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A First Step for the Molecular Characterization of Neurological Involvement of Behçet Syndrome: an Italian Pivotal Study

Maria Carmela Padula[1,2] · Pietro Leccese[1] · Nancy Lascaro[1] · Angela Anna Padula[1] · Teresa Carbone[1] · Giuseppe Martelli[2] · Salvatore D'Angelo[1]

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Abstract

Behçet syndrome (BS) is a vasculitis characterized by several clinical manifestations including the rare neurological involvement (neuro-BS, NBS). The aim of our pivotal study was to investigate the mutational status of several inflammation-related genes in a cohort of Italian patients with and without the neurological involvement (20 NBS vs 40 no-NBS patients). The preliminary *in silico* single nucleotide polymorphism (SNP) selection and primer design were performed by NCBI Primer-Blast tool. Genomic DNA was isolated and amplified using PCR. PCR amplicons were sequenced and bioinformatically analysed. Twelve tagSNPs were selected and genotyped: *ERAP1* rs30187, rs17482078, and rs27044; *IL10 rs1800872* and rs1518111, *IL12A* rs17810546, *IL23R* rs17375018, *IL23R-IL12RB2* rs924080, *STAT4* rs7572482, *CCR1* rs7616215, *KLRC4* rs2617170, and *UBAC2* rs3825427. *ERAP1* and *IL23R* SNPs showed statistically significant higher frequencies in NBS group than no-NBS. *ERAP1* rs30187 AA was more common in no-NBS patients (20.0% NBS vs 47.5% no-NBS; $p < 0.05$), while rs17482078 GA frequency was higher in NBS patients (55.0% NBS vs 22.5% no-NBS; $p < 0.05$, OR: 4.21). *IL23R* rs17375018 GG was more frequent in NBS group (65.0% NBS vs 40.0% no-NBS; $p < 0.05$), according to a previous finding. No other statistically significant differences were found. In conclusion, *ERAP1* and *IL23R* SNPs were found associated with neurological involvement of BS. Additional and larger analyses were required to verify our preliminary findings.

Keywords Behçet syndrome · ERAP1 · IL23R · Neuro-Behçet syndrome · Susceptibility

Introduction

Behçet syndrome (BS) is a vasculitis involving blood vessels of all sizes and characterized by a wide spectrum of clinical manifestations, in particular recurrent oral aphthosis, genital ulcers, skin lesions, arthritis, uveitis, and thrombosis. The gastrointestinal and the nervous system involvement were less common reported manifestations (Hatemi et al. 2016; Leccese et al. 2017). The nervous system involvement, known as "Neuro-BS" (NBS), is a rare disease manifestation and is one of the main causes of long-term morbidity and mortality in BS. NBS comes in the form of parenchymal NBS or extra-parenchymal NBS. Parenchymal NBS lesions predominantly involve the brainstem but can also affect the basal ganglia, the thalamus, the cortex and the white matter, the spinal cord, or the cranial nerves. Cerebral venous sinus thrombosis, resulting from a vasculitic process of the large veins, includes the majority of vascular NBS cases (Noel et al. 2014; Saip et al. 2014; Gheita et al. 2015; Caruso and Moretti 2018). NBS signs are more common in males, and the neurological involvement usually develops after the onset of other manifestations within 3–6 years. Neurological manifestations are the first BS manifestation in only 6% of patients (Al-Araji and Kidd, 2009; Noel et al. 2014; Saip et al. 2014; Gheita et al. 2015; Caruso and Moretti 2018; Sorgun et al. 2018).

BS frequency and clinical manifestations significantly differ from country to country, suggesting the role of both genetics and environmental factors in disease etiopathogenesis (Noel et al. 2014; Saip et al. 2014; Gheita et al. 2015; Hatemi et al. 2016; Leccese et al. 2017; Caruso and Moretti 2018). Human leukocyte antigen (HLA)-B*51 was found to be the most strongly associated genetic factor responsible for

✉ Maria Carmela Padula
mcpadula25@gmail.com

[1] Rheumatology Institute of Lucania (IReL), San Carlo Hospital, Potenza 85100, Italy

[2] Department of Science, University of Basilicata, Potenza 85100, Italy

the development of BS (Ohno et al. 1978). The prevalence of HLA-B*51 among NBS patients was found similar to that found in BS patients without neurological signs (Caruso and Moretti 2018).

