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Association between interleukin-*12B* gene polymorphisms and multiple sclerosis in turkish population

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ABSTRACT

Elevated levels of IL-12p40 have been observed in the cerebrospinal fluid and serum of MS patients, and it has been suggested that genetic variants in the IL-*12B* gene may contribute to MS susceptibility and/or pathogenesis. In this study, we investigated the association between MS and IL-*12B* gene rs17860508 and rs3212227 polymorphisms in the Turkish population. We identified two polymorphic regions in the IL-*12B* gene using bioinformatics tools and genotyped 351 MS patients and 221 healthy controls for these variants. Our results showed a significant association between the IL-*12B* rs17860508 polymorphism and MS, but no significant relationship between rs17860508 genotype distribution and allele frequency and MS age onset or subtypes. Genotype distribution and allele frequency of IL-*12B* rs3212227 polymorphism did not show any significant association with MS directly. However, AC genotype of IL-*12B* rs3212227 polymorphism in early-onset multiple sclerosis (EOMS) was a risk factor according to age of onset. Additionally, we observed a higher risk for the relapsing-remitting multiple sclerosis (RRMS) subtype in females. This is the first study to investigate the association between IL-*12B* polymorphisms and MS in the Turkish population.

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Introduction

Multiple Sclerosis (MS) is a potentially progressive autoimmune disease that affects the brain and spinal cord and initiates a demyelination process which results in neurological deficits that present with variable clinical symptoms depending on the variety of tissue damage. Although the average peak age ranges between 25 and 34 years, MS can also be seen in children and adult/older people [1-3]. And women are two to three times more likely to develop the disease than men [4]. Whereas the etiology of MS is unknown, it has been suggested that the disease is a multifactorial disorder with an underlying genetic proneness. The inflammation is mediated by an autoimmune response in genetically predisposed individuals, in whom various non-genetic factors such as; female sex, smoking, low vitamin D levels, and Epstein-Barr virus (EBV) infection could influence the development and progression of the disease [5]. Genetic predisposition is mainly mediated by the major histocompatibility complex (MHC). It has been determined that the human leukocyte antigen (HLA) region, specifically HLA class II genes, found on chromosome 6, is in a strong relationship with MS. However, HLA alone cannot fully explain the genetic effect since non-HLA genes also affect the disease [6].

Several studies suggested that further genetic factors such as non-HLA-linked single-nucleotide polymorphisms (SNPs) may underlie the pathology of MS [7-9]. Polymorphisms in the IL-12B gene have been reported to be among the gene variants associated with MS disease [8, 10, 11]. Accordingly, it is suggested that IL-12B variants may contribute to the susceptibility and/or pathogenic pathways of the disease. IL-12B gene is located at 5q33.3, has 8 exons, and the biological activities of the gene include encoding the subunit of the IL-12p40 cytokine which has a broad array of biological activities playing a role in autoimmune diseases like MS [12]. Recent studies have shown that there is a significant elevation of IL-12p40 in cerebrospinal fluid (CSF) and serum among MS patients. Moreover, the high level of this cytokine is considered a potential biomarker for MS-related neuroinflammation [13-15].

Although the association between IL-12B gene polymorphisms and MS susceptibility has been investigated widely, there is no consensus on the results of the studies carried out in different populations [8]. Considering the genetic basis of the disease, it is important to explore

the role of IL-12B SNPs in MS tendency in different communities with different ethnic backgrounds. Recently, Miteva L et al. have shown the impact of the studied polymorphisms on MS in the Bulgarian population in their study. Taking into consideration the results reported in this study, we aimed to investigate the relationship between IL-12B rs17860508 and rs3212227 polymorphisms and MS disease in the Turkish community. The first polymorphism is rs17860508, which is resulted from a 4 bp micro-insertion/deletion with an AA/GC transition and is in the 5'-untranslated region (UTR) of the gene, and the second one (rs3212227) is a single nucleotide polymorphism + 16974 A/C located in the 3'-untranslated region (UTR) of IL-12B.

Material and Methods

MS patients and control subjects

This study was performed on a total of 572 individuals consisting of 351 unrelated MS patients and 221 unrelated healthy controls. Only MS patients who have been diagnosed with clinically definite MS or laboratory-supported definite MS, based on the modified McDonald criteria [16, 17], have been included in the study.

