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CTLA-4 Gene Polymorphisms (-318C/T, +49A/G, +6230A/G) in Iranian Patients with Multiple Sclerosis

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ABSTRACT

Multiple sclerosis (MS) is a disease of the central nervous system (CNS) characterized by multiple regions of demyelination and inflammation along axons with a T cell-mediated autoimmune etiology. While the cytotoxic T lymphocyte antigen 4 (CTLA-4) gene seems to be a strong candidate gene in autoimmune diseases, we investigated its association with a group of patients with MS.

One hundred and thirty five patients with relapsing-remitting form of MS and 135 healthy subjects were enrolled in this study. Three single nucleotide polymorphisms (SNPs) (-318C/T, +49A/G, +6230A/G) of the CTLA-4 gene were assessed using PCR-RFLP method. The genotypes -318 CC (82.9% in patients vs. 76.2% in controls) and +49 AA (31.1% in patients vs. 28.1% in controls) were overrepresented in the patient group; however, these differences were not statistically significant.

In spite of some previous reports, this study did not confirm any significant association with alleles and genotypes of SNPs of the CTLA4 in Iranian MS patients. Such disparity could be due to genetic background, ethnicity and different forms of the disease.

Key words: CTLA-4; Multiple Sclerosis; RFLP; Single Nucleotide Polymorphism

INTRODUCTION

Multiple sclerosis (MS) is a disease of the central nervous system (CNS) characterized by multiple regions of demyelination and inflammation along axons with a T cell-mediated autoimmune etiology.

Although the underlying mechanisms are unknown, it seems that this is a complex autoimmune disease in which inflammation leads to demyelination of the neurons in the CNS. Susceptibility to this condition is
controlled by multiple factors such as cytokine genes and environmental factors.\(^1\)

Cytotoxic T lymphocyte Antigen-4 (CTLA-4) gene is expressed mainly in activated T-cells, which acts as a down-regulator of T lymphocytes. Therefore, it plays a significant role in maintaining peripheral self-tolerance.\(^2\)

Two possible mechanisms have been proposed for the action of CTLA-4. One mechanism is through transmitting negative signals to T cell receptor (TCR) by which it inhibits T-cell response in a process that is not clearly known.

The second mechanism postulates that CTLA-4 on activated T cell binds its ligands (CD80 and CD86) and in this way inhibits T cell activation events by counteracting with CD28, as a T cell stimulator.\(^2\)

Several single nucleotide polymorphisms (SNPs) have been identified in the CTLA-4 gene region, which is located on chromosome 2q33.\(^3\) Association of CTLA-4 SNPs has been tested in a number of autoimmune diseases such as Graves’s disease, Hashimoto Thyroiditis, Diabetes Mellitus type 1, Systemic Lupus Erythematosus, and Rheumatoid Arthritis. There were also some studies in MS, which showed inconsistent results in different populations.\(^4\)–\(^7\)

Due to the relatively high prevalence of MS in our region,\(^8\) identification of its risk factors has great value for health system. Therefore, we aimed to investigate the association of CTLA-4 SNPs (+49G/A rs231775, -318C/T rs5742909 and +6230A/G rs3087243) with MS in our population.

**MATERIALS AND METHODS**

**Patients and Controls**

One hundred and thirty five (45 male and 90 female) unrelated Iranian patients with relapsing remitting and clinically definite MS were recruited. One hundred patients were from the Iranian MS Society and thirty five patients were from Buali-Sina General Hospital, Qazvin University of Medical Sciences.

Mean age of patients was 29.47 years. Mean age of onset of the disease was 23.59 years. Patients had Expanded Disability Status Scale (EDSS) between 2.0 up 4.0. Diagnosis of MS was made according to the Poser criteria.\(^9\)

Control group consisted of 135 age, gender and ethnicity matched healthy subjects. One hundred individuals of control group were randomly selected from Tehran and the rest were recruited from Qazvin city. Informed consent was obtained from all the participants or their legal guardians. This study was performed in accordance with the Declaration of Helsinki and its subsequent revisions and approved by the ethics committee of Tehran University of Medical Sciences.

