Correlation of tumor necrosis factor-α -308G>A polymorphism with susceptibility, clinical manifestations and severity in Behçet syndrome: evidences from an Italian genetic case-control study

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Abstract

Background The tumor necrosis factor (TNF)α is a multifunctional proinflammatory cytokine, implicated in a variety of diseases, including Behçet syndrome (BS), a rare vasculitis with a wide spectrum of clinical manifestations. The aim of the present study was to investigate the association between a functional drug-response TNFα gene polymorphism (at the positions of -308; rs1800629) and both disease susceptibility and clinical manifestations in a cohort of Italian BS patients compared with healthy controls (HC).

Methods We recruited 130 Italian patients with BS consecutively seen at Rheumatology Institute of Lucania (Italy) and 100 ethnically, age and gender matched HC. Demographic and clinical features were retrieved by medical records. Specific primer pairs were designed for TNFα coverage. Genomic DNA isolation, primer-specific PCR amplification and direct sequencing were performed for genotyping our group of patients and controls. In silico analysis was downstream performed using Mutation Surveyor software (SoftGenetics, USA) and NCBI-BlastN on line tool for the query-subject similarity analysis.

Results The genotype distribution of BS patients and HC underlined a higher percentage of wild-type GG genotype in BS patients group vs HC (106/130 patients, 81.5% vs 91/100 HC, 91%; $p<0.05$), while the heterozygous genotype (GA) was identified in 24/130 patients (18.5%) vs 9/100 HC (9%) ($p<0.05$). GA genotype was significantly associated with the disease (OR=2.29, 95% CI 1.01-5.18). No significant association was recognized between the SNP and the BS clinical manifestations, as well as with disease severity (Krause's index).

Conclusions We found statistically significant higher frequency of TNFα rs1800629 GA genotype in patients than in controls. No significant association was recognized between the polymorphism and the clinical parameters, as well as between the SNP and the disease severity. Our data need to be confirmed in larger cohort of patients and matched controls.

Background

Behçet syndrome (BS) is a chronic vasculitis characterized by a wide spectrum of clinical manifestations, that shares some clinical features with well-recognized autoinflammatory diseases (AIDs) [1–4]. Some evidences of the key role of inflammasome-related genes in AIDs and BS were underlined by genetic studies [5–7]. In this context, the tumor necrosis factor (TNF)α was reported as marker of disease due to its involvement in the pathogenesis of BS. *TNFα* is located on chromosome 6p21.3 and encodes a 233-amino-acid type II transmembrane protein. TNFα is a multifunctional proinflammatory cytokine belonging to the TNF superfamily, implicated in a variety of diseases, ranging from autoimmune diseases, cardiovascular disorders, diabetes mellitus and cancer. Anti-TNFα therapies are used for the treatment of various inflammatory and autoimmune rheumatic diseases with positive but variable outcomes for the patients. The causes of inadequate response or treatment failure remain unknown and can be related to several factors, such as an alternative non-TNFα related pathway of inflammation and/or anti-drug

antibodies presence or development [8–12]. In addition, the alterations of TNFα expression can be associated with polymorphic alleles of TNFα gene having a pathogenetic role. *TNFα* –308A gene variation (rs1800629) is a single nucleotide polymorphism (SNP) located within the gene promoter consisting in the guanine (G) to adenine (A) substitution (NG_007462.1:g.4682G>A; HGVS nomenclature). The polymorphism was previously associated with increased susceptibility to and severity of rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, inflammatory bowel disease, vitiligo, multiple sclerosis, cardiomyopathy and malignancies, as well as of pre-eclampsia and celiac disease. It was reported that patients with the AA genotype and inflammatory diseases who are treated with anti-TNFα therapies may be less likely to have improvement in symptoms as compared to patients with the GG genotype [8–21]. TNFα –308A has been associated with high production of TNFα and poor response to anti-TNF therapy also in BS. The SNP frequency was analysed in various populations reporting inconsistent and/or conflicting data about the association of the polymorphism and both the susceptibility and the clinical patterns of BS [14, 22–32].

To date, these aspects were not analyzed in Italian BS population. The aim of this study was to investigate the frequency of rs1800629 and the possible association between the SNP and both disease susceptibility and clinical features and disease severity in a cohort of Italian BS patients compared with healthy controls (HC).

Methods

Study population. Consecutive patients with BS were recruited at Rheumatology Institute of Lucania (IReL) - Italy. Ethnically, age and gender matched healthy controls (HC) were also recruited among hospital and university employees without clinical signs and family history of both rheumatic diseases and AIDs. Diagnosis of BS was made according to International Study Group (ISG) for BS criteria [33]. Patients demographic and clinical data were collected by medical records. Disease severity was assessed according Krause's index [34]. Informed consent was collected for all participants involved in the study. The study was approved by the Regional Ethics Committee (Permit Number: 705/2017).