Genotyping of Polymorphisms

Genomic DNA was extracted by using the salting out method, as previously described [18]. Rs17860508 located at the promoter region, and rs3212227 polymorphism located at the 3'-UTR were amplified by a polymerase chain reaction (PCR) using specific primers designed by bioinformatic tools, shown in Table 1. PCRs were carried out by the total volume of 25 µl reaction containing 0,2 µg of genomic DNA, 1x buffer including 1,5 mM MgCl2, 0,5 µM forward primer, 0,5 µM reverse primer, 0,2 mM dNTPs and 1U Taq polymerase. The PCR cycle program was an initial denaturation at 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 sec, 65 °C for 30 sec, 72 °C for 45 sec, and a final step of 72 °C for 10 min. The PCR products for IL-12B rs17860508 were run on 1% agarose gel electrophoreses and genotyping was determined based on the fragment size of the PCR products. The PCR products are 202 bp for CTC TAA homozygote wild type, 367 bp for GC homozygote mutant, and 202 bp and 367 bp for heterozygote alleles. The PCR products for the IL-12B rs3212227 polymorphism region were digested by 10 Unit TaqI enzyme (Thermo Fisher Scientific) at 65 °C for 3 hours. The digested PCR products were run on 1.5% agarose gel electrophoresis and genotyping was done based on digested PCR products. The PCR products are 567 bp for wild type homozygote alleles, 381 bp and 186 bp for homozygote mutant alleles, and 567 bp, 381 bp, and 186 bp for heterozygote alleles.

Table 1 Primers used for amplification of polymorphism sites on the IL-*12B* gene

Polymorphism		Forward Primer	Reverse Primer
rs17860508	CTCTAA (allele-1)	TGTCTCCGAGAGAGGCTCTAATG	TTCACCCATGGAGGAAGTGGTTC
	GC (allele-2)	TGTCTCCGAGAGAGGGCTGT	AGAATCAATGTGAGGAGCCTGGAAC
rs3212227		TGATGGATCAGGTCATAAGAG	AGCAAGGAAGTAACACAATC

Statistical Analyses

Analysis of association between two polymorphisms and MS was performed using Pearson's Chi-square test, Fisher exact test, and Fisher-Freeman Halton test (Fisher-Freeman Halton Test was used when calculated in tables with more than 20 of them an expected values less than five and tables with more than 2x2 meshes). Genotype distribution and allelic frequency between case and control groups were applied by Chi-square test, and Pearson's $\chi 2$-test was used to look for significant differences in both genotype and allele frequencies between case and control groups. odds ratio (OR) and 95% confidence interval (CI) were implemented to predict the contribution of the risk factors. All statistical analyses explained above were done in RStudio 1.3 program. Haplotype analysis was performed by the SHEsis platform to calculate the frequency of haplotypes in case and control groups. Frequency below 0.03 in both controls and cases has been dropped [19]. While evaluating the results, a two-tailed *p*-value less than 0.05 was considered statistically significant.

Results

Based on the classification of the disease into subgroups by age of onset, 91.5% (321 patients) of the patients were in the adult-onset (AOMS) group and 8.5% (30 patients) were in the early-onset (EOMS) group while there was no patient to be involved in late-onset (LOMS) group. These findings align with previous reports [1, 2, 20, 21]. The distribution of patients according to clinical subgroups is: 69.5% of 351 patients are relapsing-remitting multiple sclerosis (RRMS) patients and 30.5% are secondary-progressive multiple sclerosis (SPMS) patients. No patients were recorded to belong to the clinically isolated syndrome (CIS) or primary-progressive multiple sclerosis (PPMS) groups in our study. Genotyping was performed on all participants to investigate the genotype distribution and allele frequencies of IL-12B rs17860508 and rs3212227 polymorphisms, and their associations with disease were evaluated. Notably, gender was significantly associated with MS subtypes ($p = 0.04$), with male patients exhibiting a higher frequency of SPMS and a lower occurrence of RRMS compared to female patients ($p = 0.04$, OR = 1.72, 95% CI = 1.06-2.81). Additional information on patient demographics is summarized in Table 2.

