SELPLG and SELP single-nucleotide polymorphisms in multiple sclerosis

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Abstract

P-Selectin (SELP) and P-selectin glycoprotein ligand-1 (SELPLG) constitute a receptor/ligand complex involved in the recruitment of activated lymphocytes, a critical event in the pathogenesis of multiple sclerosis (MS). In order to determine whether genetic variation in these pivotal molecules influences susceptibility to MS, we genotyped 214 Italian patients compared with 220 Italian controls for three single-nucleotide polymorphisms (SNPs): SELPLG Met62Ile, SELP C–2123G and SELP Thr715Pro. No significant differences in both SELP SNPs were found between patients and controls, whereas a decreased frequency of the Met62Ile SNP was found in patients versus controls in the Italian population (P = 0.025). To confirm these preliminary findings, the Met62Ile SNP was analysed in 938 UK trio families. This SNP did not show evidence for association with susceptibility to MS in the larger UK cohort. Therefore, none of the SNPs investigated is associated with MS, although this analysis does not conclusively exclude SELPLG and SELP as genetic risk factors for MS as much variation remains untested.

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Multiple sclerosis (MS) is a multifactorial disease with a complex aetiology in which unknown environmental factors trigger the disease in genetically susceptible individuals [6]. It is known that autoimmunity plays a major role in the disease pathogenesis and that the recruitment of activated lymphocytes across endothelial cells in the blood–brain barrier is a critical step in this process. Molecular mechanisms leading to migration of autoreactive T-cells from the bloodstream involve a series of sequential and overlapping interactions among different families of adhesion molecules. Among these, selectins and their counterligands deserve particular attention since E- and P-selectins (SELE and SELP)-dependent tethering and rolling of leukocytes under flow is the earliest observable event in the recruitment of leukocytes into inflamed tissues. SELP is a cellular adhesion molecule belonging to the lectin family which is mainly expressed by platelets and endothelial cells and plays a major role in initial phases of leukocytes adhesion to the endothelium and in the interaction between leukocytes and platelets [22]. The best-characterized selectin ligand is P-selectin glycoprotein ligand-1 (SELPLG). It is a dimeric mucin-like glycoprotein expressed on leukocyte surface, which supports SELP-dependent rolling in vitro and in vivo [11]. SELP and SELPLG are involved in the development of atherosclerosis and its complications as well as in cardiovascular artery disease [3,24] and are likely to be involved in various inflammatory processes such as MS. In this regard, Piccio et al. [19] showed that anti-PSGL-1 and anti-SELE and -SELPLG antibodies block tethering and rolling of autoreactive lymphocytes in inflamed brain vessels in experimental autoimmune encephalomyelitis, the animal model of MS. In addition, Battistini et al. [2] showed a critical role for SELPLG in the recruitment of CD8+ cells in brain vessels of patients with MS during acute attacks. The strategic role of cell–cell interaction in the pathogenesis of MS immediately suggests that genetic variations in genes involved could influence susceptibility to the disease.

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particular, recent findings demonstrate that the ITT genotype of the G967 SNP in SELE confers an increased risk to develop MS, whereas the A561C SNP is likely to act as protective factor towards the progression to Secondary Progressive (SP)-MS [8].

The C-212G variant in the distal promoter of SELP has previously been reported to be associated with myocardial infarction [1] and cardiovascular risk factors [4]. The SELP gene is located on chromosome 1q21–q24 and consists of 17 exons, all of which encode distinct structural domains. Molecular screening led to the identification of several polymorphisms including Thr715Pro in exon 13 which is believed to influence the binding of a number of transcription factors, in accordance to McDonald’s criteria [15]. The control group consisted of 220 subjects matched for ethnic background, gender and age (Table 1).

A decreased frequency of the Met62Ile SNP in Italian MS patients compared with controls was observed (8.5% vs. 13.6%, Table 1). DNA was isolated from peripheral blood leukocytes by standard procedures. The amount of DNA for each sample was determined by measuring the optical density at 260 nm wave-length using a spectophotometer (Eppendorf AG, Germany). DNA samples were stored at −20°C.

Genotyping was first carried out in the Italian case-control cohort by Polymerase Chain Reaction (PCR)-Restriction Fragment Length Polymorphism analysis (RFLP). Each PCR was carried out for all SNPs analysed in 25 μl total volume containing 25 ng genomic DNA, 1 μM of each specific primers, 0.06 μM of each dATP, dTTP, dCTP and dGTP, 1.5 U Taq DNA polymerase (Applied Biosystems, ABI). Thermocycling consisted of 5 min at 94°C, 30 s and 72°C for 30 s, 5 min for a final extension step of 72°C for 10 min. For Thr715Pro and C-212G variants determination, Psfl and BstEII restriction enzymes were used, according to the method of Carter et al. [4]. For the Met62IIe SNP, a novel Rsfl analysis was developed. Two primers were used to amplify a region of 174bp, containing the SNP. A mutation was introduced by a primer mismatch to create a restriction cut site for Rsfl in the forward primer: 5′-CCT GCC AGA AAC GGA GCC TTC AGA GTG GCC AGC TCC AGT-3′; reverse: 5′-TTC CCC AGT TGC AGC AGC AGC TCC GT-3′. The restriction site was determined using the Webcutter software program available from the web (Webcutter 2.0, http://www.firstmarket.com/cutter/cut2.html). PCR product (10 μl) was digested with 3 U of Rsfl (New England Biolabs, Beverly, MA, USA) overnight at 37°C. Digestion yields two fragments (147 and 27 bp) visualized on a 3.5% agarose gel stained with ethidium bromide (Fig. 1). The novel RFLP assay for detecting the Met62IIe SNP was validated by direct sequencing in 50 subjects. To this aim, a 530 bp fragment of exon 2 was amplified using specific primers (5′-CGT GGG CAC TGG TTG A-3′; reverse: 5′-CGT GGG CAC TGG TTG AGT GG-3′). The fragment was purified using ExoSAP-IT® Kit (usb, USA) and then direct sequence was performed with an ABI PRISM 3100 gene analyser (ABI).

