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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.

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The JLIN software (v1.6.0) (Carter et al., 2006) was used for linkage disequilibrium calculation, and the Thesias software (v3.1) (Tregouet and Garelle, 2007) for haplotype analysis. Haplotype frequencies were estimated using the SEM algorithm (Tregouet et al., 2004). The analysis of haplotype effects was performed taking the most frequent haplotype as a reference and comparing all other haplotypes with the reference simultaneously, according to additive model. The binary outcomes of the haplotype–phenotype association analysis were absence/presence of MS and relapsing–remitting/secondary progressive disease course. Association of haplotypes with the binary phenotypes was presented as odds ratio (OR) and its 95% confidence interval (CI).

Association of haplotypes with disease course was additionally estimated in the subgroups of patients with relatively slow progression (RR patients whose disease duration was >10 years (14.24 ± 4.94, mean ± SD) vs. all SP patients and vs. those with fast switch to secondary progressive form of the disease (patients who developed SP form within <10 years). In that way more precise modifying effect of haplotypes on switch of the disease course should be observed.

Statistical power of the study for the analyzed SNPs and haplotypes was calculated using the PS (v3.0.43) (Dupont and Plummer, 1990) and PGA (Menashe et al., 2008) software, respectively.

2.4. Informational spectrum method (ISM) analysis

Prediction of the effects that CX3CR1 sequence variations (V249I and T280M) could have on CX3CR1–CX3CL1 interaction was done by the informational spectrum method (ISM) as an established sequence analysis method (Veljkovic et al., 2007). In order to create an informational spectrum (IS) for each of the analyzed proteins, the amino-acid sequence was transformed into a numerical sequence. Each amino acid was represented by the value of electron–ion interaction potential (EIIP) corresponding to the average energy states of all valence electrons in a particular amino acid. The EIIP values for each amino acid were calculated using the general model of pseudopotential: $\langle k+q|w|k\rangle = 0.25Z \sin(\pi 1.04Z)/(2\pi)$ where q is a change of momentum of the delocalized electron in the interaction with potential w, and $Z = (\sum Zi)/N$ where Zi is the number of valence electrons of the i-th atom of each amino acid and N is the total number of atoms in the amino acid.

Using discrete Fourier transform (DFT), the numerical sequence was then transformed into the frequency domain to create an IS. DFT is defined as: $X(n) = \sum x(m)e^{-j(2/N)nm}$, n = 1, 2,..., N/2, where x(m) is the m-th member of a given numerical series, N is the total number of points in this series, and X(n) represents the DFT coefficients. DFT coefficients describe the amplitude, phase and frequency of sinusoids of the original signals. Absolute value of complex Fourier transform defines the amplitude spectrum and the phase spectrum. Complete information about the original sequence is contained in both spectral functions. Given this was a protein analysis, the relevant information was provided by the energy density spectrum defined as: $S(n) = X(n)X(n) = |X(n)|^2$, n = 1, 2,..., N/2. In this way, the individual sequences were considered discrete signals. Since the average distance between amino-acid residues in a polypeptide chain is 3.8°A, it is assumed that the points into each derived series are equidistant. Therefore, the distance (d) has been arbitrarily set to 1, so the maximum frequency in the spectrum has been F = 1/2 d = 0.5. In order to distinguish common spectral characteristics of two sequences, mathematical filtering was done by multiplying the conjugate complex Fourier transform by the Fourier transform of the target signal: C(n) = S1(n)S 2(n), n = 1, 2,..., N/2. The result of multiplication is cross-spectral (CS) density function. The prominent peak in CS function denotes a common frequency component of the explored proteins. According to the ISM concept, the amino-acid sequence variations affecting the amplitude at that characteristic frequency will affect the efficiency of the protein–protein interaction. The numerical series derived from analyzed sequences are normalized to zero mean and zero padded, to produce a vector equal in length to the smallest power of 2 greater than (or equal to) the largest domain in the data set. Padding with zeros is itself a standard practice in Fourier analysis when using a discrete fast Fourier transform-based algorithm, as the length of the input patterns are required to be a power of two. The zero padding induces an increase of the spectrum resolution without influencing the characteristic frequency.