Table 2 Summary of patient information

		Total patients (n = 351)	Female patients (n = 251, 72%)	Male patients (n = 100, 28%)	p value
Age of onset, mean (range, SD)		28.60 (10-48, 7.42)	28.61 (10-48, 7.62)	28.58 (16-46, 6.93)	
Age of onset	EOMS	30 (8.6)	23 (9.2)	7 (7.0)	0.85
	AOMS	321 (91.5)	8 (90.8)	93 (93.0)	
MS subtype	RRMS	244 (69.5)	3 (72.9)	61 (61.0)	**0.04**
	SPMS	107 (30.5)	68 (27.1)	39 (39.0)	

SD standart deviation, EOMS early-onset multiple sclerosis, AOMS adult-onset multiple sclerosis, RRMS relapsing-remitting multiple sclerosis, SPMS secondary-progressive multiple sclerosis. The significant p values are indicated in bold.

The distribution of IL-12B rs17860508 polymorphism genotypes was investigated both in the control subjects and MS patients. The control group displayed a genotype distribution of 20.4%, 70.1%, and 9.5% for TTAGAG/TTAGAG, TTAGAG/GC, and GC/GC, respectively, whereas the MS patients had a distribution of 11.1%, 76.1%, and 12.8% for the same genotypes. The allelic frequency for the TTAGAG and GC alleles in control subjects was 55.4% and 44.6%, respectively, compared to 49.4% and 50.6%, respectively, in MS patients. These findings revealed significant differences between the two groups with respect to both genotype distribution and allelic frequency (p=0.007 and p=0.007, respectively). However, there were no significant differences in genotype distribution or allelic frequency of the IL-12B rs17860508 polymorphism among MS subtypes or based on age of onset. Similarly, the genotype distribution of IL-12B rs3212227 polymorphism was investigated in control subjects and MS patients. The control group had a distribution of 57.9%, 38.5%, and 3.6% for AA, AC, and CC, respectively, while the MS patients had a distribution of 53%, 40.2%, and 6.8% for the same genotypes. There was no significant difference between the two groups in terms of genotype distribution or allelic frequency. There were also no significant differences in genotype distribution or allelic frequency of the IL-12B rs3212227 polymorphism among MS subtypes or based on age of onset, except for the absence of the CC genotype in the EOMS group (p=0.007). In addition, the frequency of the A allele was significantly higher in AOMS compared to EOMS (p=0.01). These results suggest that IL-12B rs17860508 and rs3212227 polymorphisms may contribute to the susceptibility of MS, and their effects may differ based on the age of onset of the disease. All data are presented in Table 3 and 4.

Table 3 Genotype distribution and allele frequencies of IL-*12B* SNPs in MS patients compared to healthy controls

IL-12B rs 17860508				**IL-12B rs 3212227**			
Genotype	**MS, *n* (%)**	**Control, *n* (%)**	***p* value**	**Genotype**	**MS, *n* (%)**	**Control, *n* (%)**	***p* value**
Total							
N	351 (100)	221 (100)	**0.007**	N	351 (100)	221 (100)	
TTAGAG/ TTAGAG	39 (11.1)	45 (20.4)		AA	186 (53.0)	128 (57.9)	0.20
TTAGAG/ GC	267 (76.1)			AC	141 (40.2)	85 (38.5)	
GC/ GC	45 (12.8)			CC	24 (6.8)	8 (3.6)	
Allele- TTAGAG	345 (49.4)		**0.007**	Allele- A	513 (73.1)	341 (77.15)	0.08
Allele- GC	357 (50.6)	197 (44.6)		Allele- C	189 (26.9)	101 (22.85)	

The significant p values are indiciated in bold.

Table 4 Genotype distribution and allele frequencies of IL-*12B* SNPs in MS subtypes and age of onset of the disease

SNP	**Subject**	**Genotypes (%)**			***p* value**	**Allele (%)**		***p* value**	***n***
		Maj./maj.	**Maj./min.**	**Min./min.**		**Maj. allele**	**Min. Allele**		
rs17860508	Total MS	39 (11.1)	267 (76.1)	45 (12.8)		345 (49.2)	357 (51.8)		351
	RRMS	29 (11.9)	183 (75)	32 (13.1)	0.76	241 (49.6)	247 (50.4)	0.76	244
	SPMS	10 (9.35)	84 (78.5)	13 (12.5)		104 (48.6)	110 (51.4)		107
	EOMS	6 (20.0)	21 (70.0)	3 (10.0)	0.26	34 (55.0)	26 (45.0)	0.25	30
	AOMS	33 (10.3)	245 (76.3)	43 (13.4)		311 (48.4)	331 (51.6)		321
rs3212227	Total MS	186 (53.0)	141 (40.2)	24 (6.8)		513 (73.1)	189 (26.9)		351
	RRMS	124 (50.8)	106 (43.45)	14 (5.75)	0.11	354 (72.5)	134 (27.5)	0.11	244
	SPMS	62 (57.95)	35 (32.7)	10 (9.55)		159 (74.3)	55 (27.5)		107
	EOMS	13 (43.3)	17 (56.6)	0 (0.0)	**0.007**	43 (71.7)	17 (28.3)	**0.01**	30
	AOMS	173 (53.9)	124 (38.6)	24 (7.5)		470 (73.2)	172 (26.8)		321

The significant p values are indicated in bold.

