to the possible role of TNFSF15 (rs3810936) polymorphism in the pathophysiology of BD and its effect on clinical presentation.

Disclosure statement

No potential conflict of interest was reported by the authors.

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