**Genomic DNA Extraction and PCR**

Whole peripheral blood sample was collected in tubes containing Ethylene Diamine Tetraacetic Acid (EDTA). Genomic DNA was extracted by standard salting-out method and stored at -20°C until use. Genotypes of the -318C/T polymorphism were determined by PCR followed by restriction fragment length polymorphisms (PCR-RFLP) analysis using tru1I enzyme. To amplify the region of the CTLA-4 gene promoter, the forward primer 5’-GCTGTCAGGGACCATAGAAGGA-3’ and reverse primer 5’-GAACACCGCTCCCATAAAGCC-3’ were used.

The PCR consisted of one cycle of initial denaturation at 95°C for 3 minutes, followed by 30 cycles of denaturation (94°C, 50 sec), annealing (57°C, 55 sec) and synthesis (72°C, 1 min). The reaction finished by a final extension of 5 min at 72°C. The PCR product was digested overnight and analyzed on 2% agarose gel. The -318C/T polymorphism was determined by a 568bp fragment (representing the C allele) or two fragments of 249bp and 319bp (representing the T allele).

**Statistical Analysis**

Statistical Analysis was performed by Statistical Package for Social Sciences (SPSS), version 11.5 for windows. Genotype and allele frequency of CTLA-4 gene (-318C/T, +49A/G, +6230A/G) polymorphisms were compared using Chi-square and Fisher’s exact test. P-value less than 0.05 was regarded as significant. The A/G49 polymorphism of the CTLA-4 gene was detected as described by Donner et al.\(^10\) and the +6230A/G SNP was analyzed as described by Torres et al.\(^11\)

**RESULTS**

Genotype distribution in control group for SNPs (-318C/T, +49A/G, +6230A/G) did not show significant difference from Hardy-Weinberg equilibrium. Genotype and allelic distributions for the examined SNPs are shown in the tables 1 and 2, respectively.
Table 1. CTLA-3 genotypes distribution in MS patients and control group

<table>
<thead>
<tr>
<th>SNP Genotypes</th>
<th>Position</th>
<th>MS patients n(%)</th>
<th>Control group n(%)</th>
<th>RR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC -318</td>
<td></td>
<td>112(82.9)</td>
<td>103(76.2)</td>
<td>1.24</td>
<td>0.88-1.74</td>
<td>0.2</td>
</tr>
<tr>
<td>TC</td>
<td>+49</td>
<td>20(14.8)</td>
<td>31(22.8)</td>
<td>0.74</td>
<td>0.51-1.07</td>
<td>0.1</td>
</tr>
<tr>
<td>TT</td>
<td></td>
<td>3(2.2)</td>
<td>1(0.74)</td>
<td>1.51</td>
<td>0.84-2.69</td>
<td>0.6</td>
</tr>
<tr>
<td>AA</td>
<td>+6230</td>
<td>42(31.1)</td>
<td>38(28.1)</td>
<td>1.07</td>
<td>0.83-1.38</td>
<td>0.6</td>
</tr>
<tr>
<td>AG</td>
<td></td>
<td>69(51.1)</td>
<td>74(54.8)</td>
<td>0.72</td>
<td>0.73-1.17</td>
<td>0.5</td>
</tr>
<tr>
<td>GG</td>
<td></td>
<td>24(17.7)</td>
<td>23(17)</td>
<td>1.02</td>
<td>0.75-1.39</td>
<td>0.34</td>
</tr>
<tr>
<td>AA +6230</td>
<td></td>
<td>23(17)</td>
<td>29(21.5)</td>
<td>0.86</td>
<td>0.61-1.19</td>
<td>0.4</td>
</tr>
<tr>
<td>AG</td>
<td></td>
<td>75(55.5)</td>
<td>62(45.9)</td>
<td>1.24</td>
<td>0.5-1.54</td>
<td>0.14</td>
</tr>
<tr>
<td>GG</td>
<td></td>
<td>37(27.4)</td>
<td>44(32.5)</td>
<td>0.88</td>
<td>0.66-1.15</td>
<td>0.42</td>
</tr>
</tbody>
</table>

Comparison between the case and control groups did not indicate any statistically significant differences in genotype frequencies. Similarly, allele frequencies showed no significant difference between the two groups. For -318 C/T SNP, the data revealed slight excess of CC genotype among patients (82.9% in patients vs. 76.2% in controls $P = 0.153$). Comparison of allelic distribution for this SNP, again revealed that the C allele was slightly more common in patients ($\chi^2 = 0.932$ and $P = 0.408$).