Molecular analysis. We designed a specific primer pair for the coverage of *TNFα* rs1800629 using NCBI Primer-Blast tool: 5'TTCCCTCCAACCCCGTTTTC3' and 5'CTGCACCTTCTGTCTCGGTT3'. Genomic DNA was isolated from all subjects whole blood using EuroGOLD Blood DNA Mini kit (Euroclone®, Italy) and quantified using NanoDrop™ 1000 spectrophotometer (NanoDrop Technologies, Inc). PCR amplification was performed using Q5 Hot Start High-Fidelity DNA Polymerase (BioLabs Inc, New England) at the following conditions: 1) initial denaturation: 98°C/5 min; 2) thermocycling: 98°C/1 min; 62°C/1 min; 72°C/2 min (30 cycles); 3) final extension: 72°C/5 min. PCR products were analyzed by gel electrophoresis (1.5% agarose gel). A negative control was used for all PCR reactions. Good-quality PCR amplicons were sequenced (Sanger method) using Microsynth AG sequencing service (Germany). *In silico* analysiswas downstream performed using Mutation Surveyor software (SoftGenetics, USA) and NCBI-Blast Nucleotide on line tool for comparing query nucleotide sequences to sequence databases (subject sequences).

Statistical analysis. We used Chi-square or Fisher's exact tests for comparing: a) genotype frequencies between patients and HC; b) genotype frequencies and BS clinical manifestations; c) genotype frequencies and disease severity score (Krause' score). The odds ratio (OR) was calculated for testing the strength of association between BS and each genotype. The 95% confidence interval (CI) was used to estimate the precision of the OR. p-values less than 0.05 were considered statistically significant.

Results

A total of 230 Italian subjects were included in the study. Of which, 130 were consecutive patients with BS and 100 were matched HC. Table 1 reported the demographic and the clinical features of BS patients. The mean age of the patients at disease onset was equal to 45.8±12.3 years. Male/Female ratio was 64:66, while for HC sex ratio was 48M:52F and mean age was 44.1±12.0 years, without statistically significant differences between patients and HC ($p>0.05$). BS patients predominant lesions were oral ulcers (100%), eye lesions (86.2%), skin lesions (72.3%), genital ulcers (57.7%), followed by joint involvement (50.0%). HLA-B51 positivity was found in 81/130 patients (62.3%).

Genotyping of *TNFα* rs1800629 showed a higher percentage of wild-type GG genotype in BS patients group (106/130 patients, 81.5%) than in HC (91/100 subjects, 91%) ($p<0.05$). The heterozygous genotype (GA) was identified in 24/130 patients (18.5% of cases) and in 9/100 HC (9%) ($p<0.05$). This genotype was significantly associated with the disease (OR=2.29, 95% CI 1.01-5.18). No AA genotype was found in patients group and in controls group (Table 2).

Table 1. Demographic features and clinical manifestations of our group of BS patients.

Demographics	BS patients, n=130
Age, years	45.8±12.3 years
Male/Female	64M:66F
Clinical manifestations	
Oral ulcers, n (%)	130 (100.0%)
Genital ulcers, n (%)	75 (57.7%)
Skin disease, n (%)	112 (72.3%)
Ocular involvement, n (%)	82 (86.2%)
Neurologic involvement, n (%)	26 (20.0%)
Vascular involvement, n (%)	25 (19.2%)
Joint involvement, n (%)	65 (50.0%)
Gastrointestinal involvement, n (%)	6 (4.6%)
HLA-B51 positivity	81 (62.3%)

Abbreviations: BS, Behçet syndrome; n, number of subjects

Table 2. Genotype frequencies of rs1800629 in BS patient and control groups.

SNP	Genotype	BS patients (n=130)	Controls (n=100)	p-value	OR (95% CI)
		n (%)	n (%)		
rs1800629	GG	106 (81.5%)	91 (91.0%)	0.0424*	0.44 (0.19-0.99)
	GA	24 (18.5%)	9 (9.0%)	0.0424*	2.29 (1.01-5.18)
	AA	0 (0.0%)	0 (0.0%)	---	---

Abbreviations: BS, Behçet syndrome; n, number of subjects; OR, odds ratio; CI, confidence interval

We compared the frequency of rs1800629 genotypes with the distribution recognized in other populations (Table 3). The data now available demonstrated that the genotype and allele frequencies of rs1800629 vary substantially among different ethnic groups of BS patients.