3. Results

3.1. Controls and MS patients

Description of the study participants is shown in Table 1.

3.2. Genotypes and alleles of the CX3CR1 polymorphisms in controls and MS patients

Strong linkage disequilibrium was found between the V249I and T280M polymorphisms, with D′ values equal to 0.96 and 0.97 in controls and patients, respectively. Genotype and allele frequencies of CX3CR1 V249I and T280M gene polymorphisms in controls and MS patients are shown in Table 3. There were no deviations from Hardy–Weinberg equilibrium for either of the two polymorphisms. The genotype and allele frequencies of V249I and T280M polymorphisms were not significantly different between control subjects and patients (Table 3). We also checked for the genotype distribution according to gender and found no differences in females compared to males, neither in controls nor in patients (results not shown). Analysis of association of these two polymorphisms with the course of disease showed higher frequencies of I249 allele in RR patients compared to SP patients (I = 0.33 vs. I = 0.24; OR = 0.65, 95%CI = 0.42–1.00, p = 0.05; respectively) (Table 3). With a cut-off of disease duration to >10 years in the RR group, and <10 years in SP similar trend in association of I allele with course of disease was found (I = 0.34 vs. I = 0.25; OR = 0.64, 95%CI = 0.33–1.24, p = 0.2).

Table 3
Genotype and allele frequencies of CX3CR1 V249I and T280M gene polymorphisms in MS patients and controls.

Genotypes	Controls n (%)	Patients RR + SP n (%)	OR (±95%CI)	p (χ^2)	RR n (%)	SP n (%)	OR (±95%CI)	p (χ^2)
V249I	278	385			319	66		
VV	134 (48.2)	174 (45.2)	Reference		144 (45.3)	36 (54.0)	Reference	
VI	125 (45.0)	176 (45.7)	1.08 (0.78–1.49)	0.65	140 (43.8)	28 (42.9)	0.80 (0.46–1.38)	0.42
II	19 (6.8)	35 (9.1)	1.42 (0.78–2.59)	0.25	35 (10.9)	2 (3.1)	0.23 (0.05–0.99)	0.05
I allele	0.29	0.32	1.14 (0.89–1.46)	0.30	0.33	0.24	0.65 (0.42–1.00)	0.05
T280M								
TT	186 (66.9)	239 (62.1)	Reference	0.16	201(62.9)	42 (63.5)	Reference	
TM	78 (28.1)	129 (33.5)	1.28 (0.91–1.79)	0.88	102 (31.6)	23 (34.9)	1.08 (0.61–1.89)	0.79
MM	14 (5.0)	17 (4.4)	0.94 (0.45–1.97)	0.25	16 (5.5)	1 (1.6)	0.30 (0.04–2.32)	
M allele	0.19	0.21	1.13 (0.86–1.48)	0.37	0.21	0.19	0.88 (0.55–1.41)	0.60

RR — relapsing–remitting, SP — secondary progressive, OR — odds ratio, and CI — confidence interval.

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Table 4
Estimation of haplotype effects of CX3CR1 V249I and T280M gene polymorphisms on susceptibility to and course of MS.

Haplotypes	Controls n (%) 278	Patients RR + SP n (%) 385	OR (±95%CI)	p (χ^2)	RR n (%) 319	SP n (%) 66	OR (±95%CI)	p (χ^2)
$V_{249}T_{280}$	195 (70.09)	260 (67.61)	Reference		213 (66.74)	49 (74.96)	Reference	
$I_{249}M_{280}$	50 (18.48)	80 (20.73)	1.16 (0.88–1.54)	0.29	65 (20.28)	13 (19.49)	0.85 (0.50–1.44)	0.54
$I_{249}T_{280}$	31 (10.84)	43 (11.22)	1.09 (0.76–1.58)	0.64	39 (12.37)	4 (5.55)	0.39 (0.17–0.92)	0.03
$V_{249}M_{280}$	2 (0.59)	2 (0.44)	–	–	2 (0.61)	0 (0.00)	–	–

RR — relapsing–remitting, SP — secondary progressive, OR — odds ratio, and CI — confidence interval.

