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Distribution of rs17482078 and rs27044 *ERAP1* polymorphisms in a group of Italian Behçet's syndrome patients: a preliminary case–control study

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Abstract
The Endoplasmic reticulum aminopeptidase protein 1 (ERAP1) trims N-terminal amino acids from epitope precursors for Major Histocompatibility Complex class I presentation. Genome-wide association studies demonstrated that *ERAP1* gene single nucleotide polymorphisms (SNPs) are associated with Behçet's syndrome (BS). This study was conducted on the two most consistently BS-associated *ERAP1* polymorphisms, rs17482078 (NG_027839.1:g.35983G>A) and rs27044 (NG_027839.1:g.35997C>G) to analyse their distribution in 55 Italian BS patients and 65 ethnically matched controls (healthy controls, HC) and to test their association with BS risk. SNPs were detected by isolation, amplification of genomic DNA and direct sequencing. SNPs functional effects were predicted by bioinformatics software. The odds ratio (OR) with 95% confidence intervals was calculated to assess the strength of BS association for genotypes and alleles, also validated by logistic regression (LR). LR was used to test the association between both SNPs and patients HLA genetic data. Bonferroni correction was also applied. Comparing patients and controls, we found a significant higher frequency of rs17482078 A allele (32.73% BS vs 17.69% HC, $p=0.007$) and AA genotype (18.18% BS vs 0% HC; $p=0.0003$) and rs27044 G allele (63.64% BS vs 46.92% HC; $p=0.0096$) in BS group after Bonferroni correction. No association was found between HLA-B*51 and both *ERAP1* SNPs. Although preliminary, our data show a stronger association of rs17482078 with BS compared to rs27044 by means of case–control genetic analysis and bioinformatics prediction of protein structure change. A larger series of patients and controls is required to confirm our preliminary findings.

Keywords Behçet's syndrome · Disease susceptibility · *ERAP1* · rs17482078 · rs27044

Introduction

Behçet's syndrome (BS; MIM 109650) is a chronic multisystemic inflammatory disorder of unclear etiology. The most significant clinical hallmarks of disease are mucocutaneous manifestations which include oral aphtosis, genital ulcers and a wide spectrum of skin lesions. Other BS features include ocular inflammation, articular, gastrointestinal, vascular and neurological involvement. BS is characterised by a peculiar geographical distribution along the "Silk road", an ancient route from Japan to the Mediterranean area. It is endemic in Eastern and Central Asia countries, especially Turkey and Iran, but is considered a rare disease in Europe, including Italy [1, 2]. Although BS etiology is still unclear, both genetics and environmental factors (dietary habits, hygienic practices, infections and pollution) may contribute to BS onset and development. An autoimmune

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reaction triggered by an environmental agent, in genetically predisposed subjects, may contribute to the dysregulation of the immune response [3]. Genome-wide association studies (GWASs) have become a significant tool in the analysis of single nucleotide polymorphisms (SNPs) in the pathogenesis of complex disorders, including BS. SNP-based GWASs for BS have been performed on multiple ethnic groups, including Turkish, Japanese, Chinese, Korean, and Iranian populations [1, 3–5]. They have identified the predominant BS susceptibility loci within the major histocompatibility complex (MHC) class I region, which includes the Human Leukocyte Antigen-B51 (HLA-B*51). HLA-B*51 is the most strongly associated risk factor for BS, confirmed in different populations. Although its association was suggested about 40 years ago [6], the HLA-B*51 role in disease pathogenesis remains elusive and only partially explains the genetics risk of BS [3, 7, 8], in fact, only 15–65% of patients are carriers depending on their genetic ancestry [3]. Non-HLA variants have also been investigated and their association with BS has been demonstrated for several genes, such as *IL10*, *IL23R-IL12RB2*, *STAT4*, *TNFα*, *CCR1*, *KLRC4*, *UBAC2, TLR4*, *MEFV*, and *NOD2* genes [4, 5, 9, 10]. In particular, the endoplasmic reticulum aminopeptidase protein 1 (*ERAP1*) gene has been recently proposed as a significant marker of BS risk. This gene is located on chromosome 5q15 and it encodes an amino-peptidase responsible for the peptides N-terminal trimming in the endoplasmic reticulum. The mechanism is a critical step of the peptide processing to optimize their length for the class I major histocompatibility complex (MHC-I) binding [11–13]. The ERAP1 ability to perform the trimming from epitope precursors depends on the amino acids sequence [11]. ERAP1 preferentially trims peptide substrates that are 9–16 residues long and its enzymatic efficacy is significantly reduced for peptides shorter than 8 residues [12]. *ERAP1* is highly polymorphic: several common polymorphisms encoding variant amino acids have been reported with genome-wide significance not only for BS, but also for other rheumatic diseases, such as ankylosing spondylitis (AS) and psoriasis. The same SNPs that are involved in BS risk are protective against AS and psoriasis: this effect on disease susceptibility has been related to the different disease-associated MHC-I interacting with ERAP1 [13]. *ERAP1* variants preferentially conferred disease risk in HLA-B*51-positive BS patients; in particular *ERAP1* rs17482078 (p.Arg725Gln) might affect the repertoire of peptides that bind to HLA-B*51 [9]. In addition to rs17482078, four other candidate SNPs were genotyped in a replication Spanish study: rs2287987 (p.Met349Val), rs30187 (p.Lys528Arg), rs10050860 (p.Asp575Asn), and rs27044 (p.Gln730Glu): data in the Spanish population were consistent with association between ERAP1 and BS as well as with an epistatic interaction between ERAP1 and HLA-B [14]. A recent Turkish study genotyped 10 missense SNPs and confirmed that only p.Arg725Gln hypoactive *ERAP1* allotype contributes to risk of BS by altering the peptides to bind to HLA-B*51 [8].

