

## Original Article

## Investigation of the association of ccl22 gene polymorphism rs4359426 with multiple sclerosis

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### Abstract

**Background:** CCL22 is a chemokine that induces the migration of Th2- and regulatory T cells to the inflammatory sites. The aim of this study was to investigate the association of a single nucleotide polymorphism (SNP), rs4359426, in CCL22 gene, with multiple sclerosis (MS) in patients from southeast of Iran. **Materials and Methods:** The blood samples collected from 150 patients with MS and 150 healthy subjects as a control group. The serum levels of CCL20 measured by ELISA and the DNA analyzed for CCL22 polymorphism using PCR-RFLP method. **Results:** There were no significant differences in the frequencies of genotypes and alleles at SNP rs4359426 in CCL22 gene between MS patients and controls. No significant differences also observed between controls and patients with RRMS, SPMS, PPMS and PRMS patterns regarding the genetic variation of rs4359426. In both MS and control groups, no significant differences were observed between subjects with CC, CA and AA genotypes or between subjects with C and A alleles concerning rs4359426 with respect to the serum levels of CCL22. **Conclusion:** These results do not show any association between the investigated genotypes and alleles at rs4359426 in CCL22 gene with MS or its patterns in MS patients. The serum levels of chemokine did not also influence by genetic variation of SNP rs4359426.

**Keywords:** Multiple sclerosis, CCL22, Chemokine, Polymorphism

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### Introduction

Multiple sclerosis (MS) is characterized as a demyelinating disease of the human central nervous system (CNS). Its etiology remains unknown, although genetic predisposition, environmental factors and autoimmune mechanisms play an important role in the pathogenesis of MS [1]. The MS clinical course is classified as relapsing-

remitting (RRMS), primary progressive (PPMS), progressive relapsing (PRMS) and secondary progressive (SPMS) [2]. The immunologic responses play key roles in the pathogenesis and complications of MS disease [3, 4]. Specifically, T-helper (Th)-dependent responses, play an important role in the pathogenesis of MS disease (3). Upon antigenic stimulation, Th cells differentiate into

several subsets such as Th1, Th2, Th17 and regulatory T (Treg) cells which have been characterized by the release of distinct cytokines profile [5, 6]. It has been reported that the Th1- and Th17-mediated responses can lead to MS disease progression and worsening of symptoms whereas the Th2- and Treg-mediated responses have been associated with reduction of inflammation and improvement of symptoms in MS patients [4, 7, 8].

The infiltration of leukocytes into the CNS is an essential step in the neuropathogenesis of MS disease that controls by chemokines [9]. Chemokines are classified into CXC, CC, CX3C or C chemokines based on the position of the conserved cysteine residues [10]. Chemokine (C-C motif) ligand 22 (CCL22) or macrophage-derived chemokine (MDC) is a chemokine that is produced by macrophages and dendritic cells (DCs). The receptor for CCL22, named CC Chemokine receptor 4 (CCR4) is preferentially expressed on Th2 and Treg cells. Accordingly, CCL22 acts as a chemo attractant for the migration and accumulation of Th2 and Treg cells into inflammatory sites [11, 12].

The genetic variations in the CCL22 gene may also influence on the serum levels of chemokine in MS patients. The gene of CCL22 chemokine has been located on the chromosome 16q13 with several SNPs in both coding and non-coding sequences, of which the SNP rs4359426 (16C/A) may influence the chemokine expression [13, 14]. The functional effect of SNP rs4359426 is changing of GAT to GCT in coding region which causes a 2 Asp to 2 Ala substitutions in the CCL22 protein [14]. We assumed that this SNP may have an association with MS disease. Accordingly, the aim of the present study was to investigate the association of SNP rs4359426 with MS disease and the serum levels of CCL22 in a population from the southeast of Iran to clarify any association.

## Methods

**Subjects.** Blood samples were collected from 150 patients with MS disease (mean age  $35.72 \pm 8.37$  years) during March to September 2013 in Shephah Hospital of Kerman (a city located in southeast of Iran). The expert neurologists confirmed the presence of MS, according to the clinical and paraclinical

findings (MRI study, oligoclonal bands in CSF, and evoked potentials) based on McDonald's criteria [15]. The patients were in the RRMS (n=76), SPMS (n=39), PPMS (n=20) or PRMS (n=15) course of disease.

The control group consisted of 150 age-matched healthy subjects, with no personal and familial history of MS disease (mean age  $36.52 \pm 8.21$ ). The healthy control subjects were recruited among blood donors of the Kerman Transfusion Organization and interviewed regarding the CNS disorders and none of them had any history of CNS or any other relevant disease.

This study was evaluated and approved by the Ethical Committee of Kerman University of Medical Sciences. Moreover, patients were recruited if they agreed for blood sampling. A peripheral blood sample (4-5 milliliter) was obtained from all participants and the sera were separated and stored at  $-70^{\circ}\text{C}$  until analysis.

