
Association of small ubiquitin-like modifier 4 (SUMO4) polymorphisms in a Tunisian population with Behçet's disease

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ABSTRACT

Objectives. An association of SUMO4 gene, which has recently been shown to be a negative feedback regulator for nuclear factor NF- κ B, has been reported in several autoimmune/inflammatory diseases. A case-control study was set up to investigate the contribution of SUMO4 locus to the genetic susceptibility to Behçet's disease (BD).

Methods. One hundred and thirty-five Tunisian BD patients and 167 healthy blood donors from the same geographical area were genotyped by polymerase chain reaction for the SUMO4 polymorphisms.

Results. The SUMO4+438 C allele frequency is significantly increased in BD patients ($p=0.03$; $\chi^2=4.71$; OR=1.44; 95% CI=1.02–2.04) and highly significantly increased in HLA-B51 positive BD patients ($p=3 \times 10^{-6}$; $\chi^2=21.62$; OR=4.44; 95% CI=2.21–8.98). Similarly, the SUMO4 -847 G allele frequency is significantly increased in BD patients ($p=0.03$; $\chi^2=4.34$; OR=1.41; 95% CI=1.01–1.97). The studied polymorphisms were also associated with disease severity, skin lesions and vascular involvement.

Conclusion. SUMO4+438 C and -847 G alleles seem to be associated with susceptibility to BD in Tunisian population. We suggest that SUMO4 gene polymorphisms may be involved in the development of skin lesions, vascular BD, as well as the severity of the disease.

Introduction

Behçet's disease (BD) is a chronic relapsing systemic inflammatory disease characterised by the presence of orogenital ulcers, cutaneous manifestations, and uveitis. The disease can also lead to vascular complications such as arterial and venous thrombosis, central nervous system vasculitis, arthritis, and gastrointestinal involvement.

(1-5). Since the aetiology of Behçet's disease is not fully understood, treatment remains insufficient and relies on non-specific immunosuppressive medications, with significant side effects. Evidence for a genetic contribution to the pathogenesis of the disease comes from strong familial aggregation, the strong predominance in patients with Mediterranean or Asian ancestry, and the confirmed association with HLA-B51 in several ethnic groups (6).

It is estimated that the association with HLA-B51 in Behçet's disease accounts for only ~20% of the relative risk in siblings of affected individuals (7). This suggests that other genetic elements outside the HLA region carry risk for developing Behçet's disease. Indeed, a genetic linkage study in a cohort of Behçet's disease multiplex families identified evidence for linkage ($p \leq 0.05$) on 16 chromosomal regions (8). Genetic association studies performed via genotyping single-nucleotide polymorphisms (SNPs) in candidate genes have been performed. These revealed genetic associations with several genes, including IRF1 (interferon regulatory factor 1) (9), TNF (tumour necrosis factor) (10), and PTPN22 (protein tyrosine phosphatase, non-receptor type 22) (11).

The precise aetiology and pathogenesis of BD remain unclear; a widely accepted hypothesis is that the autoimmune response and genetic factors are both involved in this disease. Small ubiquitin-like modifier 4 (SUMO4), located on 6q25, has been found to be involved in autoimmune and inflammatory responses through regulation of NF- κ B and the activation of heat shock transcriptional factor (12-13). Research on the SUMO system over the past decade has demonstrated that sumoylation is a remarkably versatile regulatory mechanism of protein function involved in the regulation of diverse life processes such as immune response and cell apoptosis

Competing interests: none declared.

Whether there is an association of SUMO4 with BD is not yet clear. We therefore investigated its association with BD in a well defined group of Tunisian patients. The four SNPs of SUMO4 gene studied in this report are located in the promoter region at -847 and -504, in the coding region at +163, and in the 3'-untranslated region at +438, respectively.

Our study identified two susceptible alleles with BD in Tunisian patients. SUMO4 polymorphisms could also be involved in the pathogenesis of BD and this hypothesis was therefore the subject of the study presented here.