3.3. Genotypes and clinical parameters of MS

There were no significant effects of V249I and T280M genotypes on age at onset, disease duration or MSSS (results not shown).

3.4. Haplotypes in controls and MS patients

Three predominant haplotypes were inferred: $V_{249}T_{280}$, $I_{249}M_{280}$, $I_{249}T_{280}$, and the rare, $V_{249}M_{280}$. The $V_{249}M_{280}$ haplotype was not considered to be of potential clinical significance in this study, due to its low frequency. Haplotype frequencies in controls and MS patients are represented in Table 4.

The effects of $I_{249}M_{280}$ and $I_{249}T_{280}$ haplotypes on MS susceptibility and secondary progression of the disease were estimated, taking the most frequent, $V_{249}T_{280,}$ haplotype as referent. There were no associations of the CX3CR1 haplotypes with MS susceptibility (Table 4). Association of the haplotypes with MS course was observed, showing significantly higher frequency of $I_{249}T_{280}$ in RR patients compared to SP patients ($I_{249}T_{280}$ = 12.37% vs. $I_{249}T_{280}$ = 5.55%; OR = 0.39, 95%CI = 0.17–0.92, p = 0.03, respectively). Additional analysis of haplotypes in the patient subgroup including only RR patients whose disease duration was >10 years also resulted in significantly higher frequency of $I_{249}T_{280}$ in RR patients (OR = 0.30, 95%CI = 0.11–0.79, p = 0.01, respectively) when compared to all SP patients. The most stringent comparison (RR > 10 years vs. SP < 10 years) also showed higher frequency of $I_{249}T_{280}$, but without significant difference between groups (OR = 0.53, 95%CI = 0.18–1.56, p = 0.25) (Table 5.). However, haplotype background analysis showed that I249 allele had significant protective effect on disease course switch to SP, when it is in haplotype with T280 allele compared to V249 allele ($V_{249}T_{280}$ vs. $I_{249}T_{280}$, OR = 0.6, 95%CI = 0.58–0.64, p = 0.000). All haplotype effects' OR values were subsequently adjusted to gender as a covariate. No significant differences were observed between adjusted OR and initial crude OR values (results not shown).

The allele/genotype/haplotype frequencies and their effects were not estimated in PP patients, due to low number of participants in this patient group.

3.5. Informational spectrum method (ISM) analysis of CX3CR1–CX3CL1 interaction

Fourier frequency characteristic for interaction between CX3CR1 and its ligand, CX3CL1, was identified. For that purpose, the IS of both proteins was calculated (Fig. 1a and b) and, by cross-spectral filtering, the frequency of 0.037 was found to be characteristic for their interaction (Fig. 1c). Further, we determined the amplitudes at characteristic Fourier frequency (0.037) for 4 sequence variants of CX3CR1 protein, corresponding to 4 estimated haplotypes (Fig. 1d). We found that ΔA (0.037) of I249 M280 variant was almost equal to the common isoform's, V249T280, (ΔA ~ 0.00). The ΔA (0.037) values of two other sequence variants, I249 T280 and V249M280, differed significantly in comparison to V249T280. The most prominent amplitude deviation was detected for CX3CR1 I249 T280 variant (ΔA = −0.47%) (Fig. 1d).

4. Discussion

Two common CX3CR1 gene polymorphisms, V249I and T280M, have been associated with chronic inflammatory diseases (McDermott et al., 2001; Moatti et al., 2001; Sabate et al., 2008), but their potential roles as genetic risk factors for MS susceptibility has been studied recently in a single GWA study (IMSGC&WTCCC2, 2011). Similar to this study, we did not find an association of either genotypes or alleles of these two CX3CR1 polymorphisms with MS susceptibility. Results of the studies that investigated these two polymorphisms in susceptibility to other inflammatory diseases have been inconsistent. Some have reported positive, while others found no association (McDermott et al., 2001; Moatti et al., 2001; Ghilardi et al., 2004; Norata et al., 2006; Apostolakis et al., 2007; Apostolakis et al., 2009; Kimouli et al., 2009).

