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Interleukin-10 Promoter and the CCR5 Polymorphisms in Azari Population of Iran with Multiple Sclerosis

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ABSTRACT

Background: Changes in the expression of cytokines as the result of the single nucleotide polymorphisms (SNPs), can affect the incidence of multiple sclerosis (MS).

Objective: To investigate the relationship between the frequencies of interleukin-10 (IL-10)-1082 A/G (rs1800896) and CCR5-delta32 genotypes and susceptibility to MS in the Iranian Azari population.

Methods: IL-10-1082 A/G SNP and the CCR5-delta32 were genotyped in 152 patients suffering from MS and 242 healthy non-relatives by allele specific-PCR and simple PCR methods, respectively.

Results: The frequencies of AA (37.6%) and AG (55.9%) genotypes of IL-10-1082 were significantly high in the control (P=0.021) and MS patients (P=0.015), respectively, with no statistical difference between these groups. There was no significant difference in the CCR5 gene based on the possession of wild/wild and wild/del32 genotypes between MS patients and the control group. The del32/del32 genotype was not seen in any of the investigated groups. Tobacco (cigarettes and hookahs) consumption was higher among the MS patients (P=0.004), and this has the potential to raise the risk of MS in both the individuals and their family. However, it had no significant relation with the frequency of different genotypes of the IL-10-1082 and the CCR5.

Conclusion: Our finding conclude on possible role of AA genotype of IL-10 -1082 as a protective factor in MS.

Keywords: CCR5, Delta 32, Interleukin-10, Multiple Sclerosis, Polymorphism

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INTRODUCTION

Multiple sclerosis (MS) is one of the most common diseases of the central nervous system (CNS) which affects people mostly in early adulthood. (1) Theprevalence of this disease is increasing especially in females (2) and it is different in various races and geographical areas. (3) Its prevalence in Iran is about 54.51 and its incidence is 5.87 in 100,000. (4) Its prevalence in Tehran, the capital of Iran, is increasing which is 101.39 in 100,000. (5) The main cause of this disease is still unknown but epigenetic, genetic, and environmental factors contribute to the risk of developing this disease. (6) In MS, the activated T lymphocytes and macrophages/ monocytes infiltrate the CNS.(7) Study results showed that chemokines, regulatory cytokines of the immune system, such as interleukines, are effective in the pathogenesis of MS.(8) So, we can consider various genotypes of these cytokines, and their receptors as the risk factors of this disease because of the change in the production of cytokines, and inflammation.

IL-10 is an anti-inflammatory cytokine and immune regulatory which leads to the inhibition of Th1 and production of proinflammatory cytokines of tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ). (9) The *IL-10* gene is located on chromosome1 and the promoter of this gene has three SNPs. One of the most important SNPs of which is -1082A/G (rs1800896) SNP that affects gene expression in a way that the presence of A allele is accompanied by a lower production of the *IL-10* that leads to the increased activity of MS. (10, 11) Perhaps A allele can be considered effective at the onset or relapse of the disease by raising the Th1 activity .

Chemokines are a group of pro-inflammatory cytokines that are involved in the guidance and migration of the immune cells to the site of inflammation by connecting to their receptors in the above mentioned cells. (12) C-C chemokines receptor type 5 (*CCR5*) is a chemokine receptor on the surface of leukocytes (13) which acts as an especial receptor for chemokines of macrophage inflammatory protein-1α (MIP1α; CCl3), MIP1β (CCL4) and regulated upon activation, normal T cell expressed and secreted (CCL5; RANES), and causes the absorption of T lymphocytes in the target tissue. This may be influential in inflammatory responses. The *CCR5* gene is located in chromosome number 3 and the *CCR5-delta32* is considered one of its alleles due to the deletion of 32 base-pairs (BPs) in the mentioned gene which causes the change in the function of this receptor and can lead to the disruption in inflammatory responses after the infection (14) or it can reduce the entrance of immune cells to damaged areas and have a protective effect on MS.(15) For clarifying the importance of the *IL-10*-1082 *A/G* SNP biology and the deletion of 32 bp in the *CCR5* gene in susceptibility to the MS more investigation in different populations is needed. So in this study, we have examined the possible relation among the *IL-10*-1082 *A/G* SNP and the *CCR5-del32*, and MS in the Iranian Azari population.

