Association of Methylenetetrahydrofolate Reductase Gene C677T Polymorphism With Multiple Sclerosis in Turkish Patients

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Background and Aim: Multiple sclerosis (MS) is a chronic neurodegenerative autoimmune disease of the central nervous system. Genetic risk factors are known to contribute to the etiology of MS. Methylenetetrahydrofolate reductase (MTHFR) gene C677T polymorphism has been associated with susceptibility to various autoimmune diseases. The aim of this study was to investigate a possible association between the MTHFR gene C677T polymorphism and MS in Turkish patients.

Methods: The study included 130 MS patients and 150 group-matched controls. Genomic DNA was isolated and genotyped using polymerase chain reaction–based restriction fragment length polymorphism assay for the MTHFR gene exon C677T polymorphism.

Results: The genotype and allele frequencies of C677T polymorphism showed statistically significant differences between MS patients and controls ($P = 0.002$ and $P = 0.002$; odds ratio, 1.79; 95% confidence interval, 1.23-2.63, respectively). A significant association was observed when the patients were compared with the controls according to CC genotype versus CT + TT genotypes ($P = 0.0005$; odds ratio, 2.35; 95% confidence interval, 1.45-3.82). There were no statistically significant association between MTHFR gene C677T polymorphism and baseline clinical and demographical characteristics of MS patients.

Conclusions: These results showed that T allele of C677T polymorphism was associated with MS susceptibility in Turkish population.

Key Words: multiple sclerosis, susceptibility, MTHFR, C677T, gene polymorphism

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Multiple sclerosis (MS) is a chronic neurodegenerative autoimmune disease of the central nervous system (CNS) characterized by areas of inflammation, demyelination, and axonal degeneration.[1] Multiple sclerosis affects around 2.5 million people worldwide, 80% to 85% of whom are diagnosed with a relapsing-remitting form of this disease. It is twice common in women than in men and is the most common cause of neurologic disability in young and middle-aged adults.[2] Clinical features include various neurological dysfunctions, such as visual and sensory problems, limb weakness, or gait disturbance. The clinical course can be subdivided into different subtypes: relapsing-remitting MS (RRMS), secondary progressive MS (SPMS), and primary progressive MS (PPMS). Relapsing-remitting MS is characterized by abrupt start of symptoms and acute episodes worsening with complete or partial recovery. Secondary progressive MS means that an initial relapsing-remitting course is followed by chronic progression, and PPMS is characterized by chronic progression from the beginning of the disease without clinical remission.[3]

The exact etiology of MS remains elusive with a complex interaction between environmental factors, genetic susceptibility, and viral infection.[4] The genetic structure of MS is primarily suggested by familial aggregation of cases and differences in MS risk among ethnic groups residing in the same geographical regions. Indeed, twin studies have revealed higher concordance rates (~25%–30%) in monozygotic twins compared with dizygotic twins (~3%–5%), suggesting a strong, but complex, genetic structure.[5] The human leukocyte antigen region is the strongest susceptibility locus for MS, but genome-wide association studies recently identified new susceptibility genes, each contributing a small to moderate effect to the overall disease predisposition.[6]

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme-regulating folate and homocysteine metabolism. The human MTHFR gene (encodes MTHFR enzyme) is located on chromosome 1p36.22 and contains 11 exons.[7] This genomic region has been found to be positively associated with MS in a genome-wide association study.[8] The C677T is the most common single nucleotide polymorphism of the MTHFR gene with a C (cytosine) to T (thymine) transition, located at nucleotide 677 in exon 4. This polymorphism results in the alanine-to-valine amino acid change leading to a thermolabile enzyme with reduced activity. The TT variant has only 30%, and the CT variant has 65% enzyme activity when compared with the CC variant.[9] The MTHFR enzyme catalyzes the irreversibly conversion of 5,10 methylenetetrahydrofolate (5,10-MTHF) to 5-methyltetrahydrofolate (5-MTHF). In homocysteine metabolism, 5-MTHF is necessary to remethylate the neurotoxic intermediate homocysteine to methionine, which itself serves as precursor of S-adenosylmethionine (SAM), essential for CNS myelination.[10] The MTHFR C677T polymorphism leads to mild hyperhomocysteinemia and impairs the ability to process folate and methionine.[11] Patients with MS also have higher levels of plasma and cerebrospinal fluid homocysteine.[12] Elevated homocysteine levels and reduced availability of SAM can increase the risk of extensive neuronal loss combined with diffuse demyelination. Moreover, homocysteine can directly damage CNS cells or influence macrophage activation, both of which are important aspects of MS pathology.[10,13]