This case–control genetic study focused on the analysis of two BS-related tagSNP, rs17482078 (NG_027839.1:g.35983G>A; NP_001035548.1:p.Arg-725Gln) and rs27044 (NG_027839.1:g.35997C>G; NP_001035548.1:p.Gln730Glu) in a group of Italian BS patients compared with a group of ethnically matched controls.

Materials and methods

Patients recruitment and sampling

55 consecutive Italian BS patients (33 male and 22 female with a mean age ± SD of 45.81 ± 11.94 years) followed at Rheumatology Institute of Lucania (IReL)—Rheumatology Department of Lucania were recruited and genotyped. All patients met the International Study Group (ISG) criteria [15]. Prior to enrolling in the present study, all subjects provided their written, informed consent. The Regional Ethics Committee approved the study (Permit Number: 705/2017). Demographics, clinical features and HLA genetic data were obtained from a review of medical records. The patients with any other inflammatory/autoimmune diseases were excluded from the study. Demographic and clinical characteristics are reported in Table 1. Sixty-five matched Italian healthy controls (HC) (36 male and 29 female with a mean age ± SD of 44.52 ± 12.04 years) unrelated to each others and/or to BS patients were selected among unrelated university and hospital employees and included after ruling out any history of inflammatory and autoimmune rheumatic diseases.

Table 1 Demographic features and clinical manifestations of Italian patients with Behçet's syndrome included in the study

	BS patients, $n = 55$
Demographics	
Age, years	45.81 ± 11.94
Male/female	33/22
Clinical manifestations	
Oral ulcers, *n* (%)	55 (100.00)
Genital ulcers, *n* (%)	22 (40.00)
Skin disease, *n* (%)	44 (80.00)
Ocular involvement, *n* (%)	33 (60.00)
Neurologic involvement, *n* (%)	22 (40.00)
Vascular involvement, *n* (%)	10 (18.18)
Joint involvement, *n* (%)	29 (52.72)
HLA-B51 positivity	36 (65.45)

BS Behçet's syndrome, *n* number of subjects

The a priori sample size was calculated on line (http://www.stat.ubc.ca/~rollin/stats/ssize/caco.html). The significance threshold of $\alpha = 0.05$ was considered appropriate in the analysis of a SNP as in candidate gene studies [16]. We used the minor allele frequencies (MAF) described in the European population for rs17482078 (http://www.broadinstitute.org/mammals/haploreg/haploreg.php), the odds ratio (OR) previously described [9] and a recessive model. A one-tailed test was chosen taking into account that a possible difference in our hypothesis should only be in one direction (we considered a genetic marker consisting of two distinct alleles). Our sample size was found equal to 46 subjects to have a minimum 80% power; the sample size was raised to 55 patients and 65 controls to increase the study power (86%).

Patients and controls whole blood samples were obtained by venipuncture.

