ABSTRACT

Objective. Behçet’s disease (BD) is an immune-mediated and complex disease associated with HLA class I and other genes. The aim of this study was to contribute to a better understanding of the relationship of the 32-bp deletion in the CCR5 gene (CCR5Δ32) and this disease by conducting a case-control study in the Spanish population and also a meta-analysis including all the studies available to date.

Methods. A cohort composed of 348 BD Spanish patients and 477 unrelated healthy and ethnically matched individuals were genotyped in CCR5Δ32 using polymerase chain reaction (PCR) and capillary electrophoresis with fluorescent detection. In the meta-analysis, data from a total of seven populations extracted from four previous studies along with data of the present study were included.

Results. Regarding the case-control study, no statistically significant differences were observed when the patient and control groups were compared (allelic model: 0.07 in patients versus 0.06 in controls, p=0.303). In the meta-analysis, no evidence of association of the CCR5Δ32 polymorphism with BD was observed (p meta= 0.091; OR= 1.22; 95%CI 0.98 to 1.52 in the allelic model).

Conclusion. The results of this meta-analysis discard a major role of the CCR5Δ32 polymorphism in BD.

Introduction

Behçet’s disease (BD) is a multisystemic inflammatory disorder characterised by recurrent oral and genital ulcerations; ocular affection, mainly uveitis, and skin lesions, additional manifestations which involve other organs such as joints and central nervous system are relatively common. The aetiology of BD remains elusive, although it has been suggested that the disease is the result of complex interactions between environmental factors (e.g. certain infectious agents) and genetic predisposition (1).

The evidences of genetic contribution to the pathogenesis of the disease are based on familial aggregation, predominance in patients with Mediterranean or Asian ancestry and the association with human histocompatibility complex (HLA) in several ethnic groups (2-4). The contribution of the HLA region has been estimated to represent approximately 20% of the genetic component of this disease therefore other genes should be involved in the predisposition to this pathology (5). As a result of different studies designed to establish the contribution of genes located outside the HLA class I region with the disease, a relationship of BD with IL23R, IL10 and other genes has been established in different populations (6-12).

One of the non-HLA genes proposed as a candidate in BD is the chemokine receptor type 5 (CCR5), a G protein-coupled receptor which is expressed on TH1 cells, monocytes and dendritic cells. Binding of CCR5 to its ligands mediates the migration of mononuclear cells to the inflammation site and, in addition, the CCR5 molecule is also a co-receptor of the HIV. The most extensively studied polymorphism in this gene is a deletion of 32 bp (CCR5Δ32) which results in the introduction of a premature stop codon by a reading frameshift producing, as a consequence, a truncated protein unable to
bind to the natural ligands of CCR5 (13). The CCR5Δ32 allele is related to the resistance to HIV infection (14) and it has also been associated with immune-based diseases such as rheumatoid arthritis and multiple sclerosis (15, 16). The role of the CCR5Δ32 variant in immuno-mediated diseases is not clear because in some conditions such as rheumatoid arthritis it appears as a protective factor (17) because it is associated with a reduced risk whereas in others such as multiple sclerosis it increases the risk to develop the disease (18). CCR5 mediates mononuclear cell recruitment to sites of inflammation by interacting with its ligands CCL3, CCL4 and CCL5. The lack of functional CCR5 causes up-regulation of its ligands which can exert their biological effects by engaging other available receptors (19). Thus, lymphocytes from CCR5Δ32 homozygous subjects secrete CCL5 at levels that are 5–10 times higher than the CCR5 non-mutated control subjects (20). The elevated levels of this chemokine can, by engaging other available receptors such as CCR3 and CCR1 result in increased recruitment of inflammatory cells and production of pro-inflammatory cytokines. So far, four studies investigating the association between CCR5 gene and BD have been published (21-24). Three of these studies found no association of the CCR5Δ32 variant with susceptibility to BD. Nevertheless, they include cohorts with a relatively small sample size and this fact, along with the low frequency of the variant, determines that the statistical power of the individual studies is inadequate. The aim of this study was to contribute to improve the current knowledge about the relationship of this functional variant of the CCR5 gene and BD by investigating whether the CCR5Δ32 variant is associated with BD in the Spanish population and also by conducting a meta-analysis including all the available data.

