BTNL2 gene polymorphism associations with susceptibility and phenotype expression in sarcoidosis

António Morais a,*, Bruno Lima b, Maria José Peixoto b, Helena Alves b, Agostinho Marques a, Luís Delgado c,d

a Pneumology Department, Centro Hospitalar São João, Faculdade de Medicina, Universidade do Porto, 4200-319 Porto, Portugal
b North Histocompatibility Centre, Portugal
c Immunology Department, Faculdade de Medicina, Universidade do Porto, 4200-319 Porto, Portugal
d Centre for Research in Health Technologies and Information Systems (CINTESIS), University of Porto, Portugal

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KEYWORDS
Sarcoidosis; Genetics; BTNL2; HLA; Disease evolution

Summary
A functional polymorphism within butyrophilin-like 2 (BTNL2) gene has been described as a potential risk factor for sarcoidosis. The association between chronicity and the rs2076530 SNP A allele has also been reported.

This study evaluates the BTNL2 rs2076530 G/A allele associations with sarcoidosis susceptibility and disease evolution in a Portuguese cohort of patients.

A case-control study of 151 patients and 150 controls was performed. Allele frequencies were compared with Chi-square test in a univariate analysis and with logistic regression in a multivariate analysis.

BTNL2 rs206530 A allele frequencies were significantly higher in sarcoidosis with no linkage disequilibrium with HLA-DRB1 alleles, except in the subgroup of patients with Löfgren syndrome where the determinant allele was HLA-DRB1*03. The A allele was also increased in those with isolated thoracic disease, with no differences regarding radiological stages or disease evolution. HLA-DRB1*03, besides the association with Löfgren syndrome was significantly related with disease resolution. Our data confirms the association of BTNL2 rs2076530 A allele with sarcoidosis susceptibility in a Portuguese population. We found independent genetic risk factors in clinically distinct disease phenotypes: BTNL2 rs2076530 A allele in patients without Löfgren syndrome or with isolated thoracic disease, and HLA-DRB1*03 in Löfgren syndrome or disease resolution.

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* Corresponding author. Serviço de Pneumologia, Centro Hospitalar São João, Alameda Prof. Hernani Monteiro, 4200-319, Porto, Portugal.
Tel.: +351 919175999.
E-mail address: amorais3@hotmail.com (A. Morais).

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Introduction

Sarcoidosis is a multiorgan granulomatous inflammatory disease of unknown etiology, characterized by activation of macrophages and CD4+ T cells and the formation of non-caseating granulomas, predominantly in the lungs and lymph nodes.1–4 An underlying interaction between genetic factors and unknown environmental factors is a widely accepted hypothesis for the pathogenesis of sarcoidosis.1,4–6 Familial clustering, the increased risk of relatives to develop sarcoidosis, and different clinical presentations related to distinct ethnic groups, support the role of genetic factors in sarcoidosis susceptibility and heterogeneity.5,6

Family-based and case control studies have consistently shown an association between Human Leukocyte Antigen (HLA) haplotypes and sarcoidosis, regarding both disease susceptibility and phenotype expression.7,8 However due to the high range of linkage disequilibrium (LD) within the major histocompatibility complex (MHC) it remains unclear whether the HLA genes directly determine the susceptibility or the associations are due to other genes in linkage with this region.9,10 This is the case of butyrophilin-like 2 (BTNL2) gene, a member of the immunoglobulin superfamily located at the junction of the HLA class II and class III regions.11,12

Valentonyte et al. reported for the first time a novel association with a truncating single nucleotide polymorphism (SNP) rs2076530 (G → A) of the BTNL2 gene, independently of the HLA sarcoidosis risk alleles.11 BTNL2 seems to have a role in the modulation of costimulatory receptors involved in T-cell responses, on the basis of its amino acid homology to the CD80/CD86 family of costimulatory proteins.13,14 As a consequence, dysfunction of the BTNL2 protein could impair normal T-cell regulation and response to antigens. Following the initial report, other studies of BTNL2 polymorphisms and sarcoidosis generated conflicting results in populations from different geographic areas or different disease phenotypes.15,16 On the other hand, whether BTNL2 associations are independent of HLA-DRB1 alleles remains to be elucidated, in view of contradictory data so far.11,16–19

