Evaluation of the association between ICAM-1 gene polymorphisms and sICAM-1 serum levels in multiple sclerosis (MS) patients in Southeast Iran

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Multiple sclerosis (MS) is an autoimmune nervous system disorder characterized by leukocytes recruitment into nervous system and demyelination. Intercellular adhesion molecule-1 (ICAM-1) mediates the extravasation of leukocytes and their accumulation in inflamed tissue. The aim of this study was to evaluate the probable association of ICAM-1 Exon 4 (G241R) and Exon 6 (E469K) gene polymorphisms with circulating levels of sICAM-1 in MS patients (n=78) and consecutive unrelated healthy controls (n=123). Analysis of ICAM-1 polymorphisms was performed by PCR with sequence-specific primers (SSP) and concentration of sICAM-1 in serum was performed by ELISA techniques. No significant differences were detected for allele frequencies of ICAM-1 Exon 4 and Exon 6 in MS patients than in the controls respectively (ns-P > 0.05). Moreover, baseline serum sICAM-1 concentrations to be significantly increased among patient carriers of K allele as compared with the respective non-carriers of these variants (P < 0.001). This study illustrates that K allele of the ICAM-1 codon 469 mutation might contribute to the pathogenesis of MS through increase levels of sICAM-1 and establish inflammation. Our result invites further investigation relevant to understanding the mechanisms underlying the immunopathogenesis of this autoimmune disease.

Key words: Polymorphism, intercellular adhesion molecule-1, heterogeneity, single nucleotide polymorphisms (SNP).

INTRODUCTION

The cellular and molecular mechanisms of multiple sclerosis (MS) are poorly understood and involve changes in the adhesive qualities, increase leukocytes migration and establish autoimmune reaction against myelin in central nervous system (CNS) (Polman et al., 2005). Four major clinical subtypes of MS exist, ‘relapsing, remitting (R.R, about 85%)’ when the disease exhibits only relapses and remissions; ‘secondary progressive (S.P)’ when an initial relapsing–remitting phase is followed by a progressive phase, whether super-imposed with relapses or not; ‘primary progressive (P.P)’ when the disease starts with a progressive phase and no relapse supervenes upon progression; ‘relapsing progressive (R.P, about 5%)’ when the progressive phase is present, since the onset of the disease and superimposed with relapses (Stevenson et al., 1999). Lymphocyte infiltration of the brain tissue is regulated by adhesion molecules.

Adhesion molecules are expressed on the cellular surface of endothelium in response to inflammatory stimuli and can be detected in soluble form in circulation (Albelda et al., 1994). The endothelial cell–adhesion molecules (ECAMs) are particularly important for leukocyte homing to target tissues, and can be expressed either on blood and endothelial surface (Albelda et al., 1994). ICAM-1 which is known to be involved in the process of lymphocyte migration (Springer, 1994; McMurry, 1996) and activation (Seventer et al., 1990), is expressed at increased levels in the brain microvessels of MS patients.
(Washington et al., 1994). ICAM-1 is also involved in various leukocyte functions, including extravasation into lymphoid tissues and inflamed non lymphoid tissues (Springer, 1990). Studies using monolayers of human brain microvessel endothelial cells have also highlighted the role of ICAM-1 in both adhesion and migration of the T lymphocytes across the cerebral endothelial barrier, which are distinct processes (Wong et al., 1999; Wu et al., 2000).

Its soluble form (sICAM-1) is also present at higher concentrations in the cerebrospinal fluid (Tsukada et al., 1993) and serum (Hartung et al., 1993; Jander et al., 1993) of MS patients. The human ICAM1 gene is located on chromosome 19p13.3 to 13.2 (GeneID 3383; www.ncbi.nlm.nih.gov/entrez/query).

Two nonsynonymous single nucleotide polymorphisms (SNPs) sites G241R (substitution of glycine to arginine) in exon 4 and K469E (substitution of lysine to glutamate) in exon 6 of ICAM-1 have been previously described (Mycko et al., 1998; Vora et al., 1994; Braun et al., 2001; Gaetani et al., 2002; Lee et al., 2004; Verity et al., 2000).

ICAM-1 gene polymorphism has been implicated in susceptibility to a range of degenerative and inflammatory diseases (Papa et al., 2004; Nishimura et al., 2000). Several studies examined association of the ICAM-1 gene polymorphism with MS (Mycko et al., 1998; Luomala et al., 1999; Killestein et al., 2000; Marrosu et al., 2000). Thus, ICAM-1 might contribute in the MS pathogenesis, whereas sequence variations in the ICAM-1 gene could potentially be responsible for the genetic susceptibility to MS (Barcellos et al., 2002). Genetic polymorphisms in ECAMs have been defined and studied extensively in diseases with multifactorial etiology (Vora et al., 1994; Wenzel et al., 1996).