Genotyping

A three-step molecular biology analysis was carried out: (a) genomic DNA isolation; (b) in vitro Polymerase Chain Reaction (PCR) amplification; (c) *ERAP1* amplicons sequencing. DNA extraction was performed by using a commercial kit (Nuclear Laser Medicine S.r.l., Italy) according to the manufacturer instructions. DNA purity and concentration were determined by means of NanoDrop™ 1000 spectrophotometer (NanoDrop Technologies, Inc, U.S.A.): only the samples with a 260/280 ratio between 1.7 and 2.0 and a final concentration ranging from 15 to 30 ng/µl were considered suitable for the downstream applications. After a phase of PCR conditions optimization, a total of 25 µl of PCR reaction was used in each amplification reaction: 1.5 µl $MgCl_2$, each dNTP 2 mM, 1 µl specific primers, 0.4 U/µl AmpliTaq Gold® DNA polymerase in 10X PCR buffer (100 mM tris–HCl, pH 8.3, 500 mM KCl) (Roche Molecular Systems, Inc, USA).

The conditions of reaction were the following: (1) initial denaturation: 95 °C/7 min; (2) thermocycling: 94 °C/1 min; 58 °C/1 min; 72 °C/2 min (35 cycles); (3) final extension: 72 °C/10 min. Positive and negative controls were also used in the PCR amplification. PCR products were separated by gel electrophoresis (1.5% agarose gel) stained with the ethidium bromide and photographed. Good-quality amplicons were sequenced by the GATC Biotech Sanger sequencing service.

Bioinformatics analysis

Upstream in silico analysis was conducted for target-specific primers design using NCBI Primer-Blast on the basis of *ERAP1* Gene Bank NCBI RefSeq (NG_027839.1). Primer sequences were the following: 5′-GTCCTGAAGTCTTGTTGCAT-3′ (forward) and 5′-GCAGGGGAGACACTTAAACT-3′ (reverse).

Downstream bioinformatics analysis was also performed for DNA variant analysis by means of similarity search (BlastN tool); Mutation Surveyor and Sequence Scanner software were also used for this purpose. A third bioinformatics step was carried out to investigate the functional effects of both *ERAP1* SNPs and to conduct a structural protein analysis using Polymorphism Phenotyping v2 (PolyPhen-2) prediction tool [17]. The input queries were generated by altering the polymorphic positions (p.Arg725Gln and p.Gln730Glu).

Statistical analysis

Hardy–Weinberg Equilibrium (HWE) test was performed using a Chi square goodness-of-fit test. The differences in genotype/allele frequencies between patients and controls were analysed using the Chi square test (p values < 0.05 were considered significant; degrees of freedom: 1). The strength of disease association referred to each genotype and allele was expressed by OR. We calculated both allelic OR and genotypic OR, which defines the association of the disease with the alleles and the genotypes, respectively [18]. The OR > 1 indicated a positive association; the 95% confidence interval (CI) was used to estimate the precision of the OR [19]. The Etiologic Fraction (EF) was also calculated as previously described for positive association only (OR > 1) to indicate the hypothetical genetics component of the disease; the values ranging from 0.00 to 0.99 were considered of significance [20]. Bonferroni correction was applied using the formula $pc = p/n$, where pc is the corrected p value, p is the original p value and n is the number of comparisons made ($pc = 0.05/3 = 0.017$ for the genotypes and $pc = 0.05/2 = 0.025$ for the alleles).

The logistic regression was also performed to validate the significance of the genotype frequencies differences between patients and controls, to analyse the influence of the gender on the SNPs-disease association and to test the association between HLA-B*51 and both *ERAP1* polymorphisms using R.3.2.4 revisited software [21].

Results

The distribution of genotype frequencies of the two tested SNPs conformed to HWE (p value > 0.05). No statistically significant differences in age distribution were observed when BS patients (45.81 ± 11.94 years old) and controls (44.52 ± 12.04 years old) were compared (p value 0.567). The SNPs genotypes and allele frequencies in patient and control groups are shown in Table 2. The first SNP, rs17482078, was predicted to be possible damaging by the

PolyPhen-2 prediction tool (score: 0.587; sensitivity: 0.87, specificity: 0.91), while the second SNP, rs27044, was termed as benign by the PolyPhen-2 software (score: 0.022; sensitivity: 0.95, specificity: 0.80) (Fig. 1; Table 3).