Other BS risk genetic factors are involved in the inflammation and the immunity processes. In fact, several BS susceptibility loci were highlighted outside the HLA in genome wide association studies (GWASs). Non-HLA variations have also been investigated and their association with BS has been demonstrated for several genes, such as *Endoplasmic reticulum aminopeptidase 1 (ERAP1)*, *Interleukin 10 (IL10)*, *IL-23 receptor-IL-12 receptor* β2 (*IL23R-IL12RB2), Signal transducer and activator of transcription 4 (STAT4)*, *C–C Motif Chemokine Receptor 1 (CCR1)*, *Killer Cell Lectin Like Receptor C4* (*KLRC4)*, *Ubiquitin-associated domain-containing protein 2 (UBAC2)*, *Toll-like receptor 4 (TLR4)*, *Mediterranean fever (MEFV)*, and *Nucleotide-binding oligomerization domain-containing protein 2 (NOD2)* genes (Kirino et al. 2013; Conde-Jaldon et al. 2014; Gul 2014, 2015; Ombrello et al. 2015; Sousa et al. 2015; Takeuchi et al. 2015, 2016; Burillo-Sanz et al. 2017; Jung et al. 2017; Kang et al. 2017; Wang et al. 2017; Deng et al. 2018; Padula et al. 2018, 2019a, 2019b).

To date, although BS inflammatory features were recently studied, with focus on single nucleotide polymorphisms (SNPs) of target genes (Gul 2015; Takeuchi et al. 2015; Burillo-Sanz et al. 2017; Deng et al. 2018), the mutational state of NBS patients was poorly analysed (Gheita et al. 2015). The SNP distribution in the context of the presence and absence of neurological symptoms could be an interesting topic to study in deep in the field of the modern molecular personalized medicine. In particular, Gheita and collaborators investigated the level of serum IL-23 and assessed rs17375018 *IL23R* SNP genotypes in BS Egyptians patients. The authors found that rs17375018 GG genotype was higher in BS patients with neurological involvement and uveitis. No similar data are now available for the Italian population. The aim of the present study was to genotype a SNPs panel of several inflammation-related genes, not a single polymorphism, in a group of Italian NBS patients compared with a group of BS patients without neurological involvement (no-NBS), in order to identify possible differential/discriminant markers.

Materials and Methods

Sixty Italian BS patients (36 males and 24 females) were recruited at Rheumatology Institute of Lucania (IReL)-Rheumatology Department of Lucania. Diagnosis of BS was made according to the International Study Group (ISG) for Behçet disease criteria (Weichsler et al. 1990). Written informed consent was obtained from all the patients. The study was approved by the Regional Ethics Committee (Permit Number: 705/2017). We identified a cohort of NBS patients during the routine visits at our centre. Neurological involvement was assessed according to clinical examination and neuroradiological investigations, ruling out any risk factors and etiological causes (large artery atherosclerosis, cardioaortic embolism, and small artery occlusions) that may mimic NBS.

We included in our study a control group of BS patients characterized by the absence of neurological manifestations. In detail, for each NBS patient, we recruited the two following BS patients without neurological involvement seen during the same scheduled visit.

Literature and *in silico* specific databases (dbSNP-NCBI Database) consultation were carried out to select the SNPs for genotyping. Primer pairs were designed for the coverage of the selected tagSNPs using the on line NCBI Primer-Blast tool based on the Reference Sequences of each gene.

DNA extraction was performed using a commercial kit (Nuclear Laser Medicine S.r.l., Italy) according to the manufacturer instructions. NanoDrop™ 1000 spectrophotometer (NanoDrop Technologies, Inc, USA) was used to determine DNA purity and concentration.

Primer specific-PCR amplification was performed using the Q5 Hot Start High-Fidelity DNA Polymerase (BioLabs Inc, New England) according to the manufacturer's recommendations. PCR conditions were the following: (1) initial denaturation: 98 °C for 7 min; (2) thermocycling: 98 °C for 1 min; 54–60 °C for 1 min; 72 °C for 2 min (30 cycles); and (3) final extension: 72 °C for 5 min. Each amplification reaction was in duplicate, and a negative control was also used. PCR products were separated using 1.5% agarose gel electrophoresis, and the good-quality amplicons were sequenced by the Microsynth AG sequencing service. DNA variant analysis was performed using similarity search tool (NCBI-Blast Nucleotide) and Mutation Surveyor software (SoftGenetics,USA).

The odds ratio (OR) was calculated to assess the strength of BS association for each genotype. The 95% confidence interval (CI) was used to estimate the precision of the OR (Szumilas 2010). A $p < 0.05$ was considered statistically significant.