Our purpose was to evaluate BTNL2 rs2076530 G/A allele as a putative genetic risk for sarcoidosis in a Portuguese population, before and after stratification of clinically distinct disease phenotypes and evolution. Given the variability of the published data from diverse ethnic groups, an evaluation of HLA alleles with LD analyses was also carried out.

Materials and methods

Subjects

For a case-control study with 1:1 ratio, a type 1 error rate of 5%, an 80% of power to detect a modest genotype odds ratio of 1.4 for disease susceptibility, we estimated a sample size with 123 patients and 123 controls. We included a total of 151 unrelated Caucasian patients from the north region of Portugal (mean age 38.0 ± 4.2 years, 57% women) and 150 controls. All patients had thoracic sarcoidosis, as determined by symptoms, radiology and pulmonary function tests, supported by the evidence of non-caseating epithelioid cell granuloma in biopsy specimens in 66%. All subjects without histological confirmation fulfilled the ERS/ATS/WASOG statement criteria,2 namely compatible clinical and radiographic features, a bronchoalveolar lavage fluid (BALF) lymphocyte CD4/CD8 > 4.0, and a 2 years observation period to exclude other medical conditions. Lögren’s syndrome (LS) was defined as bilateral hilar lymphadenopathy, fever, ankle arthralgia and erythema nodosum.1–3 Thoracic involvement was classified according with Scadding criteria at the time of diagnosis (stage 0-no thoracic involvement, stage I-adenopathies without lung involvement, stage II-adenopathies and lung involvement, stage III-only lung involvement, IV-lung fibrosis).20 Disease resolution was considered when a disappearance of symptoms, normalization of chest X-ray and pulmonary function tests occurred within 2 years after diagnosis.18,19,21 A control group included 150 unrelated and healthy bone marrow donors, randomly recruited from the same geographic region and from the same ethnic background. They had a mean age of 51.9 ± 14.5 years and 51% were women, no history of lung disease, respiratory symptoms or other disease by chest radiography or laboratory blood tests. Written consent was obtained from all subjects, and the study approved by our hospital Ethics Committee.

Genotyping

DNA from patients and controls was obtained from peripheral blood using standard methods. Samples were genotyped for the BTNL2 G → A transition of rs2076530 using a TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA, USA). Minor groove binding probes were labeled with the fluorescent dyes VIC and FAM, respectively. Polymerase chain reaction (PCR) was carried in a total reaction volume of 12.5 µl with TaqMan SNP Genotyping assay 1x, TaqMan Genotyping Master Mix 1x and 20 ng of genomic DNA. The amplification protocol included a denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 92 °C for 15 s and annealing and extension at 60 °C for 1 min. After PCR, the genotype was determined by allelic-specific fluorescence on the ABI PRIM 7000 Sequence Detection Systems and SDS 1.1 software for allelic discrimination (Applied Biosystems, Foster City, CA, USA).

