Lack of association of *TNFAIP3* and *JAK1* with Behçet's disease in the European population

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ABSTRACT

Objective. *Behçet's disease (BD) is an immune-mediated and complex disease which has been associated with HLA class I molecules although other genes such as IL23R and IL10 have also been involved in the susceptibility to BD. Recently, an association of variants of the JAK1 and TNFAIP3 genes with the disease has been reported in the Chinese Han population. The aim of the present work was to asses whether the association described in Asian populations is replicated in Europeans.*

Methods. *This study includes a total of 1155 Spanish subjects of European origin (372 BD and 783 unrelated healthy individuals). Patients were recruited from different hospitals and controls were collected in the same geographic regions and they matched with patients in age and gender. A total of five SNPs, two in the JAK1 gene: rs2780815 and rs310241 and the other three in the TNFAIP3: rs10499194, rs9494885 and rs610604, were included in this study. The genotyping of these SNPs was performed using a real time PCR system (TaqMan® SNP Genotyping Assays).*

Results. *No statistically significant differences were found when the patient and control groups were compared. The distribution of the risk alleles was similar in patients with and without eye manifestations and in patients with and without HLA-B*51.*

Conclusion. The association of variants of the genes JAK1 and the TNFAIP3 with BD which has been described in the Chinese population was not replicated in Europeans.

Introduction

Behçet's disease (BD) is an immune-mediated and complex disease in which certain environmental factors such as infectious agents are the triggers of the disease in genetically predisposed individuals (1). This rare disease is most common along the old route named "Silk Road", stretching from China to the Mediterranean area (2). This particular geographical distribution in addition to the familial aggregation and association with HLA class I molecules (specifically with HLA-B51) are evidences supporting genetic contribution into the pathogenesis of the disease (3, 4). The contribution of the HLA region to the genetic component has been estimated to be approximately 20% (5). Some genes such as IL23R, IL10 and others have been related with BD in different populations (6-10), whereas in other cases the association seems to be limited to one ethnic group or specific population (11-14). The complexity of this vasculitis which is clinically characterized by the presence of oral and genital ulcerations but whose manifestations are diverse and they can spread to eyes, joints, digestive system, etc. (1) is an additional difficulty to establish whether a gene is related with the disease itself or with a specific subgroup. The *JAK1* (Janus kinase 1) gene is located in 1p32.3-31.3, this gene encodes a large and widely expressed membrane-associated phosphoprotein with protein-tyrosine kinase activity, which is involved in the signal transduction pathways of interferon-alpha/beta and gamma (15). The *TNFAIP3* (Tumour necrosis factor alpha-induced protein 3) gene is located at 6q23, this gene encodes an ubiquitin-editing enzyme (also known as A20) which is involved in NF-kappa-B responses and as a consequence, it is a key regulator in different immune and inflammatory respons-

es (16). Therefore, from a functional perspective, these two genes are good candidates for association with immuno-mediated diseases; in fact, they have been related with different autoimmune diseases (17-19). Recently, an association of variants of the genes *JAK1* and *TNFAIP3* with BD has been reported in the Chinese Han population (20, 21) in patient cohorts with 100% uveitis. Nevertheless, the association of these genes with BD has been not yet investigated in Europeans neither in BD patients without ocular involvement.

The aim of the present work was to asses whether the association of *JAK1* and *TNFAIP3* described in the Chinese Han population is replicated in Europeans.

