DNA Sequencing Validation by PCR-RFLP for Evaluating Butyrophilin-Like 2 RS2076530 Polymorphism in Iranian Patients with Sarcoidosis

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Abstract. Background: Sarcoidosis is a multifactorial immune disorder with an uncertain origin. A single nucleotide polymorphism (G→A, rs2076530) in the butyrophilin-like 2 (BTNL2) gene results in the formation of truncating protein. This study aimed to genotype the predisposition of the BTNL2 rs2076530 polymorphism in Iranian patients with sarcoidosis using the RFLP technique. Materials and Methods: In this study, 80 patients with sarcoidosis and 80 healthy individuals were included. The rs2076530 polymorphism of the BTNL2 gene was genotyped using the PCR-RFLP method by AvrII restriction enzyme and confirmed by DNA sequencing (Capillary electrophoresis 3130, ABI). Results: There was a statistically significant difference between proportions of patients with AA (47.5%) and controls (27.5%) (OR=2.38, 95%CI:1.23-4.61, P=0.009). In addition, a significant difference was observed in the frequency of the A allele (62.5%) in sarcoidosis (OR=2.14, 95%CI:1.37-3.35, P=0.001). A Bonferroni correction with P<0.0038 indicates a statistical difference for genotype AA (P=0.009). In an effective model, binary logistic regression analysis indicates a statistical association between AA genotype and sarcoidosis (P=0.018 with 60% prediction). Based on the gene analysis study using DNA sequencing, all of the mentioned mutations were seen via RFLP. Conclusion: According to our findings, the BTNL2 rs2076530 A allele in the Iranian population is associated with susceptibility to sarcoidosis. This designed PCR-RFLP method for detecting SNPs is effective as DNA sequencing.

Keywords: BTNL2, Sarcoidosis, RFLP, Polymorphism, Single Nucleotide

Introduction

Sarcoidosis is an inflammatory disease characterized pathologically and immunologically by an increase in macrophages and CD4 T cells in the granulomas, affecting multiple organs such as the lungs, lymph nodes, eyes, skin, and heart (1, 2). The etiology of sarcoidosis remains uncertain(3), but different genetic predispositions and environmental factors may trigger the disease. The ACCESS study (A case-controlled ethological study in sarcoidosis) has identified some high-risk exposures to sarcoidosis, such as farming, bird-raising jobs, and woodworking. (4, 5). Also, Mycobacterium tuberculosis (MTB) may trigger sarcoidosis (6-8). Sarcoidosis has been associated with human leukocyte antigen (HLA) class
II genes including HLA-DRB1 and HLA-DQB1 genes in various racial groups (9-13). Currently, data derived from the gene map of the human leukocyte antigen (HLA) regions have revealed that BTNL2 gene closely located from the HLA-DRB1 gene on chromosome 6p21, plays a vital role in the pathogenesis of sarcoidosis (14, 15). BTNL2, a butyrophilin gene, is a member of the immunoglobulin gene superfamily and is likely associated with costimulatory activation of T-cell based B7 and B7.2 (CD80 and CD86) receptors (15). For the first time, Valentonyte et al. in 2005 reported a novel association with rs2076530, a single nucleotide polymorphism (SNP) on exon 5 of the BTNL2 gene (MIM 606000), with a non-HLA-DRB1 function in a white German cohort (16). The existence of rs2076530 G to A transition will create an alternative splice site leading to form a truncating protein in patients with sarcoidosis (17).

Optimal T-cell activation requires antigen engagement of the T-cell receptor with additional costimulatory interactions. CD28, expressed in T cells, binds to either the B7.1 or B7.2 counterreceptors on antigen-presenting cells (18, 19). Dysfunctional BTNL2 could interfere with reasonable T-cell regulation (20). Rybicki et al. investigated the polymorphism of BTNL2 as well as nine additional variants in an African-American family cohort and two other case-control studies, one in African-American families and one in white families in 2004 (12). They confirmed the association between the rs2076530 A allele and increased risk for sarcoidosis in only white subjects. Valentonyte also found that the rs2076530 A allele was associated with the disease and subsequently showed that this was associated with a functional change (17).