It have been reported that MTHFR gene C677T polymorphism may influence susceptibility to several neuropsychiatry disorders such as depression, schizophrenia, neural tube defects, stroke, cognitive impairment, dementia, and amyotrophic

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TABLE 1. The Demographical Characteristics of Patients With MS and Healthy Controls

Demographic Characteristics	MS Patients (n = 130)	Healthy Controls (n = 150)	*P*
Age, mean (SD), y	36.95 (9.629)	35.93 (8.201)	0.343
Sex, male/female, n (%)	37/93 (28.5/71.5)	54/96 (36.0/64.0)	0.202

Data were analyzed by ANOVA and χ^2 tests.

lateral sclerosis.[14–16] Previous studies have also provided evidence that high prevalence of MTHFR gene polymorphisms was frequently detected in patients with autoimmune diseases, suggesting a novel genetic association with autoimmune disorders.[17,18] Because autoimmunity and genetic factors are both common in the etiopathogenesis of MS, we aimed to design a case-control study to investigate the possible association between the MTHFR gene C677T polymorphism and MS susceptibility in a Turkish population.

MATERIALS AND METHODS

Subjects

The study population comprised 130 unrelated patients (37 male and 93 female patients; mean [SD] age, 36.95 [6.629] years) with a clinical diagnosis of MS recruited consecutively and prospectively from those whom were treated and followed up in the Neurology Department of Gaziosmanpasa University Research Hospital, Tokat, Turkey. The diagnosis of MS was based on the 2005 Revised McDonald Multiple Sclerosis criteria for classification.[19] A total of 150 unrelated healthy subjects (54 male and 96 female patients; mean [SD] age, 35.93 [8.201] years) were recruited consecutively. All participants, patients and healthy controls, were of Turkish origin from the inner Central Black Sea region of Turkey. The healthy controls matched for age and sex with MS patients ($P = 0.343$ and $P = 0.202$, respectively; Table 1) and free from another inflammatory-demyelinating disease. The protocol of this study was approved by the institutional ethics committee, and all participants gave 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 MTHFR C677T (rs1801133) polymorphism was analyzed by polymerase chain reaction (PCR)–based restriction fragment length polymorphism assay as described previously.[9] The amplification conditions consisted of an initial melting step of 5 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 61°C, and 30 seconds at 72°C, and a final elongation step of 5 minutes at 72°C. The sequences of PCR primers were 5′-TGA AGG AGA AGG TGT CTG CGG GA-3′ and 5′-AGG ACG GTG CGG TGA GAG TG-3′. Polymerase chain reaction was carried out in a total volume of 25 μL reaction containing 100 ng of genomic DNA, 2.5 μL of 10X PCR buffer, 200 μM dNTP, 10 pM each primers, and 1 unit of Taq DNA polymerase. After amplification, the 198-bp PCR product was digested with HinfI in a 15-μL reaction solution containing 10 μL of PCR product, 1.5 μL of 10X buffer, and 2 units of HinfI at 37°C overnight. The digestion products were separated on 3% agarose gels, and fragments stained with the ethidium bromide were photographed on an UV transilluminator. Wild type (CC) individuals were identified by only a 198-bp fragment, heterozygotes (CT) by both the 175/23 bp and 198 bp, and homozygote variants (TT) by the 175/23 bp. The second PCR and RFLP were performed to confirm the results.