Results

NBS patients subset consisted of 20 patients, 15 males and 5 females with mean age equal to 44.68 ± 10.64 years. The mean age at NBS onset was 30.00 ± 10.95 years. Average BS disease duration (DD) was 22.50 ± 12.03 years. Parenchymal involvement occurred in 14 patients (70% of NBS cases), of which 3 patients (15% of NBS cases) showed optic neuritis, while non-parenchymal involvement

was found in 6 patients (30% of NBS cases). Neurological signs were the first BS manifestation in 2/20 NBS patients (10%). A total of 6/20 (30%) NBS patients were treated with cyclosporine at neurological involvement onset.

No-NBS patients subset included 40 patients, 21 males and 19 females with mean age of 44.52 ± 12.04 years. Average DD of this group was 27.64 ± 16.56 years. In this group, 17/40 (42.5%) patients were treated with cyclosporine during their follow-up.

Neurological involvement was more common in males than in females, but no statistically significant difference was found in gender distribution ($p = 0.0935$), as well as in age ($p = 0.9612$), when we compared NBS and no-NBS patients.

A statistically significant difference was found for the HLA-B*51 positivity, more frequently recognized in NBS (84.21%) than in no-NBS patients (44.00%) ($p < 0.01$).

Twelve SNPs of 9 genes were considered eligible for molecular analysis and genotyped: rs30187, rs17482078, and rs27044 of *ERAP1*; rs1800872 and rs1518111 of *IL10*; rs17810546 of *IL12A*; rs17375018 of *IL23R*; rs924080 of *IL23R-IL12RB2*; rs7572482 of *STAT4*; rs7616215 of *CCR1*; rs2617170 of *KLRC4*; and rs3825427 of *UBAC2*. Their distribution in NBS and no-NBS cohorts was reported in Table 1.

We found a statistically significant difference in the frequencies of rs30187 and rs17482078 *ERAP1* and rs17375018 *IL23R* SNPs between NBS and no-NBS groups.

Table 1 Genotype frequencies of selected tagSNPs in BS patients with (NBS) and without (no-NBS) neurological involvement.

Gene	SNP	Genotype	NBS patients (n=20); n (%)	no-NBS patients (n=40); n (%)	p-value	OR (95% CI)
ERAP1	rs30187	AA	4 (20.00)	19 (47.50)	0.0389*	0.28 (0.08–0.97)
		AG	7 (35.00)	8 (20.00)	0.2059	2.15 (0.65–7.16)
		GG	9 (45.00)	13 (32.50)	0.3436	1.70 (0.56–5.11)
	rs17482078	GG	7 (35.00)	23 (57.50)	0.1003	0.40 (0.13–1.21)
		GA	11 (55.00)	9 (22.50)	0.0118*	4.21 (1.22–13.32)
		AA	2 (10.00)	8 (20.00)	0.3272	0.44 (0.09–2.32)
	rs27044	CC	5 (25.00)	12 (30.00)	0.6854	0.78 (0.23–2.63)
		CG	5 (25.00)	12 (30.00)	0.6854	0.78 (0.23–2.63)
		GG	10 (50.00)	16 (40.00)	0.4612	1.50 (0.51–4.42)
IL10	rs1800872	AA	2 (10.00)	9 (22.50)	0.2382	0.38 (0.07–1.97)
		AC	4 (20.00)	10 (25.00)	0.6660	0.75 (0.20–2.78)
		CC	14 (70.00)	21 (52.50)	0.1949	2.22 (0.68–6.60)
	rs1518111	AA	7 (35.00)	17 (42.50)	0.1330	0.41 (0.13–1.32)
		AG	2 (10.00)	11 (27.50)	0.1209	0.29 (0.06–1.48)
		GG	11 (55.00)	12 (30.00)	0.0604	2.85 (0.94–8.66)
IL12A	rs17810546	AA	15 (75.00)	33 (82.50)	0.4936	0.64 (0.17–2.33)
		AG	2 (10.00)	3 (7.50)	0.7412	1.37 (0.21–8.94)
		GG	3 (15.00)	4 (10.00)	0.5695	1.59 (0.32–7.90)
IL23R	rs17375018	AA	1 (5.00)	4 (10.00)	0.5089	0.47 (0.05–4.54)
		AG	6 (30.00)	21 (50.00)	0.0986	0.39 (0.12–1.21)
		GG	13 (65.00)	15 (40.00)	0.0441*	3.10 (1.01–9.49)
IL23R-IL12RB2	rs924080	TT	14 (70.00)	21 (52.50)	0.1949	2.11 (0.68–6.60)
		TC	5 (25.00)	16 (40.00)	0.2508	0.50 (0.15–1.65)
		CC	1 (5.00)	3 (7.50)	0.7144	0.65 (0.65–6.67)
STAT4	rs7572482	TT	6 (30.00)	7 (17.50)	0.2679	2.02 (0.57–7.10)
		TC	9 (45.00)	12 (30.00)	0.2508	1.91 (0.63–5.80)
		CC	5 (25.00)	21 (52.50)	0.0427	0.30 (0.09–0.99)
CCR1	rs7616215	CC	3 (15.00)	7 (17.50)	0.8065	0.83 (0.19–3.63)
		CT	7 (35.00)	13 (32.50)	0.8465	1.12 (0.36–3.37)
		TT	10 (50.00)	20 (50.00)	1.000	1.00 (0.34–2.93)
KLRC4	rs2617170	TT	7 (35.00)	16 (40.00)	0.7073	0.81 (0.26–2.46)
		TC	4 (20.00)	15 (37.50)	0.1695	0.42 (0.12–1.48)
		CC	9 (45.00)	9 (22.50)	0.0730	2.82 (0.89–8.92)
UBAC2	rs3825427	CC	17 (85.00)	31 (77.50)	0.4936	1.65 (0.39–6.90)
		CA	2 (10.00)	8 (20.00)	0.3272	0.44 (0.09–2.32)
		CC	1 (5.00)	1 (2.50)	0.6111	2.05 (0.12–34.63)