MATERIALS AND METHODS

Study Population

152 MS patients and 242 healthy non-relative people of the Azari population of the northwest Iran participated in this study as a control group. A demographic questionnaire for the patients and the healthy groups was prepared that included: age, the onset of the disease, its duration, the type of MS, and tobacco consumption by the people themselves and others around them, their financial situation, and residential pollution briefly presented in Table 1. Inclusion criteria for the control group were the lack of neurological and autoimmune diseases and inflammatory disorders in the person himself/herself and the lack of MS in the individuals' first-degree relatives. MS disease was confirmed in the chosen patients by a

Table 1. Demografic charasteristics of Multiple sclerosis patients and healthy controls in Northwest of Iran

Characteristics	Frequency of MS patients (%)	Frequency of healthy controls (%)	P value
Age (year) (meanSD)	32.18±6.52	31.02±7.44	
Duration (year) (meanSD)	6.51±5.11	-	
Age at onset (year) (meanSD)	25.04±4.67	-	
Smoking habit			
Smoker	20 (13.2)	12 (5%)	0.004
Non-smoker	71 (46.7)	151 (62.4%)	0.002
Relatives around them	61 (40.1)	79 (32.6)	0.13
Financial situation			
High	72 (47.4)	116 (47.9)	0.91
Median	49 (32.2)	106 (43.8)	0.02
Low	31 (20.4)	20 (8.2)	0.0001
Residential pollution			
Clean	81 (53.3)	118 (48.76)	
Traffic pollution	24 (15.8)	44 (18.18)	0.65
Dust	22 (14.5)	43 (17.76)	
Noise	25 (16.4)	37 (15.3)	

neurologist specialist based on the clinical and paraclinical parameters. A consent form was taken from all the people. This study was confirmed by the ethics committee of Tabriz University of Medical Sciences (Project number 1082.

DNA Extraction and Purification

Genomic DNA was extracted by a method that Asgharzadeh et al had explained. (16) 150 µl of TE buffer (10 mM Tris-cl, 1mM EDTA, pH 8) was added to 300 µl of buffy-coat, and then was mixed. Next, 60 µl of sodium dodecyl sulfate (SDS) 10% and 10 µl of proteinase K (20 mg/ml, Sinaclon, Iran) was added to it and after being mixing, was incubated overnight at 60°C. Afterward, 100 µl of NaCl 5M was added and mixed and then 80 µl of warm solution of N-cetyl-N, N, N - trimethylammonium bromide (CTAB)/ NaCl (10% CTAB+0.7 M NaCl) with the temperature of 65°C was added, stirred and was incubated for ten minutes in the temperature of 65°C. In the next stage, about 700 µl of chloroform/isoamyl alcohol (24:1) was added to the mixture and it was mixed for 20 seconds and centrifuged for 8 minutes in 11,000 g and finally, the supernatant solution was transferred to another microtube. 0.6 of solution volume of 2-propanol was added which was mixed gently and put at -20°C for 30 minutes and then, it was centrifuged in 12,000 g for 15 minutes. For washing the DNA pellet, 1ml of 70% ethanol was used, and finally, the produced pellet was dissolved in 60 µl of distilled water, and the DNA was kept at -20°C.

Genotyping

The *IL-10*-1082 *A/G* SNP was genotyped in promoter region with allele-specific PCR method, mentioned earlier above. (17) The primers used for genotyping are of the sequences mentioned below:

5'-CAG TGC CAA CTG AGA ATT TGG -3' (common primer), 5'-ACT ACT AAG GCT TCT TTG GGA A-3' (A primer), 5'-CTA CTA AGG CTT CTT TGG GAG-3' (G primer).