Statistical Analysis

Statistical analysis was performed using the Statistical Package Program for the Social Sciences (SPSS 20) and the OpenEpi Info software package version 3.01 (www.openepi.com). Results were given as mean (SD). The 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 C677T polymorphism and the clinical and demographical characteristics of patients were analyzed by using the χ^2 test or the analysis of variance (ANOVA) statistics. Odds ratio (OR) and 95% confidence interval (CI) were used for the assessment of risk

TABLE 2. Clinical and Demographical Characteristics of Patients Stratified According to MTHFR Gene C677T Polymorphism

Characteristic	Total (n = 130)	CC (n = 55)	CT + TT (n = 75)	*P*
Sex, male/female, n (%)	37/93 (28.5/71.5)	17/38 (30.9/69.1)	20/55 (26.7/73.3)	0.695
Age, mean (SD), y	36.95 (6.629)	38.00 (10.112)	36.17 (9.251)	0.287
Age of onset, mean (SD), y	29.41 (8.967)	30.16 (8.717)	28.85 (9.165)	0.413
Disease duration, mean (SD), y	7.52 (6.570)	7.80 (6.795)	7.32 (6.438)	0.683
EDSS score, mean (SD)	3.05 (1.994)	3.15 (2.105)	2.98 (1.920)	0.642
Mean time to reach EDSS 3, mean (SD) (n = 76),y	5.86 (5.581)	5.71 (5.385)	5.95 (5.755)	0.854
Mean time to reach EDSS 6, mean (SD) (n = 49), y	10.11 (7.219)	10.52 (7.621)	9.74 (6.967)	0.706
MS types, n (%)				0.637
RRMS + SPMS	126 (96.9)	54 (98.2)	72 (96.0)	
PPMS	4 (3.1)	1 (1.8)	3 (4.0)	
Family history, n (%)	5 (3.8)	4 (80.0)	1 (20.0)	0.162

Data were analyzed by ANOVA and χ^2 tests. Mean (SD) values are presented for all variables, except sex, family history, and MS types.

TABLE 3. Genotype and Allele Frequencies of C677T Polymorphism of MTHFR Gene in Patient and Control Groups

MTHFR (C677T)	MS Patients (n = 130), %	Healthy Controls (n = 150), %	*P*	OR (CI 95%)
Genotypes				
CC	55 (42.3)	95 (63.3)	**0.002**	
CT	66 (50.8)	47 (31.3)		
TT	9 (6.9)	8 (5.3)		
CC + CT : TT	121 (93.1) : 9 (6.9)	142 (94.7) : 8 (5.3)	0.589	1.32 (0.48–3.66)
CC : CT + TT	55 (42.3) : 75 (57.7)	95 (63.3) : 55 (36.7)	**0.0005**	**2.35 (1.45–3.82)**
Alleles				
C	176 (67.7)	237 (79.0)	**0.002**	**1.79 (1.23–2.63)**
T	84 (32.3)	63 (21.0)		

The results that are statistically significant are shown in boldface.

factors. All *P* values were 2-tailed, and *P* values less than 0.05 were considered as significant.

RESULTS

The baseline clinical and demographical characteristics of the study patients with MS stratified according to MTHFR gene C677T polymorphism were shown in Table 2. Sex, age, age of onset, disease duration, Expanded Disability Status Scale (EDSS) score, and family history of MS patients were analyzed, and any statistically significant association was not observed between clinical and demographical characteristics of MS patients and MTHFR gene C677T polymorphism (Table 2). We also analyzed the probability of MS patients to reach EDSS: 3 or 6 during the disease course in relation to the C677T polymorphism and no association was found (Table 2). Allelic and genotypic distributions of the MTHFR gene C677T polymorphism in patients and controls were shown in Table 3. The genotype and allele frequencies of C677T polymorphism showed statistically significant differences between MS patients and controls ($P = 0.002$ and $P = 0.002$; OR, 1.79; 95% CI, 1.23–2.63, respectively). A significant association was observed when the patients were compared with the controls according to CC genotype versus CT + TT genotypes ($P = 0.0005$; OR, 2.35; 95% CI, 1.45-3.82). These results showed that T allele of C677T polymorphism was associated with MS susceptibility in Turkish population. The observed and expected frequencies of the polymorphism in both patient and control group were in Hardy-Weinberg equilibrium.