Table 3. rs1800629 frequency in patients with BS and controls in different ethnic groups.

rs1800629				BS Patients			Controls		
Author	Ethnicity	Patients (n)	Controls (n)	GG n (%)	GA n (%)	AA n (%)	GG n (%)	GA n (%)	AA n (%)
Abdolmohammadi and Bonyadi, 2017 [22]	Iranian Azer Turks	65	96	4 (6.2%)	61 (93.8%)	0 (0.0%)	6 (6.2%)	90 (93.8%)	0 (0.0%)
Al-Okaily et al, 2015 [14]	Saudi	61	211	2 (3.3%)	59 (96.7%)	0 (0.0%)	116 (55.0%)	80 (37.9%)	15 (7.1%)
Ates A et al, 2006 [23]	Tukish	107	102	86 (80.4%)	21 (19.6%)	0 (0.0%)	84 (82.4%)	16 (15.7%)	2 (1.9%)
Ates O et al, 2010 [24]	Tukish	102	102	76 (74.5%)	26 (25.5%)	0 (0.0%)	85 (83.3%)	17 (16.7%)	0 (0.0%)
Bonyadi et al, 2009 [25]	Iranian Azer Turks	53	79	48 (90.6%)	5 (9.4%)	0 (0.0%)	63 (79.8%)	14 (17.7%)	2 (2.5%)
Duymaz-Tozkir et al, 2003 [26]	Tukish	99	96	79 (79.8%)	18 (18.2%)	2 (2.0%)	67 (69.8%)	26 (27.1%)	3 (3.1%)
Kamoun et al, 2007 [27]	Tunisian	89	157	67 (75.3%)	18 (20.2%)	3 (3.4%)	122 (77.7%)	29 (18.5%)	6 (3.8%)
Lee et al, 2003 [28]	Korean	94	94	83 (88.3%)	10 (10.6%)	1 (1.1%)	86 (91.5%)	8 (8.5%)	0 (0.0%)
Park et al, 2006 [29]	Korean	254	344	227 (89.4%)	27 (10.6%)	0 (0.0%)	283 (82.3%)	58 (16.8%)	3 (0.9%)
Radouane et al, 2012 [30]	Moroccon	120	112	82 (68.3%)	38 (31.7%)	0 (0.0%)	68 (60.7%)	37 (33.0%)	7 (6.3%)
Stork et al, 2008 [31]	German	92	51	78 (84.8%)	14 (15.2%)	0 (0.0%)	44 (86.3%)	7 (13.7%)	0 (0.0%)
Stork et al, 2008 [31]	Turkish	30	20	29 (96.7%)	1 (3.3%)	0 (0.0%)	18 (90.0%)	2 (10.0%)	0 (0.0%)

A subset analysis was carried out for investigating the difference in genotype frequencies in the clinical subsets of BS. No statistically significant differences were found when both GG and GA frequencies were analyzed comparing the presence and the absence of each clinical manifestation ($p>0.05$) (Table 4). We also compared the SNP genotype frequencies according to Krause' severity index: no statistically significant differences were found when the score was calculated for the patient group with GG genotype (Krause's score=5.095±2.528) and for the patient group with GA genotype (Krause's score=5.081±2.430) ($p>0.05$).

Table 4. The association between genotype frequencies and BS clinical manifestations. All p-values were >0.05.

Clinical manifestations		Genotypes	
		GG	GA
Genital ulcers	With (n=75)	65 (86.7%)	10 (13.3%)
	Without (n=55)	41 (74.5%)	14 (25.5%)
Skin disease	With (n=112)	92 (82.1%)	20 (17.9%)
	Without (n=18)	14 (77.8%)	4 (22.2%)
Ocular involvement	With (n=82)	66 (80.5%)	16 (19.5%)
	Without (n=48)	40 (83.3%)	8 (16.7%)
Neurologic involvement	With (n=26)	20 (76.9%)	6 (23.1%)
	Without (n=104)	86 (82.7%)	18 (17.3%)
Vascular involvement	With (n=25)	21 (84.0%)	4 (16.0%)
	Without (n=105)	85 (81.0%)	20 (19.0%)
Joint involvement	With (n=65)	54 (83.1%)	11 (16.9%)
	Without (n=65)	52 (80.0%)	13 (20.0%)
Gastrointestinal involvement	With (n=6)	4 (66.7%)	2 (33.3%)
	Without (n=124)	102 (82.3%)	22 (17.7%)

Discussion

This is the first report of assessment of *TNF-α* –308G/A allele promoter SNP distribution and its association with BS clinical manifestations in patients from South Italy compared with sex-age and ethnically-matched HC. Our sample size is higher than other populations analyzed in European studies.