DNA samples were also genotyped for HLA-A, B, Cw, DRB1 and DQB1 by PCR-Sequence Specific Oligonucleotides (PCR-SSO) with Luminex technology (Tepnel Lifecodes Kits, Stamford). DNA amplification was carried in a total volume of 25 µl with 7.5 µl of Master Mix specific for each locus, 1.25 U of GoTaq Hot Start Polymerase (Promega, Madison WI) and 50 ng of genomic DNA. PCR was run with the program: 5 min at 95 °C, followed by 8 cycles of 95 °C 30s, 60 °C 45s, 72 °C 45s, with an increment of 32 cycles of 95 °C 30s, 63 °C 45s, 72 °C 45s and a final extension hold of 72 °C for 15 min. After DNA amplification, 2.5 µl of PCR product was mixed with 7.5 µl of pre-warmed Luminex probes (58 °C) and hybridized with the following protocol: 97 °C for 5 min, 47 °C for 30 min and 56 °C for 10 min, prior to Luminex reading, the hybridization stage stopped at 56 °C with 100 µl of a dilution solution combined with 0.4 µl of Streptavidin-PE (Tepnel Lifecodes, Stamford). HLA typing was analyzed with the software Quicktype LifeMatch v2.5 (Tepnel Lifecodes).
Allelic and genotypic distributions were statistically analyzed with the χ²-test (or Fisher exact test when appropriate) in univariate analysis and with logistic regression in multivariate analysis. Relative Risks or Odds ratios (OR) and their 95% confidence intervals (95% CIs) were calculated as association measures. The software used was the Statcalc program (EpiInfo 2002, Centers for Disease Control and Prevention, Atlanta, GA, USA). Regression analyses where performed in SPSS v13 statistical software. p Values less than 0.05 were considered statistically significant. p Values where corrected (pc) for multiple comparisons using Bonferroni method. HLA data and BTN2L2 haplotypes were analyzed by carriage frequency, while BTN2L2 allele’s data by allele frequency unless otherwise stated. Due to the strong LD between many HLA alleles and BTN2L2, logistic regression analysis was performed to determine the potential for confounding and effect modification of BTN2L2 allelic in relation to HLA class II risk alleles. For haplotype analysis, pairwise LD measures were investigated and HLA-DRB1*–DQB1*–BTN2L2 haplotypes constructed with the expectation-maximization (EM) algorithm implemented in Arlequin software.²⁵ LD between selected pairs of loci was tested using a likelihood test for the significance of the association between pairs of loci, where the likelihood of the sample evaluated under the hypothesis of no association between loci (linkage equilibrium) is compared to the likelihood of the sample when association is allowed. We tested LD between two alleles from two different loci, constructing a 2 × 2 contingency table with the individual (observed) values cross-classified by level in the two different attributes, i.e., two alleles from two different loci.

Results

According with Scadding criteria 49 (32.4%) patients were in stage I, 67 (44.3%) in stage II, 14 (9.2%) in stage III and 21 (13.9%) in stage IV. Sixty one (40.3%) patients presented extra-thoracic disease, with skin and liver as the most frequent involved structures, both with 21 (13.9%) patients. Löfgren’s syndrome was observed in 29 (19.2%) cases. A consistent clinical course was established in 144 patients (95.4%), 66 (43.7%) presenting disease resolution and 78 (51.7%) chronic forms; among these, 41 (52.6%) had chronic stable and 37 (47.4%) progressive disease.

Disease susceptibility

BTN2L2 rs2076530 genotype frequencies and HLA allele frequencies were in Hardy–Weinberg equilibrium across both studied populations, cases and controls. Genotype and allele frequencies were similar to those reported for both studied populations, cases and controls. Genotype and allele frequencies in Hardy–Weinberg equilibrium across both studied populations, cases and controls. Genotype and allele frequencies were similar to those reported for

