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Lack of Association Between *MIF* Gene -173G>C Polymorphism with Multiple Sclerosis

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Abstract. *Aim: The aim of the present study was to investigate a possible association between the MIF -173G>C polymorphism and MS in Turkish patients. Materials and Methods: The study included 153 MS-patients and 210 controls. Genomic DNA was isolated and genotyped using PCR-RFLP assay for the MIF -173G>C promoter polymorphism (rs755622). Results: There was no statistically significant difference in allele and genotype frequencies between MS-patients and controls (p=0.227 and p=0.157, respectively). Accordingly, no association was observed when the patients were compared against controls in terms of GG versus GC+CC genotypes and GG+GC versus CC genotypes (p=0.324 and p=0.179, respectively). Also, there was no statistically significant association between MIF-173G>C polymorphism and clinical and demographic characteristics of MS-patients. Conlusion: The results of the present study suggest no relation between MS susceptibility and MIF gene -173G>C polymorphism in the examined Turkish population.*

Multiple sclerosis (MS) is a chronic, neurodegenerative autoimmune disease of the central nervous system (CNS) characterized by areas of inflammation, demyelination and axonal damage (1). MS affects around 2.5 million people worldwide, 80-85% of whom are diagnosed with a relapsing-remitting (RR) form of the disease. It typically affects young adults and leads to significant physical and cognitive disabilities (1, 2). MS is triggered by environmental factors in individuals with complex genetic-risk profiles. Although the human leukocyte antigen region is the first and major genetic risk factor, genome-wide association studies identified new susceptibility genes including some cytokine genes (3).

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine that plays a critical role in the regulation of macrophage effector functions and T-cell activation (4). Human *MIF* gene is located on chromosome 22q11.2. The *MIF* -173G>C (rs755622) (a guanine-to-cytosine transition at position-173 of the *MIF* gene) polymorphism is located in the promoter region, and has been indicated to correlate with increased MIF production both *in vitro* and *in vivo* (5). Elevated levels of MIF have been observed in the cerebrospinal fluid (CSF) and sera of MS patients during relapses, and a number of lymphocytes strongly-expressing MIF infiltrate into the pathogenic lesions (MS plaques) (7-9). Recently, anti-MIF treatments of mice with experimental autoimmune encephalomyelitis (EAE), an animal model of MS, improved disease severity and accelerated recovery (9, 10). These results suggest that MIF plays a critical role in the physiopathology of the MS. *MIF* -173G>C polymorphism has been associated with disease course and susceptibility to various inflammatory and autoimmune diseases such as rheumatoid arthritis (11), juvenile idiopathic arthritis (12), sarcoidosis (13), psoriasis (14), scleroderma (15), glomerulonephritis (16), asthma (17), and inflammatory bowel disease (18) in various populations. All these data may suggest a novel genetic association with autoimmune disorders and *MIF* -173G>C polymorphism. Since certain cytokine gene polymorphisms are known to influence susceptibility to, and clinical phenotype of MS (19), we designed a case-control study to investigate the possible association between the *MIF* gene -173G>C polymorphism and susceptibility and/or clinical features of MS in a Turkish population.

Patients and Methods

Subjects. The study population comprised of 153 unrelated patients (45 males and 108 females; mean age (±SD)=36.84±9.412 years) with a clinical diagnosis of MS recruited consecutively and prospectively from those who were treated and followed-up in the Neurology Department of Gaziosmanpasa University Research

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Hospital, Tokat, Turkey. The diagnosis of MS was based on the 2005 Revised McDonald Multiple Sclerosis criteria for classification (20). A total of 210 unrelated healthy subjects (80 male and 130 female; mean age=36.36±10.933 SD years) were recruited consecutively. All participants, patients and healthy controls, were of Turkish origin, from inner Central Black Sea region of Turkey. The healthy controls matched for age and gender with MS patients (p=0.658 and p=0.094, respectively) (Table I) and free from another inflammatory-demyelinating disease. The protocol of this study was approved by the Institutional Ethics Committee and all participants gave their written informed consent before entering the study.

Genotyping. Genomic DNA was extracted from whole venous blood samples using a commercial DNA isolation kit (Sigma-Aldrich, Taufkirchen, Germany). The *MIF* -173G>C promoter polymorphism (rs755622) was analyzed by polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) assay as described previously (21). The sequences of forward and reverse PCR primers were 5'-ACT AAG AAA GAC CCG AGG C-3' and 5'-GGG GCA CGT TGG TGT TTA C -3', respectively. After amplification, the 366 bp PCR product was digested with *Alu*I in a 15 μl reaction solution containing 10 μl of PCR product, 1.5 μl of 10× buffer, and three units of *Alu*I at 37˚C overnight. The digestion products were separated on 3% agarose gels, and fragments stained with the ethidium bromide were photographed on an ultraviolet transilluminator. 2 fragments (268 and 97 bp) were seen when G allele was present at position -173 and 3 fragments (206, 97, and 62 bp) when C allele was present.