Despite the large body of literature on the expression and function of the cellular adhesion molecules, the biological properties and function of the circulating form of these molecules remain unclear (Gearing et al., 1993). Therefore, any discussion of the potential mechanisms relating circulating adhesion molecule levels to MS is necessarily speculative. Based on this information, it is hypothesized that genetic polymorphisms in cell adhesion molecules modulate cell to cell interactions and may therefore contribute to pathogenesis of MS. Therefore, the present study was designed to test, for the first time, the relationship between SNPs in ICAM-1 gene and concentration of sICAM-1 in southeast Iranian patients with MS.

**MATERIALS AND METHODS**

**Study population**

The study, approved by the University of Zabol Multiple Institutional Review Board, was conducted with all clinical samples from MS patients who were treated at the Department of Neurology, Ali-ebn Abitaleb Hospital, Zahedan, Iran, and Healthy blood donors that voluntary submitted for research at the central medical laboratory of Zahedan from December 2008 through July 2009. MS patients (in southeast of Iran) who had been diagnosed with magnetic resonance imaging (MRI) and McDonald criteria were collected (Polman et al., 2005). We analyzed 201 different samples; 78 were from patients and 123 were from healthy controls. In the patient group, there were 22 men (mean age, 28.8 years; age range, 17 to 48 years) and 56 women (mean age, 30.3 years; age range, 16 to 52 years). In the control group of healthy blood donors, there were 34 men (mean age, 26.4 years; age range, 17 to 42 years) and 89 women (mean age, 26.0 years; age range, 17 to 50 years). Patients adjusted in definite RRMS (n=46; 10 men and 36 women), SPMS (n=11; 2 men and 9 women), PPMS (n=10; 5 men and 5 women), and RPMS (n=11; 5 men and 6 women) subtypes.

**Genotyping of ICAM-1 gene polymorphisms**

Genomic DNA was extracted and purified from whole blood lymphocytes using a blood DNA Kit (Takara, Japan) according to the manufacturer’s instructions. PCR with sequence-specific primers (SSP) was used to detect the ICAM-1 polymorphisms at Exon 4 (G241R) and Exon 6 (E469K) as described elsewhere (Dorra et al., 2009). For G241R in exon 4, two sequence-specific forward primers: 5’-GTGGTCTGTTCCCTGGACG-3’(G241) and 5’-GTGGTCTGTTCCCTGGACA-3’ (R241), and for K469E (exon 6) two sequence-specific reverse primers: 5’-GACATTACGGTCACCT-3’ (K469) and 5’-GACATTACGGTCACCT-3’ (E469) were used. Each combination of the four primers contained one forward primer and one reverse primer for the 927 bp fragment of sequence-specific amplifications, respectively.

For the positive internal control, the primers 5’-GAAGGGTAAGGTCGGATG-3’(forward) and 5’-GAAGATGTAAGGATGATGTTTCTC-3’ (reverse) coding for the 225 bp fragment of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were used. DNA was amplified by one rounds of PCR performed with 5 µl of DNA in a 25 µl reaction mixture containing 1 U of Smar Taq DNA Polymerase (Takara, Japan), 0.5 mM of each primers, 240 µM of each dNTPs, 20 mM of Tris-HCl 3 mM MgCl2, 50 mM KCl and 20 mM ammonium sulfate. Thermal cycling conditions were as follows: denaturation of 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. The amplification was followed by a final extension step at 72°C for 5 min. PCR products purified by precipitation with 2.5 M ammonium acetate and isopropyl alcohol and subsequently washed with 70% ethanol to remove the dNTPs and excess primers. The pellet was diluted in 20 µl of DNase-free sterile water, and a 10 µl aliquot was analyzed by conventional (1.5% agarose) gel electrophoresis containing ethidium bromide (Sigma, Germany).

**Quantitative determination of sICAM-1 levels using ELISA**

Serum was separated from 5 ml whole blood by centrifuge for 15 min at 150 g at 20°C and then stored at -20°C until the experiment was performed. Soluble ICAM-1 in serum was quantified using the ELISA kit (invitrogen Corp. USA), which based on a sandwich format (Sanadgol et al., 2010). The data presented were means of triplicate determinations.
Statistical analysis

Each polymorphism was tested in controls to ensure the fitting with Hardy-Weinberg equilibrium. To test the hypothesis of association between genetic polymorphisms and MS, multivariate methods based on logistic regression analyses were used. Allele and genotype frequencies in all subjects were calculated by direct counting. Hardy-Weinberg equilibrium was tested using the Fisher's exact test. The strength of the gene-MS associations was measured by odds ratio (OR) and its 95% confidence interval (CI). P < 0.05 was considered statistically significant. The SPSS was used in the statistical analysis.