### *DNA Extraction and Genotype Analysis.*

DNA was extracted from peripheral blood leukocytes by salting out method as previously described by Miller et al. [16]. The quantity and the purity of DNA samples were determined by measuring the optical density at 260 and 280 nm wavelengths using spectrophotometry (Ependorf, Germany). DNA samples were stored at  $-20^{\circ}\text{C}$  until use. Genotypes at position 16C/A in CCL22 (rs4359426; Asp2Ala) were determined by polymerase chain reaction–restriction length polymorphism (PCR–RFLP) method.

The forward primer (5'-CAAGTGACTCTGGGCACCCCA-3') and reverse primer (5'-CTGCTCTTGGTCCAGTGCTTGTC-3') were used to amplify the region around 16C/A SNP. PCR reaction mixture was made up of addition of the following reagents to a 0.2 ml microcentrifuge tube on ice: 2.5  $\mu\text{l}$  of Taq DNA polymerase buffer (10 $\times$ ), 0.5  $\mu\text{l}$  of MgCl<sub>2</sub> (stock concentration 1.5 mM), 0.5  $\mu\text{l}$  of each dNTP [dATP, dCTP, dGTP, and dTTP (stock concentration of 10 mM)], 1  $\mu\text{l}$  of each primer, 1  $\mu\text{l}$  of prepared DNA, and sterile double-distilled water to a final volume of 25  $\mu\text{l}$ . The amplification was performed with the following program: one cycle of  $94^{\circ}\text{C}$  for 5 min, followed by 30 cycles of  $94^{\circ}\text{C}$  for 30 s,  $64^{\circ}\text{C}$  for 20 s and  $72^{\circ}\text{C}$  for 30 s. The amplified PCR product of CCL20 gene covers rs4359426 with a

molecular size of 297 bp.

The amplified products were then underwent restriction reaction in the presence of 5 IU of MboI restriction enzyme (Fermentas, Lithuania) overnight at 37°C. The digested products were electrophoresed on a 2.5% agarose gel after adding 4 µl of loading buffer (Cinnagen, Iran) and studied on Chemi-Doc model XRS (Bio-Rad, USA) after staining with ethidium bromide. The 297 bp product from 16C/A loci was cut into 164 and 133 bp fragments when A allele existed, but remained undigested if C allele was present at this position. In case of heterozygotic form (C/A), three different fragments with 297, 164 and 133 bp are then visible. In homozygotic form, a 297 bp fragment [without any digestion (C/C)] or two 164 and 133 bp [digesting both alleles (A/A)] was observed.

**Chemokine Assay.** The serum levels of CCL22 were measured by ELISA (R&D systems, UK) in patients and healthy controls according to the manufacturer guidelines. The intra- and inter assay coefficient variations of test were <5% and 8%, respectively.

**Statistical Analysis.** Hardy–Weinberg equilibrium was assessed using genotype data. Allele and genotype frequencies were calculated in patients and healthy controls by direct gene counting. Differences in variables were analyzed using Student t, ANOVA and  $\chi^2$  tests as appropriate and P values of less than 0.05 were considered significant. The data were analyzed by SPSS 15 (SPSS Inc, Chicago, IL, USA) and Epi Info 2000 (CDC, Atlanta, Georgia, USA) software packages.

## Results

Table 1 summarizes the frequencies of genotypes and alleles at SNP rs4359426 in CCL22 gene in patients with MS and healthy control group. Statistical analysis showed no deviation of genotype frequencies from Hardy-Weinberg equilibrium, neither in the patients nor in the controls. Statistical analysis also revealed no significant differences in the frequencies of genotypes (CC, CA and AA genotypes) and alleles (C and A alleles) at SNP rs4359426 in CCL22 gene between MS patients and controls.

The frequencies of genotypes and alleles at

**Table 1:** The frequency of CCL22 gene polymorphism in MS patients and controls.

Genotype	MS patients	Healthy subjects	P value
	No. (%)	No. (%)	
CC	126 (84.0)	127 (84.7)	0.87
CA	24 (16.0)	20 (13.3)	0.51
AA	0 (0.0)	3 (2.0)	0.08
C	276 (92.0)	274 (91.4)	0.67
A	24 (8.0)	26 (8.6)	0.67

SNP rs4359426 in CCL22 gene in patients with different patterns of MS disease have been summarized in Table 2. Statistical analysis showed no significant differences between patients with RRMS, SPMS, PPMS and PRMS patterns regarding the frequencies of genotypes (CC, CA and AA genotypes) and alleles (C and A alleles) at SNP rs4359426 in CCL22 gene.

The levels of chemokine CCL22 according to the genetic variations at SNP rs4359426 in CCL22 gene have been demonstrated in Table 3. In both MS patients and healthy control group, no significant differences were observed between subjects with CC, CA and AA genotypes or between subjects with C and A alleles at SNP rs4359426 with respect to the mean serum levels of CCL22.