**Clinical presentation**

When patients with (n = 29, 19.2%) and without (n = 114, 75.4%) Löfgren’s syndrome were analyzed, we didn’t find significant differences between BTN2L2 rs2076530 A allele frequencies (Table 2). The association found between BTN2L2 rs2076530 A allele and sarcoidosis was statistically significant for both groups: Löfgren’s syndrome (OR = 2.09, 95% CI [1.88; 2.27], p < 0.01) remained significantly associated with disease risk. DRB1*14 allele was still overrepresented in the patient group although not significantly so after adjusting for the BTN2L2 allele (OR = 3.29, 95% CI = [0.88; 12.21], p = 0.08). The effect modification was also tested in a regression model with terms for the rs2076530 AA genotype, DRB1*14 allele and a cross-product interaction term. This interaction did not reach statistical significance.
Table 1  BTNL2 and HLA alleles associated with sarcoidosis susceptibility.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Sarcoidosis</th>
<th>Controls</th>
<th>p value</th>
<th>pc</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 302 (%)</td>
<td>n = 300 (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs2076530 A</td>
<td>197 (65.2%)</td>
<td>167 (55.7%)</td>
<td>0.01</td>
<td>—</td>
<td>1.49</td>
<td>1.06–2.10</td>
</tr>
<tr>
<td>rs2076530 G</td>
<td>105 (34.8%)</td>
<td>133 (44.3%)</td>
<td>0.01</td>
<td>—</td>
<td>0.67</td>
<td>0.48–0.94</td>
</tr>
<tr>
<td>A*03</td>
<td>38 (12.6%)</td>
<td>23 (7.7%)</td>
<td>0.04</td>
<td>0.72</td>
<td>1.73</td>
<td>0.97–3.10</td>
</tr>
<tr>
<td>A*29</td>
<td>11 (3.6%)</td>
<td>23 (7.7%)</td>
<td>0.03</td>
<td>0.54</td>
<td>0.46</td>
<td>0.20–1.00</td>
</tr>
<tr>
<td>B*07</td>
<td>23 (7.6%)</td>
<td>11 (3.7%)</td>
<td>0.03</td>
<td>0.84</td>
<td>2.17</td>
<td>0.99–4.83</td>
</tr>
<tr>
<td>B*44</td>
<td>28 (9.3%)</td>
<td>52 (17.3%)</td>
<td>0.003</td>
<td>0.08</td>
<td>0.49</td>
<td>0.29–0.82</td>
</tr>
<tr>
<td>C*16</td>
<td>13 (4.3%)</td>
<td>34 (11.3%)</td>
<td>0.001</td>
<td>0.01</td>
<td>0.35</td>
<td>0.17–0.71</td>
</tr>
<tr>
<td>DRB1*07</td>
<td>33 (10.9%)</td>
<td>50 (16.7%)</td>
<td>0.04</td>
<td>0.52</td>
<td>0.61</td>
<td>0.37–1.01</td>
</tr>
<tr>
<td>DRB1*08</td>
<td>5 (1.7%)</td>
<td>17 (5.7%)</td>
<td>0.01</td>
<td>0.12</td>
<td>0.28</td>
<td>0.08–0.81</td>
</tr>
<tr>
<td>DRB1*14</td>
<td>11 (3.6%)</td>
<td>3 (1.0%)</td>
<td>0.03</td>
<td>0.39</td>
<td>3.74</td>
<td>0.97–21.06</td>
</tr>
</tbody>
</table>

a Allele frequencies; OR — Odds Ratio; CI — Confidence Interval; pc — corrected p-value for multiple comparisons.

Evolution

Comparison of rs206530 allele frequencies between chronic patients and those who had disease resolution didn’t show any statistically significant differences (Table 3). No differences were also seen comparing patients with resolution with those with chronic stable or progressive disease (data not shown). Moreover, comparison between these two groups of chronic patients didn’t found any differences regarding BTNL2 rs2076530 A SNP (64.6% vs 62.2%, RR = 1.05; 95% CI for RR = [0.77; 1.44]; p = 0.75). However, regarding HLA alleles, disease resolution was significantly associated with DRB1*03 (RR = 1.61; 95% CI = [1.23; 2.1]; p = 0.004).

Discussion

In this study, we evaluated a single nucleotide polymorphism of the BTNL2 gene, as a putative genetic risk factor for sarcoidosis in a Portuguese population. The significant increase of the BTNL2 rs2076530 A allele observed, corroborates the role of this truncating SNP in disease susceptibility. Analyzing the subgroup presenting with Löfgren’s syndrome, this association disappeared after HLA class II allele adjustment, since the determinant allele was HLA-DRB1*03; nevertheless, the association persists in the subgroup of patients without Löfgren syndrome even after adjustment for HLA-DRB1. An increased frequency of A allele was also noticed in those with isolated thoracic disease, although with no differences regarding radiological stages, disease resolution or a chronic course over at least 2 years of follow-up. HLA-DRB1*03 allele, besides its association with Löfgren syndrome, was also significantly related with disease resolution.