Regarding the gender of the patients, analysis did not show any significant differences in the genotype frequency ($P = 0.23$) and allele frequency ($P = 0.31$) in female subjects as compared with female controls. Similarly genotype ($P = 0.46$) and allele ($P = 0.2$) frequency distributions revealed no significant difference between male patients and controls.

### DISCUSSION

In Caucasian population of Northern European descent, Major Histocompatibility Complex (MHC) class II alleles, most notably DRB1*1501, have been proposed as the most consistent genetic findings in MS.12

In a case control study on Iranian MS patients, a positive association with the DRB1*1503, DQA1*0102, and DQB1*0602 haplotype was also reported.13 Furthermore, study on Iranian optic neuritis and MS patients demonstrated increased frequency of HLA-DRB1*15 and DRB1*04 in MS patients, while the frequency of HLA-DRB1*07 and *11 were much higher in patients with optic neuritis.14

However, the consensus is that susceptibility to MS is influenced by multiple genes, including CTLA-4. CTLA-4, as a down-regulator of T lymphocytes, inhibits T cell activation events possibly by binding to its ligands (CD80 and CD86) and counteracting to CD28, as a T cell inducer. CTLA-4 also transmits negative signals to T cell receptor (TCR) and inhibits T-cell response through mechanism which is not clearly identified. CTLA-4 has received widespread attention as a candidate gene for MS susceptibility.15-18

The most frequently studied polymorphisms are a G/A transition in exon 1 at position +49, a C/T transition in -318 position of the promoter sequence and more recently an A/G transition (+6230A/G) within the 3′UTR region.19-22 These SNPs modulate the expression or function of CTLA-4 and are reported to be associated with a variety of autoimmune diseases.7

We performed this study to investigate whether these SNPs influence the risk of developing MS in Iranian population. Although Tehran city is a mixed population of different ethnicities, we recruited 35 people of control group from Qazvin city, in order to avoid population stratification problem.

The allele and genotype frequencies of +49G/A, -318C/T and +6230A/G polymorphisms did not differ significantly between either of the MS group and the healthy control in this population. Therefore, our results indicated that the examined polymorphisms are not associated with MS development. Our results are in consistent with the majority of studies conducted on populations from Denmark,23 Canada,24 Netherlands,25 Russia,16 Japan and China.26 Relatively recently, a study conducted in an Iranian population, also reported lack of association between CTLA-4 (+49G/A) polymorphism and presentation of MS.27 However, studies in populations from France,28 USA,29 Sweden30 and Finland31 indicated strong association with MS.
Table 2. CTLA-4 polymorphisms allelic distribution in MS patient and control groups

<table>
<thead>
<tr>
<th>SNP alleles</th>
<th>MS patients n(%)</th>
<th>Control group n(%)</th>
<th>OR (CI 95%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>-318</td>
<td>C 244(90.3)</td>
<td>234(86.6)</td>
<td>1.307</td>
<td>0.408</td>
</tr>
<tr>
<td></td>
<td>T 269.6)</td>
<td>23(8.5)</td>
<td>(0.758-2.252)</td>
<td></td>
</tr>
<tr>
<td>+49</td>
<td>A 153(56.5)</td>
<td>150(55.5)</td>
<td>0.956</td>
<td>0.862</td>
</tr>
<tr>
<td></td>
<td>G 117(43.4)</td>
<td>120(44.4)</td>
<td>(0.680-1.343)</td>
<td></td>
</tr>
<tr>
<td>+6230</td>
<td>A 149(55.1)</td>
<td>150(55.5)</td>
<td>1.015</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>G 121(44.8)</td>
<td>120(44.4)</td>
<td>(0.723-1.425)</td>
<td></td>
</tr>
</tbody>
</table>

It is noteworthy to mention that frequencies of C and A alleles (although not statistically significant) in -318 C/T and +49 A/G SNPs in our study were higher in the patient group, having the same trend reported in previous studies. It implies that in case of performing the study on a large enough sample size it is likely that we could detect the minor effects of these alleles towards susceptibility to MS.

The inconsistency of association studies could result from differences between population samples, ethnic backgrounds and selection criteria for affected patients. On the other hand, disease heterogeneity may affect involvement and nature of genetic susceptibility factors. Therefore, the possible relationships between clinical subtypes and the role of CTLA-4 polymorphisms should be further investigated in larger populations.

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