RESEARCH ARTICLE

Genetic Polymorphisms of CD28 in Iraqi Patients with Behcet's Syndrome

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ABSTRACT

Background: Behçet disease (BD) is a long-lasting systemic inflammatory disease considered through repeated cutaneous, ocular, and urogenital ulcers, vascular, gastrointestinal, neurovascular, and arthritic participation. Immune checkpoints are membrane molecules regulators of immune activation in usual biological conditions, preserving homeostasis of the immune system and preventing auto-immunity as CD28 and CTLA4. The study's goal was to clarify the correlation between CD28 genetic polymorphisms in Iraqi patients and BD susceptibility and the impact of genetic polymorphism on soluble CD28 in Behçet's.

Methods: A case-control study on 50 BD patients with 40 controls was conducted to determine the correlation between CD28 gene polymorphisms (*rs3116496* C/T) using primers specific probe and RT-PCR amplification and estimation serum level of soluble CD28 by ELISA technique.

Results: The BD patients showed an elevated level of soluble CD28. The odds ratio and confidence interval (CI) 95% CI for the CC was 1.83 (0.80–4.21), TC was 0.52 (0.22–1.20), and TT genotypes was 1.21 (0.20–7.47) respectively with no significant differences between patients and controls in genotype and allele frequencies of *rs3116496* SNP ($p > 0.05$).

Conclusions: Higher levels of soluble CD28 support their role as positive T-cell regulator molecules and therapeutic target. Genetic polymorphisms in CD28 showed no association with BD. To support these results, additional cases should be investigated in further studies.

Keywords: Behçet's Disease, CD28, Enzyme-linked immunoassay (ELISA), *rs3116496* SNP, Reverse transcription–polymerase chain reaction (RT-PCR).

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INTRODUCTION

Behçet's disease (BD) is a systemic disorder and multifactorial genetic background with innate and adaptive immune responses that cause various clinical manifestations.[1,2]

Immune-checkpoints are membrane molecules regulators of immune activation under normal physiological conditions, and vital companies in maintaining immune homeostasis and avoiding auto-immunity and membrane molecules.[3,4]

The immune response is controlled by stability between costimulatory and repressive which are essential for preserving self-tolerance and host protective from tissue damage.[5]

Immune-checkpoints can increase repressive or stimulatory signals via associates of the ligands with analogous receptors placed on the marked and effector cells.[6,7] Human CD28 has 4 exons coding 220 amino acids that are expressed on the cell's surface as a glycosylated, disulfide-linked homo-dimer of Forty-four kDa. The CD28 related family shares several common features. These receptors consist of paired V-set immunoglobulin superfamily (IgSF) domains attached to single transmembrane domains and cytoplasmic domains that contain critical signaling motifs.[8] CD28 plays a critical role in the T-cell stimulation, development, and homeostasis of Treg cells. CD28 contests with CTLA-4 to bind B7.1 (CD-80) and B7.2 (CD86) on APCs, and controls the T-cell activation by various processes, through the formation of the immunological synapse, the post-translational alteration of several indicator proteins, and actin renovation of the cytoskeleton, forming a complex transcriptional system in T-cells populations.[9,10] This study was aimed to estimate the serum level of sCD28 and detect the genetic polymorphisms of CD28 in Iraqi patients with BD and healthy populations.

SUBJECTS, MATERIALS AND METHODS

Subjects

A retrospective study was done on 50 Behçet's patients (34 males and 16 females) who were collected and clinically diagnosed from the dermatology department Baghdad teaching hospital, Iraq and their age ranged was 22 to 65 years. Also, 30% of patients gave positive results to the pathergy test while 70% showed negative. For comparison, 40 healthy individuals

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(24 males and 16 females) were also registered in the study, and their age ranged was 20 to 60 years and showed negative results for related diseases.

MATERIALS AND METHOD

Measurement of Serum Level of Soluble CD28

Human CD28 sandwich ELISA kit, Thermofisher, Invitrogen, Vienna, Austria was used to measure the level of CD28 in serum of BD patients.

Genomic DNA Extraction

Quick-gDNA™ Blood MiniPrep (D3072 and D3073), ZYMO, USA extracted DNA from blood samples. The purity and concentration of the DNA were determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA.