BTNL2 is a member of the butyrophilin-like molecules (BTNLs) sharing structural homology with CD80/CD86 (B7) family of costimulatory molecules, expressed on antigen-presenting cells (APCs) and critical for effector immune responses. BTNLs have a restricted expression in human cells and BTNL2, along with some epithelial cells of the mucosal barriers, is expressed on dendritic cells (spleen and lymph nodes) and peripheral B cells, consistent with a role in the modulation of APCs function. In fact, BTNL2 has been shown to inhibit T cell proliferation and IL-2 production, also diminishing the production of pro-inflammatory cytokines in T cell cultures. The single nucleotide polymorphism that we studied (rs2076530 G > A), results in a mutated BTNL2 gene leading to a truncated protein and disruption of its membrane localization. Although the precise functional implications of this mutation has not been established, the putative inappropriate/inadequate membrane expression of a molecule inducing a negative signal to T-lymphocytes, could result in an uncontrolled high state of activation of T cells, a known pathological feature of sarcoidosis. One possible limitation of our study is the number of included cases. However, it represents the population currently referred to our university hospital center, which gives respiratory care to a total of 3 million inhabitants in the North region of Portugal (a country with 9 million inhabitants). This is a relatively homogeneous population and our controls were from the same geographic and genetic background. Another limitation concerns a sarcoidosis cohort in a tertiary care setting, potentially excluding less sever forms. The majority (59.7%) of our patients had

<table>
<thead>
<tr>
<th>Allele</th>
<th>With LS</th>
<th>Without LS</th>
<th>p</th>
<th>pc</th>
<th>RR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 58 (%)</td>
<td>n = 228 (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs2076530 A</td>
<td>42 (72.4%)</td>
<td>151 (66.2%)</td>
<td>0.4</td>
<td>—</td>
<td>1.26</td>
<td>0.75–2.13</td>
</tr>
<tr>
<td>DRB1*03</td>
<td>16 (27.6%)</td>
<td>21 (9.2%)</td>
<td>0.0002</td>
<td>0.003</td>
<td>2.56</td>
<td>1.62–4.06</td>
</tr>
<tr>
<td>DRB1*04</td>
<td>1 (1.7%)</td>
<td>30 (13.2%)</td>
<td>0.01</td>
<td>0.13</td>
<td>0.14</td>
<td>0.02–1.01</td>
</tr>
<tr>
<td>DQB1*02</td>
<td>24 (41.4%)</td>
<td>42 (18.4%)</td>
<td>0.0002</td>
<td>0.001</td>
<td>2.35</td>
<td>1.51–3.67</td>
</tr>
</tbody>
</table>

a Allele frequencies; RR — relative risk; CI — confidence interval; pc — corrected p-value for multiple comparisons.
isolated thoracic involvement, 19.2% Löfgren’s syndrome, 43% disease resolution, 26.4% chronic stable disease and 25.1% chronic progressive disease, which seems in accordance with the usual presentations of Caucasians followed in specialized care settings. Although not age matched to the patients, our controls were unrelated healthy individuals, recruited from the same geographic and ethnic background. Their comparatively higher mean age gives additional confidence, since a possible sarcoidosis diagnosis in their lifetime will be less likely.

Several studies have been published in different populations and ethnic groups, with different approaches regarding clinical phenotyping or HLA allele’s adjustment. Although with different conclusions, an overall evaluation of the available data supports a role of this SNP in the disease susceptibility. In our cohort there is no apparent influence from HLA alleles. This study also suggested an influence of race in this genetic risk as the association with BTNL2 was independent of the HLA class II genes in whites but interacted antagonistically in African Americans, with no apparent influence from HLA alleles. Both a different genetic background and patient’s selection may account for these discrepancies, as our patients were consecutively recruited from a tertiary reference center, a cohort more similar to the one reported by Valentonyte group. Otherwise, the association of HLA-DRB1*03 with Löfgren syndrome has been described in several populations and different DRB1*03 frequencies may even explain the rarity of this clinical presentation in Japan and the higher prevalence in Scandinavian countries.