Allelic and genotypic frequencies were obtained by direct counting. Hardy–Weinberg equilibrium was tested using a χ2 goodness-of-fit test. Fisher’s exact test was used for differences in allele frequency distribution. The odds ratio was calculated according to McDonald’s criteria [15]. An informed consent to participate in this study was given by all individuals.

Mean age at onset ± S.D., years (range) 32.1 ± 10.6 (13–67) 39.1 ± 9.5 (26–57) 38.5 ± 11.1 (27–51)
Fig. 1. SELPLG polymorphism determination by PCR-RFLP. Amplification fragment: 174 bp; forward primer introduces a mismatch which creates a restriction site for \( Rsa \)\(_1\) when mutation occurs, yielding 27 and 147 bp fragments. Lane 1: DNA weight marker VIII (Boehringer-Roche); lane 2: GG; lane 3: GA; lane 4: AA.

\( P = 0.025, \text{OR: 0.56, CI: 0.35–0.90; Table 2} \), whereas no differences were observed for both SELP SNPs (Table 2). Stratifying patients according to the course of the disease, no differences were found. Allelic and genotypic frequencies were not influenced by gender as well.

Given the borderline significance obtained for the Met62Ile SNP, it was further tested in the larger UK population of trio families by using the Taqman methodology. Each Taqman 5′-nuclease assay employed 25 ng of genomic DNA as template. The Assay-on-demand product (ABI assay ID: C1617099310) was used for genotyping the SELPLG variant (NCBI ID: rs2228315). Assays were performed in 4/9262 reactions in 384-well plates using an ABI PRISM \textsuperscript{®}7900HT instrument (ABI). Thermocycling consisted of 10 min at 95°C for AmpliTaqGold activation, and 40 cycles of 95°C for 15 s and 60°C for 1 min.

The pedigree check revealed that no Mendelian inconsistencies or duplicate typing errors occurred and genotype frequencies were found to be in Hardy–Weinberg equilibrium. The Met62Ile SNP did not show an association with MS on testing (Table 3).

This study represents the first attempt to screen a MS population for the genetic variant Met62Ile in SELPG as well as for Thr715Pro and C-2123G SNPs in SELP. A number of polymorphisms in these genes have been reported so far, although only the above-mentioned allelic variants are considered to have a potential functional importance.

All these SNPs influence the transcription rate of the respective protein [1,4,17,24] and have been associated with coronary heart disease and myocardial infarction [3,10]. Moreover, these genetic variants are located in regions previously considered as potential susceptibility loci to autoimmune diseases, for example the SELP gene lies on chromosome 1q23.2, a region that has been reported as a potential locus for type 2 diabetes susceptibility in a number of studies [7,26]. Similarly, the SELPLG marker falls in a region previously identified in various autoimmune disease linkage scans [9,14,23,27].

So far, association studies with candidate genes have been widely used for the study of complex diseases; however, this approach has been criticized because results are rarely replicated. To avoid false positive conclusions we employed two different study cohorts. Moreover, different technical approaches

### Table 3

<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphism</th>
<th>Heterozygosity (%)</th>
<th>Genotyping success rate</th>
<th>Minor allele frequency</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SELPLG</td>
<td>Met62Ile</td>
<td>10.2</td>
<td>10.3</td>
<td>99</td>
<td>6</td>
</tr>
</tbody>
</table>

Values are expressed as % (n). OR: 0.56, CI: 0.35–0.90, patients vs. controls. \( P=0.025 \).
were applied, including sequencing, PCR-RFLP and allelic discrimination by TaqMan system. Notably, a new PCR-RFLP protocol to easily detect the Met62Ile SELPLG variant was successfully developed, greatly decreasing chances of detecting mutations other than the Met62Ile. In the Italian population-based sample of affected and unaffected individuals (the case-control study), none of the variants analysed in the SELP gene revealed association with MS. On the contrary, a decreased frequency of the Met62Ile variant was observed in MS patients (Table 2), suggesting a possible protective role of the Met62 variant. Nevertheless, at this stage no definitive conclusions could be drawn. As the trend was not significant because of the small sample size, the required statistical power was not reached due to a type 1 error. Therefore, to better clarify these preliminary findings, a larger association study based on 938 UK trio families (an index patient and both parents) was performed on the Met62Ile SNP. The apparent trend was not replicated, as this SNP did not show evidence for association with susceptibility to MS in UK population. However, other polymorphisms in these genes need to be explored to conclusively exclude SELP and SELPLG as potential factors in genetic predisposition to MS. Moreover, the possible functional role of these variants should be investigated since the Met62Ile variant is located at the border of the binding region of SELPLG to SELP [11] and it is possible that the mutated allele modifies the affinity of SELPLG to SELP, influencing the amount of activated lymphocytes entering into the CNS. Besides, the Thr715Pro variant for SELP might lead to conformational changes in the molecule, resulting in an alteration of its function. This hypothesis is supported by studies conducted both in vivo and in vitro by Maland et al. [13], demonstrating that the application of a monoclonal antibody against the SELP’s domain in which the Thr715Pro variant occurs, gives rise to reduced adhesion activity, therefore, indicating that it might play a functional role in SELP leukocyte interactions.

In conclusion, although we failed to find any evidence for association of MS with SELP and SELPLG variants considered, further studies focused on other variation influencing functional activity in the recruitment of lymphocytes need to be further performed before these genes can be confidently excluded as candidate susceptibility loci.

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