As far as the genetic relationship between BTNL2 genes and sarcoidosis is concerned, this study attempts to study the genetic changes in BTNL2 in the Iranian population by using the newly designed RFLP technique, whose results have been confirmed through DNA direct sequencing. Single nucleotide polymorphism and sarcoidosis could be better understood by comparing other ethnic groups.

**Materials and Methods**

**Patients**

The present study included 80 Iranian patients with sarcoidosis, who registered or were referred to Massih Daneshvari Hospital, a third-level pulmonary hospital in Tehran, Iran. These patients were recruited from January 2016 to January 2017. Sarcoidosis was diagnosed by clinical and radiological findings, along with histological and serological evidence compatible with the ATS definition of sarcoidosis (21), excluding other causes of granulomatous diseases such as tuberculosis that is fully described in our previous publication (22). A healthy control group consisted of 80 non-dependent age and sex-matched volunteers. The control subjects had no history of chronic or inflammatory disease in the last six months. This study was done according to the Ethics Committee protocol of Masih Daneshvari hospital in Tehran, Iran (IR.SBMU.NRITLD.REC.1394.151).

**DNA extraction, BTNL2 RFLP, and BTNL2 gene sequencing**

Sequence-specific primers–polymerase chain reaction amplified a single nucleotide polymorphism (SNP), rs2076530 G to A on exon 5 of the BTNL2 gene. Genomic DNA was extracted from 3 ml of peripheral blood leukocytes by QIAamp DNA Blood Mini kit (Qiagen) according to the manufacturing structure. For BTNL2 RFLP, each PCR experiment was performed in 200 µl micro-tube containing 25 µl PCR reactions including 20 pmol of each forward and reverse primer: F: 5’GCCAGTTTGGATCTGAAGGTCCTA3’ and R: 5’ACAGTTTCACACACTGGAGGATTTG 3’, 10 µl MasterMix Red, 10 µl genomic DNA, and 3 µl distilled water and then cycled to thermocycler condition as the following: denaturation for 5 min at 94°C and 45 cycles with 30 seconds at 95°C as the second denaturation, and 67°C for 45 seconds, 72°C for 30 seconds followed by the final extension step at 72°C for 5 min (ABI thermal-cycler 5020). Only 10 µl of the amplified PCR products were loaded on a 2% agarose gel. Once a Gel Doc Viber Transilluminator detected the DNA band corresponding to 200bp, the PCR
product was then digested using the AvrII restriction enzyme (Thermo scientific Lot: 00336905). The bands corresponding to 174 bp AA, 174/153/21 bp AG, and 153/21 bp GG were reported (Figure 1).

Direct DNA sequencing for those patients and controls was performed to confirm the RFLP method, the same as earlier the amplification of all PCR reactions were achieved in 200 µl micro-tubes

Figure 1. (A) It shows the BTNL2 gene reference sequence (NG_054759.1) and rs2076530 position. (B) PCR mutagenesis for AvrII restriction enzyme site design using the change of GG nucleotides in 17837-17838 position to CC, and (C) following PCR mutagenesis, AvrII digests G in rs2076530 position of controls and not digests A in all individual patients.
and cycled in the program temp control system ABI thermal-cycler 5020. The micro-tubes contained a 25 µl PCR reaction consisting of 10 µl of isolated DNA, 10 10 µl MasterMix Red, and 20 pmol of each forward and reverse primer: BTS-1: 5’-ACAGTTCACACACTGGAGGATTTG-3’ and BTS-2: 5’-CTAACCTGTACCTGCTC-CCC-3’. Thermo cycler condition was as follows: denaturation for 5 min at 94°C and 35 cycles with 30 seconds at 94°C, 1 min at annealing temperature 62°C with 30 seconds, and 72°C for 30 seconds followed by the final extension step at 72°C for 5 min. Only 10 µl of the amplified products were loaded on a 2% agarose gel. Next, 20 µl of the amplified PCR product was sequenced using ABI 3130 automated sequencer (XL Genetic Analyzer) by the Big-Dye Terminator Version 3.1 Cycle. Finally, the evaluation of BTNL2 gene mutation, from rs2076530 G to A on exon 5, for all patients and controls was reported as A/A homozygosity, A/G heterozygosity.