Interestingly, associations between BTNL2 rs2076530 A allele and a group of different diseases such as multiple sclerosis, leprosy, type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus or Graves disease, were actually due to LD with other genes from this region, namely no differences were seen among the four radiothoracic involvement. To our knowledge, this association has never been reported or addressed in previous studies, although no differences were seen among the four radiological Scadding stages, a finding also described by Spagnolo et al.

Li et al. described for the first time an association between BTNL2 rs2076530 A SNP and a chronic disease course, in a German population. However no linkage disequilibrium analysis to DRB1 alleles was carried out, and the type of evolution of the chronic forms was not assessed. Coudurier et al. described the pathogenic variant of BTNL2 on both alleles (AA homozygote) in three related patients, all with severe forms, although they also did not address HLA-DR linkage. Collectively, these results do suggest an association of the variant allele with more severe cases with progression to fibrosis.

Generally we can consider three forms of evolution in sarcoidosis: disease resolution, chronic stable and chronic progressive disease (needing persistent therapeutic intervention). In our cohort we stratified these different outcomes with the hypothesis that they might be related with different genetic risk factors. However, we didn’t find any significant association between BTNL2 and outcomes, neither with those who had disease resolution nor with who become chronic. Moreover, no differences between chronic stable or chronic progressive disease

<table>
<thead>
<tr>
<th>Allele</th>
<th>Resolution</th>
<th>Chronic</th>
<th>p</th>
<th>pc</th>
<th>RR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2076530 A</td>
<td>91 (68.9%)</td>
<td>99 (63.5%)</td>
<td>0.32</td>
<td>–</td>
<td>1.14</td>
<td>0.87–1.51</td>
</tr>
<tr>
<td>DRB1*01</td>
<td>18 (13.6%)</td>
<td>10 (6.4%)</td>
<td>0.04</td>
<td>0.48</td>
<td>1.47</td>
<td>1.08; 2.00</td>
</tr>
<tr>
<td>DRB1*03</td>
<td>24 (18.2%)</td>
<td>11 (7.1%)</td>
<td>0.004</td>
<td>0.05</td>
<td>1.61</td>
<td>1.23–2.10</td>
</tr>
</tbody>
</table>

* Allele frequencies; RR — relative risk; CI — confidence interval; pc — corrected p-value for multiple comparisons.

**Table 3** BTNL2 and HLA class II alleles associated with disease outcome in sarcoidosis.
were found. On the other hand, disease resolution was significantly associated with DRB1*03, an association also seen in various Caucasian populations. 30–32 In contrast with our observations, Wijnen et al. described that BTN2L rs2076530 variant allele was more frequent in patients with chronic persistent (progressive) disease than chronic non-persistent (stable) disease, independently from DRB1 allele’s influence. 19 In their larger Dutch cohort, the A-allele almost doubled the risk of a progressive course of sarcoidosis. 19

In conclusion, our study of a Portuguese sarcoidosis cohort at a tertiary reference centre showed a significant increase of the BTN2L rs2076530 A allele frequency, with an even stronger association to the BTN2L rs2076530 AA genotype, corroborating the role of this truncating SNP in the genetic risk of sarcoidosis in our population. Taking into account BTN2L and HLA-DR linkage disequilibrium, we also uncovered two independent genetic risk factors for different clinical presentations of sarcoidosis: the BTN2L rs2076530 A allele in patients without Löfgren syndrome or with isolated thoracic disease, and the HLA-DRB1*03 allele in those presenting Löfgren syndrome or disease resolution within 2 years of follow-up. The immunogenetic base of these associations deserves further investigation.

Conflict of interest statement

The authors have no conflict of interest.

References