Materials and methods

Study populations

A case-control study was conducted to investigate SUMO4 polymorphisms in 135 native Tunisian BD patients (93 male and 42 female) and 167 healthy age- and sex-matched subjects who were unrelated volunteer blood donors. This study group was recruited from La Rabta Hospital (Department of Internal Medicine). All patients gave informed consent according to a protocol approved by the local ethics committee. Then, they were classified according to the international diagnostic criteria (14). The genotype frequencies of BD patients and healthy control group are conformed to Hardy-Weinberg equilibrium.

Genotyping

Genomic DNA was extracted from EDTA anti-coagulated whole blood using conventional salting-out procedure as we have recently reported (15-16). SUMO4 gene was genotyped using polymerase chain reaction (PCR) followed by restriction analysis. The PCR primers for the SUMO4 -847 G/A, -504 A/G, +163 A/G and +438 C/T polymorphisms were (Forward, 5'-TCC CAA CCA ATA ATA GCA AGT CT-3'; Reverse, 5'-ATG CCT GGA TCA AAA CAC ACA-3'), (Forward, 5'-TGT GTG TTT TGA TCC AGG CAT TA-3'; Reverse, 5'-TGT TTT GCT CCT CTT TTC CTC TT-3'), (Forward, 5'-ATT GTG AAC CAC GGG GAT TGT TA-3'; Reverse, 5'-CAG CGT TCT GGA GTA AAG AAG-3') and (Forward, 5'-ATA CCA GTT ACT TCA TGT ATA

ATA GA-3'; Reverse, 5'-AGA TTA CTG CAT TCT CAA TTA G -3'), respectively. The PCR was carried out in 25 µl reaction volume containing 100 - 200 ng template DNA, 1x Taq polymerase buffer, 200 µM each of dATP, dCTP, dGTP, dTTP (Fermentas, Lithuania), 2mM MgCl₂, 0.5µM of each primer, and 1Unit Taq DNA Polymerase (Fermentas, Lithuania).

Genotypes were determined by restriction fragment length polymorphism (RFLP). Amplified DNA was digested with 1-3U of endonucleases for 4h to overnight at optimal temperatures which were indicated by the manufacturers (Fermentas, Lithuania). PCR products of -847 G/A, -504A/G, +163A/G, and +438C/T loci were incubated with SspI at 37°C, Alw21I at 37°C, MseI at 65°C, and MnlI at 37°C. The PCR products were separated by electrophoresis through a 3% agarose gel containing ethidium bromide.

HLA typing

Serologic HLA class I typing was performed using peripheral blood lymphocytes and the standard microlymphocytotoxicity technique.

Determination of disease severity

All patients were divided into three severity groups according to the BD total activity index described by Gül *et al.* (17):

Mild: patients with only mucocutaneous findings or acute attacks of arthritis.

Moderate: patients with one of the following symptoms; (i) uveitis and ≥ 0.5 best corrected residual visual acuity on the Snellen scale, (ii) deep vein thrombosis of the lower extremities, or (iii) chronic articular disease.

Severe: patients with one of the following symptoms; (i) one or more attacks of uveitis in a year, resulting in < 0.5 residual visual acuity on the Snellen scale or total loss of vision, (ii) neurological involvement, including sinus thrombosis, (iii) thrombosis of the superior and/or inferior *vena cavae*, including the hepatic veins, (iv) arterial aneurysms and occlusions, or (v) secondary amyloidosis.

Statistical analysis

Association analyses in this study were performed using standard chi-squared test (Epistat statistical package, Epi Info version 6) to detect differences in distribution among groups. If any cell number in the 2x2 contingency table has < 5 subjects, Fisher's exact test is used. The strength of a gene association is indicated by the odds ratio (OR). The odds ratio and the 95% confidence intervals (CI) were calculated whenever applicable. Probability values of 0.05 or less were regarded as statistically significant.

Results

In the present report, we compared the distribution of four SUMO4 polymorphisms between Tunisian patient's group and healthy controls in order to investigate a potential association of these polymorphisms with BD susceptibility, BD severity, the presence of HLA-B51 and clinical features such as skin lesions as well as vascular, ocular and neurologic involvements. The clinical characteristics of the patients and the demographic features of the controls are summarised in Table I.