Material and methods
Subject of study
The study includes 372 BD patients (161 males and 211 females) with a mean age at onset (years) ± SD of 48.22±12.19 who fulfilled the criteria of 1990 of the International Study Group for the classification of Behçet's disease (22) and 783 unrelated healthy individuals recruited in the same geographic regions who matched in age and gender with BD patients. All the subjects were Spanish from European origin and they were recruited from different Spanish hospitals. The study was approved by the local ethics committees of all the participant hospitals, A Coruña (CHU A Coruña), Almería (H. Torredecárdenas), Barcelona (H. Clinic, Vall d'Hebron and Mútua Terrassa), Granada (H. Clínico San Cecilio), Madrid (H. de la Princesa), Málaga (H. Carlos Haya), Palma de Mallorca (H, Universitari Son Espases), Pamplona (H. Virgen del Camino), Santander (H. Marques de Valdecilla) and Sevilla (H. Virgen del Rocío and H. Virgen de Valme) and a written informed consent was obtained from all participants. Clinical features of the patient group were: 100% had oral ulcers, 66% genital ulcers, 59% uveitis, 51.6% arthritis, and 22% vascular, 23% neurological and 20% gastrointestinal involvement.

DNA extraction
Peripheral blood or saliva were used as the starting material. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Quiagen, Barcelona, Spain) according to the manufacturer's recommendations and stored at -20ºC until use. The purity of DNA was determined using NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Only those DNA samples having a 260/280 ratio between 1.7 and 2.0 and a final concentration of 10-20 ng/µl were genotyped.

SNP selection and genotyping
Regarding the *JAK1* gene, we selected two out of the three SNPs whose association to BD has been reported in the Chinese Han population (20). The minor allele frequency (MAF) values of the two selected SNPs, rs2780815 and rs310241, in Europeans are 0.47 and 0.24, respectively. The other SNP associated in the Chinese population, rs3790532, was excluded because it is not polymorphic in Europeans (MAF is zero) and, in addition, it is in a complete linkage disequilibrium (LD) with the rs310241 (r2=1; D'=1) in the Asian population (http://www.1000genomes.org). Concerning the *TNFAIP3* gene, three SNPs: were found associated with BD in the Chinese Han population, rs10499194, rs7753873 and rs9494885 (21). From these, we choose rs10499194 and rs9494885 (MAF 0.26 and 0.08, respectively in Europeans). The last is in a strong LD with rs7753873 (r2=1; D'=1) in Asian and European populations. Another SNP in the *TNFAIP3* was also included in the present study, rs610604. This SNP was genotyped in the Chinese cohort and statistically significant differences in the distribution of frequencies in patients and controls were not found. Nevertheless, we decided to include this SNP because the MAF in Europeans is higher than in Asians (0.33 *vs*. 0.09) and, as a consequence, the statistical power for this SNP is higher in our population assuming that the rest of the conditions were similar. Genotyping of these SNPs was performed using TaqMan® SNP Genotyping Assays (Applied Byosistems, Barcelona, Spain) in a LightCycler 480 (Roche, Barcelona, Spain). About 10% of the samples were genotyped twice to verify the genotyping consistency, showing identical genotypes in 99% of the cases. Patients and controls had been previously genotyped in HLA-B using PCR-SSOP Luminex method with LABType SSO (One Lambda Inc., Canoga Park, CA), following the manufacturer's instructions (23).

Statistical analysis
The Linux software PLINK v.1.07 (http://pngu.mgh.harvard.edu/purcell/plink/) was used to carry out all the statistical analyses (24). The χ^2 test was used to test Hardy-Weinberg equilibrium (HWE) and to compare genotype distribution in control and patient groups. Univariate analysis of the distribution of the frequencies of genotypes and alleles were performed in 2×2 contingency tables using the χ^2 or Fisher exact tests, when appropriate. The *p*-values<0.05 were considered statistically significant and the odds ratios (ORs) and 95% CIs were calculated according to the Woolf method. The statistical powers to detect associations with ORs ranging from 1.1 to 1.6 were calculated in the allelic model taking into account the frequency of each variant in our population with the Statistical Power test of the Research Tool Kit of the DSS Research Software https://www.dssresearch.com/KnowledgeCenter/toolkitcalculators/statisticalpowercalculators.aspx).