A lower frequency of the wild-type rs17482078 GG genotype was found within BS patients (52.72%) than controls (64.62%). This difference was not statistically significant (p value: 0.187; OR 0.61). Similarly, the frequency of SNP in heterogyzosity state showed no statically significant differences when patients and controls were compared (p value 0.463). The rs17482078 AA genotype was interestingly absent in the control group, while its frequency was

Table 2 Genotype and allele frequencies of rs17482078 and rs27044 in BS patients and controls

SNP	Genotype/allele	BS patients ($n=55$) n (%)	Controls ($n=65$) n (%)	p value	OR (95% CI)	EF[a]
rs17482078	GG	29 (52.72)	42 (64.62)	0.187	0.61 (0.29–1.27)	NA
	GA	16 (29.09)	23 (35.38)	0.463	0.75 (0.35–1.62)	NA
	AA	10 (18.18)	0 (0.00)	0.0003*	144.44 (0.28–7421.30)	0.993
	G	74 (67.27)	107 (82.31)	0.0070*	0.44 (0.24–0.81)	NA
	A	36 (32.73)	23 (17.69)	0.0070*	2.26 (1.24–4.13)	0.558
rs27044	CC	11 (20.00)	22 (33.85)	0.091	0.49 (0.21–1.13)	NA
	CG	18 (32.73)	25 (38.46)	0.514	0.78 (0.37–1.65)	NA
	GG	26 (47.27)	18 (27.69)	0.0266	2.34 (1.10–5.00)	0.573
	C	40 (36.36)	69 (53.08)	0.0096*	0.51 (0.30–0.85)	NA
	G	70 (63.64)	61 (46.92)	0.0096*	1.98 (1.18–3.33)	0.495

BS Behçet's syndrome, *n* number of subjects, *OR* odds ratio, *CI* confidence interval, *EF* etiologic fraction

Statistically significant ($p<$pc)

[a]EF was calculated only for positive association (OR > 1)

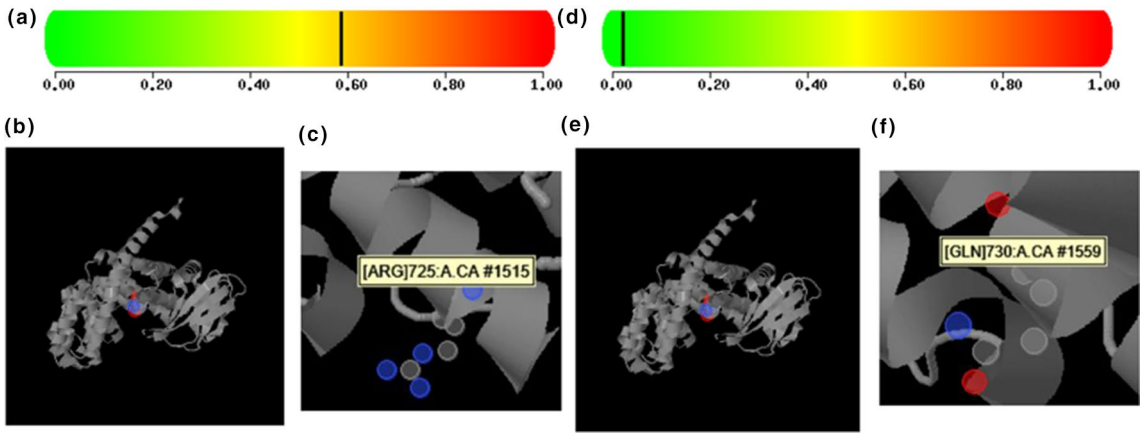

Fig. 1 PolyPhen-2 bioinformatics prediction of rs17482078 and rs27044 functional effect and mutant ERAP1 protein structure. **a** Prediction output showing rs17482078 pathogenicity score (0.587); **b** structure of the human ERAP1 protein and **c** location of the p.Arg725Gln variation obtained using PolyPhen-2 software; **d** PolyPhen-2 prediction output showing rs27044 pathogenicity score (0.022); **e** structure of the human ERAP1 protein and **f** location of the p.Gln730Gln variation obtained using PolyPhen-2 software

Table 3 Predicted effects of *ERAP1* rs17482078 and rs27044 tested SNPs

SNP	Nucleotide variation	Amino acids variation	PolyPhen-2 prediction	Score
rs17482078	g.35983G > A	p.Arg725Gln	Possible damaging	0.587
rs27044	g.35997C > G	p.Gln730Glu	Benign	0.022