Statistical analysis. Statistical analysis was performed by using the Statistical Package Program for the Social Sciences (IBM SPSS, version 2.0) and the OpenEpi Info software package version 3.01 (www.openepi.com). Results were expressed as mean±SD. The chi-square (χ^2) test was used to evaluate the Hardy-Weinberg equilibrium for the distribution of the genotypes of the patients and the controls. The relationships between *MIF* -173G>C polymorphism and the clinical and demographic characteristics of patients were analyzed by using χ^2 test, Fischer exact test or analysis of variance (ANOVA) statistics. Odds ratio (OR) and 95% confidence interval (CI) were used for the assessment of risk factors. All p-values were 2-tailed and p-values less than 0.05 were considered as significant.

Results

The baseline clinical and demographic characteristics of the study patients with MS stratified according to *MIF* gene -173G>C polymorphism are shown in Table II. Gender, age, age of onset, disease duration, EDSS (expanded disability status scale) score, frequency of attacks, number of lesions on magnetic resonance imaging and family history of MS patients were analyzed and no statistically significant association was observed between clinical and demographic characteristics of MS patients and *MIF* gene -173G>C polymorphism (Table II). We also analyzed the probability of MS patients to reach an EDSS score of 3 or 6 during the disease course in relation to the -173G>C polymorphism and no association was found (Table II). Allelic and genotypic distributions of the *MIF* gene -173G>C polymorphism in patients and controls are shown in Table III. The genotype and allele frequencies of -173G>C polymorphism did not show any statistically significant differences between MS patients and controls (p=0.227 and p=0.157 OR=0.74, 95%CI=0.48-1.12, respectively). Also, no significant association was observed when the patients were compared with the controls according to GG genotype *versus* GC+CC genotypes and GG+GC *versus* CC genotypes (p=0.324 and p=0.179, respectively). Results of the present study suggest no relation between MS susceptibility and *MIF* gene -173G>C polymorphism in the present Turkish population. The observed and expected frequencies of the polymorphism in both patient and control groups were in Hardy-Weinberg equilibrium.

Discussion

Although the etiopathology of MS remains unknown, it is considered to trigger an inflammatory cascade resulting in axonal and neuronal degeneration in CNS induced by myelin-reactive $CD4^+$ T-lymphocytes crossing the blood-brain barrier after being activated in peripheral tissues (2, 9). The initial phase of the disease includes adaptive immunity and $CD4^+$ T-helper (Th)1, Th-17 and $CD8^+$ T-cells, all of which are modulated by T-regulator, $CD4^+$ Th2, B cells, macrophages, microglia and natural killer cells. On the other hand, disease progression includes the innate immunity (22). MIF is a pleiotropic pro-inflammatory cytokine involved in the regulation of both innate and adaptive immunity (23). It is mainly secreted by activated T-lymphocytes and monocyte/macrophages, up-regulates the pro-inflammatory actions of these cells, and stimulates them to produce a wide range of pro-inflammatory molecules that play a vital role in the MS pathogenesis, including interleukin (IL)-1beta, IL-6, IL-8, interferon-gamma, tumor necrosis factor-alfa, prostaglandin E2, nitric oxide, cytosolic phospholipase A2 and cyclooxygenase-2 (23-26). MIF inhibits the random migration of macrophages, concentrating macrophages at the inflammatory site and it is thought to play an important role in cell-mediated immunity. In addition, MIF enhances Toll-like receptor-4 expression on the macrophage surface, increases phagocytosis and intracellular killing. It up-regulates the expression of intracellular adhesion molecule-1 and vascular cell adhesion molecule-1, and matrix metalloproteinases, leading to enhanced blood-brain barrier permeability (10, 23, 24, 27). Thus, MIF induces the accumulation of neuroantigen-reactive T-cells and other inflammatory cells in the CNS, facilitating the development of EAE and MS. MIF reduces the population of regulatory T-cells, which suppresses the immune functions of other cells, allowing inflammation in the context of MS in both the

Table I. *The demographic characteristics of MS patients and healthy controls.*

Demographic characteristics	MS patients n=153	Healthy controls n=210	*p*-Value
Age, mean±SD (years)	36.84±9.412	36.36±10.933	0.658
Gender, male/female, n (%)	45/108 (29.4/70.6)	80/130 (38.1/61.9)	0.094

Data were analyzed by analysis of variance and χ^2 test. MS: Multiple sclerosis, SD: standard deviation.

