CC chemokine receptor 5 polymorphism in Italian patients with Behc¸ et’s disease

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Abstract

Objective. To evaluate the potential role of CC chemokine receptor 5 (CCR5) D32 polymorphism in the susceptibility to and clinical expression of Behc¸ et’s disease (BD) in a cohort of Italian patients.

Methods. One hundred and ninety-six consecutive Italian patients satisfying the ISG criteria for BD were followed up for 8 years, and 180 healthy age- and sex-matched blood donors were molecularly genotyped for the CCR5D32 polymorphism. A standard microlymphocytotoxicity technique was used to serotype HLA-B51. The patients were subgrouped on the basis of the presence or absence of clinical manifestations.

Results. The distribution of the CCR5D32 genotype differed between BD patients and controls (P = 0.02). The CCR5D32 allele was more common in BD patients than in controls [P = 0.02, odds ratio (OR) 2.28 (95% CI 1.1, 4.8)]. Carriers of the CCR5D32 allele (D32/D32 + CCR5/D32) were significantly more common in BD patients than in controls [P = 0.02, OR 2.37 (95% CI 1.1, 5.1)]. Population-attributable risk was 7.1%. In categorizing patients according to gender, the association between CCR5D32 polymorphism and BD was similar in females and males (ORs 2.76 and 2.0, respectively). No significant differences were found when the frequencies of clinical manifestations were compared between CCR5D32 allele carriers and non-carriers.

Conclusion. CCR5D32 polymorphism is associated with an increased susceptibility to develop BD. Chemokines may have a role in the pathophysiology of BD.

Key words: Behc¸ et’s disease, CC chemokine receptor 5 Δ32 polymorphism, disease manifestations, chemokines.

Introduction

Behc¸ et’s disease (BD) is a primary systemic vasculitis of unknown aetiology that may affect venous and arterial vessels of any size [1]. The hallmark manifestation of BD is oral aphthosis, often associated with genital aphthae and various skin lesions [1]. Vascular, ocular and internal organ involvement occurs less frequently, but contributes substantially to morbidity and mortality [1]. Approximately one-third of BD patients develop thrombophlebitis of the deep or superficial veins (usually of the lower extremities), whereas arterial disease is distinctly less common (<5% of cases) [1, 2].

The pathogenesis of BD is still debated, but is likely to involve complex interactions between T cells, neutrophils and antigen-presenting cells [3]. In particular, mechanisms that may be operating include neutrophil hyperactivity [3], alteration of innate and adaptive immunity such as T helper 1 (Th1) [3, 4] and Th17 polarization [5] and excessive cytokine [4, 5] and chemokine production [4]. Chemokines and their receptors play an important role...
in the selective recruitment of various subsets of leucocytes to affected sites in various autoimmune conditions [6].

In BD, increased expression of the Th1-associated CC chemokine receptor type 5 (CCR5) and CXCR3 has been demonstrated in mucosal samples, suggesting a pathogenic role for Th1 lymphocytes [4]. CCR5 is a G protein-coupled receptor expressed on Th1 cells, monocytes and dendritic cells [6]. CCR5 ligands include the chemokines CCL3 [chemokine (C-C motif) ligand 3, also known as macrophage inflammatory protein-1α], CCL4 (also known as macrophage inflammatory protein-1β) and CCL5 [also known as RANTES (regulated on activation, normal T cell expressed and secreted)] [6]. CCR5 engagement by its ligands mediates mononuclear cell migration to sites of inflammation and has been implicated in the pathogenesis of various immune-mediated diseases [6]. CCR5 is also a major port of entry for macrophage-tropic HIV strains into host cells in association with CD4 [6]. The CCR5 gene polymorphism (a 32-bp deletion in the CCR5 gene CCR5Δ32) leads to a non-functional surface receptor that is unable to bind to its natural ligands [6].