## Discussion

In this study, the association of the SNP rs4359426 in CCL22 gene with MS disease has been investigated in MS patients from the southeast of Iran. Our results indicated that CC genotype is the most common genotype among patients and controls; with no significant different between these groups. The frequencies of other genotypes CA and AA in patient and control groups were also not significantly different. The minor allele frequency (MAF) in our population was also 0.02. Consistent with our study, the results of a study in Italian patients with MS indicate no significant association between SNP rs4359426 and MS disease; although, in the consideration of its relation with CCL17 polymorphism, AT haplotype (CCL22 A, CCL17 T) was significantly lower in MS patients than control group [17]. The MAF in this Italian sample was 0.04 which is slightly upper than our study. The results of the present study also showed no

**Table 2:** The chemokine gene polymorphism in patients with MS according to disease patterns.

Diseases pattern	Genotypes [No. (%)]			Total	Alleles [No. (%)]	
	CC	CA	AA		C	A
RRMS	64 (84.2)	12 (15.8)	0 (00.0)	76 (100.0)	140 (92.1)	11 (7.9)
SPMS	33 (84.6)	6 (15.4)	0 (00.0)	39 (100.0)	72 (92.3)	5 (7.7)
PPMS	16 (80.0)	4 (20.0)	0 (00.0)	20 (100.0)	36 (90.0)	4 (10.0)
PRMS	13 (86.7)	2 (13.3)	0 (00.0)	15 (100.0)	28 (93.3)	2 (6.7)
Healthy	127 (84.7)	20 (13.3)	3 (2.0)	150 (100.0)	274 (91.4)	26 (8.6)

**Table 3:** Serum levels of CCL22 in MS patients and healthy subjects according to the chemokine gene polymorphism.

Group	Genotypes /Alleles	No. (percent)	CCL22 levels* (mean $\pm$ SD)	P value
MS patients	CC	126 (84.0)	410.6 $\pm$ 24.51	0.24
	CA	24 (16.0)	338.33 $\pm$ 58.63	
	Total	150 (100.0)	399.08 $\pm$ 22.65	
	C	276 (92.0)	398.76 $\pm$ 22.80	0.44
	A	24 (8.0)	338.33 $\pm$ 58.63	
Healthy control	CC	127 (84.7)	419.88 $\pm$ 22.55	0.60
	CA	20 (13.3)	362.83 $\pm$ 45.56	
	AA	3 (2.0)	370.00 $\pm$ 110.0	
	Total	150 (100.0)	411.26 $\pm$ 20.11	0.45
	C	274 (91.4)	419.88 $\pm$ 22.55	
A	26 (8.6)	363.85 $\pm$ 40.49		

\*The serum levels of chemokine expressed as Pg/ml.

The P values represent the differences between different genotypes or alleles in a particular group.

significant differences between patients with RRMS, SPMS, PPMS and PRMS patterns regarding the frequencies of genotypes (CC, CA and AA genotypes) and alleles (C and A alleles) SNP rs4359426 in CCL22 gene. Accordingly, we could not find any association between different genotypes at SNP rs4359426 and the MS disease or its pattern.

The association of the SNP rs4359426 (16C/A) with several diseases has been investigated in a number of studies. Recently, in two studies in southern Iranian population no correlation was found between the SNP rs4359426 in CCL22 gene with susceptibility to, and progression of breast cancer or colorectal cancer [18, 19]. The results of a study in a population from China demonstrated a 2.3 times increase in the risk of gastric carcinoma among subjects who inherited Ala/Ala (AA) genotypes at SNP rs4359426 in CCL22 gene in comparison with other genotypes [14]. Janssen et al.

were not able to indicate the association of the SNP rs4359426 with respiratory syncytial virus bronchiolitis in a population from Netherlands [20]. The discrepancy between the results of the different studies may be due to the differences in the etiopathological mechanisms of the investigated diseases, the differences in the study design, study sample size and finally the different genetic background of the investigated populations.

It has been reported that C to A substitution at position 16 in exon 1 of CCL22 results in the substitution of 2 aspartate (2Asp) to 2 alanine (2Ala) in the N-terminal of the signal peptide of the CCL22 protein [21]. The substitution of Asp by Ala at the N-terminal of signal peptide has been suggested to increase the net positive charge of the N-terminus, which potentially may lead to a higher level of CCL22 expression [22]. Moreover, as a negatively charged amino acid is replaced with a non-polar one,

an effect on the protein function would be conceivable which in turn affects its interaction with its CCR4 receptor. Accordingly, the variations in CCL22 may directly influence the activity of the protein.

The association of this SNP in CCL22 gene with serum levels of CCL22 has been evaluated in the present investigation. However, our results indicated that in both MS and healthy control groups, no significant differences were observed between subjects with different genotypes or alleles at SNP rs4359426 with respect to the mean serum levels of chemokine CCL22. These data represent that the serum levels of CCL22 were not influenced by genetic variation at SNP rs4359426.

In conclusion, the results of the present study do not indicate the association of SNP rs4359426 (16C/A) in CCL22 gene with MS disease or its pattern in patients from southeast of Iran. The serum levels of chemokine did not also influence by genetic variation at SNP rs4359426.

## Conflicts of Interest

The authors declare that there are no conflicts of interest.

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