Table II. *Clinical and demographic characteristics of patients stratified according to MIF gene -173G>C polymorphism.*

Characteristic	Total n=153	GG n=115	GC+CC n=38	*p*-Value
Gender, male/female, n (%)	45/105 (29.4/70.6)	34/81 (29.6/70.4)	11/27 (28.9/71.1)	0.942
Age (years)	36.84±9.412	37.31±9.598	35.42±8.791	0.284
Age of onset (years)	29.79±8.933	30.15±9.434	28.74±7.251	0.401
Disease duration (years)	7.19±6.307	7.36±6.640	6.68±5.238	0.570
EDSS score	2.94±1.895	2.98±1.792	2.80±2.195	0.615
Mean time to reach EDSS 3 (years) (n=99)	5.73±5.246	5.73±5.589	5.71±4.231	0.982
Mean time to reach EDSS 6 (years) (n=52)	9.97±7.215	10.33±7.415	8.80±6.669	0.526
MS types, n (%)				1.000
RRMS + SPMS	147 (97.4)	110 (97.3)	37 (97.4)	
PPMS	4 (2.6)	3 (2.7)	1 (2.6)	
Family history, n (%)	6 (4.0)	4 (66.7)	2 (33.3)	0.642
Lesion number in brain MRI, n (%)				0.227
<9	26 (17.3)	22 (84.6)	4 (15.4)	
>9	124 (82.7)	90 (72.6)	34 (27.4)	
Frequency of attacks				0.474
No relaps in last 2 years	40 (27.0)	29 (72.5)	11 (27.5)	
1 relapse in last year	78 (52.7)	57 (73.1)	21 (26.9)	
2 relapses in last year	24 (16.2)	19 (79.2)	5 (20.8)	
≥ 3 relapses in last year	6 (4.1)	6 (100)	0	

Data were analyzed by analysis of variance, χ^2 or Fischer's exact test. Mean plus standard deviation values are presented for all variables, except gender, family history and MS types. EDSS: expanded disability status scale, *MIF*: macrophage migration inhibitory factor, MRI: magnetic resonance imaging, MS: multiple sclerosis, RRMS: relapsing-remitting MS, SPMS: secondary progressive MS, PPMS: primary progressive MS.

brain and spinal cord (4, 10). Furthermore, it inhibits p53-dependent apoptosis of lymphocytes and macrophages. A failure of autoreactive T-cells to undergo apoptosis due to higher levels of MIF expression may lead to inappropriate persistence of these cells and cause harmful immunoreactivity in MS (8, 10). MIF also stimulates the antibody production in B-cells, which have been demonstrated to exhibit clonal enlargement (22). As a neuro-endocrine mediator, MIF is secreted by anterior pituitary cells and counter-regulates the immunosuppressive and anti-inflammatory actions of glucocorticoids. Hence, genetic predisposition to higher MIF production may increase the magnitude, duration and possibly the chronicity of immune and inflammatory responses (5, 27, 28).

MIF is necessary for the progression of EAE but is not required for initial activation of auto-reactive lymphocytes. It has been demonstrated that the detection of infiltrating monocytes in the CNS of EAE mice is correlated with substantial clinical disability (10, 29). MIF synthesis is known to be significantly increased in active MS plaques constituted mainly by T lymphocytes, macrophages and microglia (9, 10). To further implicate a role for MIF in MS, Denkinger *et al*. (4) have demonstrated that anti-MIF treatment in mice with acute EAE decreases accumulation of encephalitogenic T-cells and other inflammatory cells into the CNS and expands a population of regulatory T lymphocytes, reducing the severity of clinical signs and accelerating recovery from the disease. It has also been suggested that inhibition of MIF reduces relapses of disease in a relapsing/remitting model of EAE (10). MIF levels have been found to increase in the CSF and sera/blood of patients with active/relapsed multiple sclerosis and have therefore been associated with the disease

Table III. *Genotype and allele frequencies of* -173G>*C polymorphism of MIF gene in patient and control groups.*

Polymorphism	Patients n=153 (%)	Controls n=210 (%)	*p*-Value	OR (CI 95%)
Genotypes				
GG	115 (75.2)	148 (70.5)	0.227	
GC	36 (23.5)	53 (25.2)		
CC	2 (1.3)	9 (4.3)		
GG:GC+CC	115 (75.2):38 (24.8)	148 (70.5):62 (29.5)	0.324	0.79 (0.49-1.26)
GG+GC:CC	151 (98.7):2 (1.3)	201 (95.7):9 (4.3)	0.179	0.29 (0.04-1.27)
Alleles				
G	266 (86.9)	349 (83.1)	0.157	0.74 (0.48-1.12)
C	40 (13.1)	71 (16.9)		