We found a statistically significant higher frequency of GA genotype in patients group than in HC. No association between rs1800629 genotypes and any clinical hallmark was recognized, as well as no relationship was found considering the disease severity according Krause's index.

We focused our attention on *TNF-α* due to the role of its polymorphisms as genetic contributors to BS immunopathology. Genetic factors that predispose to BS are considered to have a significant role in the

disease development, so various loci were described as susceptibility markers, beside the HLA-B51, known as the major BS risk allele [1, 5–7, 35–41]. *TNF-α* is encoded in the HLA complex on chromosome 6, a region that has been known to be associated with BS. In particular, *TNF-α* rs1800629 is localized on the gene transcriptional start site and is related to the specific regulation of TNF-α synthesis. In fact, the *TNF-α* regulatory regions may play a role in cytokine production, and the binding of inducible cytoplasmatic factors may result in the translational blockade, leading to the dysregulated cytokine production. Genotype-phenotype correlation studies reported that G allele conferred two-fold lower effects on the transcription level than A allele [8–21, 26].

In our cohort, the association between GA genotype and disease risk could be explained taking into account that the presence of A allele could enhance the inflammatory reactivity influencing the binding of the transcriptional factors and contribute to the inflammation signature of BS.

Similarly to our investigation, several studies assessed the influence of the promoter polymorphism on the expression of TNF-α in BS or its susceptibility or its severity and clinical features, as well as its role as drug-response allele [14, 22–32]. The effect of geographical differences, rather than the high genetic variation, are important factors for the adaptability and the evolution of any population.

The low frequency of AA genotype is a common finding in several studies. The frequency of rs1800629 found in our cohort was very similar to the distribution reported in Turkish population by Duymaz-Tozkir and colleagues [26] (79.8% for GG genotype, 18.2% for GA genotype) and Ates and collaborators (80.4% for GG genotype, 19.6% for GA genotype) [23]. AA genotype was absent in both groups. The SNP frequency was previously studied by another research group in the same Turkish population with comparable results (74.5% for GG genotype, 25.5% for GA genotype and 0.0% for AA genotype) [24]. The frequencies of our group of controls were very similar to the distribution found in the Korean [28] and Turkish [31] populations.

About the correlation between the SNP and the clinical manifestations of BS, few studies are now available. In agreement with our data, Ates and collaborators reported no association of the polymorphism and both BS clinical hallmark and severity [23]. The polymorphism did not show any association with clinical findings also in Tukish population [22, 25]. Lee and colleagues studied the SNP genotypes according to the presence of uveitis, recognising that the presence of rs1800629 in patients with uveitis were not different from those without uveitis [28]. The comparison between patients with severe and mild uveitis was also investigated in Duymaz-Tozkir et al's paper; the authors reported the haplotypic distribution (−308G/−376A) in the disease severity groups did not show significant differences, while the comparison between patients with severe and mild uveitis showed higher frequency of the same haplotype in severe cases than mild cases [26].

Conclusions

Here we studied the distribution of *TNFα* rs1800629 in a group of Italian BS patients compared with HC and the relation of the SNP with disease clinical hallmarks and severity. We found statistically significant

higher frequency of GA genotype in patients group than in controls. No significant association was recognized between the polymorphism and the clinical parameters, as well as between the SNP and the disease severity. About the clinical significance of our findings, we underline that the SNP is a promoter variation that could affect the auto-inflammatory response and have genetically and functionally implication in BS etiopathogenesis. Analyses of a larger cohort of patients and matched controls are need to confirm our preliminary data and to clarify the SNP role.

List Of Abbreviations

AIDs, autoinflammatory diseases

BS, Behçet syndrome

CI, confidence interval

HLA, Human Leukocyte Antigen

ISG, International Study Group

MHC, Major Histocompatibility Complex

GWAS, Genome-wide association study

OR, odds ratio

ISG, International Study Group

PCR, Polymerase Chain Reaction

SNP, single nucleotide polymorphism

TNFα, tumor necrosis factor alpha

Declarations

Ethics approval and consent to participate. All subjects included in the study provided their written, informed consent to participate. The Regional Ethics Committee approved the study (Permit Number: 705/2017).

Consent for publication. Not applicable.

Data availability. The data that support the findings of this study are available from the corresponding author PL on reasonable request.

Competing interest. The authors declare no financial and non-financial competiting interests.

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Authors' contributions. MCP, PL and GM conceived and designed the study. MCP, GS, RPR, ARL and TC performed the experiments. MCP, SD, PL, GM and AAP participated in the analysis and interpretation of the data. PL, NL, MCP, GS, RPR, ARL and GM contributed in the acquisition of data. MCP wrote the first paper draft. All authors were involved in drafting, reading and revising the paper and approved the final version.

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