TaqMan®R MGB-Probes and the Primers used for RT-PCR Amplification

The CD28 gene (*rs3116496* C/T), ThermoFisher scientific, USA, was used to investigate the SNP. The probes were characterized with FAM and HEX dyes to signify the two diverse alleles, correspondingly, and the allelic class was measured automatically using the Sequence Detection System SaCycler-96 Real-Time PCR System, Sacace, Italy rendering to the concentration of the VIC and FAM dyes.

Statistical Analysis

A substantial departure from HWE was predictable online using H-W calculator for two alleles www.dr-petrek.eu/documents/HWE.xls. The genotypes of SNPs were given as incidences ratio, and statistically significant differences among their distributions in BD controls and patients were evaluated by Fisher's exact probability (p). The risk association between the genotype and susceptibility was estimated by the calculation of OR (odds ratio) and CI (confidence intervals) (95% CI). The OR rate can vary from less than one, considered a negative association, to more than one, which is considered a positive association.[11,12] These assessments were intended using the software WINPEPI for epidemiologists at www.dr-petrek.eu/documents/HWE.xls.[13-15]

Table 1: Distribution of Behcet's patients according to gender and age

Factors		*No. (Total = 50)*	*Percentage (%)*	*p-value*
Gender	Male	34	68.00	0.0109 **
	Female	16	32.00	
Age (year)		50	33.92 ± 1.56	—
	Least than 20	3	6.00	0.0001 **
	20-30	15	30.00	
	More than 30	32	64.00	

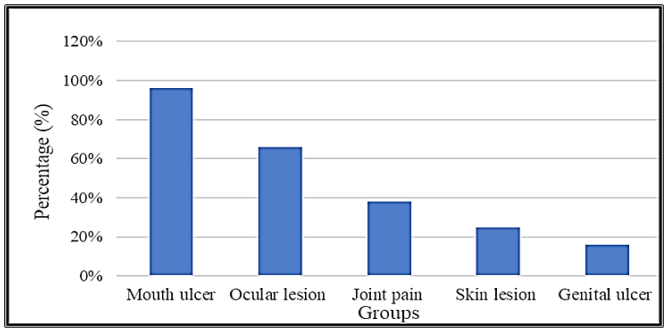

Figure 1: Clinical features of Behçet's disease patients

RESULTS

Demographic Characteristics of Behçet's Patients and Control

Results exhibited that men were more affected (68%) with BD than females (32%), and disease activity score showed higher rates (64%) at the age group more than 30 years and 15% at age group (20–30) years Table 1.

According to clinical examination, all patients were suffered from mouth ulcer, ocular lesion, genital ulcer, skin lesion and joint pain as these symptoms were intertwined with each other as shown in Figure 1. Forty-eight (96%) patients were found with mouth ulcer as a symptom followed by ocular lesion in 33 (66%), joint pain in 19 (38%), skin lesion in 12 (24%), and genital ulcer in 8 (16%), respectively.

Soluble CD28

Investigation of variance presented a statistically significant difference ($p<0.05$), with admiration to soluble CD28 levels amongst the experimental variants of BD patients. The levels were significantly increased in BD patients compared to healthy controls (46.48 ± 3.27 pg/mL vs. 5.33 ± 0.7 pg/mL). While CD28 level in Behçet's males was (41.2 ± 3.63 pg/mL vs. 5.95 ± 0.91 pg/mL) compared to control males (Figure 2). Increased amounts of CD28 were detected in Behçet's females likened to control females (56.84 ± 6.22 pg/mL vs. 4.95 ± 1.09 pg/mL).

SNP rs3116496

Figure 3 showed that the melting curve represented a pure, single amplicon for each sample, and the specificity of amplification was considered great with intercalating dye assay. Based on the amplicon length, melting temperature (T_m) varied between the alleles and was used to discriminate the alleles.

