

Association between Multiple Sclerosis and *FOXP3* Gene promoter region mutations

Multiple Skleroz ile *FOXP3* geni promotor bölge mutasyonları arasındaki ilişki

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SUMMARY

Regulatory T-Cells (Treg Cells), as one of the immune system components, have been highly effective in the autoimmune diseases prevention, particularly multiple sclerosis (MS). MS is a chronic inflammatory and autoimmune disease characterized by immune infiltration and inflammation in the central nervous system. Regulatory T (Treg) cells play an important role in the control of autoimmunity. Expression and action of the transcription factor *FOXP3* controls the development and function of Treg cell. The aim of this study was to investigate the association between MS and *FOXP3* gene promoter region polymorphisms rs2232365 (-924A/G) and rs3761548 (-3279A/C) in a Turkish population. In this case-control study we investigated these polymorphisms in 80 MS patients and 80 healthy controls using PCR-RFLP methods.

Results of our study showed that while there is significant correlation between MS and *FOXP3* rs3761548 polymorphism ($p=0.031$), *FOXP3* rs2232365 polymorphism, has not been found to be associated with the disease ($p=0.31$). As *FOXP3* gene is one of the most important genes in the regulation of the immune cells, it may be concluded that the expression of this gene is important in MS patients. As this SNP is located in the promoter region of the gene, it may affect the expression level of *FOXP3* protein.

Keywords: Multiple sclerosis, *FOXP3*, polymorphism

ÖZET

Amaç: Bağışıklık sistemi bileşenlerinden biri olan düzenleyici T (Treg) hücreleri, özellikle multiple skleroz (MS) gibi otoimmün hastalıkların önlenmesinde oldukça etkilidir. Multiple skleroz (MS), santral sinir sisteminde immün hücre infiltrasyonu ve inflamasyonla karakterize kronik inflamatuvar, otoimmün bir hastalıktır. Treg hücreleri otoimmüitenin kontrolünde önemli bir rol oynar. Treg'lerin gelişimi ve işlevi, transkripsiyon faktörü *FOXP3*'ün (Transkripsiyon Faktörü Forkhead Box P3) uygun ifadesine ve hareketine bağlıdır. Bu bağlamda çalışmamızın amacı, MS ve *FOXP3* geni yaygın promotor bölge mutasyonlarından rs2232365 (-924 A/G) ve rs3761548 (-3279 A/C) arasındaki ilişkinin bir Türk popülasyonunda araştırılması olarak belirlenmiştir.

Yöntem: Çalışma kapsamında 80 MS hastası ve 80 sağlıklı kontrol bireyinden alınan kan örnekleri kullanılmıştır. Foxp3 genotipleri PCR-RFLP yöntemi kullanılarak belirlenmiştir.

Bulgular: Elde edilen sonuçlar istatistiksel olarak değerlendirildiğinde, MS ve *FOXP3* rs3761548 polimorfizmi arasında anlamlı bir ilişki gözlenmiştir ($p= 0.031$). Bunun aksine araştırılan diğer polimorfizm, rs2232365, MS hastalarında hastalıkla ilişkili bulunmamıştır ($p= 0.31$).

Sonuç: *FOXP3* geni, bağışıklık hücrelerinin düzenlenmesinde en önemli genlerden biri olduğundan, MS hastalarında bu genin ekspresyonunun önemli olduğu sonucuna varılabilir. Bu SNP (Tek Nükleotit Polimorfizmi), genin promotor bölgesinde yer aldığından *FOXP3* proteinin ekspresyon seviyesini etkileyebilir.

Anahtar sözcükler: Multiple skleroz, *FOXP3*, polimorfizm

INTRODUCTION

MS is a complex and multifactorial disease. A large number of genetic and environmental factors together play roles in its immunopathogenesis¹⁻³. A series of auto immunities leading to pathology in the central nervous system and the process leading to the clinical picture is thought to begin in the peripheral immune system⁴. However, since a clear and homogeneous transmission pattern has not been established for MS, the classical genetic epidemiologic techniques have not been applied in studies⁵.

The regulatory T (Treg) cells comprise a subset of CD4+ lymphocytes that play an important role in regulating the immune response with their ability to suppress Th cells. Any defect that occurs in the Treg cells leads to the development of autoimmune diseases⁶. Treg cells also have important roles in the pathogenesis of autoimmune inflammation of the central nervous system; because altered Treg function and homeostasis are dangerous in terms of MS pathogenesis^{7,8} and it is known that MS is a Th1/Th17-mediated autoimmune disease⁹. Although there is no difference in peripheral blood and cerebrospinal fluid of Treg cells in MS patients, there is considerable evidence that these cells are a problem in suppressing autoimmunity⁷.

The majority of the MS risk variants are located within immunologically related genes^{10,11}. But known variants are sufficient to account for roughly 30% of the genetic basis of the disease. Many explanations have been proposed for this incomplete heritability of MS, including neglecting the analysis of sex chromosomes^{12,13}.