In Serbian population, strong linkage disequilibrium was found between V249I and T280M polymorphisms and that was in accordance with the previous population studies' results (Faure et al., 2000). According to HapMap data, V249I and T280M are located in a unique LD block. Regions of strong LD could facilitate the discovery of genes related to common diseases through association studies. Aside from genotype analysis, CX3CR1 haplotypes were inferred in order to increase the power for detection of the polymorphisms' effects on susceptibility to MS and course of the disease. None of the haplotypes showed any significant impact on MS susceptibility. Our result is concordant with the result of haplotype analysis in the study of coronary artery disease (CAD) (Matzhold et al., 2009). In contrast, haplotype CAD meta-analysis revealed a significant predominance of $I_{249}M_{280}$ haplotype in controls compared to CAD patients (Apostolakis et al., 2009). In another study, only the individuals carrying $I_{249}M_{280}$ haplotype were at decreased risk of recurrent headaches, which are known to be caused by neuroinflammation and impaired vascular activity (Combadière et al., 2008).

Table 5
Estimation of haplotype effects of CX3CR1 V249I and T280M gene polymorphisms on course of MS with stringent selection of patients with relatively slow vs. fast switch to secondary progressive form of disease.

Haplotypes	RR n (%)	SP n (%)	OR (±95%CI)	p (χ^2)	RR n (%)	SP n (%)	OR (±95%CI)	p (χ^2)
	RR > 10 yrs, n = 70	all SP, n = 66			RR > 10 yrs, n = 70	SP < 10 yrs, n = 32		
$V_{249}T_{280}$	46 (65.72)	49 (74.96)	reference		46 (65.72)	24 (75.00)	reference	
$I_{249}M_{280}$	14 (20.71)	13 (19.49)	0.74 (0.39–1.41)	0.37	14 (20.71)	5 (15.63)	0.65 (0.29–1.46)	0.30
$I_{249}T_{280}$	10 (13.57)	4 (5.55)	0.30 (0.11–0.79)	0.01	10 (13.57)	3 (9.37)	0.53 (0.18–1.56)	0.25
$V_{249}M_{280}$	0 (0.00)	0 (0.00)	–	–	0 (0.00)	0 (0.00)	–	–

Left: selected RR patients with disease duration of > 10 years vs. all SP patients; right: selected RR patients with disease duration of > 10 years vs. SP patients with disease duration of < 10 years, RR — relapsing–remitting, SP — secondary progressive, OR — odds ratio, and CI — confidence interval.

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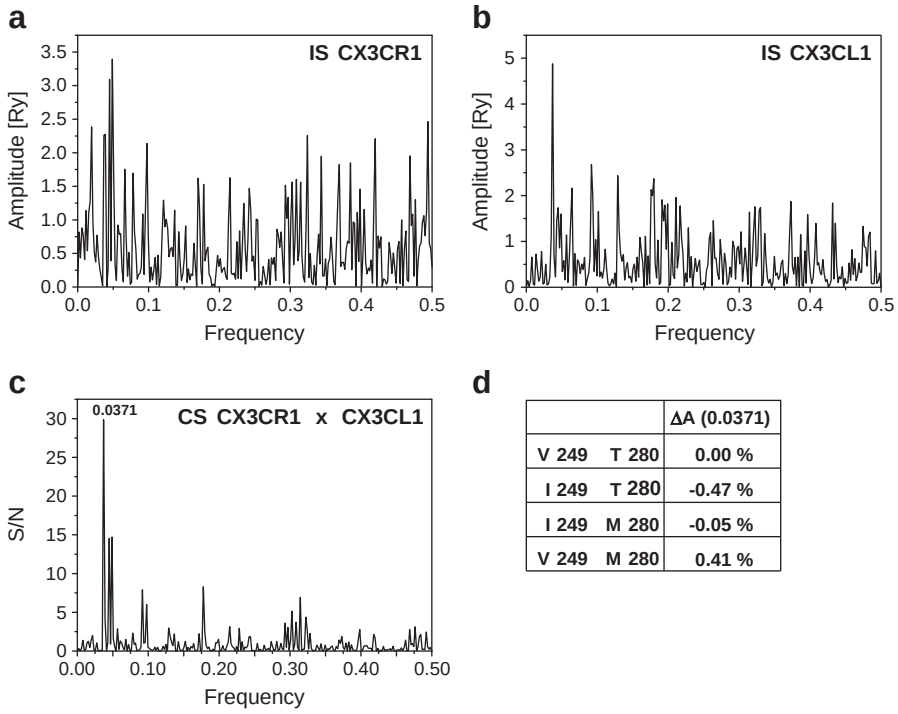