Results
In the cohort included in our study, the successful rate of genotyping was 95%, the study population was found to be in the Hardy-Weinberg equilibrium (*p*>0.05) and distribution of the variants in *JAK1* and *TNFAIP3* in the cohorts from different hospitals was not significantly different. Considering the allelic model, the statistical power values for the 2 SNPs of *JAK1* (rs2780815 and rs310241) and the 3 SNPs of *TNFAIP3* (rs10499194, rs610604 and rs9494885) were greater than 80% to detect an OR=1.3 (Table I).

The distribution of the genotypes and MAFs of the five SNPs in the patient and control groups are displayed in Table II. The MAFs of the five SNPs in our control population were not significant different to those described

in 1000 Genome Project database for the European population. According to these data, no statistically significant differences were found when the patient and control groups were compared. The Table II also displays that the distribution of the MAFs of these SNPs in the groups of patient with ocular involvement and HLA-B*51 positive are not different to those observed in the whole group.

Discussion

The conclusion of the present study is that the association recently described in the Asian populations between the two genes included in this study, *JAK1* and *TNFAIP3*, and BD is not replicated in Europeans. The frequencies of the five SNPs included in our study were not significant different in the Spanish population when patients and controls were compared. The consolidation of the findings of genetic association studies in complex diseases requires replication in different cohorts to avoid different types of bias. It is necessary to discard a false negative result in our study (type II error) in order to conclude that these genes are not associated in the Spanish population, therefore it is important to demonstrate an adequate statistical power. In this sense, it is unlikely that the lack of association detected in our study was due to a false negative result because it had a statistical power greater than 80%, to detect similar ORs to those described in the Chinese Han population at a 5% significance level. Discarded type II errors, discrepancies may be originated by false positive results in the original study (type I errors). In this sense, we note that the findings in the Chinese population were published in two different reports with a design of cases and controls and using a candidate gene approach but only in the case of the *JAK1* gene the study included two cohorts (20), in the case of the *TNFAIP3* gene, the results were not confirmed in a second cohort (21). Even in the case of the *JAK1* the discovery and replication cohorts were from the same population (20). The lack of association is also supported by the genotyping studies performed in BD using high throughput platforms. Although these

Table I. Statistical power at the 5% significance level, taking into account the allelic frequencies in our population for a set of values of ORs from 1.1 to 1.6. The values for all the SNPs included in this study are shown in this table.

	Statistical power for each OR value					
	1.1	1.2	1.3	1.4	1.5	1.6
TNFAIP3						
rs10499194	26	61	87	97	99	100
rs610604	27	62	88	98	99	100
rs9494885	18	40	65	84	93	98
JAK1						
rs2780815	28	65	90	98	99	100
rs310241	25	59	86	97	99	100

Table II. Minor allele frequencies (MAFs) of the five SNPs included in this study in Spanish BD patients and healthy controls. MAFs of these five SNPs in the subgroup of patients with ocular involvement and HLA-B*51 positive are also displayed.

		BD Patients			Controls	
	Allele	Whole group n=372	Ocular involvement n=219	B*51 + n=153*	Whole group n=783*	B*51 + n=48*
JAK1						
rs2780815	G	0.49	0.47	0.46	0.47	0.48
rs310241	G	0.27	0.28	0.27	0.28	0.29
TNFAIP3						
rs10499194	T	0.32	0.34	0.37	0.30	0.35
rs61064	G	0.36	0.34	0.35	0.33	0.31
rs9494885	C	0.11	0.11	0.13	0.12	0.11

*Not all the individuals were genotyped in HLA-B. A total of 356 patients and 357 controls were genotyped. Comparisons made were patients vs. controls; patients with ocular involvement *vs.* patients without this manifestation; patients with ocular involvement *vs.* controls; patients B51 positive *vs.* patients B51 negative; patients B51 positive vs. controls B51 positive. The p-values were greater than 0.05 in all the cases.