Abbreviations: SNP, single nucleotide polymorphism; NBS, Neuro Behçet syndrome; n, number of subjects; OR, odds ratio; CI,

In particular, rs30187 wild-type AA genotype was more frequent in no-NBS than NBS patients ($p = 0.0389$). On the contrary, rs30187 AG genotype and GG genotype frequencies were higher in NBS patients without statistical significance (35.0% NBS vs 20.0% no-NBS and 45.0% NBS vs 32.5% no-NBS, respectively; $p > 0.05$). rs17482078 GA genotype occurred more frequently in NBS patients (55.0%) than in no-NBS (22.5% ($p = 0.0118$). The OR value for the heterozygous genotype was 4.21, indicating a strong association between the heterozygous genotype and the neurological manifestation.

We found lower OR for the wild-type genotypes in NBS group for all interleukin family genes (AA genotype of both *IL10* rs1800872 and rs1518111, AA genotype of *IL12A* rs17810546, AA genotype of *IL23R* rs17375018), except for *IL23R-IL12RB2*. A statistical significance was found for GG genotype of *IL23R* rs17375018, more frequently determined in NBS (65.0%) than in no-NBS (40.0%) group ($p = 0.0441$). No statistically significance was found for the other genes analysed: *KLRC4* and *UBAC2* minor genotypes were more frequent in NBS group, while *STAT4* rs7572482 minor genotype occurred less frequently in NBS than in no-NBS group.

Discussion

This is a monocentric comparative study assessing the mutational state of several SNPs of inflammatory genes in a group of NBS versus no-NBS subjects. We investigated the inflammatory-related genomic variability between the two groups to identify genotypes and connect them to different phenotypes. In our knowledge, BS-related SNP distribution was not previously analysed in the context of the presence or absence of neurological features. We found that *ERAP1* and *IL23R* SNPs of our gene panel showed statistically significant differences in the frequency distribution between the groups.

ERAP1 encodes an amino-peptidase responsible for processing N-terminally extended antigenic precursors for optimal loading onto HLA molecules. The enzyme trimming efficiency has been related to the presence of SNPs influencing the dynamics of the open-closed conformational change (Kirino et al. 2013; Conde-Jaldon et al. 2014; Gul 2014, 2015; Ombrello et al. 2015; Takeuchi et al. 2015, 2016; Wang et al. 2017; Padula et al. 2018, 2019a, 2019b).

The association between *ERAP1* and BS was previously reported in various populations for several SNPs, in particular for known rs30187 (p.Lys528Arg), rs17482078 (p.Arg725Gln), and rs27044 (p.Gln730Glu) polymorphisms (Kirino et al. 2013; Conde-Jaldon et al. 2014; Gul 2014, 2015; Ombrello et al. 2015; Takeuchi et al. 2015, 2016; Wang et al. 2017; Padula et al. 2019b). rs30187 and rs17482078 were reported as two functional SNPs able to affect the ERAP1 protein structure and the enzymatic activity (Kirino et al. 2013; Takeuchi et al. 2016; Wang et al. 2017). rs30187 SNP was reported as an influencing factor on the substrate-binding affinity with a 30–40% reduction in enzymatic activity than the wild-type protein (Wang et al. 2017). rs17482078 AA genotype of *ERAP1* was found to be a risk for BS, in particular if associated with other protein coding variants. The haplotype was responsible for the reduction of the peptide trimming activity and the alteration of the peptides available for HLA binding (Kirino et al. 2013; Ombrello et al. 2015; Takeuchi et al. 2016; Wang et al. 2017).