	ΔA (0.0371)
V 249 T 280	0.00 %
I 249 T 280	-0.47 %
I 249 M 280	-0.05 %
V 249 M 280	0.41 %

Fig. 1. ISM and CS analyses of CX3CR1 and CX3CL1. a) IS of CX3CR; b) IS of CX3CL1; c) CS of CX3CR1 and CX3CL1, the dominant frequency is 0.037. Frequencies from the Fourier transform of the sequence of electron–ion interaction potential corresponding to the amino-acid sequence of the protein are put in abscissa axis. The lowest frequency is 0.0 and the highest is 0.5. The amplitudes, in arbitrary units corresponding to each frequency component in the informational spectrum, are put in ordinate axis. d) Relative amplitude change corresponding to the characteristic frequency 0.037 in IS of four polymorphic variants of CX3CR1.

It is known that genes could be implicated in pathophysiology of disease as susceptibility factors or modifying factors that affect course of disease after it has been initiated. The recent GWA study found no evidence for genetic associations with clinical course of MS (IMSGC&WTCCC2, 2011). We also didn't find the association of CX3CR1 polymorphisms analyzed solely with MS course, but instead in haplotype analysis we found significantly lower frequency of $I_{249}T_{280}$ haplotype in SP patients compared to RR patients. We didn't find the significant association of haplotype with MSSS, known as a marker of MS progression. The explanation could be in the fact that there are patients in RR phase of the disease with relatively high MSSS. Thus, our results suggest a protective effect of $I_{249}T_{280}$ haplotype for switching to secondary progressive form of disease, rather than for continual progression of the disease. Yet, comparison of patients with relatively slow (RR > 10 years) and those with fast switch to SP form (SP < 10), the protective OR of 0.5 for $I_{249}T_{280}$ haplotype was no longer significant. We could speculate that either the $I_{249}T_{280}$ haplotype is not significant predictor of slower switch to SP form, or the significance of the result was lost due to truncation of SP group in half (only 32 patients fulfilled criteria of SP < 10). This indicates a need for multicentre replication study with higher number of patients in dissected groups. Even better, we would suggest the analysis of haplotypes between benign (RR > 20) and highly progressive form of MS (SP < 5 years).

The ISM analysis of CX3CR1 protein sequence variations' revealed that, out of three common protein (haplotype) variants, the I249 T280 protein variant could have significantly different interaction with CX3CL1 in comparison to wild-type, V249T280. This may explain the potential association of $I_{249}T_{280}$ haplotype with the disease course. In line with the current ISM analysis is the result from another study that showed V249T280 and I249 M280 variants being indistinguishable in receptor binding capacity when soluble form of CX3CL1 was used for measuring (Daoudi et al., 2004). However, other studies have reported significantly reduced binding affinity of fractalkine to CX3CR1 I249 M280 compared to wild-type, V249T280, variant (Faure et al., 2000; McDermott et al., 2003). This discrepancy might be related to a particular effect of the CX3CL1 form (membrane-bound or soluble) on distinguishing the functional properties of these two CX3CR1 protein variants (Daoudi et al., 2004).