Statistical analysis

Genotype and allelic distributions were compared between the two patients and healthy groups by utilizing Pearson’s chi-squared in univariate analysis. A Bonferroni correction (a multiple-comparison correction) was applied to all associations and a P value was reported both with Bonferroni correction and after adjustment. As a measure of association, relative risks or odds ratios (ORs) were calculated along with the 95% confidence intervals (95% CIs). Binary logistic regression analysis was employed to test the association between the selected genotype and disease susceptibility. Statistical analysis was performed by IBM SPSS Statistics for Windows, version 23 (IBM Corp., Armonk, N.Y., USA).

Results

A total of 80 patients with sarcoidosis and 80 healthy controls were registered in this study. Of these patients, 46 (57.5%) cases were females. RFLP technique was first used for mutation analysis. RFLP results showed 38 (47.5%) patients with AA homozygote mutant in comparison to 22 (27.5%) AA individuals (OR=2.38, 95%CI: 1.235-4.607, P=0.009) in controls, while there are 24 (30%) patients with AG heterozygote and 18 (22.5%) patients with GG homozygote. A Bonferroni correction with P<0.0038 shows a statistical difference for genotype AA (P=0.009) (Table 1).

Also, a significant difference was statistically found in allele distribution for the A (62.5%) allele (OR=2.14, 95%CI: 1.37-3.35, P=0.001). Binary logistic regression analysis indicates a statistical association of AA genotype and sarcoidosis (P=0.018 with 60% prediction) in an effective model. Through DNA sequencing, all of the mentioned mutations G→A, rs2076530 were found and their patterns of homozygote mutants,

| Table 1. BTNL2 rs2076530 polymorphism genotypes and allele frequencies in sarcoidosis patients and healthy controls |
|---------------------------------|------------------|-----------------|-----------------|------------------|------------------|
|                                | Genotypes                | Alleles          |                  |                  |                  |
|                                | AA             | AG             | GG             | A               | G               |
|                                | Number (%)     | Number (%)     | Number (%)     | Number (%)     | Number (%)     |
| Sarcoidosis                    | Count (%)      |                  |                |                  |                  |
|                                | 38 (47.5)      | 24 (30)        | 18(22.5)       | 100(62.5)       | 60(37.5)        |
|                                | P value*       | 0.0090         | 0.7338         | 0.0168          |                  |
|                                | P value**      | 0.0270         | 2.2014         | 0.0504          |                  |
| Healthy Control                | Count (%)      |                  |                |                  |                  |
|                                | 22(27.5)       | 26(32.5)       | 32(40)         | 90(56.25)       | 70(43.75)       |
|                                | P value*       | 0.0090         | 0.7338         | 0.0168          |                  |
|                                | P value**      | 0.0270         | 2.2014         | 0.0504          | 0.001           |

*P value with Bonferroni correction (P<0.0083), **adjusted P value (P<0.05)
heterozygotes, and standard homozygotes were identified via RFLP (Figure 2). The point mutations were also analyzed and confirmed using DNA direct sequencing (Figure 3).

