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The association of V249I and T280M fractalkine receptor haplotypes with disease course of multiple sclerosis

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ABSTRACT

We investigated the association of CX3CR1 genotypes/haplotypes with MS and performed the prediction analysis of protein sequence variants' effects on CX3CL1/CX3CR1 interaction. We found no association of CX3CR1 with MS susceptibility. Frequency of $I_{249}T_{280}$ haplotype was significantly lower in SP compared to RR patients (RR > 10 years, OR = 0.30, 95%CI = 0.11–0.79, p = 0.01; OR = 0.53, 95%CI = 0.18–1.56, p = 0.2, in SP < 10 years vs. RR > 10 years). Prediction analysis showed that I249 T280 protein variant would significantly affect CX3CL1/CX3CR1 interaction. Our results suggest that CX3CR1 $I_{249}T_{280}$ haplotype could have protective effect for switch to SP MS. Further research is warranted to validate and replicate currently observed results.

1. Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS). The disease is characterized by inflammation, demyelination and axonal injury, leading to formation of sclerotic plaques (Compston and Coles, 2002). It is thought that initial recruitment and extravasation of systemic inflammatory cells into the CNS drives the subsequent development of the lesions (Piccio et al., 2002).

Chemokines and their receptors play an important role in molecular mechanisms of inflammation. Fractalkine (CX3CL1) is a unique CX3C chemokine, which is synthesized as a transmembrane molecule, while soluble form of CX3CL1 can be released from the cell surface by proteolysis (Bazan et al., 1997; Imai et al., 1997). Therefore, CX3CL1 displays properties of both chemokines and adhesion molecules, acting as a chemoattractant for cells implicated as the most important in inflammation. Fractalkine is produced by endothelial cells activated by proinflammatory cytokines (Umehara et al., 2004). Unlike other chemokines, CX3CL1 interacts with a single receptor. Fractalkine receptor, CX3CR1, is a 7-transmembrane-domain G-protein-coupled receptor expressed on the surface of multiple cell types including monocytes, T-lymphocytes, natural killer cells, vascular endothelial cells (Bazan et al., 1997) as well as astrocytes and microglial cells (Hulshof et al., 2003).

Studies of chemokine receptors in MS have shown that their expression may be used for immunologic staging of MS: relapsing–remitting (RR) and secondary progressive (SP) disease (Balashov et al., 1999; Infante-Duarte et al., 2005). The constitutive and regulated expression of CX3CL1 and its receptor, CX3CR1, by neurons/astrocytes and microglia, has been shown within the normal and inflamed rat brain (Sunnemark et al., 2005). Significantly different CX3CR1 expression in leukocytes of MS patients compared to healthy individuals has been demonstrated (Infante-Duarte et al., 2005). Additionally, a correlation between disease activity and frequency of CX3CR1-positive natural killer cells in relapsing–remitting MS patients has also been revealed (Infante-Duarte et al., 2005). These findings suggest the role of CX3CR1 in development of MS as well as in disease course.

Two common single nucleotide polymorphisms (SNP), V249I (rs3732379) and T280M (rs3732378), were identified in CX3CR1 coding sequence (Faure et al., 2000) and located in the sixth and seventh transmembrane domains of the CX3CR1 protein, respectively. It was implicated that they alter fractalkine-receptor binding affinity (McDermott et al., 2003) as well as expression level of CX3CR1 (Chan et al., 2005). These SNPs are in strong linkage disequilibrium (Faure et al., 2000), forming three common haplotypes: $V_{249}T_{280}$ (wild-type), $I_{249}T_{280}$, and $I_{249}M_{280}$ containing both rare alleles. Their association with inflammatory diseases including Crohn's disease (Sabate et al.,

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2008), atherosclerosis and coronary artery disease (McDermott et al., 2001; Moatti et al., 2001), has been demonstrated. The $I_{249}M_{280}$ haplotype was associated with the decreased endothelial reactivity (Faure et al., 2000; Daoudi et al., 2004) and its protective roles in atherosclerosis and acute coronary events have been suggested (McDermott et al., 2001; Moatti et al., 2001; Apostolakis et al., 2009).

Although the roles of CX3CR1 were indicated in development of MS and disease activity, the association of CX3CR1 gene polymorphisms with MS susceptibility has been examined recently in a single GWA (genome wide association) study (IMSGC&WTCCC2, 2011). The aim of this study was to investigate if CX3CR1 V249I and T280M genotypes and haplotypes are associated with MS susceptibility and course of the disease. We also performed the analysis of CX3CR1 protein sequence variations to examine the possible effects of these two non-synonymous polymorphisms on CX3CL1/CX3CR1 protein interaction efficiency.