The distribution of genotype and allele frequencies of SUMO4 gene was compared between Tunisian BD patients and healthy controls (Table II). No significant associations were found when comparing the distribution of allelic and genotypic frequencies of SUMO4 -504 A/G and +163 A/G polymorphisms. However, the distribution of SUMO4 +438 C/C genotype ($p=0.01$; $\chi^2=8.82$), +438 C allele ($p=0.03$; $\chi^2=4.71$; OR=1.44; 95% CI=1.02-2.04) and -847 G allele ($p=0.03$; $\chi^2=4.34$; OR=1.41; 95% CI=1.01-1.97) frequencies were significantly higher in BD patients when compared with healthy controls.

When data were analysed according to the presence or the absence of HLA-B51 in BD patients, an interesting highly significant difference was found in the presence of HLA-B51 regarding the distribution of SUMO4 +438 C allele. Indeed, this allele frequency was highly significantly increased in the HLA-B51 positive BD patients ($p=3 \times 10^{-6}$; $\chi^2=21.62$; OR=4.44; 95% CI=2.21-8.98) (Table III).

Table I. Clinical characteristics of patients with Behçet's disease and demographic features of healthy controls.

| Characteristics | Number of patients (%) | Number of controls (%) |
|--|------------------------|------------------------|
| Age (mean ± SD, years) | 39.5 ± 13 | 40.2 ± 11 |
| Sex (Male) | 93 (68.8) | 117 (70) |
| Duration of the disease (mean ± SD, years) | 8.69 ± 4 | – |
| Familial antecedent | 14 (10.3) | – |
| HLA-B51 | 31 (31) | – |
| Frequencies of clinical manifestations | | |
| Oral ulceration | 135 (100) | – |
| Genital ulceration | 109 (80.7) | – |
| Skin lesions | | |
| Erythema nodosum | 16 (11.8) | – |
| Folliculitis/acne | 65 (48.1) | – |
| Skin pathergy response | 29 (21.5) | – |
| Ocular inflammation | | |
| Uveitis | 62 (45.9) | – |
| Retinal Vasculitis | 21 (15.5) | – |
| Vascular involvement | | |
| Vascular involvement | 28 (20.7) | – |
| Phlebitis | 19 (14.0) | – |
| Neurologic involvement | | |
| Arthritis | 26 (19.2) | – |

The BD patient's group was divided according to each symptom of the disease. The distribution of the selected SUMO4 polymorphisms was explored in each sub-group. The data was analysed according to BD severity and to each one of the following symptoms; skin lesions (erythema nodosum, folliculitis/acne, and skin pathergy response), ocular inflammation (uveitis, retinal vasculitis), vascular involvement and neurological involvement. Only positive associations have been

reported in Table IV. According to the severity status of BD, a unique statistically significant difference was found for the SUMO4 +438C/T polymorphism. In fact, C/C genotype frequency increases in patients with severe BD when compared with those with mild/moderate BD ($p=0.01$; $\chi^2=6.19$). The C allele frequency of the same polymorphism was also significantly higher in patients with severe BD ($p=0.01$; $\chi^2=5.82$; OR=1.88; 95% CI=1.09–3.26).

When skin lesions were taken into account, two significant associations were found (Table IV); SUMO4 +438 C/C genotype ($p=0.06$; $\chi^2=3.49$) and C allele frequencies ($p=0.02$; $\chi^2=5.03$; OR=1.82; 95% CI=1.04–3.19) were significantly increased in patients with skin lesions. However SUMO4 -847G/G genotype ($p=0.02$; $\chi^2=5.19$) and G allele frequencies ($p=0.02$; $\chi^2=5.25$; OR=0.56; 95% CI=0.33–0.95) were significantly more prevalent in patients without skin lesions.

Vascular involvement was positively associated with SUMO4 -504 A/G polymorphism (Table IV). Indeed, SUMO4 -504 A/A genotype ($p=0.008$; $\chi^2=6.85$) and A allele frequencies ($p=0.01$; $\chi^2=6.13$; OR=3; 95%CI=1.15–8.27) were significantly higher in patients with vascular involvement.

Discussion

Behçet's disease is characterised by a peculiar geographical distribution, association with HLA-B51 allele (18-20) and a familial aggregation (21, 22). These features have been regarded as evidence supporting a genetic influence on the pathogenesis of BD (23, 24). Nevertheless, the really disease-susceptible gene has not yet been determined. SUMO4 gene, located on 6q25, seems to be implicated as a functional candidate gene in the pathogenesis of BD. A number of polymorphisms in the SUMO4 gene have been identified.