The SNP of the CD28 gene (3116496 C/T) was observed to have 3 genotypes (CC, TC, and TT) that match 2 alleles (C and T) (Table 2). In the BD patients' group, the three

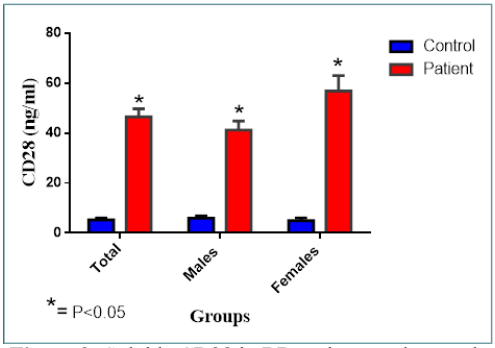

Figure 2: Soluble CD28 in BD patients and controls

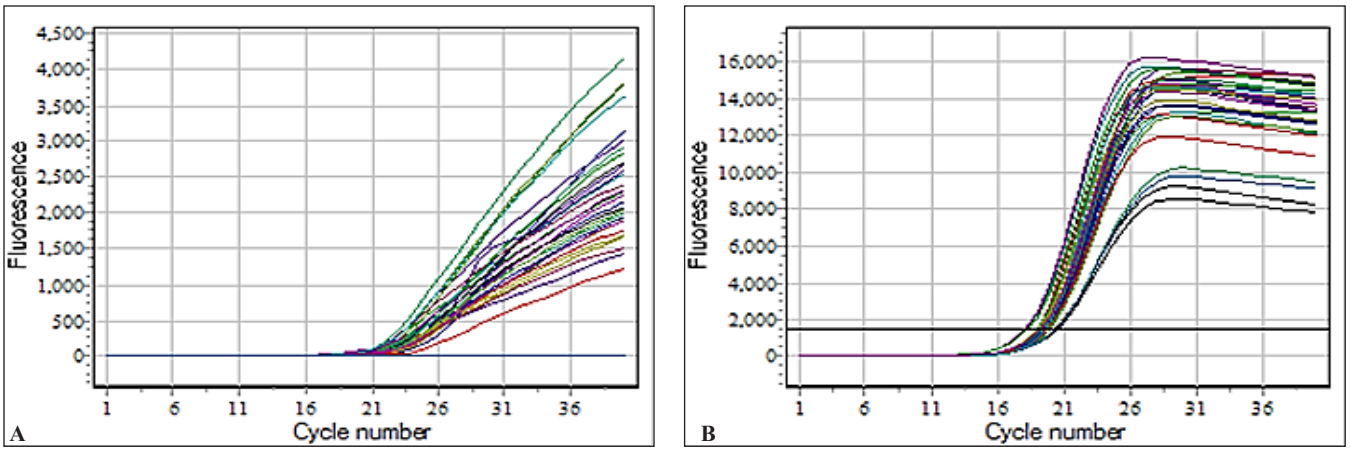

Figure 3: Melting-curve analysis for the SNP *rs3116496* (C/T) showing the wmelting ranges of genotypes; CC genotype, CT genotype and TT genotype in BD patients and control subjects. A) FAM channel, B) HEX channel.

Table 2: The distribution of the observed SNP *rs3116496* genotype and allele incidences in BD patients and control

Genotype Frequency (%)					
Genotype	*BD patients n = 50*	*Control n = 40*	*p-value*	*Odds Ratio*	*95% Cl*
CC	30 (60%)	18 (45%)	0.203	1.83	0.80 to 4.21
TC	17 (34%)	20 (50%)	0.138	0.52	0.22 to 1.20
TT	3 (6%)	2 (4%)	1	1.21	0.20 to 7.47
HWE-P	0.776808	0.228328			
Allele frequency (%)					
Allele	*BD patients n = 50*	*Controls n = 40*	*p-value*	*Odds ratio*	*95% Cl*
C	77 (77 %)	56 (70%)	0.309	1.43	0.74 to 2.79
T	23 (23 %)	24 (30%)	0.309	0.70	0.36 to 1.35

Table 3: Soluble CD28 according to *rs3116496* genotype frequency in patient and control

CD28 rs 3116496	*CD28 rs3116496 genotype frequency*			
	CC	*TC*	*TT*	*p-value*
Control (No. = 40)	4.85 ± 1.03	5.61 ± 1.01	7.14 ± 4.80	0.743 NS
Patients (No. = 50)	47.01 ± 4.57	44.94 ± 5.11	49.87 ± 11.65	0.928
p-value	0.0001 **	0.0001 **	0.0001 **	—