Through haplotype analysis, it was found that the GCC haplotype is significantly associated with susceptibility to MS, including RRMS and SPMS, with a *p*-value of 0.0008, an odds ratio of 2.28, and a 95% confidence interval of 1.39-3.74; 0.002, OR = 2.22, 95% CI = 1.32–3.74; and 0.005, OR = 2.35, 95% CI = 1.28–4.32, respectively. Additionally, the GCC haplotype is significantly associated with increased proneness for both EOMS and AOMS, with a *p*-value of 0.00005, OR = 4.48, 95% CI = 2.06–9.74; and 0.0011, OR = 2.26, 95% CI =1.37–3.73, respectively. The GCA haplotype is also associated with a protective effect against EOMS, with a *p*-value of 0.044, OR = 0.54, 95% CI = 0.29–0.990. Finally, the haplotype of TTAGAGC did not show a significant association with AOMS, but its frequency of 16% was decreased almost by half among EOMS patients, with a *p*-value of 0.103, OR = 0.48, and 95% CI = 0.196–1.18. All results can be found in Table 5.

Table 5 Frequency of haplotypes in IL-12B SNPs in MS patients and control individuals

Haplotype												
rs17860508	rs3212227	All Controls (2*n* = 442)	All MS (2*n* = 702)	*p* value (OR/ 95% CI)	RRMS (2*n* = 488)	*p* value (OR/ 95% CI)	SPMS (2*n* = 214)	*p* value (OR/ 95% CI)	EOMS (2*n* = 60)	*p* value (OR/ 95% CI)	AOMS (2*n* = 642)	p value (OR/ 95% CI)
TTAGAG	A	166 (37%)	230 (33%)	0.10 (0.81/ 0.63 - 1.04)	157 (32%)	0.09 (0.79/ 0.61 - 1.04)	72 (34%)	0.35 (0.85/ 0.6 - 1.20)	27 (45%)	0.23 (1.39/ 0.81 - 2.40)	207 (32%)	0.08 (0.80/ 0.62 - 1.03)
TTAGAG	C	79 (18%)	115 (16%)	0.49 (0.90/ 0.65 - 1.23)	84 (17%)	0.75 (0.95/ 0.68 - 1.33)	32 (15%)	0.33 (0.79/ 0.51 - 1.25)	6 (9.5%)	**0.103** (0.48/ 0.20 - 1.18)	105 (16%)	0.48 (0.90/ 0.65 - 1.23)
GC	A	175(40%)	283 (40%)	0.83 (1.03/ 0.81 - 1.31)	197 (40%)	0.84 (1.03/ 0.79 - 1.34)	87 (41%)	0.82 (1.04/ 0.75 - 1.45)	16 (26%)	**0.044** (0.54/ 0.29 - 0.99)	263 (41%)	0.68 (1.05/ 0.82 - 1.35)
GC	C	22 (5%)	74 (11%)	**0.0008** (2.28/ 1.39 - 3.74)	50 (10%)	**0.0021** (2.22/ 1.32 - 3.74)	23 (11%)	0.00495 (2.35/ 1.28 - 4.32)	11 (19%)	**0.000047** (4.48/ 2.06 - 9.74)	67 (10.5%)	**0.0011** (2.26/ 1.37 - 3.73)

Frequency < 0.03 in both control and case has been dropped. Threshold of significance $p \leq 0.05$. Significant values are indicated in bold. For the distribution of all haplotypes in all patients vs all controls *p* value is 0.006.

Discussion

Previous studies have shown that genetic variations in the IL-12B gene, which encodes for the p40 subunit, are associated with the disease course of several autoimmune diseases, including Rheumatoid Arthritis, Behçet's Disease, Ankylosing Spondylitis, Psoriatic Arthritis [22-25]. This association is believed to be attributed to the critical function of IL-12B in stimulating Th1 cell responses.

