Genetic Susceptibility to Multiple Sclerosis: The Role of FOXP3 Gene Polymorphism

Multipl Skleroza Genetik Yatkınlık: FOXP3 Gen Polimorfizminin Rolü

Nihal IŞIK¹, Nüket YILDIZ MANUKYAN², İlnur AYDIN CANTÜRK¹, Fatma CANDAN¹, Ayşen ÜNSAL ÇAKMAK¹, Güher SARU HAN DİRESKENELİ³

¹Clinic of Neurology, Medeniyet University Göztepe Training and Research Hospital, İstanbul, Turkey

²Clinic of Neurology, Ministry Health Fatih Sultan Mehmet Training and Research Hospital, İstanbul, Turkey

³Department of Neurology, İstanbul University İstanbul Faculty of Medicine, İstanbul, Turkey

ABSTRACT

Introduction: It is well recognized that both genetic and environmental factors play an important role in the pathogenesis of multiple sclerosis (MS). Immune pathogenesis of MS focuses on pathogenic CD4+ T lymphocytes. CD4+CD25+ regulatory T cells have suppressive function in this cell group. FOXP3 (forkhead boxP3) transcription factor is a key structure in the development and function of regulatory cells. Functional alterations in FOXP3 gene expression have been observed in various autoimmune diseases.

Methods: We screened a non-synonymous coding single nucleotide polymorphism (exon +2710 C/T) (rs2232369) of human FOXP3 gene in 148 MS patients (118 with Relapsing Remitting MS, 30 with Secondary Progressive MS) and 102 age- and sex-matched healthy controls. The association of polymorphisms with susceptibility, and course of the disease was evaluated.

Results: We could not detect any single nucleotide polymorphism in MS patients, however, polymorphic allele was detected in 3% of the control group. Consequently, a genetic association between the FOXP3 gene polymorphism and MS was not revealed.

Conclusion: The distribution of this polymorphism has not been screened in any other MS populations before. Although we could not succeed to find any association between susceptibility to MS and screened FOXP3 gene polymorphisms, we suggest that this particular polymorphism is not appropriate for these kind of studies in the future. (*Archives of Neuropsychiatry 2014; 51: 69-73*)

Key words: Multiple Sclerosis, FOXP3, polymorphism, genotype

Conflict of interest: The authors reported no conflict of interest related to this article.

ÖZET

Amaç: Multipl Skleroz (MS) patogenezinde genetik ve çevresel faktörlerin önemi iyi bilinmektedir. Hastalığın immün patolojisi, patojen CD4+T lenfositlerine odaklanmıştır. Bu hücrelerin aktivitesi, CD4+CD25+ T regülatör hücreler tarafından kontrol edilmektedir. FOXP3 (forkhead boxP3) transkripsiyon faktörünün ise regülatör hücrelerin gelişimi ve fonksiyonu üzerinde kilit rolü vardır. Çeşitli otoimmün hastalıklarda, FOXP3 gen ekspresyonunda fonksiyonel değişiklikler saptanmıştır.

Yöntem: Yaş ve cinsiyet açısından uyumlu 102 sağlıklı kontrol ve 148 Multipl Skleroz (118 Relapsing Remitting MS ve 30 Sekonder Progressif MS) hastasında, FOXP3 genine ait ekzon +2710 C/T (rs2232369) tek nükleotid polimorfizmi tarandı. Saptanan polimorfik alelin, hastalığa yatkınlık ve hastalık tipi ile bağlantısı araştırıldı.

Bulgular: Hasta grubunda tek nükleotid polimorfizmi saptanmadı ancak kontrol grubunda %3 oranında polimorfik alel mevcuttu. Bu bulgularla, FOXP3 gen polimorfizmi ile hastalığa yatkınlık arasında bir bağlantı saptanmadı.

Sonuç: Bu polimorfizm daha önce başka bir Multipl Skleroz hasta grubunda taranmamıştır. Sonuç olarak, hastalıkla polimorfik alel arasında bir bağlantı saptanamasa da, hastalığın patogenezinde son derece önemli bir yapı olan FOXP3 ile ilgili bundan sonraki genetik çalışmalarda, bu kodlanan bölgenin iyi bir aday olmadığı söylenebilir. (*Archives of Neuropsychiatry 2014; 51: 69-73*)

Anahtar kelimeler: Multipl Skleroz, FOXP3, polimorfizm, genotip

Çıkar Çatışması: Yazarlar bu makale ile ilgili olarak herhangi bir çıkar çatışması bildirmemişlerdir.

Introduction

Multiple sclerosis (MS) is a chronic, inflammatory, autoimmune disease characterized with immune cell infiltration and inflammation in the central nervous system (CNS) (1,2).

