AIF-1 gene does not confer susceptibility to Behçet’s disease: Analysis of extended haplotypes in Sardinian population

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Abstract

Background

Behçet’s disease (BD) is a polygenic immune-mediated disorder characterized by a close association with the HLA-B*51 allele. The HLA region has a strong linkage disequilibrium (LD) and carries several genetic variants (e.g. MIC-A, TNF-α genes) identified as associated to BD because of their LD with HLA-B*51. In fact, the HLA-B*51 is inherited as part of extended HLA haplotypes which are well preserved in patients with BD. Sardinian population is highly differentiated from other Mediterranean populations because of a distinctive genetic structure with very highly preserved HLA haplotypes.

Patients and methods

In order to identify other genes of susceptibility to BD within the HLA region we investigated the distribution of human Allograft Inflammatory Factor-1 (AIF-1) gene variants among BD patients and healthy controls from Sardinia. Six (rs2736182; rs2259571; rs2269475; rs2857597; rs13195276; rs4711274) AIF-1 single nucleotide polymorphisms (SNPs) and related extended haplotypes have been investigated as well as their LD within the HLA region and with HLA-B*51. Overall, 64 BD patients, 43 HLA-B*51 positive healthy controls (HC) and 70 random HC were enrolled in the study.

Results

HLA-B*51 was the only allele with significantly higher frequency (p_c = 0.0021) in BD patients (40.6%) than in HC (9.8%). The rs2259571T AIF-1 variant had a significantly reduced phenotypic, but not allelic frequency in BD patients (72.1%; p_c = 0.014) compared to healthy population (91.3%). That was likely due to the LD between HLA-B*51 and rs2259571G (p_c =
9E-5), even though the rs2259571 distribution did not significantly differ between BD patients and HC.

**Conclusion**

No significant difference in distribution of AIF-1 SNPs haplotypes was observed between BD patients and HC and between HLA-B*51 positive BD patients and HLA-B*51 positive HC. Taken together, these results suggest that AIF-1 gene is not associated with susceptibility to BD in Sardinia.

**Introduction**

Behçet’s disease (BD) is a chronic vasculitis characterized by recurrent oral ulcers, genital ulcers, ocular and skin manifestations with involvement of arteries and veins of all sizes. BD clusters in an area between latitudes 30˚ N and 45˚ N spanning from the far Eastern Asia to the Mediterranean basin [1,2]. Such a distinctive clustering seems related to geographical distribution of genetic susceptibility factors among general population [3,4]. As described in detail previously [5,6], several lines of evidence suggest that host genetic factors play a pivotal role in determining susceptibility to BD and its close association with the HLA-B*51 allele represents the clearest evidence of a genetic contribution to the disease. However, HLA-B*51 alone is neither necessary nor sufficient to BD development and other susceptibility genes, whose products are responsible for inflammatory and immune-mediated mechanisms, have been identified both outside and within the HLA region [6–8]. Genome wide association studies (GWAS) identified a strong association between BD and the HLA region comprehensive of HLA-B*51 within an extended haplotype [9]. These findings were suggestive of the presence of additional genes within the HLA region conferring susceptibility to BD. Actually, additional genes within the region such as other HLA class I alleles (e.g. B*15, B*57, A*26), the TNF-α and the MHC Class I chain-related gene A (MIC-A) have been associated to an increased risk of BD [10–12].

We previously pointed out that [13], in Sardinia, the BD-associated HLA-B*51:01 allele is inherited as part of a haplotype which is different from that characterizing the B*51:01-positive healthy controls. The HLA haplotype distribution in Sardinians, compared to other Mediterranean populations, is characterised by a small number of preserved and highly frequent haplotypes and by a very high number of rare haplotypes [14,15]. Therefore, the peculiar genetic background of Sardinians represents a valuable source for studying HLA-related genetic and epigenetic associations to BD [16].

Human Allograft Inflammatory Factor-1 (AIF-1) is a 143 amino acid, 17 kDa, cytoplasmic calcium-binding protein, encoded within the HLA class III region on chromosome 6p21 which is densely clustered with genes involved in the inflammatory responses including TNF-α. Because its pro-inflammatory role, AIF-1 is involved in various inflammatory pathological processes such as allograft rejection, autoimmune diseases, inflammatory central nervous system injury. Several single-nucleotide polymorphisms (SNPs) have been identified in the AIF-1 gene as associated with autoimmune diseases [17,18]. Considering its position within the HLA region, between the TNF-α gene promoter and the HLA-B locus, and its pro-inflammatory activity we deemed interesting to study AIF-1 as a candidate gene for BD susceptibility.

The objective of this study was to investigate the association of selected AIF-1 SNPs with susceptibility to BD in Sardinian and their distribution within distinct HLA extended haplotypes harbouring the HLA-B*51 allele.
Materials and methods

Patients and controls

Overall, 64 unrelated consecutive Sardinian patients with BD referring to the Rheumatology Unit of Cagliari and classified according to the 1990 International Study Group criteria, were enrolled in this study between January 2014 and December 2016 (Table 1).