Ethical considerations

The study conformed to the Helsinki Declaration and was reviewed and approved by the local Research Committee; written informed consent was obtained from all subjects.

RESULTS

No significant differences in allele were observed for ICAM-1 K469E, and G241R polymorphisms between MS patients, and controls (P < 0.05). The frequencies of the three possible genotypes of the ICAM-1 K469E polymorphism in this population are shown in Table 1. The frequency of ICAM-1 E469 homozygosity was relatively lower in MS patients than in the controls (6.42% vs 14.63% subsequently, P < 0.14) (Table 1). Moreover, ICAM-1 K469 allele was more frequent in MS patients than in controls (78.21% vs 70.73% subsequently, P < 0.097) (Table 1). The frequencies of the three possible genotypes of the ICAM-1 G241R polymorphism in this population are shown in Table 2. The ICAM-1 G241R genotype and allele frequencies of MS patients and healthy volunteers were approximately similar (P < 0.93) (Table 2). The distribution of these two SNPs between MS and controls was not significantly different (P < 0.105).

Higher levels of sICAM-1 were observed for the patients with K469 homozygosity (995 ng/ml) and lower levels of sICAM-1 were observed for those with R241 homozygosity (368 ng/ml) but the sICAM-1 distributions were not notably different between controls with different ICAM-1 polymorphism (Figure 1). Patients who are carrying the polymorphic R241 allele showed markedly lower sICAM-1 serum levels than carriers of the homozygous GG wild type (P < 0.001) (Figure 1). In contrast, no significant difference in the sICAM-1 serum levels was seen regarding the 241 genotype distribution in controls (Figure 1). Patients who are carrying the polymorphic K469 allele showed markedly higher sICAM-1 serum levels than carriers of the homozygous EE wild type (P < 0.001) (Figure 1).

There was not any statistically significant difference between distributions of ICAM-1 polymorphisms in MS subtypes, but patient with SPMS showed higher concentration of sICAM-1 in contrast with other subtypes. Our results identified ICAM-1 polymorphisms that were significantly associated with sICAM-1 level but not risk of MS.

We generally observed increase sICAM-1 levels in MS patients in contrast with controls. In conclusion, this study revealed a significant association between the K469 and R241 ICAM-1 polymorphism and serum sICAM-1 levels, probably due to the impairment in binding of ICAM-1 to leukocyte integrin Mac-1 protein.

DISCUSSION

ICAM-1 is a cytokine-inducible or, in certain cell types, a constitutively expressed but cytokine-up-regulatable single chain transmembrane glycoprotein expressed on a variety of cells of various lineages (van der Stolpe and van der Saag, 1996; Hayflick et al., 1998). ICAM-1, as a surface glycoprotein, is expressed on vascular endothelium, macrophages, and activated lymphocytes, and mediates leukocyte circulation and extravasation from the blood into the areas of inflammation and macrophage differentiation. A recent experimental study on the functional significance of ICAM-1 polymorphisms, confirmed that the genotype G241/E469 was associated with greater cell surface expression, leading to greater adhesion of leukocytes (Holder et al., 2008). The molecule is also shed from the cell, most likely through proteolytic cleavage and is present as a soluble form (sICAM-1) in plasma and other biological fluids (van der Stolpe and van der Saag, 1996). The surface form plays a key role in trans-endothelial migration of neutrophils and in lymphocyte activation, throughout its interaction with two leukocyte integrins, leukocyte function associated antigen-1 (LFA-1) and Mac-1 (Diamond et al., 1991; van der Stolpe and van der Saag, 1996; Hayflick et al., 1998). Circulating forms of adhesion molecules or soluble receptors may be released from cells as a consequence of activation, and may be useful markers for inflammation in the CNS. Circulating forms of ICAM-1, ICAM-3, VCAM-1, and L-selectin were increased in serum and CSF from most MS patients (Hartung et al., 1995). These raised levels of circulating ICAM-1 and VCAM-1 are believed to reflect disease exacerbation and blood–brain barrier (BBB) disturbance (Rieckmann et al., 1998) but other studies do not show such elevated concentrations in MS blood (Giovannoni et al., 1997).