Xp11.23 localized *FOXP3* gene is a central molecule in the function of the Treg cells that play a role in the immunoregulation^{14,15}. In various regions of *FOXP3* gene, such as promoter, exon and intron, more than 100 SNPs (single nucleotide polymorphism) have been identified that are associated with gene expression, miRNA expression, gene splice mechanism or encoded protein structure and activity¹⁶. The role of

FOXP3 gene polymorphisms in MS genetic susceptibility is still unclear due to the inconsistency of the result of the association studies¹⁷. In order to understand the role of *FOXP3* in genetic susceptibility to MS, these inconsistencies need to be clarified. *FOXP3* is an important gene, because it is located on the X chromosome and it encodes an effective transcription factor in the immune system. In this context, we investigated the association between MS and *FOXP3* rs3761548 and rs2232365 promoter region polymorphisms in a Turkish population.

MATERIAL AND METHODS

Patients

The case group consisted of 80 patients diagnosed with MS according to McDonald criteria¹⁸ in the Neurology department of Cumhuriyet University Hospital, Sivas, Turkey. The control group was selected from 80 healthy individuals without MS and without MS in their family. The study was approved by ***. An approval form was taken from the patients and control group individuals involved in the study.

Genotyping

DNA isolation was performed using the phenol-chloroform method¹⁹ from 10 ml blood samples taken from each individual. The *FOXP3* promoter region was amplified by polymerase chain reaction (PCR) to include the region -924. Forward and reverse primer sequences were 5'-TGGAGGGCTTTCAAGGTGAGGA-3' and 5'-GGGGAGTTGGATTGGGTGCA-3' respectively. The method for PCR included an initial denaturing at 94°C for 4 min, followed by 30 cycles at 94°C for 1 min, 58°C for 30 s, 72°C for 30 s with a final extension at 72°C for 10 min. The resulting 374 bp amplicons were incubated with *BsMI* (New England Biolabs) restriction endonuclease enzyme at 37°C for 1 hour and then separated on 3% agarose gel. For the AG genotype 374+188+186 bp fragments, for the GG

genotype 188+186 bp fragments and for AA genotype 374 bp fragment were obtained following restriction digestions and agarose gel electrophoresis separation.

For *FOXP3* -3279 SNP, forward and reverse primer sequences were 5'-CTGACATGCCTCCATCAT-3' and 5'-TGCAGGGCTTCAAGTTGACAG-3' respectively. The method for PCR included an initial denaturing at 94°C for 4 min, followed by 30 cycles at 94°C for 1 min, 55°C for 30 s, 72°C for 30 s with a final extension at 72°C for 10 min. The resulting 361 bp amplicons were incubated with *Pst*I (Nzytech) restriction endonuclease enzyme at 55°C for 1 hour and then separated on 3% agarose gel. For the CC genotype 138+223 bp fragments, for the CA genotype 361+138+223 bp fragments and for the AA genotype 361 bp fragment were obtained following restriction digestion and agarose gel electrophoresis separation.

Statistical Analysis

The data were analyzed using the SPSS 22.0 (SPSS, Chicago, IL, USA) program. Chi square and Fisher's exact tests were used to compare the data. OR and 95% confidence intervals for obtained results were given. $p < 0.05$ was considered as significant in all tests.

RESULTS

Demographic data of the patient and control group were summarized in the Table 1. According to Table 1, there was no statistically significant difference in age and gender between the MS group and the control group. Only patients with bout-onset MS were included in the study: 66 individuals were classified as having relapsing-remitting (RS) MS, and 14 had secondary-progressive (SP) form.

Table 1: Demographic data of MS patients and controls.

| Demographic data | Control (n=80) | Patients (n=80) |
|--------------------------|----------------|-----------------|
| Age | 37.9±8 | 40.9±10.6 |
| Gender | | |
| Female | 52 (%65) | 55 (%68.75) |
| Male | 28 (%35) | 25 (%31.25) |
| Disease duration (years) | | 9±5 |
| MS form | | |
| RR | | 66 (%82.5) |
| SP | | 14 (%17.5) |

MS: multiple sclerosis, RR: relapsing-remitting, SP: secondary progressive

When genotype distribution of the patient and control group is examined in terms of *FOXP3* gene promoter region rs2232365 polymorphism, there was no statistically significant difference in

genotype and allele frequency between the patient and control group for this polymorphism. The distributions for rs2232365 obtained from MS patients and healthy control group are summarized in Table 2.

Table 2: Distribution of genotypes and allele frequencies of *FOXP3* gene promoter region polymorphism rs2232365 among patients with MS and healthy subjects

| | Control (n=80) | Case (n=80) | p value | OR(%95CI) |
|-----------------|----------------|-------------|---------|---------------------|
| Genotype | | | | |
| AA | 44 (%55) | 34 (%42.5) | | |
| AG | 20 (%25) | 23 (%28.75) | 0.3 | 1.483(0.699-3.169) |
| GG | 16 (%20) | 23 (%28.75) | 0.12 | 1.85(0.8474-4.102) |
| Genotype | | | | |
| AA | 44 (%55) | 34 (%42.5) | | |
| AA+AG | 36 (%45) | 46 (%57.5) | 0.12 | 1.648(0.8815-3.102) |
| Allele | | | | |
| A | 108 (%67.5) | 91 (%56.87) | | |
| G | 52 (%32.5) | 69 (%43.13) | 0.05 | 1.573(0.9974-2.488) |

When the case and control groups were compared in terms of rs3761548, it was determined that heterozygous AC genotype and homozygous CC genotype showed a significant difference between

MS patients and healthy individuals. The association between *FOXP3* rs3761548 polymorphism and MS disease is shown in Table 3.