The CCR5Δ32 allele has been linked to a number of immunological diseases, including RA and sarcoidosis [7–8]. With regard to BD, no association with the CCR5Δ32 polymorphism was found in British, Turkish or Palestinian patients [9], whereas an increased frequency of this polymorphic variant was found in Iranian patients [10]. The aim of our study was to investigate the potential impact of the CCR5Δ32 polymorphism on the susceptibility to and clinical expression of BD in a cohort of Italian patients.

Materials and methods

Study population

The study comprises 196 BD patients, recruited consecutively, who were followed in nine different Italian referral centres for 8 years (1999–2007). All patients fulfilled the International Study Group for BD (ISG) criteria [11]. The control group consisted of 180 healthy, age- and gender-matched, unrelated blood donors with a mean age of 42 ± 13 years.

All study subjects were Caucasians who had been resident in Italy for at least one generation. There were no ethnic differences between patients and controls. The diagnosis of subcutaneous thrombophlebitis (ST) and deep vein thrombosis (DVT) was based on clinical data and confirmed in all patients by ultrasonography or contrast venography. In most of the patients with erythema nodosum, ultrasonographic examination was performed to help in the differential diagnosis between superficial thrombophlebitis and erythema nodosum. The study was approved by the ethics committees of Reggio Emilia, and written informed consent was obtained from patients and controls before study entry.

HLA class I typing

A standard microlymphocytotoxicity technique was used to serotype HLA class I alleles in peripheral blood lymphocytes. One hundred and ninety-four of the 196 patients were typed for the HLA-B51 allele. The control group consisted of the same 180 healthy unrelated blood donors used for the CCR5Δ32 assays.

Molecular analysis of CCR5Δ32 polymorphism

DNA extraction was performed using a Genomic DNA Purification Kit (Gentra Systems, Inc., Minneapolis, MN, USA) from peripheral white blood cells. Genotyping of CCR5Δ32 polymorphism comprised a one-step PCR method with forward primer 5'-TCTTATTACACCTGCA GCTC-3' and reverse primer 5'-CTCACAGCCCTTGCC TCTTC-3' flanking of the region containing the 32-bp deletion. The PCR products were analysed by 2.5% agarose gel electrophoresis. The normal allele was detected as a 137-bp fragment and the d32 allele was detected as a 105-bp fragment [12]. In each analysis, three samples with known genotype were added; the genotype control samples were confirmed each time, demonstrating that the procedure was highly reproducible.

Statistical analysis

Statistical analysis was done using the SPSS statistical package (SPSS Inc., Chicago, IL, USA; version 14.0, 2006). The frequencies of the alleles and genotypes among the case patients and control group were compared by \( \chi^2 \) test. Fisher’s exact test was used when the minimum expected value was <5. Odds ratios (ORs) were calculated together with their 95% CI. To determine P-values we used asymptotic lookup distribution with continuity correction. The cases and controls were tested for conformity to the Hardy–Weinberg equilibrium using a 2 × 2 \( \chi^2 \) test between observed and expected numbers. To identify potential genotype–phenotype correlates, we compared patients with and without specific clinical manifestations according to CCR5Δ32 carrier status and compared with healthy controls.

The population-attributable risk percentage (PAR%) for the risk genotype (Δ32/Δ32 and CCR5Δ32) was estimated with the following formula:

\[
PAR\% = \frac{Pe(RR - 1)}{[Pe(RR - 1) + 1]},
\]

where Pe represents the risk genotype frequency in the population and RR represents the relative risk of the risk genotype [13]. Given the low prevalence of BD, Pe can be estimated based on the genotype frequencies in healthy controls and RR can be approximated by OR for the risk genotypes. Power testing was performed using PAWE software: at a significance level of 0.05, power was 0.89 for the allelic test and 0.82 for the genotypic test (PAWE; http://linkage.rockefeller.edu/pawe/pawe.cgi).