PCR was conducted in a total volume of 20 µl containing about 100 ng of genomic DNA, 100 µM of each dNTP and 0.5 µM of each primer, 50mM KCl, 20 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 1.25 unit of recombinant Taq DNA polymerase (Sinaclon, Iran) cycling was performed by master cycler gradient (Eppendorf, Germany) as follows: Initial denaturation step at 94°C for 7 min followed by 35 cycles of denaturation at 94°C

for 45 Sec, annealing at 59°C for 45 Sec and extension at 72°C for 50 Sec, and completed by 7 min at 72°C as a final extension. After performing all the steps of PCR, the products were followed by 1.5% agarose gel (Cinaclon, Iran) electrophoresis and they were investigated under UV light after being stained in Ethidium Bromide (0.5 µg/ml). The size of PCR products was 258 bp determined in comparison with 100 bp DNA ladder plus (Fermentas, Lithuania).

For genotyping the *CCR5* (the wild and mutant delta32), PCR method was applied, already described by Mack et al. (18) The primers applied for PCR had the sequences below :

5'-TTT ACC AGA TCT CAA AAA GAA G-3' (forward primer), 5'-GGA GAA GGA CAA TGT TGT AGG-3' (reverse primer). The DNA samples were amplified with cycling parameters like the previous gene except that 200 µM of dNTP was added instead of 100 µM and also annealing temperature was 60°C for 50 Sec. The sizes of PCR products for the wild type of *CCR5* and allele of the delta32 were 274 and 242 bp, respectively.

Statistical Analysis

The distribution of alleles and genotypes among the patients and the healthy control group, and the relation of genotypes with the duration of the disease, and also the demographic features among the patients and the healthy people was compared by Chi-square and $P<0.05$ was considered to be statistically significant. All statistical analyses were conducted using SPSS software (version 18).

RESULTS

Genotypic and Allelic Frequency of IL-10-1082

The frequency of *AA* genotype had a significant difference in the patients and, in the healthy control group (P=0.021) and *AG* genotype was observed in 55.9% of patients in contrast to 43.4% of the healthy people (Table 2). The frequency of A and G alleles in the IL-*10*-1082 position had no significant difference in the patients and the control group (P=0.165) (Table 2).

Genotypic and Allelic Frequency of CCR5

In terms of possessing *wild/*wild and *wild/del32* genotypes in the *CCR5* gene, no significant difference was observed in MS patients and the control group. It was not seen in any of the *del32/del32* genotype. (Table 2). The wild-type allele was the dominant one in the patients and the healthy people and the

Table 2. Genotype and allele frequencies of the *IL-10* and *CCR5* in MS patients and controls

Gene		Frequency (%)		P value
		MS patients	Controls	
IL-10-**1082**	Genotype			
	AA	40 (26.3)	91 (37.6)	0.021
	AG	85 (55.9)	105 (43.4)	0.015
	GG	27 (17.8)	46 (19)	0.760
	Alleles			
	A	165 (54.28)	287 (59.3)	
	G	139 (45.72)	197 (40.7)	0.165
CCR5	Genotype			
	wild/wild	144 (94.7)	224 (92.6)	0.397
	wild/del32	8 (5.3)	18 (7.4)	0.397
	del32/del32	0 (0)	0 (0)	-
	Alleles			
	wild	296 (97.37)	466 (96.28)	
	del32	8 (2.63)	18 (3.72)	0.4

Table 3. Relationship between duration & age at onset of the disease and genotype in MS patients