PolyPhen-2 software predicted that rs17482018 is a possible damaging variant, while no functional effect was underlined for rs27044. Both genomics and amino acids variations were reported according to the Human Genome Variation Society (HGVS) nomenclature

equal to 18.18% for the patient group; both the OR and EF of this genotype were very high (p value: 0.0003; EF: 99.3%). This difference survived to Bonferroni correction (p value < 0.017). In addition, a statistically significantly higher frequency of A allele was found in patients (32.73%) compared with controls (17.69%) (p value 0.0070), confirmed after Bonferroni correction (p value < 0.025). The OR value for G allele and A allele was 0.44 and 2.26, respectively, indicating a moderate association between the A mutant allele and BS. Out of 55 BS patients, 11 subjects (20.00%) showed the rs27044 wild-type CC genotype, while 22/65 (33.85%) of controls revealed the same genotype; the difference between the two groups was not statistically significant (p value 0.091). No statistically significant difference was found for the heterozygous CG genotype frequency in the patients (32.73%) and the controls (38.46%) (p value 0.514). The frequency of the GG genotype significantly varied between patients (47.27%) and controls (27.69%) (p value 0.0266). The significance was lost after Bonferroni correction (p value > 0.025). The C allele was identified 40 times (36.36%) in the BS group and 69 times (53.08) in the control group; this difference was statistically significant (p value 0.0096), also after Bonferroni correction (p value < 0.017). This allele showed an OR value of 0.51, putting in evidence no association between the allele and BS. The OR value of the G allele was found equal to 1.98, suggesting a moderate effect of the allele. In fact, the frequency of G allele was significantly higher in patients (63.64%) than in controls (46.92%) (p value 0.0096), confirmed after Bonferroni correction (p value < 0.017). The association between the disease susceptibility and rs17482078 was confirmed by logistic regression analysis (p value 0.006576).

No statistical difference was found when the genotypes were analyzed regarding the gender and HLA-B*51 status: logistic regression data showed no influence of the gender on the disease risk for both rs17482078 (p value 0.401692) and rs27044 (p value 0.28458) and no association between HLA-B*51 and both rs17482078 (p value 0.3134) and rs27044 (p value 0.607).

Discussion

Growing literature evidences suggest that *ERAP1* is a promising candidate gene to clarify the genetics contribution to the risk of BS. *ERAP1* polymorphisms affect the protein trimming activity. The protein influences the cytotoxic T and natural killer cells function and is involved in the pathogenesis of several immune-mediated diseases associated to HLA class I with a partially unknown mechanism [3, 8, 9, 11–14].

Molecular, bioinformatics and statistical approaches were integrated in this study to characterize two *ERAP1* SNPs, rs17482078 and rs27044, previously associated with poor protein trimming function [11]. We found a significantly higher frequency of rs17482078 A allele and AA genotype in BS patients than in controls and a strong association between AA genotype and BS. In addition, the EF result showed that AA genotype had a large effect on the disease risk. About the second polymorphism, rs27044 G allele and GG genotype were higher in patients compared with controls, but we obtained statistical significant only for the allele and not for genotype after Bonferroni correction. No functional effects were found for rs27044, which was predicted as a neutral change. The higher OR and EF and the predicted functional effect of rs17482078 suggest a major role of this polymorphism in our BS cohort. The association between rs17482078 and BS susceptibility was previously described in particular in Iranian [5, 7], Turkish [8, 9] and Spanish [14] populations with a different strength of association. A genetic epistasis between rs17482078 and HLA-B*51 was also reported [9, 14]. We found no association between both *ERAP1* SNPs and HLA-B*51, probably due to our small sample size. No statistically significant differences in OR values were found in Spanish population when BS patients with HLA-B risk factors (HLA-B*51 and HLA-B*57) and BS patients without HLA-B risk factors were compared, although higher OR values were observed in the first group [14].

The bioinformatics analysis highlighted that rs17482078 had a moderate impact of amino acid substitutions on the protein stability and function. Our results agreed with a recent study conducting a bioinformatics analysis of several *ERAP1* SNPs in AS: the authors demonstrated that the substitution may be responsible for the disruption of two hydrogen bound between p.Arg725 and p.Asp766 residues in the ERAP1 active state, affecting the protein C-terminal stability and its enzymatic activity [22].

In conclusion, our data were in agreement with previous studies reporting the SNPs role as BS markers [3, 8, 9, 11–14] with a discrepancy in the genotype distribution and in the strength of association. This observation could be related to two factors: our small sample size and the substantial variance in gene allelic frequencies among populations of different ethnic groups and different genetic ancestry.

Although our results report for the first time the distribution of the most significant *ERAP1* genotypes and alleles in a cohort of Italian BS patients, these ones must be interpreted with caution as the number of patients is low. So, the analysis of a larger series of patients and controls is required to verify our preliminary findings.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Statements on human and animal rights All procedures performed in the study (involving human participants) were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was collected for all human participants involved in the study.

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