Overall, 43 consecutive HLA-B*51 positive and 70 unselected healthy bone marrow donors served as controls. All patients and controls, matched for gender, came from various areas of Sardinia and were representative of the islander population distribution. Both patients and controls gave their written informed consent to the study which protocol was specifically approved by the local ethics committee "Comitato Etico Indipendente AOU Cagliari" (n. 224/CE).

Genotyping

Peripheral blood from BD patients and HC was drawn in EDTA-containing vials and genomic DNA was extracted using the Nucleic Acid Extraction and Cell Separation Instrument (Manufacturer: DiaSorin Inc.). The amount of DNA was determined using the Qubit fluorometric quantitation that comprises the Qubit 3.0 Fluorometer and sensitive, specific Qubit quantitation DNA assay (Thermo Fisher Scientific). All patients and controls were genotyped for 6 different SNPs of the AIF-1 gene (Table 2) by the reverse sequence-specific oligonucleotide polymerase chain reaction (PCR) technique using TaqMan SNP Genotyping Assays from Life Technologies according to the manufacturer’s protocol.

Patients and controls were also typed for HLA-A, B, C, DRB1, DQA1 and DQB1 using commercial kits (HLA SSP kits; Biotest, Dreieich, Germany) in order to identify a different distribution of the AIF-1 SNPs in the extended HLA haplotypes.

It is well known that choosing preliminary candidate SNPs is critical for candidate gene association studies. The chosen SNPs were based on previously described associations in various immune-mediated diseases [17,18], as well as according to functional features deemed interesting by the authors.

Statistical analysis

Hardy–Weinberg equilibrium (HWE) was tested using the Chi-square test. To assess differences in the proportions of AIF-1 polymorphic alleles and disease associations in healthy

Table 1. Cumulative features of patients suffering from Behcet’s disease enrolled in this study.

| Features              | N (%) |
|-----------------------|-------|
| Gender                | F/M = 2/1 |
| Age at diagnosis (mean ± SD) | 31.0 ± 9.7 |
| Oral ulcers           | 64 (100) |
| Genital ulcers        | 41 (64) |
| Cutaneous involvement | 36 (56.2) |
| Ocular involvement    | 33 (51.4) |
| Neurological involvement | 6 (9.4) |
| Vascular involvement  | 14 (21.8) |
| Musculoskeletal involvement | 21 (32.8) |
| Pathergy test positive| 7 (10.9) |

Unless otherwise specified, numbers are absolute values, number in brackets are percentage.

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controls (HC) versus BD patients, chi-square test or two-tailed Fisher’s exact test, for low frequency, were performed using MedCalc software (version 16.8.4, Mariakerke, Belgium). The strength of association was estimated by calculating the odds ratio (OR) with 95% Confidence Interval (95% CI). Under the assumption of independence, a value of $p < 0.05$ was considered statistically significant where Bonferroni correction was applied for multiple comparisons to all novel associations, with a correction factor derived from the number of alleles examined; $p_c$ indicates where the Bonferroni correction was applied. The LD among the 6 SNPs of the $AIF-1$ gene and between single SNPs and HLA-B*51 was calculated using the HaploView 4.2 software.

**Results**

HLA-B*51 phenotype frequency was significantly higher ($p_c = 0.0021$; OR = 6.2; 95%CI 2.5 to 15.8) in BD patients (40.6%) than in HC (9.8%). No other HLA class I and II alleles were independently associated with BD.

Six SNPs in $AIF-1$ were determined in 64 BD patients, 43 HLA-B*51 positive HC and 70 HC (Table 3). Five out of 6 $AIF-1$ SNPs (rs2736182; rs2269475; rs2857597; rs13195276; rs4711274) did not show different allelic and phenotypic distribution between patients and HC. Only the rs2259571 SNP had a significantly decreased phenotypic, but not allelic, frequency of the rs2259571T variant in BD patients (72.1%, Chi squared 9.31, $p_c = 0.014$) compared to healthy population (91.3%) without a significantly different phenotypic distribution of the rs2259571G variant between BD patients and HC despite its LD with the HLA-B*51 ($p_c = 9E-5$). Noteworthy, the rs2259571T phenotypic frequency distribution did not significantly differ between HLA-B*51 positive BD patients (56.5%) and HLA-B*51 positive HC (62.8%).

Analysing the distribution of $AIF-1$ SNPs haplotypes, no significantly different haplotype distribution between BD patients and HC was detected (Fig 1). The GGTCA (haplotype frequency 42.0% in BD and 48.0% in HC) and GGGCA (haplotype frequency 47.6% in BD and 35.5% in HC) were the most frequently detected $AIF-1$ SNP haplotypes. As effect of the LD between rs2259571G and HLA-B*51 the GGGCA was found at a higher frequency in HLA-B*51 positive subjects (56.3%) and the GGTCA was most frequently carried by HLA-B*51 negative subjects (52.9%) irrespective of the disease status.

Finally, HLA-B*51 was harboured in two HLA-B-HLA-DR distinct haplotypes: B*51-DR*11 in 14/26 (53.8%) of HLA-B*51 positive BD patients and in 14/37 (37.8%) of HLA-B*51 positive HC ($p = 0.231$); and B*51-DR*4 in 1/26 (3.8%) of HLA-B*51 positive BD patients and in 14/37 (37.8%) of HLA-B*51 positive HC ($p = 0.013$; $p_c = 0.065$ OR 0.06 95%CI 0.01–0.54) according to, but not fully confirming, the previous observation of a lack of association.