Genotype	MS patients Frequency (%)	Duration (mean±SD) (year)	P value	Age at onset	P value
GG+AA	67 (44.1)	6.13±5.03	-	24.62±4.73	-
AG	85 (55.9)	6.8±5.14	0.422	25.43±4.17	0.43
wild/wild	144 (94.7)	6.34±5.18	-	25.02±4.61	-
Wild/del32	8 (5.3)	9.63±3.8	0.079	27.9±3.48	0.31
wild/wild+AG	81 (95.29)	6.35±4.28	-	25.17±4.4	-
Wild/del32+AG	4 (4.71)	8.25±3.3	0.11	29.25±4.19	0.20

del32 allele was rarely observed. A mutant allele of the *del32* was slightly more in the healthy people (Table 2). The duration of the disease was 9.6 years in the individuals with the *wild/del32* genotype, which was higher in comparison with the patients with the *wild/wild* genotype, but it was not statistically significant. (P=0.079). (Table 3)

The frequency of genotype *IL-10*-1082 *A/G* was higher in patients than in the control group (P<0.05). When the individual had the *IL-10*-1082 *A/G* genotype and the CCR5 *wild/del32* concurrently, he had MS at an older age and the damage caused by the disease started later in the body. As shown in Table 1, tobacco intake in MS patients (13.2%) was more than the healthy people (5%) (P=0.0004). And also, tabocco consumption in the patients' relatives (40.1%) was more than in the control group (32.6%), which was not significant statistically (P=0.13). Tobacco intake (cigarettes and hookahs) had no significant relation with different genotypes and alleles of the *IL-10*-1082 and the *CCR5* in the patients, and the healthy control group (P≥0.05). In the patients and the chosen control group the number of individuals with a higher income was more, but the individuals with a lower income were more in the patients' group (P=0.0001). No essential difference was observed between the two groups based on the residential pollution (Table 1). In Hardy Weinberg's equilibrium, the population of the CCR5 and the *IL-10*-1082 genes are approximately the same as the primary population; therefore, in these genes, the above mentioned equilibrium is established.

DISCUSSION

In this study -1082, *A/G* SNP of the *IL-10* and the *CCR5* genes was investigated among patients and the healthy people to determine the possible relation between certain alleles and the susceptibility to MS. When the individual had the *IL-10*-1082 *A/G* genotype and the *CCR5 wild/*del*32* concurrently, symptoms of the disease, as well as the damage to the body, began later. In the –1082 position of the *IL-10* promoter, the *AA* genotype was observed in 26.3% of patients, and the *AG* genotype was considerably more in the patients in comparison with the healthy people.

The *AA* genotype was more in the healthy control group accompanied by lower production of the *IL-10*. Based on the allelic frequency in the patients, the *G* allele was more than in the healthy people and in themselves; the *A* allele was insignificantly more than in the patients (Table 2). In the studies below just like ours, the frequency of the *AG* genotype in the patients was more than in the healthy people: Poland 55%, (19) Finland 53%, (20) Germany 51%, (21) Bulgaria 49%. (9) Unlike ours and many other studies, the *AG* genotype in the healthy people was more than in the patients in Norway (22) and in Iran, Fars province. (23) In general, the *AG* genotype was more in comparison to the healthy people; therefore, it seems the

AG genotype along with other cytokine SNPs and with other predisposing environmental factors can be considered as a risk factor in developing MS. Patients with MS appear to have inappropriate cytokine regulation as Th1 activity is more protective than Th2. (9) The *IL-10* as a cytokine is influential in the inhibition : Th1 and antigen-presenting cells activity, and macrophages functions, therefore, it can be effective in disease severity and the degree of disease damage, and also, it can be protective of the intense MS. This issue needs more simultaneous study of more effective genes in the immune responses in several sufficient patients and a control group.