Discussion

In the present study, a single nucleotide polymorphism of the BTNL2 gene was evaluated as a putative genetic risk factor for sarcoidosis using the newly developed RFLP technique which is confirmed by DNA sequencing. The study reveals a statistical difference of AA genotype in sarcoidosis, using chi-square analysis, with Bonferroni correction. Sarcoidosis risk is significantly influenced by AA genotype and A allele frequency, respectively, as determined by univariate analysis. Coming back to the literature, the first gene region to show a genetic predisposition to sarcoidosis was identified by Valentonyte et al., who searched a 16.4-Mb locus on 16p21 and found a 15-kb section of BTNL2 associated with sarcoidosis in German patients (17). Nguyen et al. published the first results of the BTNL2 gene function, which is involved in T-cell activation based on its homology with B7-1. They reported the highest expression of BTNL2 in mouse lymphoid tissues, as well as intestines, and enhanced the appearance of its putative receptors on B and T cells (23). These expression analyses showed that the BTNL2 molecule inhibits T cell activation, which has a significant impact on immune diseases such as sarcoidosis. It is becoming more apparent that the gene function of BTNL2 may play an essential role in the pathophysiology of sarcoidosis. Additionally, the accumulation of familial cases suggests that there is a genetic predisposition to the disease. Various studies have been conducted in different ethnic groups to determine genetic predisposition, but diverse clinical manifestations have been observed. In white and African-American populations, Rybicki et al. carried out family-based and case-control studies (12). They reported the most influential association of rs2076530 SNP with sarcoidosis. They also emphasized that the higher association between the rs2076530A allele and sarcoidosis was predominantly seen in the white population. In another study, sarcoidosis patients and control subjects were recruited from two European countries (17). In these populations when analyzed as a group, allele A of BTNL2 was associated with an increased risk of sarcoidosis. However, it was not associated with sarcoidosis when the exclusion criterion was present, which clinically and genetically represented a distinct disease subset (17). Li et al. carried out a case-control study, including German patients, similar to the survey by Valentonyte et al. (17, 24). According to their results, the A allele is associated with an about two-fold higher risk of sarcoidosis in both a co-dominant and a dominant model, but not in a recessive model. Another critical study performed by Milman et al. was added to the studies of different ethnic groups (25). The comprising of Danish patients was significant in terms of ethnic data and different levels
Figure 3. Direct DNA sequencing patterns of AA, AG, and GG point mutations. (A) a single point mutation AA, (B) AG point mutation and (C) GG point mutation were found and confirmed corresponded to the RFLP technique for the patients.
of risk for sarcoidosis. The BTNL2 A allele variant was very frequent in Danish patients with sarcoidosis, and the AA genotype was associated with a 3.1-fold higher risk of sarcoidosis than the GG genotype. Morais et al. evaluated the BTNL2 rs2076530 G/A allele associations with sarcoidosis susceptibility in a Portuguese population (18). Their data replicated the association of the BTNL2 rs2076530 A allele with sarcoidosis(26). Suzuki et al. performed a study to assess whether the BTNL2 association was independently present in Japanese patients with sarcoidosis which the BTNL2 rs2076530 A allele was associated with an increased risk of sarcoidosis (OR = 1.84) in their study, but the OR was lower than in other ethnic groups(27).

To interpret the results of this study, we should recognize that sarcoidosis is an unknown etiology and multi-factor disease involving possible gene–gene and gene-environment interactions. The present study, however, only examined the association between BTNL2 polymorphism rs2076530 and sarcoidosis susceptibility. On the other hand, as well described in a meta-analysis by Lin et al. (28), most of the related studies were from Caucasians, so the results may only apply to this ethnic population, considering the study of Suzuki et al. in Japan(27).

In conclusion, our study as an Iranian sarcoidosis tertiary referral center cohort showed a significant increase in the frequency of the BTNL2 rs2076530 A allele, supporting a role for this SNP in the genetic risk of sarcoidosis in our population. This study could be added to ethnographic survey information such as Asians, Africans, and Latinos to describe BTNL2 rs2076530 polymorphism and sarcoidosis susceptibility. By adding this ethnographic information to clinical courses and clinical outcomes, these genetic studies can help us to improve our knowledge of the etiology, treatment, and disease progression.

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