Table 3: Distribution of genotypes and allele frequencies of *FOXP3* gene promoter region polymorphism rs3761548 among patients with MS and healthy subjects

| | Control (n=80) | Case (n=80) | p value | OR(%95CI) |
|-----------------|----------------|-------------|--------------|--------------------|
| Genotype | | | | |
| AA | 44 (%55) | 12 (%15) | | |
| AC | 12 (%15) | 28 (%35) | 0.026 | 7.671(1.248-60.19) |
| CC | 24 (%30) | 40 (%50) | 0.031 | 5.714(1.157-34.97) |
| Genotype | | | | |
| AA | 44 (%55) | 12 (%15) | | |
| CC+AC | 36 (%45) | 68 (%85) | 0.01 | 6.568(1.511-36.03) |
| Allele | | | | |
| A | 52 (%32.5) | 100 (%62.5) | | |
| C | 108 (%67.5) | 60 (%37.5) | 0.008 | 3.405(1.362-8.806) |

DISCUSSION

More than hundred new MS risk SNPs in more than 100 loci have been discovered by development of genome wide association studies (GWAS) and subsequent Immuno-chip based

studies in recent years²⁰. In the pathogenesis and complications of MS disease, immune system, the appearance of autoimmunity in the pathogenesis, damage to peripheral immunological tolerance, play important roles^{21,22}. In some mouse models,

Treg cells have been shown to provide a critical level of protection against autoimmunity, transplant rejection and lymph-proliferative diseases²³. *FOXP3* gene encodes a transcription factor believed to be crucial for the development and function of the Treg cells¹⁴. Recent studies have confirmed that the suppressive function of regulatory T cells (Treg) is impaired in MS patients and that the *FOXP3* gene is a crucial transcription factor in the regulation of CD4⁺CD25⁺FOXP3⁺ Treg cells. Polymorphisms in the promoter region of the *FOXP3* gene may alter the gene expression level and, therefore, contribute to the disease susceptibility^{5,24,25}.

In our study, the genetic association between two common polymorphisms of the *FOXP3* gene promoter region (rs2232365 and rs3761548) and susceptibility to MS disease was evaluated in a Turkish population. As a result, while there was no significant difference in the rs2232365 polymorphism, for rs3761548 the homozygous genotype, heterozygous genotype and polymorphic allele were found to be higher in MS patients than in healthy controls (see Table 3).

In 2005, Huan and colleagues examined the level of *FOXP3* expression in MS patients and found that MS patients expressed *FOXP3* at varying low levels²⁶. In following years, Jafarzadeh and colleagues conducted a study on *FOXP3* polymorphisms in Iranian population and rs3761548 AA and AC genotype frequencies were found to be statistically significantly higher in MS patients compared to healthy control subjects. When they examined in terms of allele frequencies, in MS patients, the frequency of A allele was significantly higher and the frequency of C allele was found to be significantly lower than control subjects²⁷. Similarly, in the study of polymorphism in Iranian populations, Eftekharian et al reported that rs3761548 polymorphism may be associated with MS susceptibility²⁸. In our study, like another studies, there is an association between rs3761548 polymorphism and MS phenotype. In contrast to these data, the another study with Iranian population did not show a significant association between rs3761548 and MS disease susceptibility²⁹. And also, in a study conducted in Slovak population in 2017, it was also stated that there was no significant association between rs3761548 polymorphism and genetic predisposition to MS disease. Furthermore, they found that polymorphism allele frequencies did not significantly differ between patients and control groups independent from gender. Gajdobsechova and colleagues pointed out that there is no evidence that *FOXP3* gene

polymorphisms are associated with MS in European origin Caucasian population, consistent with the results of these studies, with reference to two GWAS studies in MS for their study results¹⁷.

The last two studies in Iranian population in case of disagreement about rs3761548 polymorphism have suggested that rs2232365 polymorphism may support MS susceptibility^{28,29}. In contrast these findings, in our study, there is no association between rs2232365 polymorphism and MS susceptibility while rs3761548 polymorphism supported MS phenotype.

These inconsistent observations between different populations and even the same ethnic groups are due to various factors such as especially the genetic differences among ethnic groups, inadequate sample size, the power of statistical analysis, the risk of false positive results, differences between the inclusion or exclusion criteria for selected individuals in the patients and control groups, the design of the study and the statistical approach it explained. Therefore, to better understand the actual effects of *FOXP3* variants on the disease susceptibility and process, in a larger sample, there is a need for further research that evaluates in terms of functional and genetic, considering both other polymorphisms in the promoter region and other regions of the gene, as well as other parameters of the disease.

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