Previous studies indicated CX3CR1 $I_{249}M_{280}$ haplotype as protective for susceptibility to some of the chronic inflammatory diseases (de Bakker et al., 2005; Apostolakis et al., 2009). Based on the current as well as the previous studies' results (Combadière et al., 2008; Apostolakis et al., 2009), it is suggested that the possible protective roles of I249 allele in inflammatory disease susceptibility and disease progression depend on this allele's haplotype background. Functionally, protective effects of I249 allele might be related to CX3CR1 structure or expression alterations, which could affect the progression of inflammation. In this study, we observed the lower frequency of I249 allele in SP patients compared to RR. Moreover, in haplotype analysis V249I polymorphism had significantly different effect on switch to SP form depending on haplotype background. Through T280 allele haplotypic background the I249 allele has shown significant protective effect compared to V249 allele. This result remained significant even after stringent dissection of patients on those with duration of RR phase > 10 years and those who became SP in less than 10 years. Significantly lower fractalkine-binding site density was observed on PBMC from individuals carrying the I249 allele in heterozygous state, either VI-TT or VI-TM, compared to PBMC from individuals carrying VV-TT genotype (Moatti et al., 2001). This would be expected to reduce CX3CR1-mediated monocyte adhesion to endothelium expressing CX3CL1, and therefore represents a potential mechanism for reducing the progression of inflammation. However, similar to inconsistent results of the genetic association studies, functional consequences of CX3CR1 V249I and T280M polymorphisms remain controversial (Moatti et al., 2001; McDermott et al., 2003; Daoudi et al., 2004), thus requiring further research. According to the Tagger software (de Bakker et al., 2005), two analyzed CX3CR1 SNPs, V249I and T280M, are located in a unique LD block and capture 44% of the non-typed SNPs of the reference panel.

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Therefore further analysis should include 4 other tag SNPs at the $r^2 > 0.8$, according to Tagger software, in order to capture 100% of this LD block variability.

In conclusion, results of this study point to a possible protective effect of CX3CR1 I249 allele on secondary progressive MS, when linked with T280 allele in $I_{249}T_{280}$ haplotype. Although the retrospectively calculated power for the significant association in this study (40%) should be higher as well as the number of SP patients, this is the first study that has been investigated the fractalkine receptor haplotypes in MS. Therefore, we believe that despite of these limitations, this study presents interesting finding associated to MS progression through the change of the disease course. As we know that the most important strategy in the research and therapy of MS is to keep patients as long as possible in the slowly progressive phase of the disease, our task is to explore every possible result that can help us to achieve this goal. Also, this work suggests that bioinformatics prediction of specific haplotype effects of receptor/ligand interaction could be useful tool in further research. Replication and validation in diverse and larger populations are warranted before a conclusion can be drawn, regarding the effects of CX3CR1 gene polymorphisms on MS disease course.

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References

Apostolakis, S., Amanatidou, V., Papadakis, E.G., Spandidos, D.A., 2009. Genetic diversity of CX3CR1 gene and coronary artery disease: new insights through a meta-analysis. Atherosclerosis 207, 8–15.

Apostolakis, S., Baritaki, S., Kochiadakis, G.E., Igoumenidis, N.E., Panutsopulos, D., Spandidos, D.A., 2007. Effects of polymorphisms in chemokine ligands and receptors on susceptibility to coronary artery disease. Thromb. Res. 119, 63–71.

Balashov, K.E., Rottman, J.B., Weiner, H.L., Hancock, W.W., 1999. CCR5(+) and CXCR3 (+) T cells are increased in multiple sclerosis and their ligands MIP-1 alpha and IP-10 are expressed in demyelinating brain lesions. Proc. Natl. Acad. Sci. U. S. A. 96, 6873–6878.

Bazan, J.F., Bacon, K.B., Hardiman, G., Wang, W., Soo, K., Rossi, D., Greaves, D.R., Zlotnik, A., Schall, T.J., 1997. A new class of membrane-bound chemokine with a CX3C motif. Nature 385, 640–644.

Carter, K.W., McCaskie, P.A., Palmer, L.J., 2006. JLIN: a java based linkage disequilibrium plotter. BMC Bioinformatics 7, 60.

Chan, C.C., Tuo, J., Bojanowski, C.M., Csaky, K.G., Green, W.R., 2005. Detection of CX3CR1 single nucleotide polymorphism and expression on archived eyes with age-related macular degeneration. Histol. Histopathol. 20, 857–863.

Combadière, C., Godin, O., Vidal, C., Cangialosi, A., Proust, C., Tzourio, C., 2008. Common CX3CR1 alleles are associated with a reduced risk of headaches. Headache 48, 1061–1066.

Compston, A., Coles, A., 2002. Multiple sclerosis. Lancet 359, 1221–1231.