**Material and methods**

**Case-control study**

This part of the study includes 348 BD patients (153 males and 195 females) with a mean age at onset (years) ± SD of 48.22±12.19 who fulfilled the 1990 International Study Group classification criteria for Behçet’s disease (25) and 477 unrelated healthy individuals recruited in the same geographic regions and matched by age and gender with BD patients. All the subjects were Spanish from European origin, patient group was partially included in the Registry of the Spanish network of Behçet’s disease (26) 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. Torrecárdenas), Barcelona (H. Clínica, 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. Marqués de Valdecilla) and Sevilla (H. Virgen del Rocío y 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. Peripheral blood or saliva were used as the starting material. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Barcelona, Spain) according to the manufacturer’s recommendations and stored at -20°C 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. Genotyping was performed by polymerase chain reaction (PCR) using primers spanning the region of the 32 bp deletion in the CCR5 gene. The sequence of the primers were those previously described 5‘CTCTCATCATCCTCTCTTGCATCG3’ (sense) and 5’GAC-CAGCCCAAGTTGACTATC3’ (anti-sense) (27), labelling the sense primer with a fluorochrome (FAM) at the 5’ end. The PCR products were separated by capillary electrophoresis using a 3130/3130xl Genetic Analyzer (Applied Biosystems, Barcelona, Spain) with fluorescent detection. The expected size of the amplified DNA fragments for the wild-type and deletion were 262 bp and 230 bp respectively. About 5% of the samples were studied in duplicate to verify genotyping consistency which was identical in all the cases. The statistical powers were calculated with the Statistical Power test of the Research Tool Kit of the DSS Research Software (https://www.dssresearch.com/KnowledgeCenter/toolkitcalculators/statisticalpowercalculators.aspx) to detect associations with odds ratios (OR) greater than 1.5 in the allelic model and taking into account the allelic frequency of the variant in each population. All the statistical analyses were performed with PLINK v.1.07 (http://pngu.mgh.harvard.edu/purcell/plink/). The χ² test was used to test Hardy Weinberg equilibrium (HWE). To check association of the variant CCR5Δ32 with disease, the distribution of alleles and genotypes in patient and control groups were compared using χ² (or Fisher exact test when appropriated) from 2x2 contingency tables. Odds ratios (ORs) and 95% confidence intervals (CI 95%) were calculated according to the Woolf method.

**Meta-analysis**

We conducted a meta-analysis which includes the data of the present study together with those previously published regarding the relationship of the CCR5Δ32 variant and BD. Literature included in the analysis was selected using the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/) and Scopus (http://www.scopus.com) searching for “CCR5 or chemokine receptor type 5” and “polymorphism” and “Behçet’s disease”. References from the retrieved papers were also checked. Papers published up to January 2015 were considered to be included in the meta-analysis. The eligible studies were included when they met the following criteria: association studies written in English, in which commonly accepted classification criteria for Behçet’s disease were used, reporting data of the population under study and displaying genotype distribution for the CCR5Δ32 polymorphism both in the patient and control groups. Exclusion criteria were HWE.
deviation, insufficient information and redundant or overlapping results.