Table II. Comparison of SUMO4 polymorphisms between Tunisian BD patients and healthy controls.

| Allele | Genotype frequency (%) | | | χ^2 (<i>p</i> -value) | Allele frequency (%) | | χ^2 (<i>p</i> -value) |
|----------------|------------------------|-----------|-----------|--------------------------------|----------------------|------------|--------------------------------|
| | A/A | A/G | G/G | | A | G | |
| +163A/G | | | | | | | |
| Patients n=135 | 41 (30.3) | 69 (51.1) | 25 (18.5) | (NS) | 151 (55.9) | 119 (44.1) | (NS) |
| Controls n=167 | 33 (19.8) | 97 (58.1) | 37 (22.1) | | 163 (48.8) | 171 (51.2) | |
| +438C/T | | | | | | | |
| Patients n=135 | 63 (46.5) | 51 (38) | 21 (15.5) | 8.82 | 177 (65.5) | 93 (34.5) | 4.71 |
| Controls n=167 | 51 (30.5) | 88 (52.7) | 28 (16.8) | (0.01) | 190 (56.9) | 144 (43.1) | (0.03) |
| -847G/A | | | | | | | |
| Patients n=135 | 37 (27.4) | 58 (43) | 40 (29.6) | (NS) | 132 (48.9) | 138 (51.1) | 4.34 |
| Controls n=167 | 29 (17.4) | 77 (46.1) | 61 (36.5) | | 135 (40.4) | 199 (59.6) | (0.03) |
| -504A/G | | | | | | | |
| Patients n=135 | 94 (69.6) | 35 (25.9) | 6 (4.5) | (NS) | 223 (82.6) | 47 (17.4) | (NS) |
| Controls n=167 | 127 (76) | 35 (21) | 5 (3) | | 289 (86.5) | 45 (13.5) | |

Table III. Distribution of SUMO4 polymorphisms for BD patients according to the presence or the absence of HLA-B51.

| Allele | Genotype frequency (%) | | χ^2 | Allele frequency (%) | | χ^2 |
|-----------------|------------------------|-----------|-----------------------|----------------------|-----------|-----------------------|
| +163A/G | A/A | A/G+G/G | (p-value) | A | G | (p-value) |
| HLA-B51(+) n=31 | 11 (35.5) | 20 (64.5) | (NS) | 36 (58.1) | 26 (41.9) | (NS) |
| HLA-B51(-) n=69 | 22 (31.9) | 47 (68.1) | | 76 (55.1) | 62 (44.9) | |
| +438C/T | C/C | C/T+T/T | | C | T | |
| HLA-B51(+) n=31 | 18 (58.1) | 13 (41.9) | 15.39 | 44 (71) | 18 (29) | 21.62 |
| HLA-B51(-) n=69 | 13 (18.8) | 56 (81.2) | (8 10 ⁻⁵) | 49 (35.5) | 89 (64.5) | (3 10 ⁻⁶) |
| -847G/A | G/G | G/A+A/A | | G | A | |
| HLA-B51(+) n=31 | 3 (9.7) | 28 (90.3) | (NS) | 26 (41.9) | 36 (58.1) | (NS) |
| HLA-B51(-) n=69 | 20 (29) | 49 (71) | | 71 (51.4) | 67 (48.6) | |
| -504A/G | A/A | A/G+G/G | | A | G | |
| HLA-B51(+) n=31 | 21 (67.7) | 10 (32.3) | (NS) | 49 (79) | 13 (21) | (NS) |
| HLA-B51(-) n=69 | 51 (73.9) | 18 (26.1) | | 118 (85.5) | 20 (14.5) | |

Table IV. Distribution of SUMO4 polymorphisms for BD patients according to the presence or the absence of severity and clinical manifestations.