Daoudi, M., Lavergne, E., Garin, A., Tarantino, N., Debré, P., Pincet, F., Combadière, C., Deterre, P., 2004. Enhanced adhesive capacities of the naturally occuring Ile249-Met280 variant of the chemokine receptor CX3CR1. J. Biol. Chem. 279, 19649–19657.

de Bakker, P.I.W., Yelensky, R., Pe'er, I., Gabriel, S.B., Daly, M.J., Altshuler, D., 2005. Efficiency and power in genetic association studies. Nat. Genet. 37, 1217–1223.

Dupont, W.D., Plummer, W.D., 1990. Power and sample size calculations: a review and computer program. Control. Clin. Trials 11, 116–128.

Faure, S., Meyer, L., Costagliola, D., Vaneensberghe, C., Genin, E., Autran, B., Delfraissy, J.F., McDermott, D.H., Murphy, P.M., Debré, P., Théodorou, I., Combadière, C., 2000. Rapid progression to AIDS in HIV + individuals with a structural variant of the chemokine receptor CX3CR1. Science 287, 2274–2277.

Ghilardi, G., Biondi, M.L., Turri, O., Guagnellini, E., Scorza, R., 2004. Internal carotid artery occlusive disease and polymorphisms of fractalkine receptor CX3CR1: a genetic risk factor. Stroke 35, 1276–1279.

Hulshof, S., Van Haastert, E.S., Kuipers, H.F., Van Den Elsen, P.J., De Groot, C.J., Van Der Valk, P., Ravid, R., Biber, K., 2003. CX3CL1 and CX3CR1 expression in human brain tissue: noninflammatory control versus multiple sclerosis. J. Neuropathol. Exp. Neurol. 62, 9.

Imai, T., Hieshima, K., Haskell, C., Baba, M., Nagira, M., Nishimura, M., Kakizaki, M., Takagi, S., Nomiyama, H., Schall, T.J., Yoshie, O., 1997. Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. Cell 91, 521–530.

Infante-Duarte, C., Weber, A., Krätzschmar, J., Prozorovski, T., Pikol, S., Hamann, I., Bellmann-Strobl, J., Aktas, O., Dörr, J., Wuerfel, J., Stürzebecher, C.S., Zipp, F., 2005. Frequency of blood CX3CR1-positive natural killer cells correlates with disease activity in multiple sclerosis patients. FASEB J. 19, 1902–1904.

International Multiple Sclerosis Genetics Consortium; Wellcome Trust Case Control Consortium, 2011. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature 476, 214–219.

Kimouli, M., Miyakis, S., Georgakopoulos, P., Neofytou, E., Achimastos, A.D., Spandidos, D.A., 2009. Polymorphisms of fractalkine receptor CX3CR1 gene in patients with symptomatic and asymptomatic carotid artery stenosis. J. Atheroscler. Thromb. 16, 604–610.

Kurtzke, J.F., 1983. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). Neurology 33, 1444–1452.

Lublin, F.D., Reingold, S.C., 1996. Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on clinical trials of new agents in multiple sclerosis. Neurology 46, 907–911.

Matzhold, E.M., Trummer, O., Grünbacher, G., Zulus, B., Boehm, B.O., März, W., Renner, W., 2009. Association of polymorphisms in the chemokine receptor CX3CR1 gene with coronary artery disease. Cytokine 47, 224–227.

McDermott, D.H., Fong, A.M., Yang, Q., Sechler, J.M., Cupples, L.A., Merrell, M.N., Wilson, P.W., D'Agostino, R.B., O'Donnell, C.J., Patel, D.D., Murphy, P.M., 2003. Chemokine receptor mutant CX3CR1–M280 has impaired adhesive function and correlates with protection from cardiovascular disease in humans. J. Clin. Invest. 111, 1241–1250.

McDermott, D.H., Halcox, J.P., Schenke, W.H., Waclawiw, M.A., Merrell, M.N., Epstein, N., Quyyumi, A.A., Murphy, P.M., 2001. Association between polymorphism in the chemokine receptor CX3CR1 and coronary vascular endothelial dysfunction and atherosclerosis. Circ. Res. 89, 401–407.

Menashe, I., Rosenberg, P.S., Chen, B.E., 2008. PGA: power calculator for case–control genetic association analyses. BMC Genet. 9, 36.