The combined data were summarised in two by two tables. The Brewslow-Day method, the Cochran \( \chi^2 \)-based Q test and the inconsistency index (I\(^2\)) were used to assess heterogeneity in the different populations. Brewslow-Day and Q test with \( p \)-values (\( P_{BD} \) and \( P_o \)) lower than 0.05 were considered statistically significant. Those \( I^2 \) values from 0 to 25% were considered non heterogeneity, 25–50% moderate, 50–75% large and 75–100% extreme heterogeneity. The ORs were pooled using the Mantel-Haenszel, Robins-Breslow-Greenland methods for fixed effects because non significant heterogeneity was detected and \( p \)-values (\( P_{M-H} \)) lower than 0.05 were considered significant. All associations were tested under the allelic and the dominant model and the meta-analysis was conducted using StatsDirect v. 2.6.6 (StatsDirect, Altrincham,UK) software. In addition, to examine the degree to which an individual study affects the overall estimate, sensitivity analyses were conducted by removing one study at a time and analysing the change of the pooled effect.

**Results**

**Case-control study**

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 CCR5\( \Delta32 \) variant in the cohorts from different hospitals was not significantly different. Table I displays the data of genotyping of CCR5\( \Delta32 \) in our cohort of BD patients and healthy controls. Frequency of the CCR5\( \Delta32 \) variant in our control population was 0.06 similar to that described in other South European populations and lower than that found in the North of Europe where the highest frequency of the CCR5\( \Delta32 \) variant has been described. No statistically significant differences were observed when the patient and control groups were compared under the allelic model (0.07 in patients vs. 0.06 in controls, \( p=0.303 \)). Regarding the genotypes distribution, no significant differences were found comparing the distribution in patients and controls under the dominant model (\( p=0.390 \)). Statistical analyses of the other inheritance models were not performed because of the low number of individuals CCR5\( \Delta32 \)/CCR5\( \Delta32 \). Regarding gender and HLA-B*51 subgroups, frequencies of the variant in the cohort were 0.07 in males vs. 0.06 in females (\( p=0.688 \)) and 0.06 in the subjects B*51 positive vs. 0.07 in those negative (\( p=0.551 \)). Therefore, no significant differences attributable to gender neither to the presence of B*51 were found. The possible association between the CCR5\( \Delta32 \) variant and the main clinical characteristics of BD were analysed but no significant differences were found (data not shown).

**Meta-analysis**

To avoid the low statistical power of the individual studies published to date regarding the relationship of the CCR5\( \Delta32 \) polymorphism and BD (see Table II), a meta-analysis was conducted. This meta-analysis includes the data pooled from the four papers previously published (all of them fulfilled the above-mentioned criteria) together with data of the present study. A summary of the data of all these studies are displayed in Table II. No heterogeneity was detected in the analysis of this polymorphism in the homogeneity analysis (\( P_{B_H}=0.1881 \), \( P_o=0.2089 \) and \( I^2=28.7\% \)). Therefore, the subsequent meta-analysis was performed using the fixed effect method with the pooled data from a total of 1114 patients and 1786 healthy controls from seven different populations. The statistical power of the pooled data to detect an association with \( OR \geq 1.5 \) is 93%. As a result of this meta-analysis, no evidence of association of the CCR5\( \Delta32 \) polymorphism with BD was observed (\( P_{B_H}=0.091 \); \( OR=1.22 \); 95%CI 0.98 to 1.52 in the allelic model) (Fig. 1). The results obtained considering a dominant model were very similar to those obtained with the allelic (data not shown).

**Discussion**

The results of the present study suggest no association of the CCR5\( \Delta32 \) with the disease in the Spanish population. Most of the four individual studies published until the present regarding the association of this polymorphism have reported the same conclusion. In fact, only one study performed in an Italian cohort reported CCR5\( \Delta32 \) as a risk factor for BD (23). Although most of the studies discarded influence of this variant in the susceptibility to the disease, the statistical power of the individual studies, including the present, is low to reach a definitive conclusion. In fact, in all the populations except in the UK and the Portuguese populations (21-24) the frequency of the variant is higher in patients and therefore, differences could not reach significance as a consequence of limitation in size. Nevertheless, according to data of the meta-analysis presented in this study, which is well powered, a major role of the CCR5\( \Delta32 \) polymorphism in BD is discarded. In some of the previous studies, authors suggested association of this variant with a specific subgroup. Thereby, in a study in an Iranian population, the authors suggested association be-