Data were analyzed by χ^2 or Fischer's exact test. MIF: macrophage migration inhibitory factor.

activity (7, 8, 30, 31). Measurement of MIF levels in healthy subjects and in patients with auto-immune inflammatory diseases, such as rheumatoid arthritis and juvenile idiopathic arthritis showed that serum and tissue MIF levels are significantly higher in *MIF* gene -173*C allele carriers than in *MIF* -173GG homozygous subjects. Increasing data from literature have linked *MIF* gene -173G>C polymorphism with susceptibility to or severity and outcome of inflammatory and/or autoimmune diseases, in which increased systemic or local MIF concentrations have been associated with severe clinical manifestations, high severity scores, and often poor outcome (6, 12, 28). These findings suggest that *MIF* may be a common risk gene for a number of autoimmune disorders. Therefore, we assumed that the *MIF* gene -173G>C polymorphism may be also associated with MS. To validate the hypotheses, we examined the association of *MIF* -173G>C polymorphism with MS and found that allele and genotype frequencies of this polymorphism do not show any statistically significant difference between MS patients and controls. There was no significant association observed when the patients were compared with the controls according to GG genotype *versus* GC+CC genotypes and GG+GC *versus* CC genotypes (Table III). Also, there as no statistically significant association between *MIF* -173G>C polymorphism and baseline clinical and demographic characteristics of MS patients (Table II). Our findings conflict with the previous data reported by Akcali *et al*. (32). In that study including 120 RRMS patients and 120 control subjects from Turkish population, *MIF*-173 CC and GG genotypes were found to be statistically higher and lower in MS patients, respectively ($p<0.001$ and $p=0.004$, respectively). Moreover, patients with the *MIF*-173 CC genotype had a significantly lower age of disease onset compared to those with the *MIF*-173 CG and *MIF* -173GG genotypes. In the study of Akcali *et al*. on *MIF*-173 gene polymorphism frequencies, GG genotype was 37% *vs*. 55%, GC genotype was 43% *vs*. 41%, and CC genotype was 20% *vs*. 4.2% in MS patients *vs*. control subjects, respectively. In the present study, the frequency of GG genotype was 75.2% *vs*. 70.5%, GC genotype was 23.5% *vs*. 25.2%, and CC genotype was 1.3% *vs*. 4.3%. There is a remarkable difference between these two groups in terms of genotype and allele frequencies of *MIF* gene -173G>C polymorphism. One possible explanation for the conflicting results of the previous report could be the population heterogeneity. Because the study of Akcali *et al*. and the present study included individuals from the Southeastern Anatolia *vs*. Central Black Sea regions, conflicting results might be attributed to the differences between these two populations or unknown genetic variations.

The etiology of MS is complex and includes the interaction of multiple environmental and genetic factors. Both genetic and environmental factors may affect disease susceptibility and phenotypic characteristics, but we cannot not yet understand their exact roles. Among the environmental factors most commonly emphasized factors are the Epstein-Barr virus presence, vitamin D deficiency, ultraviolet exposure and smoking. MS is a polygenic inherited disease. Because the odds ratio for the many genes out of HLA region –the major risk factor for MS- is very low, the size of data set needed for finding an association increases, decreasing the importance of a single gene in genetic predisposition to MS. All these facts can explain the negative association found between MS and *MIF* -173G>C polymorphism in the present study (22). On the other hand, although *MIF* -173G>C polymorphism is considered as a risk factor for many inflammatory and autoimmune diseases, studies on some inflammatory and autoimmune diseases such as giant cell arthritis and cutaneous vasculitis have failed to find an association (33, 34). This suggests that the physiopathological processes may vary between autoimmune diseases and that we may have not understood/identified the effects/roles of the MIF and *MIF* gene polymorphisms in

inflammatory diseases completely. The present study suffers several limitations; the sample size was small, which may result in false-positive and false-negative results in genetic association studies by decreasing the statistical power of the study. Moreover, another limitation was the fact that serum and/or CSF MIF levels were not measured in the patient and control groups.

In conclusion, the results of the present study suggest that *MIF* gene -173G>C promoter polymorphism is not associated with MS susceptibility or clinical presentation in the present Turkish population, but our study has a preliminary character and should be extended on a larger population.

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