Results

Table 1 shows the demographic and clinical characteristics of the 196 Italian patients with BD. A total of 50
patients (25.5%) had thrombosis, 35 of whom had DVT of the legs (17.9%) and 20 who had ST (10.25%). Two patients had isolated intracardiac thrombosis, and one had Budd-Chiari syndrome as well as extensive inferior vena cava and leg vein thromboses. None of the patients had arterial involvement. There were no significant differences in the demographic and clinical characteristics of the patients with and without DVT (data not shown).

Populations of controls and cases were tested for Hardy-Weinberg equilibrium: genotype frequencies of all populations did not reject Hardy-Weinberg equilibrium.

The allele and genotype frequencies of the CCR5Δ32 polymorphism in BD patients and in healthy controls are shown in Table 1. The distribution of the CCR5Δ32 genotype differed significantly between BD patients and controls (P = 0.024). The distribution of the genotype in the CCR5Δ32 polymorphism indicated that the differences in allele distribution were related to a higher frequency of CCR5Δ32 heterozygosity in BD patients as compared with controls. The Δ32/Δ32 homozygosity was not observed in either BD patients or in the control group. The CCR5Δ32 allele was significantly more common in BD patients than in controls (P = 0.020; OR 2.28 [95% CI 1.1, 4.8]). Carriers of the CCR5Δ32 allele (Δ32/Δ32 + CCR5Δ32) were significantly more common in the BD females than in the control group (14% vs 5.6%, P = 0.018, OR 2.76 [95% CI 1.2, 6.6]). Carriers of the CCR5Δ32 allele were also more common in BD males than in controls, but the difference was not statistically significant [10.7% vs 5.6%, P = 0.091, OR 2.0 (95% CI 0.8, 5.0)].

The possible associations between the CCR5Δ32 polymorphism and the clinical manifestations of BD shown in Table 1 were evaluated in the 196 BD patients by comparing the frequencies of clinical manifestations between CCR5Δ32 allele carriers and non-carriers; no significant differences were found. ST was more common in the carriers of the CCR5Δ32 allele, but the difference was not statistically significant. HLA-B51 allele frequency was significantly higher in BD patients compared with healthy controls [67.0% vs 18.8%, P = 0.0001, OR 8.72 (95% CI 5.4, 14.0)].

### Discussion

The CCR5 gene has a 32-bp deletion (Δ32) polymorphism in its promoter region resulting in a non-functional CCR5 protein [6]. CCR5Δ32 homozygous subjects do not express the receptor on the cell surface, whereas heterozygotes express lower amounts of the receptor than wild-type homozygotes [6]. In humans, CCR5Δ32 polymorphism has been linked to a wide range of diseases.
For some diseases, CCR5Δ32 carriage appears to be a protective factor, being associated with a reduced risk in some rheumatic disease such as primary SS [14] and RA [7]. In contrast, other studies have mapped CCR5Δ32 to increased susceptibility to or greater severity of inflammatory conditions such as chronic periaortitis [15], granulomatosis with polyangiitis [16] and sarcoidosis [8].

With regard to BD, one study found no association between the CCR5Δ32 polymorphism and BD in British, Turkish or Palestinian patients [9], whereas another study reported an association between CCR5Δ32 and BD in Iranian women, but not in men [10].

In our study, we found a positive association between the CCR5Δ32 allele and BD in a large cohort of Italian patients. In categorizing patients according to gender, the association between CCR5Δ32 polymorphism and BD was similar in females and males (ORs 2.76 and 2.0, respectively). The reason for the discrepancies in the above findings is not entirely clear, but may be related to the sizes and types of the study populations. Sample size is a major issue in many studies investigating polymorphisms, with both type 1 and 2 errors being more likely to occur in smaller study populations. On the other hand, the association between the CCR5Δ32 allele and BD may be a genuine finding, but limited to one or some ethnic groups. Either way, caution is advised when interpreting our results and those from previous studies [9, 10].