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<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients (n=348)</th>
<th>Controls (n=477)</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Delta32/\Delta32 )</td>
<td>1</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>( \Delta32/wt )</td>
<td>46</td>
<td>13.2</td>
<td>54</td>
</tr>
<tr>
<td>wt/wt</td>
<td>301</td>
<td>86.4</td>
<td>423</td>
</tr>
<tr>
<td>Allelic Model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \Delta32 )</td>
<td>48</td>
<td>6.9</td>
<td>54</td>
</tr>
<tr>
<td>wt</td>
<td>648</td>
<td>93.1</td>
<td>900</td>
</tr>
<tr>
<td>Dominant model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \Delta32/\Delta32 + \Delta32/wt )</td>
<td>47</td>
<td>13.5</td>
<td>54</td>
</tr>
<tr>
<td>wt/wt</td>
<td>301</td>
<td>86.5</td>
<td>423</td>
</tr>
</tbody>
</table>
between CCR5Δ32 and BD but only in the case of female patients (22). Nevertheless, data in our population, consistently with the study in the Portuguese population (24), do not confirm an influence of the CCR5Δ32 variant which would be dependent on gender because the distribution of the variant was not different in males and females. Additionally, data in the UK population, suggest that the frequency of the CCR5Δ32 variant is increased among patients B*51 positive (21). This fact could reflect association only among individuals B*51 and/or a higher homogeneity of the patients with this HLA risk factor. On this sense, a previous meta-analysis studying the influence of this CCR5 variant in BD and other diseases, suggested association of the CCR5Δ32 among B*51 carriers (28). Nevertheless, this association was not found in the Portuguese population (24) neither in the present study in the Spanish population. The global distribution of this CCR5 variant is well known mainly because of its interest in the outcome of the HIV infection. In general, this mutation is more common among people with European ancestry but in the southern Europe, including Mediterranean populations, the frequency is usually lower than in the northern Europe. Frequencies that are found in the Middle East populations are similar to those found in the Mediterranean area and its presence is sporadic in the Asian populations (29). For this reason, although BD has a relatively high incidence in the Japanese and Chinese populations the influence of this variant in susceptibility to this disease in Asian populations did not have investigated because this variant practically does not exist in these populations. The question about association of CCR5 remains open in Asian populations because the influence of other functional variants of this gene which are present in these populations has not been yet investigated.

In conclusion, according to data of the meta-analysis presented in this study, a major role of the CCR5Δ32 polymorphism in BD can be discarded.

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Table II. Characteristics of the studies included in the meta-analysis.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Population</th>
<th>Genotyping methods</th>
<th>Sample Allelic frequency of the Δ32 variant % SP* p-value OR (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yang <em>et al.</em> 2003</td>
<td>UK</td>
<td>PCR and agarose gel electrophoresis Turkish 131 325 0.13 0.14 67.3 &gt;0.05</td>
<td>109 96 0.06 0.04 21.7 &gt;0.05 110 98 0.02 0.005 9.0 &gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Mojtahedi <em>et al.</em> 2006</td>
<td>Iranian PCR and agarose gel electrophoresis Turkish 100 380 0.09 0.05 37.2 &gt;0.05 2196 180 0.06 0.03 25.0 0.02 2.28 (1.1-4.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bettencourt <em>et al.</em> 2015</td>
<td>Portuguese PCR and agarose gel electrophoresis Turkish 122 230 0.07 0.08 27 &gt;0.05 348 477 0.07 0.06 68.2 &gt;0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*SP-Statistical power was calculated taking into account the allelic frequency and the sample size of each population and an expected OR ≥1.5.

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**Fig. 1.** Forest plots displaying the odds ratios and 95% confidence intervals of CCR5Δ32 polymorphism pooling the six populations included in the meta-analysis. The meta-analysis was conducted under fixed-effects and considering an allelic inheritance model.
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