studies can not be considered replication studies because data about whether these gene regions were excluded from the final analysis after applying the quality criteria are not available, none of these studies has identified *TNFAIP3* or *JAK1* as risk factors for BD in Europeans (6,7, 25-27). Other factor to take into account is that the genotyping method may influence the ratio of typing errors. Our study was performed using real time PCR whereas in the two original studies, typing were performed using the IPLEX system in the case of *JAK1* (20) and PCR followed by restriction fragment length polymorphism analysis in the case of *TNFAIP3* (21). Although, theoretically, the number of errors attributable to the method would be the same in the patient and control cohorts if the same genotyping method is used; actually, the percentage of error in each group (patients and

controls) may be different if the quality of the DNA is different. Moreover, when the primary association is mainly related with clinical manifestations, the discrepancies could be due to the different features of the cohorts. Regarding this point, in the Chinese cohorts, 100% of the patients have eye manifestations, therefore association with the disease is indistinguishable from association with ocular involvement in these studies. In our cohort 59% of patients have ocular manifestations but our results do not support association with this clinical feature because the distribution of the MAFs of these SNPs in patients with and without ocular manifestations was the same. Some times the association with a specific factor depends on the presence of other genetic factor, if only because this factor defines a more homogeneous group of patients. For this reason, our cohort was stratified ac-

cording to the presence of HLA-B*51 but no differences in the distribution of the MAFs were observed. Finally, the set of genes influencing a specific pathology may have differences among populations. There are multiple examples of this fact, for example, the regions *IL23R-IL12RB2* and *IL-10* have been associated with BD in several studies (6, 7). However, Lee *et al*. reported no association (11).

In conclusion, the association described in the Chinese population between the the *JAK1* and the *TNFAIP3* genes and Behçet's disease was not replicated in Europeans.