| Allele | Genotype frequency (%) | | χ^2 | Allele frequency (%) | | χ^2 |
|-----------------------|------------------------|-----------|-----------|----------------------|-----------|-----------|
| +438C/T | C/C | C/T+T/T | (p-value) | C | T | (p-value) |
| Severe BD n=65 | 39 (60) | 26 (40) | 6.19 | 96 (73.8) | 34 (26.2) | 5.82 |
| Mild/moderate BD n=70 | 27 (38.6) | 43 (61.4) | (0.01) | 84 (60) | 56 (40) | (0.01) |
| +438C/T | C/C | C/T+T/T | | C | T | |
| Skin lesions (+) n=83 | 44 (53) | 39 (47) | 3.49 | 108 (68.4) | 50 (31.6) | 5.03 |
| Skin lesions (-) n=52 | 19 (36.5) | 33 (63.5) | (0.06) | 51 (54.3) | 43 (45.7) | (0.02) |
| -847G/A | G/G | G/A+A/A | | G | A | |
| Skin lesions (+) n=83 | 17 (20.5) | 66 (79.5) | 5.19 | 72 (43.4) | 94 (56.6) | 5.25 |
| Skin lesions (-) n=52 | 20 (38.5) | 32 (61.5) | (0.02) | 60 (57.7) | 44 (42.3) | (0.02) |
| -504A/G | A/A | A/G+G/G | | A | G | |
| Vascular Inv(+) n=37 | 32 (86.5) | 5 (13.5) | 6.85 | 68 (91.9) | 6 (8.1) | 6.13 |
| Vascular Inv (-) n=98 | 62 (63.3) | 36 (36.7) | (0.008) | 155 (79.1) | 41 (20.9) | (0.01) |

However, the role of SUMO4 gene polymorphisms in the pathogenesis of BD has not been researched enough. This case-control study was realised to investigate the potential association between SUMO4 polymorphisms and BD susceptibility, BD severity as well as the presence or the absence of HLA-B51. Demographic and clinical characteristics of BD patients were also analysed in association with SUMO4 polymorphisms.

Our study identified two susceptible SUMO4 +438C and -847G alleles with BD. SUMO4 +438C allele is highly associated with the presence of HLA-B51. The C allele frequency of the same polymorphism was also higher in patients with severe BD and in those with skin lesions whereas SUMO4 -847G allele frequency was more prevalent in pa-

tients without skin lesions. Another association was found between SUMO4 -504A allele and vascular involvement of BD.

Recently, the association of SUMO4 +438C allele with BD has been found in Chinese population (25). This association was only found in the negative HLA-B51 BD patient's group. They have also identified two possibly protective haplotypes with BD. Our results were in accordance with those reported in China suggesting that SUMO4 +438C allele is positively associated with BD. We propose that this allele is associated with BD susceptibility, the presence of HLA-B51, BD severity and with skin lesions. Nevertheless, the association of SUMO4 polymorphisms with BD is not yet fully understood.

The association of SUMO4 +163G

allele with other autoimmune diseases like type 1 diabetes has been found in Asian populations whereas discrepancies' results were observed in Caucasians (26-28). Here, we investigated the potential association of this polymorphism with BD. We found no significant association between the distribution of SUMO4 +163G/A polymorphism and susceptibility with BD. Our result is consistent with that reported previously with BD, Graves' disease, rheumatoid arthritis and systemic lupus erythematosus (SLE) (25, 29-31).

We have also identified for the first time a novel susceptible allele for BD: SUMO4 -847G allele. This allele was associated with susceptibility to BD and with skin lesions. Concerning the SUMO4 -504A/G polymorphism, we reported that this polymorphism was

not associated with BD susceptibility but could be implicated in the vascular involvement of the disease.

SUMO4 has been shown to be involved in the regulation of NF- κ B, an important transcription factor in autoimmune diseases. It has been reported that the SUMO4+163A/G polymorphism is an essential polymorphism involved in regulating its own sumoylation. This polymorphism as well as SUMO4+438C/G have been shown to be associated with several autoimmune diseases. These results suggest that SUMO4 gene could be a susceptibility gene shared by certain autoimmune diseases. Therefore, SUMO4 gene may be a general autoimmunity gene (13).

It would be very interesting to investigate the implication of SUMO4 polymorphisms in BD in other ethnic groups at large samples. This should contribute to better understanding the role of these polymorphisms in BD. The lacks of data regarding SUMO4 polymorphisms indicate the need for larger studies to clarify the role of these polymorphisms in infectious and/or autoimmune diseases.

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