MS-associated allelic variants in the IL-12B gene have been recently demonstrated to be associated with MS by several studies [8, 10, 11], while no association was found between the same polymorphisms and the disease by some other studies [26, 27]. The difference between studies may be due to ethnic background or the number of patients enrolled. Our aim in this project was to investigate whether there is a relationship between IL-12B rs17860508 and rs3212227 polymorphisms and Multiple Sclerosis disease in the Turkish population, which was not previously reported in the Turkish population, with a large MS patient group.

According to the results of our study, a significant relationship was found between IL-12B rs17860508 TTAGAG/TTAGAG genotype distribution and TTAGAG allele frequency between the patient and control groups. According to this statistically significant relationship, the TTAGAG/TTAGAG genotype was found to be 20.4% in the control group, and 11.1% in MS patients ($p = 0.007$, OR = 0.40, 95% CI = 0.35 – 0.79). And the TTAGAG allele was 55.4% in the control group and 49.4% in MS patients ($p = 0.007$, OR = 0.78, 95% CI = 0.61 – 0.98). Previously, the effects of the promoter rs17860508 on IL-12B on IL-12p40 expression levels have been studied by several groups [28-33]. Accordingly, higher transcription activity of the GC allele than that of the TTAGAG allele, and higher levels of IL-12p70 and IL-12p40 protein levels in GC/GC genotype have been found. These data suggested that GC/GC genotype may upregulate IL-12B expression at the transcriptional level and thus increase the risk of MS disease [28-33].

In our study, no significant association was found between genotype distribution and allelic frequency and the patient and control groups in the IL-12B rs3212227. While 57.9% of the control group had an AA genotype; It was found to be 53% in MS patients. Similarly, CC genotype was found in similar percentages in both patient and control groups (3.6% control; 6.8% MS patients). Previously, in the region of IL-12B rs3212227, the CC genotype and C allele were shown to be protective in the Dutch community, a risk factor in Iran and Bulgarian patients and was not associated in Italian, Chinese, Asian and Caucasian populations [8, 10, 11, 27, 26, 34, 35]. The fact that discordant results were observed in these studies carried out in different populations, suggests that distinct ethnic backgrounds may be related to the differences in the genetic studies.

When we examined the haplotype frequencies, the IL-12B GCC haplotype showed a higher risk of MS. Considering the significantly increased frequencies of the haplotype GCC in MS of either subtype (RRMS, SPMS) and the groups based on the age of onset (EOMS, AOMS) compared to the control group, it is suggested that GCC haplotype is a risk factor for MS regardless of the subtypes or age of onset. The association between the haplotype and the age of onset were not investigated by other studies. When we compared the haplotypes with the age of onset, we found that the haplotype of GCA had decreased frequency in EOMS patients. Thus, the haplotype of GCA was protective in EOMS. The fact that IL-12B TTAGAGC haplotype had decreased almost by half among EOMS patients although it showed no significant association with AOMS suggests that the haplotype TTAGAGC may be protective in EOMS.

In conclusion, this was the first study to investigate the association between IL-12B promoter region polymorphisms and MS disease in the Turkish population. According to the results, we found a significant relationship between IL-12B promoter region polymorphism rs17860508 and MS disease, while no significant relationship was found between the 3'UTR region polymorphism rs3212227 and MS disease. The difference between studies may be due to ethnic background or the number of patients enrolled.

Therefore, additional studies in larger populations from different ethnicities are necessary to have a better insight into the relation between MS disease and IL-12B gene polymorphisms. However, we may conclude that: (1) The IL-12B rs17860508 TTAGAG/TTAGAG genotype and TTAGAG allele were high in healthy individuals, which is thought to have a protective effect in healthy individuals. (2) IL-12B polymorphisms (rs17860508 and rs3212227) have a gender-dependent effect on susceptibility to RRMS. (3) IL-12B GCA and TTAGAGC haplotypes showed decreased frequency among EOMS patients, while the haplotype GCC had an increased frequency in MS patients.

Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical Approval: The study was designed and performed in agreement with the Declaration of Helsinki and was approved by the Ethics Committee of Marmara University School of Medicine, Istanbul, Turkey (protocol no. 09.2019.683).

Informed Consent: Informed consent was obtained.

Author Contributions: Conceptualization and design of the study: NC, SKA and AA; Data collection: KA, AA, NC, OK, EC; Data analyses and interpretation: NC, AA; Writing original draft preparation: NC; Manuscript review and editing: AA. All authors have read and agreed to the published version of the manuscript.

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