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References

1. MENDES D, CORREIA M, BARBEDO M *et al*.: Behçet's disease – a contemporary review. *J Autoimmun* 2009; 32: 178-88.
2. SAKANE T, TAKENO M, SUZUKI N, INABA G: Behçet's disease. *N Engl J Med* 1999; 341: 1284-91.
3. KONÉ-PAUT I, GEISLER I, WECHSLER B *et al*.: Familial aggregation in Behçet's disease: high frequency in siblings and parents of pediatric probands. *J Pediatr* 1999; 135: 89-93.
4. MENTHON DE M, LAVALLEY MP, MALDINI C, GUILLEVIN L, MAHR A: HLA-B51/B5 and the risk of Behçet's disease: a systematic review and meta-analysis of case-control genetic association studies. *Arthritis Rheum* 2009; 61: 1287-96.
5. YAZICI H, FRESKO I, YURDAKUL S: Behçet's syndrome: disease manifestations, management and advances in treatment. *Nat Clin Pract Rheumatol* 2007; 3: 148-55.
6. REMMERS EF, COSAN F, KIRINO Y *et al*.: Genome-wide association study identifies variants in the MHC class I, IL10, and IL23R-IL12RB2 regions associated with Behçet's disease. *Nat Genet* 2010; 42: 698-702.
7. MIZUKI N, MEGURO A, OTA M *et al*.: Genome-wide association studies identify IL23R-IL12RB2 and IL10 as Behçet's disease susceptibility loci. *Nat Genet* 2010; 42: 703-6.
8. HATEMI G, SEYAHI E, FRESKO I, TALARICO R, HAMURYUDAN V: Behçet's syndrome: a critical digest of the 2013-2014 literature. *Clin Exp Rheumatol* 2014; 32 (Suppl. 84): S112-22.
9. KANG EH, CHOI JY, LEE YJ, LEE EY, LEE EB, SONG YW: Single nucleotide polymorphisms in IL-10-mediated signalling pathways in Korean patients with Behçet's disease. *Clin Exp Rheumatol* 2014; 32 (Suppl. 84): S27-32.
10. TURUNE G, COSKUN D, ALIBAZ-ONER F *et al*.: TIR-domain-containing adaptor protein gene TIRAP S180L polymorphism is not increased in Behçet's disease patients in two ethnic cohorts. *Clin Exp Rheumatol* 2013; 31: 54-6.
11. LEE YJ, HORIE Y, WALLACE GR *et al*.: Genome-wide association study identifies GIMAP as a novel susceptibility locus for Behçet's disease. *Ann Rheum Dis* 2013; 72: 1510-6.
12. ORTIZ-FERNÁNDEZ L, CONDE-JALDÓN M, GARCÍA-LOZANO JR *et al*.: GIMAP and Behçet's disease: no association in the European population. *Ann Rheum Dis* 2014; 73: 1433-4.
13. BETTENCOURT A, LEAL B, CARVALHO C *et al*.: CC chemokine receptor polymorphism CCR5Δ32 in Portuguese Behçet's disease patients. *Clin Exp Rheumatol* 2014; 32 (Suppl. 84): S72-4.
14. WU Z, CHEN H, SUN F *et al*.: PTPN22 rs1893217 single-nucleotide polymorphism is associated with risk of Behçet's disease in a Chinese Han population. *Clin Exp Rheumatol* 2014; 32 (Suppl. 84): S20-6.
15. BABON JJ, LUCET IS, MURPHY JM, NICOLA NA, VARGHESE LN: The molecular regulation of Janus Kinase (JAK) activation. *Biochem J* 2014; 462: 1-13.
16. BOONE DL, TURER EE, LEE EG *et al*.: The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. *Nat Immunol* 2004; 5: 1052-60.
17. DIEUDÉ P, GUEDJ M, WIPFF J *et al*.: Association of the *TNFAIP3* rs5029939 variant with systemic sclerosis in the European Caucasian population. *Ann Rheum Dis* 2010; 69: 1958-64.
18. ZHANG X, LI W, ZHANG X *et al*.: Single nucleotide polymorphisms in *TNFAIP3* were associated with the risks of rheumatoid arthritis in northern Chinese Han population. *BMC Medical Genetics* 2014; 15: 56.
19. HU K, HOU S, LI F, XIANG Q, KIJLSTRA A, YANG P: JAK1, but not JAK2 and STAT3, confers susceptibility to Vogt-Koyanagi-Harada (VKH) syndrome in a Han Chinese population. *Invest Ophthalmol Vis Sci* 2013; 54: 3360-5.
20. HOU S, QI J, ZHANG Q *et al*.: Genetic variants in the *JAK1* gene confer higher risk of Behçet's disease with ocular involvement in Han Chinese. *Hum Genet* 2013; 132: 1049-58.
21. LI H, LIU Q, HOU S *et al*.: *TNFAIP3* gene polymorphisms confer risk for Behçet's disease in a Chinese Han population. *Hum Genet* 2013; 132: 293-300.
22. INTERNATIONAL STUDY GROUP FOR BEHÇET'S DISEASE: Criteria for diagnosis of Behçet's disease. *Lancet* 1990; 335:1078-80.
23. MONTES-CANO MA, CONDE-JALDÓN M, GARCÍA-LOZANO JR *et al*.: HLA and non-HLA genes in Behçet's disease: a multicentric study in the Spanish population. *Arthritis Res Ther* 2013; 15: R145.
24. PURCELL S, NEALE B, TODD-BROWN K *et al*.: PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 2007; 81: 559-75.
25. KARASNEH J, GÜL A, OLLIER WE, SILMAN AJ, WORTHINGTON J: Whole-genome screening for susceptibility genes in multicase families with Behçet's disease. *Arthritis Rheum* 2005; 52: 1836-42.
26. FEI Y, WEBB R, COBB BL, DIRESKENELI H, SARUHAN-DIRESKENELI, SAWALHA AH: Identification of novel genetic susceptibility loci for Behçet's disease using a genome-wide association study. *Arthritis Res Ther* 2009; 11: R66.
27. KIRINO Y, BERTSIAS G, ISHIGATSUBO Y *et al*.: Genome-wide association analysis identifies new susceptibility loci for Behçet's disease and epistasis between HLA-B*51 and ERAP1. *Nat Genet* 2013; 45: 202-7.