** ($p \leq 0.01$), non-Significant.

genotypes CC, TC, and TT account for 30 (60%), 17 (34%), and 3 (6%), respectively, compared to 18 (45%), 20 (50%) and 2 (4%) respectively, in the healthy control group. The genotypes showed a good contract with HWE in BD patients and controls. There was no significant difference between the experiential and predictable genotype frequencies. The odds ratio and 95% CI for the CC genotype was 1.83 (0.80–4.21), TC was 0.52 (0.22–1.20) and TT genotypes 1.21 (0.20–7.47), respectively. The analysis of results revealed there were non-significant ($p > 0.05$) differences for the three genotypes (Table 2). The analysis of the allele frequencies of this SNP exposed non-significant differences in the frequency of the C allele between BD patients and controls 77(77%) and 56 (70%), respectively. A similar finding was attained for the frequency of T allele in the two groups 23 (23%) and 24 (30%), respectively. The odds ratio and 95% CI were 1.43 (0.74–2.79) for the C allele and 0.70 (0.36–1.35) for the T allele. Accordingly, there are no significant differences between BD patients and controls in distributing these genotypes and alleles.

Impact of CD28 *rs3116496* SNP on Serum CTLA-4 Concentrations in BD Patients and Control.

A significant correlation with the analysis of the correlation between the serum level of CD28 and (CC, TC and TT) genotypes in BD patients (47.01 ± 4.57, 44.94 ± 5.11 and 49.87 ± 11.65) ng/mL compared to the controls (4.85 ± 1.03, 5.61 ± 1.01 and 7.14 ± 4.80) ($p \leq 0.01$), respectively Table 3.

DISCUSSION

Demographics are a factor that might affect the frequency and severity of clinical disease indicators. Though, different characteristics of these features have been stated in several studies. Although BD males are more affected in many countries that showed an analogous distribution of BD across

both male and female populations.[12].Kalın *et al.*[13] described that BD was more predominant in males which was consistent with other published studies from the demographic population and ages were less pre-dominantly at the age group less than 20. Erkek Tüfek[14] found the age groups, clinical disease activity scores were significantly higher in patients from the youngest age group and characterized by its onset during the second and third decades of life.[15,16] Co-stimulation is a vital procedure in the development of BD immune pathology. The CD28 is the major costimulatory molecule for T-cells stimulation and promotes T-cell persistence and populations.[17] A result of a study showed a higher significant increase in the soluble form of CD28 concentration among patients.[18] The increases of sCD28 may be produced either by flaking the membrane arrangement or from another mRNA splicing.[19,20] Fenoglio *et al.*[21] reported the detection of soluble CD28 in the serum of patients with BD and RA and correlated with disease activity that might propose a controlling mechanism to recompense the stimulation in T-cell populations and reflect an inadequate T cell activation. It could be contributing to the loss of tolerance, influencing the severity of autoimmune diseases.[22-25] Several previous studies[26,27] exposed the particular mechanisms fundamental to the up-regulation of the expression of circulating soluble pro-inflammatory proteins, including cytokines and chemokines, or uncontrolled initiation of intercellular signaling pathways. The SNP (IVS3 + 17T/C) of the CD28 gene, with a T/C replacement at location (+17) in the third intron, is located in the 2q33 region. This polymorphism has been associated with BD but has not been exposed to other autoimmune diseases like RA.[28,29] The soluble CD28 levels might present as an inhibitory molecule, that prevents the reciprocal action between CD28 on the surface membrane and their target receptors. This proposes that soluble CD28 attraction for CD80/86 is inferior to CD28 on the membrane surface. Besides, the CD28 signal depends on the affinity of the ligand and proposed that inactive T-cells can express sCD28 and mCD28, and together can share stimulatory and inhibitory actions to control the activating T-cells.[30] Wang *et al.*[31] found that IVS3 +17TC genetic constitution incidence of the CD28 gene is substantially higher in BD patients than in healthy controls, suggesting that this genotypic variant appears to have another risk factor for the progression of BD and might consequence in increasing of T-cell stimulation in BD patients by stimulating the expression and production of CD28 molecule on T cells.[32-34]

CONCLUSIONS

This study revealed elevation levels of sCD28 with no significant association of *rs3116496* SNP with BD. Future studies may need to investigate more CD28 SNPs in a larger sample to identify those SNPs with a minor or moderate effect on BD. Higher levels of soluble CD28 support their role as positive T-cell regulator molecules and therapeutic target. Genetic polymorphisms in CD28 showed no association with BD. To support these results, additional cases should be investigated in further studies.

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