In our study, rs30187 wild-type genotype was more frequent in no-NBS group with a low OR value, suggesting the association between the polymorphism and the absence of neurological signs. The presence of the minor G allele was more frequently recognized in NBS patients both in heterozygous and in homozygous state.

A lower frequency was found for wild-type GG genotype of rs17482078 in NBS group, while a higher frequency of rs17482078 GA genotype was observed in NBS patients than in no-NBS group. The OR value for the heterozygous genotype was 4.21, indicating a strong association between the genotype and the neurological manifestation. This association was not confirmed for AA genotype.

No differences were found for rs27044, identified as a not damaging variant when its functional role was in silico predicted (Padula et al. 2019b).

IL-23 receptor (IL-23R) is expressed on the surface of Th17 cells and macrophages. *IL-23R* and its polymorphisms influence the breakdown of self-tolerance and have been associated with several chronic inflammatory diseases, including inflammatory bowel disease, ankylosing spondylitis, and psoriasis (Gul 2014; Takeuchi et al. 2015; Jung et al. 2017).

In our data, *IL23R* rs17375018 was more frequently found in NBS than in no-NBS group with statistical significance for GG genotype. Recent studies have also investigated the association of several *IL-23R* SNPs and the uveitis in BS with conflicting results summarized in Jung et al.'s meta-analysis (Jung et al. 2017), but no data were reported for the neurological involvement.

Gheita and collaborators analysed the clinical significance of serum IL23 and *IL23R* rs17375018 SNP and reported the possible role of the interleukin and the receptor polymorphism in NBS besides uveitis (Gheita et al. 2015). The authors investigated the *IL23R* rs17375018 in 50 BS patients recruited from Cairo University Hospitals' outpatient clinics. They reported a frequency of AG genotype equal to 36% (18/50 of patients) and a frequency of GG genotype equal to 48% (24/50 of patients). In our cohort, we found a 45% of frequency for AG genotype (27/60 of patients) and an about

47% of frequency for GG genotype (28/60 of cases), higher in the first case and very similar in the second case compared with Gheita et al.'s data (Gheita et al. 2015). Considering only the NBS group, a different distribution was underlined for both heterozygous and homozygous genotypes in our group of patients than the previous results (Gheita et al. 2015): we found a lower frequency of AG genotype (30.0% vs 36.8%, respectively) and a higher frequency of GG genotype (65.0% vs 57.9%, respectively), confirming that the last genotype could be significantly related to the neurological manifestations of BS. Our data confirmed the role of *IL23R* rs17375018 in NBS susceptibility in our ethnic group.

Our results reported for the first time the genotype distribution of several susceptibility loci in a group of Italian BS patients with neurological involvement compared with BS patients without neurological manifestations. Studying in deep the molecular profile of patients with NBS could provide a tool to identify the neurological signature of disease useful for further disease stratification and for the early diagnosis of the major organ (nervous system) involvement. Our data suggested a possible association between the rs17482078 heterozygous genotype of *ERAP1* and the rs17375018 of *IL23R* polymorphic genotype and NBS. Both genotypes seem to increase the neurological risk, probably affecting the peptide trimming and the breakdown of self-tolerance in the complex nervous system molecular mechanisms, leading to the central symptoms. Understanding the exact role of these SNPs in influencing the contribution to the neurological manifestations and failure could be an interesting point to address in order to obtain a screening tool to predict the neurological involvement at the time of BS detection. For these reasons, although our data showed significant differences for *ERAP1* and *IL23R* SNPs, the analysis of larger cohorts of patients and healthy controls, as well as functional studies, is required for confirming the findings and better defining the genetic contribution to the development of neurological phenotype of BS, as well as for clarifying the clinical significance.

Authors' Contributions GM, MCP, SD, PL, and AAP conceived and designed the study. MCP and TC performed the experiments. MCP, GM, SD, NL, and PL participated in the analysis and interpretation of the data. NL, TC, AAP, and PL 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.

Compliance with Ethical Standards

Competing Interest The authors declare that they have no competing interests.

Ethics Approval and Consent to Participate All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (Regional Ethics Committee Permit Number: 705/2017) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent for Publication Not applicable.

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

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