We could ask how the CCR5Δ32 allele impacts on the susceptibility to develop BD. In wild-type individuals, CCR5 mediates mononuclear cell recruitment to sites of inflammation by interacting with its ligands CCL3, CCL4 and CCL5. However, these ligands are also able to bind to other receptors. Specifically, CCL3 can also bind to CCR1 and CCR4, CCL4 to CCR1 and CCR8, and CCL5 to CCR1, CCR3 and CCR4 [6]. It has been shown that in the absence of a functional CCR5, its ligands can undergo up-regulation and exert their biological effects by engaging other available receptors. For instance, in nephrotoxic serum nephritis, CCR5-deficient (CCR5−/−) mice express higher intrarenal levels than wild-type strains of CCL3 and CCL5, which appear to correlate with the infiltration of interferon-γ-producing CD4+ Th1 cells in the kidneys [17]. This Th1 response can be abrogated by CCR1 blockade, suggesting that both CCL3 and CCL5 act by engaging CCR1 [17–20]. Similarly, increased CCL4 synthesis has been described in CCR5−/− mice with collagen-induced arthritis [18], whereas in CCR5−/− mice with autoimmune uveoretinitis, overexpression of CCL5 has been linked to augmented intra-ocular production of IL-6 and greater neutrophil infiltration compared with wild-type mice [19]. Furthermore, it has been demonstrated that lymphocytes from CCR5Δ32 homozygous subjects secrete RANTES at levels that are 5–10 times higher than those of control CCR5 homozygous 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 within the inflammatory reactions. These findings are potentially relevant to the pathogenesis of BD, in which both a Th1-driven response and hyperactivation of neutrophils are thought to be crucially involved [3, 4]. Taken together, these data suggest that a genetically determined up-regulation of CCR5 ligands may be implicated in the pathogenesis of vascular inflammation in BD.

A second aim of this study was to determine whether CCR5Δ32 polymorphism might be associated with the clinical expression of BD in our cohort of Italian patients. However, when the frequencies of clinical manifestations were compared between CCR5Δ32 allele carriers and non-carriers, no differences were found, although our study is probably not sufficiently powered to detect significant associations between this CCR5 polymorphism and clinical manifestations.

In conclusion, the results of this study suggest that the CCR5Δ32 polymorphism may increase the susceptibility to BD in Italian patients. Further studies are required to replicate our findings in other populations and to better elucidate the role of chemokines in the inflammatory events leading to vascular injury in BD.

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### Table 2: Frequency of alleles, genotypes and carriage rates of CCR5Δ32 polymorphisms in patients with BD and in controls

<table>
<thead>
<tr>
<th>Variable</th>
<th>BD (n = 196)</th>
<th>Controls (n = 180)</th>
<th>P</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ32</td>
<td>24/392 (6.1)</td>
<td>10/360 (2.8)</td>
<td>0.020</td>
<td>2.28 (1.1, 4.8)</td>
</tr>
<tr>
<td>CCR5</td>
<td>368/392 (93.9)</td>
<td>350/360 (97.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ32/Δ32</td>
<td>0/196</td>
<td>0/180</td>
<td>0.024</td>
<td></td>
</tr>
<tr>
<td>CCR5/Δ32</td>
<td>24/196 (12.2)</td>
<td>10/180 (5.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR5/CCR5</td>
<td>172/196 (87.8)</td>
<td>170/180 (94.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carriage rate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ32/Δ32 + CCR5/Δ32</td>
<td>24/196 (12.2)</td>
<td>10/180 (5.6)</td>
<td>0.024</td>
<td>2.37 (1.1, 5.1)</td>
</tr>
<tr>
<td>CCR5/CCR5</td>
<td>172/196 (87.8)</td>
<td>170/180 (94.4)</td>
<td></td>
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</table>

Values are the number/total number examined (%).
The CCR5-D32 polymorphism may be associated with an increased risk of developing Behçet’s disease (BD) in the Italian population.

An association of CCR5-D32 with subcutaneous thrombophlebitis in patients with BD has been found but was not statistically significant.

Chemokines may have a role in the pathophysiology of BD.

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References