Many studies have described associations between ICAM-1 polymorphisms and concentration of sICAM-1 in MS (Mycko et al., 1998; Acar et al., 2005; Correale et al., 2003; Baraczka et al., 2001). Another study claimed that not only s-ICAM-1, but also s-VCAM-1 and s-E-selectin serum levels were significantly elevated in primary progressive MS compared with all other MS groups: no associations are apparent between their concentrations and the extent of disease duration and disability (McDonnell et al., 1999). Patients with relapsing-progressive disease had the highest levels of s-ICAM-1 whereas patients with progressive disease had the highest...
Table 1. Allele and genotype frequencies of the ICAM-1 K469E polymorphisms in MS cases and controls.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>MS (n=78) (%)</th>
<th>Controls (n=123) (%)</th>
<th>Fisher’s exact test</th>
<th>Chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p</td>
<td>OR (95% CI)</td>
<td>$x^2$</td>
<td>DF</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KK</td>
<td>49 (62.82)</td>
<td>69 (56.1)</td>
<td>0.380$^a$</td>
<td>1.32 (0.74-2.36)$^b$</td>
</tr>
<tr>
<td>KE</td>
<td>24 (30.76)</td>
<td>36 (29.27)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EE</td>
<td>5 (6.42)</td>
<td>18 (14.63)</td>
<td></td>
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</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>122 (78.21)</td>
<td>174 (70.73)</td>
<td>0.105</td>
<td>1.48 (0.93-2.37)$^c$</td>
</tr>
<tr>
<td>E</td>
<td>34 (21.79)</td>
<td>72 (29.27)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

OR, odds ratio; CI, confidence interval; DF, degrees of freedom; a, Genotypes: KK vs KE+EE; b, OR for KK vs KE+EE genotypes in MS; c, OR for K vs E allele in MS.

Table 2. Allele and genotype frequencies of the ICAM-1 G241R polymorphisms in MS cases and controls.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>MS (n=78) (%)</th>
<th>Controls (n=123) (%)</th>
<th>Fisher’s exact test</th>
<th>Chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p</td>
<td>OR (95% CI)</td>
<td>$x^2$</td>
<td>DF</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>23 (29.49)</td>
<td>39 (31.71)</td>
<td>0.757$^a$</td>
<td>0.90 (0.49-1.67)$^b$</td>
</tr>
<tr>
<td>GR</td>
<td>32 (41.02)</td>
<td>48 (39.02)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RR</td>
<td>23 (29.49)</td>
<td>36 (29.27)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>78 (50.00)</td>
<td>126 (51.22)</td>
<td>0.838</td>
<td>0.95 (0.64-1.42)$^c$</td>
</tr>
<tr>
<td>R</td>
<td>78 (50.00)</td>
<td>120 (48.78)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

OR, odds ratio; CI, confidence interval; DF, degrees of freedom; a, Genotypes: GG vs GR+RR; b, OR for GG vs GR+RR genotypes in MS; c, OR for G vs R allele in MS.

Figure 1. Means of sICAM-1 concentration among individuals carrying K, E, G and R alleles in MS cases and controls. Expression of sICAM-1 was analyzed with ELISA assay. Each column shows the mean ±S. D. of triplicate determinations.
levels of s-TNF-Rs (Khoury et al., 1999). The observed allele and genotype frequencies for the G241/E469 variants in the present study are some controversy with those previously reported in other sample populations (Li et al., 2005; Ponthieux et al., 2003; Cournu-Rebeix et al., 2003; Kronig et al., 2005). But, the observed association with reduced plasma sICAM1 concentrations in G241R variant are in concordance with those previously reported elsewhere (Ponthieux et al., 2003; Kronig et al., 2005; Yang et al., 2004).

To the best of our knowledge, the present study is the first to demonstrate a significant association of the E469K variant with increased plasma sICAM1 concentrations in MS patients. It is difficult to evaluate the significance of soluble adhesion molecules in MS pathology, because of conflicting reports of their levels in serum and also interpretation of their functional significance. However, as noted by others (Ponthieux et al., 2003; Li et al., 2005), the observed association of the variants tested with reduced plasma sICAM1 concentrations may be due to linkage disequilibrium with other (non-synonymous or functional) genetic polymorphism(s) that affect sICAM1 concentrations. Although it is unclear how the ICAM-1 polymorphism contributes to the pathogenesis of MS, we found that the increase in sICAM-1 expression was accompanied by well-differentiation in serum of KK genotype patients. Thus, s-ICAM-1 might be a useful tool to evaluate disease progression. These findings provide a possible reason why increasing of sICAM-1 expression occurs in MS patients.

Conclusion

Our study herein provides a potential genetic factor for the differentiation of MS that correlates with ICAM-1 K469E polymorphisms because of different sICAM1 expression. However, we are unable to define the association of the ICAM-1 polymorphisms with MS risk owing to the limitations of the development of the MS and control populations in the present study. Our findings may help to evaluate the prognosis of MS according to the individual genetic background.

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REFERENCES