Moatti, D., Faure, S., Fumeron, F., Amara, M.W., Seknadji, P., McDermott, D.H., Debré, P., Aumont, M.C., Murphy, P.M., de Prost, D., Combadière, C., 2001. Polymorphism in the fractalkine receptor CX3CR1 as a genetic risk factor for coronary artery disease. Blood 97, 1925–1928.

Norata, G.D., Garlaschelli, K., Ongari, M., Raselli, S., Grigore, L., Catapano, A.L., 2006. Effects of fractalkine receptor variants on common carotid artery intima–media thickness. Stroke 37, 1558–1561.

Piccio, L., Rossi, B., Scarpini, E., Laudanna, C., Giagulli, C., Issekutz, A.C., Vestweber, D., Butcher, E.C., Constantin, G., 2002. Molecular mechanisms involved in lymphocyte recruitment in inflamed brain microvessels: critical roles for P-selectin glycoprotein ligand-1 and heterotrimeric G(i)-linked receptors. J. Immunol. 168, 1940–1949.

Polman, C.H., Reingold, S.C., Banwell, B., Clanet, M., Cohen, J.A., Filippi, M., Fujihara, K., Havrdova, E., Hutchinson, M., Kappos, L., Lublin, F.D., Montalban, X., O'Connor, P., Sandberg-Wollheim, M., Thompson, A.J., Waubant, E., Weinshenker, B., Wolinsky, J.S., 2011. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. Ann. Neurol. 69, 292–302.

Roxburgh, R.H., Seaman, S.R., Masterman, T., Hensiek, A.E., Sawcer, S.J., Vukusic, S., Achiti, I., Confavreux, C., Coustans, M., le Page, E., Edan, G., McDonnell, G.V., Hawkins, S., Trojano, M., Liguori, M., Cocco, E., Marrosu, M.G., Tesser, F., Leone, M.A., Weber, A., Zipp, F., Miterski, B., Epplen, J.T., Oturai, A., Sorensen, P.S., Celius, E.G., Lara, N.T., Montalban, X., Villoslada, P., Silva, A.M., Marta, M., Leite, I., Dubois, B., Rubio, J., Butzkueven, H., Kilpatrick, T., Mycko, M.P., Selmaj, K.W., Rio, M.E., Sa, M., Salemi, G., Savettieri, G., Hillert, J., Compston, D.A., 2005. Multiple sclerosis severity score: using disability and disease duration to rate disease severity. Neurology 64, 1144–1151.

Sabate, J.M., Ameziane, N., Lamoril, J., Jouet, P., Farmachidi, J.P., Soule, J.C., Harnois, F., Sobhani, I., Jian, R., Deybach, J.C., de Prost, D., Coffin, B., 2008. The V249I polymorphism of the CX3CR1 gene is associated with fibrostenotic disease behavior in patients with Crohn's disease. Eur. J. Gastroenterol. Hepatol. 20, 748–755.

Sunnemark, D., Eltayeb, S., Nilsson, M., Wallström, E., Lassmann, H., Olsson, T., Berg, A.L., Ericsson-Dahlstrand, A., 2005. CX3CL1 (fractalkine) and CX3CR1 expression in myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis: kinetics and cellular origin. J. Neuroinflammation 2, 17.

Tregouet, D.A., Escolano, S., Tiret, L., Mallet, A., Golmard, J.L., 2004. A new maximum likelihood algorithm for haplotype-based association analysis: the SEM algorithm. Ann. Hum. Genet. 68, 165–177.

Tregouet, D.A., Garelle, V., 2007. A new JAVA interface implementation of THESIAS: testing haplotype effects in association studies. Bioinformatics 23, 1038–1039.

Umehara, H., Bloom, E.T., Okazaki, T., Nagano, Y., Yoshie, O., Imai, T., 2004. Fractalkine in vascular biology: from basic research to clinical disease. Arteriocler. Thromb. Vasc. Biol. 24, 34–40.

Veljkovic, V., Veljkovic, N., Esté, J.A., Hüther, A., Dietrich, U., 2007. Application of the EIIP/ISM bioinformatics concept in development of new drugs. Curr. Med. Chem. 14, 441–453.

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