2. Materials and methods

2.1. Subjects

Three hundred and ninety seven (397) unrelated patients with relapsing–remitting (RR), secondary progressive (SP) and primary progressive (PP) multiple sclerosis, of Serbian origin, were recruited from the Neurology Clinic of the Military Medical Academy (MMA), Serbia. We had the study power of 80% to reveal the association of both CX3CR1 polymorphisms' rare alleles, either separately or in haplotype, with an OR = 0.5 at the significance level of 0.05. We based the study power calculation on previously reported associations of the rare alleles of the V249I and T280M polymorphisms in CX3CR1 with the atherosclerosis, the disease characterized by chronic inflammation (McDermott et al., 2001; Moatti et al., 2001). All patients fulfilled the criteria for clinically definite MS (Polman et al., 2011) and the course of the disease was determined based on clinical data (Lublin and Reingold, 1996). Disease severity was estimated using the Multiple Sclerosis Severity Score (MSSS) (Roxburgh et al., 2005), which represents the Expanded Disability Status Scale (EDSS) (Kurtzke, 1983) corrected for disease duration. Global MSSS values were calculated according to clinical data at the same time when the blood samples for genetic analysis were taken. The patients were not on immunomodulatory treatment at the time of MSSS estimation.

The patient group consisted of 249 females and 148 males, of mean age of 41.5 ± 9.9 years and mean disease onset age of 33.8 ± 9.0 years (Table 1). The control group consisted of 147 female and 131 male healthy volunteers from the MMA staff of mean age of 40.9 ± 15.0 years (Table 1). They were of the same ethnical origin as the MS patients.

The Ethical Committee of the MMA approved this study. Each participant gave written informed consent to participate in the study.

2.2. Determination of genotypes

Genomic DNA was isolated from whole peripheral blood samples collected with EDTA, using the ABI PRISM™ 6100 Nucleic Acid PrepStation DNA BloodPrep™ kit (Applied Biosystems, Foster City, CA).

Table 2
Lengths of the digestion products for genotyping of CX3CR1 V249I and T280M polymorphisms by PIRA-PCR RFLP method.

Genotypes	RFLP products
II MM	294 bp
II TM	294, 270 bp
II TT	270 bp
VV TT	175, 95 bp
VV TM	175, 119, 95 bp
VV MM	175, 119 bp (rare)
VI MM	294, 175, 119 bp
VI TT	270, 175, 95 bp
VI TM	294, 270, 175, 119, 95 bp

In most studies until now, genotyping of V249I and T280M polymorphisms in CX3CR1 gene was performed by separate PCR-RFLP analyses (McDermott et al., 2001; Moatti et al., 2001). Our group developed a new and effective Primer Introduced Restriction Analysis PCR (PIRA-PCR) based RFLP method, with use of a single restriction enzyme, *Tai*I (*Mae*II), for genotyping of both investigated polymorphisms. Naturally occurring restriction site for *Tai*I (*Mae*II) enzyme, ACGT↓, corresponds to V in codon 249. We introduced de novo restriction site for *Tai*I in sequence coding for T in codon 280, in order to perform restriction analysis for both polymorphisms using the same enzyme. The forward primer sequence was: 5′GCAATGTGGAAACAAATTTTCTTGGCTT3′ and the reverse primer sequence with designed mismatch (underlined nucleotide at 3′ end) was: 5′TCAGGCAACAATGGCTAAATGCAA**A**C3′. Optimal PCR mix contained: PCR buffer (750 mM Tris–HCl, pH 8.0), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 12.5 pmol of each primer, 0.5 U Taq polymerase (Fermentas, Lithuania) and 200 ng of template DNA, in a final reaction volume of 25 μL. PCR temperature conditions were: 95 °C 5 min of initial denaturation, followed by 30 cycles of denaturation at 95 °C 1 min, hybridization at 58 °C 1 min and elongation at 72 °C 1 min, and final extension at 72 °C 2 min. The PCR product, whose length was 294 base pairs (bp) (visualized by 2% agarose gel electrophoresis), underwent incubation with 5U *Tai*I restriction endonuclease, at 65 °C, overnight. The length of restriction products for genotyping of both V249I and T280M polymorphisms, which were visualized by 8% PAA electrophoresis and silver-staining, are shown in Table 2.

Genotyping of V249I and T280M polymorphisms by PIRA-PCR based RFLP method was validated with a previously known standard technique (Moatti et al., 2001) and concordance was 100%.

2.3. Statistical analysis

Statistical analysis of alleles and genotypes was performed using the Statistica software (v5.0, Stat Soft Inc., 1997). In all tests performed, differences with two-tailed alpha-probability (p)<0.05 were considered significant. Differences in allele and genotype frequency distribution between the studied groups as well as deviation from Hardy–Weinberg equilibrium were estimated by chi-square (χ^2) test. Relation between the genotypes and continuous variables was tested by analysis of variance (ANOVA).

Table 1
Characteristics of MS patients and controls.

Parameter	RR (n = 319)	SP (n = 66)	PP (n = 12)	RR + SP (n = 385)	Controls (n = 278)
Gender (female/male)	204/115	39/27	6/6	243/142	147/131
Age (years)	36.3 ± 9.5	42.5 ± 11.0	45.8 ± 9.3	37.6 ± 10.1	40.9 ± 15.0
Disease onset age (years)	29.3 ± 8.7	29.7 ± 8.7	42.3 ± 9.6	29.5 ± 8.7	–
Disease duration (years)	7.1 ± 5.5	12.8 ± 7.2	3.5 ± 2.5	10.0 ± 6.2	–
MSSS	4.3 ± 2.3	7.0 ± 2.3	7.0 ± 2.0	5.6 ± 2.5	–

Values are expressed as means ± SD; MS patients: RR — relapsing–remitting, SP — secondary progressive, and PP — primary progressive.