DISCUSSION

Multiple sclerosis remains a model of complex autoimmune disease with variable clinical expression and unpredictable course. Disease susceptibility and phenotypic representation are probably influenced by variations in numerous genes and complex interactions of these genes with environmental factors.[4,5] In the present study, statistically significant difference was observed between MS patients and controls in terms of allele frequencies of MTHFR gene C677T polymorphism. A significant association was observed when the patients were compared with the controls according to CC genotype versus CT + TT genotypes (Table 3). In addition, any statistically significant association was not observed between clinical and demographical characteristics of MS patients, and MTHFR C677T polymorphisms were compared (Table 2).

Association between MS and C677T polymorphism has been studied in different populations, and diverse results have been found.[8,10,20–22] A study concerning 150 MS (RRMS, SPMS, and PPMS) patients and 95 controls from Sweden did not show any association between MTHFR gene C677T polymorphism and MS.[20] Tajouri et al[21] studied the C677T polymorphism in 104 Australian MS (RRMS, SPMS, and PPMS) patients and 104 group-matched controls, and they found that the frequency of T allele was not different between MS patients and control group. In addition, comparison of the genotype distributions showed that TT genotype was slightly higher, but not statistically significant, in MS patients (16% vs 11%). Like our study, their study groups were composed of 3 MS types (RRMS, SPMS, and PPMS). In contrast to our results, they could not find a statistically significant association between C677T polymorphism and MS.

In another study, in a German population, including 138 RRMS patients and 138 controls, no association was observed between MTHFR C677T polymorphism and MS.[22] Similarly, no significant differences were found in the frequency of the MTHFR C677T polymorphism between Tunisian 80 RRMS patients and 200 healthy controls.[10] In this study, the frequency of heterozygotes (CT) were slightly higher in MS group than in control group (47.5% vs 39.5%) and that TT genotype was more prevalent in control group than in patient group (8.5% vs 3.75%); however, this trend did not reach statistical significance. In fact, the frequency of the T allele was nearly equal in MS group compared with the control group (27.5% vs 28.25%).[10] In their study, only RRMS patients were included; however, in our study, 3 subgroups of MS patients were presented. Despite all these negative results, we argued that association between MTHFR and MS cannot be completely excluded because association tests only examine a single point of a gene and also the relatively small tested population reduced the power to detect a significant association if it exists.[8,21]

Alatab et al[8] investigated the association of MS and MTHFR C677T polymorphism in 194 Iranian MS (RRMS, SPMS, and PPMS) patients and 230 controls and found that T allele was 1.7 times more present in the patients group. Moreover, they found that subjects with T allele developed MS disease almost 4 years sooner than the other genotypes, and they concluded that carrying the T allele of C677T polymorphism might be a predisposition to earlier onset of MS. We did not replicate this result.

The MTHFR enzyme converts 5,10-MTHF to 5-MTHF, which is a cofactor for remethylation of homocysteine to methionine. Reduced MTHFR enzyme activity due to C677T polymorphism can lower the synthesis of methionine by 5-MTHF–dependent methionine synthase leading to decreased availability of "active" methionine, that is, SAM. It is an important methyl donor in many methylation reactions that are important for neuronal homeostasis. Methylation of the myelin

basic protein (MBP)–arginine and membrane phospholipids are important for the hydrophobicity of those proteins and thereby influence myelin stability during demyelination or remyelination. Hypomethylation of MBP-arginine decreases the hydrophobicity of MBP, destabilizing myelin structures and enhancing degeneration of the myelin sheath. As a result, SAM has anti-inflammatory properties and is necessary for CNS (re)myelination.[13,22] In methionine metabolism, mutant variants of cystathionine β-synthase and reduced folate carrier 1 were associated with an earlier age of onset of MS.[23]