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**Table 2. Features of the six SNPs of the $AIF-1$ gene typed.**

| SNP ID | Location | Polymorphism | Molecular Consequences |
|--------|----------|--------------|------------------------|
| rs2736182 | Chr.6: 31615535 | A/G, Transition Substitution | 2KB upstream variant, missense variant, 5' UTR variant |
| rs2259571 | Chr.6: 31616050 | G/T; Transversion substitution | Intron variant 5' UTR variant |
| rs2269475 | Chr.6: 31616134 | C/T, Transition Substitution | Intron variant, missense variant |
| rs2857597 | Chr.6: 31617223 | A/T, Transversion Substitution | 500B downstream variant |
| rs13195276 | Chr.6: 31616317 | C/T, Transition Substitution | intron variant, missense variant |
| rs4711274 | Chr.6: 31615389 | A/G, Transition Substitution | 2KB upstream variant, intron variant |

Source: Database of Short Genetic Variation (dbSNP)–NCBI- NIH.

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between B*51-DR*4 and BD susceptibility in Sardinians (13). No significant difference in the distribution of \textit{AIF-1} single or combined SNPs was observed between the B*51-DR*11 and B*51-DR*4 extended haplotypes (Fig 2).

### Discussion

The present study firstly reports on the association of six \textit{AIF-1} SNPs with BD susceptibility. The results showed no significant association between each investigated SNP or SNP haplotypes and BD in Sardinia. Despite of the LD between rs2259571\textsuperscript{G} and HLA-B*51, the frequency of each SNP and the related GGGCA haplotype was not significantly increased in BD patients. As an effect of the LD with HLA-B*51, the rs2259571\textsuperscript{G} was harboured in both B*51-DR*11 and B*51-DR*4 haplotypes, the latter having different distribution in BD patients and HC from Sardinia. These results suggest that polymorphisms of \textit{AIF-1} are not associated with the susceptibility to BD in the Sardinian population.
BD is considered as a complex polygenic disorder with a mixed autoinflammatory and autoimmune pathogenesis [19]. GWAS identified several susceptibility loci associated with BD susceptibility [9,20–23], but always confirmed the major role of the HLA region and especially of the HLA-B*51 allele in BD susceptibility [9,20–23]. Nevertheless, the highest contribution of HLA-B*51 to the overall BD genetic susceptibility was estimated to be only 19% [24]. GWAS also confirmed a strong LD in the HLA region of BD patients, mainly due to the fact that HLA-B*51 was found almost exclusively on a single extended haplotype [9]. In Sardinia, two distinct extended haplotypes harbouring HLA-B*51:01 were identified: A2-Cw2-B*5101-DRB1*11-DQA1*05-DQB1*03, which marks the B*51 positive patients with BD in Sardinia, and A2-Cw2-B*5101-DRB1*04-DQA1*03-DQB1*03, which is significantly more frequent in Sardinian HC than in BD [13]. Considering the high LD in the HLA region, it is conceivable that genes besides HLA-B*51, somewhat involved in the innate and adaptive immune responses and inherited as part of distinct HLA-B*51:01 haplotypes, may play a role in BD susceptibility.
Because of its position within the HLA class III region, between the HLA-B and HLA-DR loci, and its pro-inflammatory effect, the AIF-1 gene was deemed as a possible determinant of genetic susceptibility to BD. In humans, in fact, AIF-1 influences the immune system at several key points and boosts the expression of inflammatory mediators such as cytokines (IL6, TNF-α), chemokines, inducible nitric oxide synthase and promotes inflammatory cell proliferation and migration [25]. Moreover, AIF-1 is involved in some model of autoimmune diseases such as experimental autoimmune uveitis, encephalomyelitis and neuritis [26,27]. The role of AIF-1 in rheumatoid arthritis and systemic sclerosis has been investigated and the rs2269475 SNP was found associated with an increased risk of developing both diseases [28,29]. Although preliminary data pointed to a possible role of AIF-1 in BD susceptibility, we did not find any suggestion for this in our study population.

To the best of our knowledge, this was the first study investigating the role of AIF-1 in BD. With the aim to elucidate the genetic basis of BD we set a candidate gene case-control association study and we tested six different AIF-1 SNPs. Major strengths are represented by the
peculiar genetic background of Sardinians and by the enrolment of two different control populations allowing to identify a different distribution of AIF-1 in patients and in controls but also in HLA-B*51 carriers versus other subjects. A major limitation is related to sample size, therefore caution is advised when interpreting results as they may be related to the small size of the population under study.

In conclusion, the present study does not support the hypothesis that a genetically determined regulation of AIF-1 expression or change in protein structure may predispose to the development of BD in Sardinian patients. Further, larger studies are required to confirm our findings in other populations.

Supporting information

S1 Table. Minimal anonymized dataset. Genotyping results for AIF1 are reported here. (XLSX)

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