CCR5 as a chemokines receptor can take part in the migration of the involved cells in the inflammation in MS in a way that the increase of the *CCR5*+ cells in the damaged area is observed in the above mentioned patients (24) and the del*32* mutation can lead to the creation of non-functional receptor. Heterozygote individuals have a remarkable decrease in the *CCR5* expression on the surface of T cells, (25) but our results demonstrated that the *del32* allele had no significant difference between the MS patients and the healthy control group ($P \geq 0.05$) and merely in the patients, the frequency of this allele is slightly less than in the healthy people (Table 2). Different results have been reported about the frequency of the *del32* allele in MS patients, as in the Basque population of Spain it has been reported that the *del32* allele has a protective function against MS, (15) whereas Shahbazi et al (26) have declared that the *del32* is a risk factor for MS disease in Iran's population, and Ristic et al in Croatian and Slovenian populations, (27) Török et al in Hungary and Serbia populations (12) just like our study, observed no significant difference in the frequency of the *CCR5* genotypes, the alleles in MS patients and the healthy control group. The difference in the reported results of the *del32* allelic frequency in MS patients may be due to the differences in national and geographical origins of the individuals of the study, sample size, selecting the investigated community, the difference in the design of the study, and various environmental factors that can be influential in getting MS. It seems that the *CCR5* has a partial impact on the migration of T cells from the blood-brain barrier (BBB). So, the main receptor controlling the entrance of T cells to CNS in the mentioned patients will be the main issue in the prevention and treatment of MS. The onset of the disease was 3 years later in the individuals with the *del32* allele and when the person possessed the *AG* heterozygote genotype for the *IL-10*-1082 and the *wild/del32* heterozygote genotype, the onset of the disease was 4 years later. Also, the duration of the disease in the individuals with the *del32* allele was longer than in $CCR5^{+}$ people (9.63 in contrast to 6.43). In other words, the individuals with the *del32* allele, have less inflammation and damageand live longer due to the delay in the onset of the disease. Therefore, we can claim that the *del32* allele is not the main index of the protection of MS in the investigated population. But it can have a synergistic effect on other factors like anti-inflammatory interleukins such as the *IL-10* and human leukocyte antigen and cause the slow or delayed function of the immune system by Th1.

Environmental factors like toxic environmental agents are regarded as the probable risk factors of MS, one of the important ones of which is the cigarette smoke. In this study, the cigarette consumption was more in MS patients and their relatives than in the healthy control group (Table 1). In another survey in Iran by Asadollahi et al., (28) it was shown that smokers, and non-smokers disposed to tobacco smoke increase their risk of getting infected with MS (29). Cigarette smoke, and by its indirect effect on macrophages and T lymphocytes, and also the change in DNA methylation in cells can have an impact on the innate and adaptive immune system (6). It can also change the function of BBB by inducing oxidative stress and activating pro-inflammatory responses (30). The individuals will be prone to this disease or develop it as a result of this issue. By

noticing the fact that tobacco use (cigarettes and hookahs) is increasing among the youth, especially women, this can be influential in increasing the disease in Iran. The individuals with an average income among the patients were less than in the healthy control group in this study (Table 1). It seems that people with a lower income, and due to some deficiencies and more cigarette intake among them, and the rich due to their more consumption of high-fat foods and meat, electrical devices, and sleeping late, are susceptible to this disease. This claim needs more study.

This survey had limitations including first, it contained some MS patients from the northwest Iran. Second, the level of the *IL-10* was not determined in these individuals. Third, the degree of the *CCR5* expression in T cells was not clarified. Fourth, the relation of the above genes' polymorphisms with other life habits such as food consumption, fast food consumption, the stress level in life, and maintaining a vigil was not determined.

It is suggested that the immunogenetics of gene polymorphism of more chemokines, interleukins and their receptors be surveyed in the investigated people. Also, the effect of cigarettes and their ingredients in disrupting BBB should be examined. Epidemiological examination of life habits changes of today's youth and comparing it with that of the adults can be helpful in this regard.

We can conclude that the *CCR5 del32* allele is not the risk factor of MS disease. Given the results obtained in the Azari population of the northwest Iran, probably the *AA* genotype of the *IL-10*-1082 acts as a protective factor, and the *AG* genotype may play a role in causing susceptibility to MS.

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