C677T polymorphism of MTHFR is the most common genetic cause of hyperhomocysteinemia.[11] High levels of homocysteine have been encountered in some, but not all, MS patients.[21] Studies indicate that elevated homocysteine levels can cause damage to CNS cells multiple neurotoxic mechanisms, thus leading to the pathogenesis of MS.[11,24] Homocysteine is rapidly taken up by neurons via a specific membrane transporter, a mechanism that results in accumulation of relatively high concentrations of homocysteine within the cell.[25] High homocysteine levels can induce excitotoxicity by overstimulation of *N*-methyl-D-aspartate receptors, resulting in neuronal DNA damage hence triggering apoptosis due to excessive Ca^{2+} influx and induction of reactive oxygen species.[13] In addition, hyperhomocysteinemia may trigger oxidation of low-density lipoproteins extending to lipid peroxidation and atherosclerosis stages and so may be responsible for the sensitization of neurons to oxidative stress.[13,25] Reactive oxygen species, leading to oxidative stress, have been implicated as mediators of demyelization and axonal damage in both MS and experimental autoimmune encephalomyelitis. Excess reactive oxygen species cause damage at the level of cellular components such as lipids, nucleic acids, and proteins, resulting in cell death.[26]

Homocysteine influences the response of T and B lymphocytes, natural killer cells, cytokines, and adhesion molecules, so it may sensitize patients to the viral and immunological mechanisms of MS.[13] Homocysteine is a potential T cell activator, promoting cellular activation and differentiation as well as increasing activation-induced cell death and cellular apoptosis.[27] Homocysteine adds free cysteinyl residues to proteins and actives molecules and can cleave disulfide bridges by damaging the folding pattern of them. This homocysteinylation can lead to loss or degradation of the biological function of the immune system mediators, multiple enzymes, receptors, growth factors, and structural proteins. Hence, chronic moderate hyperhomocysteinemia in MS patients may have negative effects on immune system regulators.[28]

The MTHFR polymorphisms affect both nucleotide synthesis and DNA methylation. The substrate of MTHFR, 5,10-MTHF, is necessary for nucleic acid synthesis. Reduced MTHFR enzyme activity was associated with T allele of MTHFR C677T polymorphism, putatively resulting in higher availability of 5,10-MTHF for nucleic acid synthesis and thus for cell proliferation under inflammatory conditions.[21]In addition, the presence of the T allele is associated with a significantly decrease in 5-MTHF, which is the predominant circulating form of folate and an increase in other folate forms, such as methylenetetrahydrofolate and other nonmethyl forms. The redistribution of folates could affect thymidine or purine synthesis, with consequent effects on DNA synthesis or repair.[29] Antoniades and his coworkers[30] showed that 5-MTHF, generated by MTHFR, rapidly improves endothelial function and decreases superoxide production in vessels from patients with coronary artery disease by mechanisms that seem independent of homocysteine lowering. The effects of 5-MTHF are due in part to direct scavenging of the oxidant radical peroxynitrite. By this mechanism, 5-MTHF improves bioavailability of the endothelial nitric oxide synthase cofactor tetrahydrobiopterin, which leads to and a decrease in superoxide production. In addition, hyperhomocysteinemia has been associated with an increased risk of atherosclerosis and thrombosis, and MS patients may have an increased risk of concomitant vascular disease.[11]

The limitation of the present study is the absence of homocysteine levels of patients and controls. It would be better to measure homocysteine levels of all subjects to see the effect of this polymorphism on homocysteine levels in our study group. Because MTHFR C677T polymorphism is considered the most common genetic cause of elevated homocysteine levels,[21] we discussed our results according to this information.

In conclusion, our findings suggest that T allele of MTHFR gene C677T polymorphism was associated with MS susceptibility in a Turkish population. Our findings provide additional support to a genetic basis for MS development. The number of patients included in this study is limited, and therefore the results have to be regarded as preliminary. However, further work of this polymorphism in MS, throughout the world, could help to understand the contributions of this polymorphism in MS susceptibility.

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