While the immune system protects the organism against many potential pathogen microorganisms, it avoids immune reaction against its own tissues. This self-tolerance is enabled by

abolishment or inactivation of the auto-reactive lymphocytes during development in the thymus. Despite this clonal inactivation, pathogenic T cells escaping into the peripheral circulation are actively suppressed by CD4+ regulator T cells (Treg). Most of the Treg cells structurally express CD25 (interleukin (IL)-2 receptor alpha chain) (3,4,5,6,7).

In the study of Sakaguchi et al. which was a milestone in the issue of function of Treg cells, it was found that various systemic

Correspondence Address

Dr. Nihal Işık, Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi, Noroloji Kliniği, İstanbul, Türkiye

Gsm: +90 532 225 79 07 E-mail: nihal-isik@hotmail.com Received: 18.02.2013 Accepted: 12.03.2013

©Copyright 2015 by Turkish Association of Neuropsychiatry / ©Telif Hakkı 2014 Türk Nöropsikiyatri Derneği

autoimmune diseases occurred in mice whose CD4+CD25+ Treg cells were abolished (8). In studies conducted in recent years, it has been shown that disruption in the tolerance mechanisms controlling growth of pathogenic T cells directed to myelin or other "self" tissue antigens might lead to occurrence of MS and other autoimmune diseases (9,10). Controversial results have been obtained in the few number of human studies (11,12,13).

Recent studies have shown that FoxP3 (forkhead boxP3-scurfin) transcription factor is important in realizing the regulatory function of Treg cells (6,14,15,16).

Single nucleotide polymorphisms (SNP) are the most simple DNA differences observed between individuals. SNP may change the amino acid encoded by the gene, may stay silent or may be present in the regions where encoding does not occur. Therefore, it may play a significant role in development of autoimmune diseases by affecting gene production, mRNA formation or protein production (17).

In this study, FOXP3 gene polymorphisms defined in MS patients and healthy controls were investigated and it was investigated if these mutations were related with predisposition to MS and other phenotypic properties in our community.

Methods

Patients and Control Groups

Our patient group was composed of 148 patients who were being followed up in the Multiple Sclerosis Outpatient Clinic and had a definite diagnosis of MS according to MacDonald criteria (18). 118 of the patients had relapsing remitting (RR) MS and 30 had secondary progressive (SP) MS. 102 age and gender matched healthy controls (68 women and 34 men) who had no history of MS, no signs of MS and no familial history of MS constituted the control group.

Before blood samples were obtained the participants were given information about the study and informed consent form was filled in.

DNA isolation

10 cc venous blood were obtained from the patients and controls and placed in EDTA tubes. After the necessary procedures required for separation of DNA (separation of leukocytes, cell explosion, nucleus explosion, abolishment of proteins) were performed, DNA strands which became visible with addition of ethanol were placed in a sterile DNA tube. The obtained DNA was homogenized with 200 µl sterile distilled water and rediluted at a ratio of 1:50. Their optic densities were measured by spectrophotometer (at 260 nm) and their amounts were specified. According to the values obtained, the DNAs were diluted as 30 µg/ml and kept at +4°C until they were studied.

Polymerase chain reaction (PCR)

+2710 C/T (rs2232369, 220.aa A/V) SNP which is found in the encoding region on the FOXP3 gene (exon) and has been reported to cause to amino acid alternation was screened by way of amplification of the targeted genomic region by PCR and specification of the pattern of cutting with restriction enzyme

(polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)). The method was based on loss of the property of the FOXP3 gene of being cut with the appropriate enzyme selected according to the nucleotide differences at the point of polymorphism investigated. Primer3 (Whitehead Institute for Biomedical Research) program was used for this study. Amplification was performed with 5'-CTGAGGGGCATGTGTTA-AGG and 5'-TTTGC GCACTATCCCTATCC primers and presence of C or T allele was identified by cutting the product with a length of 360 base pairs with *EciI* enzyme. The C allele constituted a cutting point for the restriction enzyme.

Statistical analysis

The allele and genotype frequencies found in the patient and control groups were compared using χ^2 test. Probable haplotype frequencies were calculated using Arlequin program.

Results

The demographic properties of the study group is summarized in Table 1. There was no difference between the patient and control groups in terms of age and gender. The distribution obtained for FOXP3 gene +2710 (C/T) polymorphism in MS and healthy controls is shown in Table 2.

When this polymorphism distribution which was identified but not screened in any population before was examined, polymorphic allele was shown with a rate of 3% only in the control group. When the allele frequencies found in MS patients and healthy controls were compared, no significant difference of distribution was observed between the two groups (Table 2).

Discussion

Although the etiology of MS which is an autoimmune disease is not known exactly, it is thought that it probably has a heterogeneous structure and both genes and environmental factors are important in development of the disease. However, a clear and homogeneous type of inheritance could not be found. Therefore, classical genetic epidemiological techniques can not be applied in studies. In all diseases with complex inheritance, the most appropriate approach for gene studies is primarily detecting the chromosomal region of the genomic effect by linkage analyses. The secondary step is to demonstrate the degree of variability in certain parts of the candidate region (exon, promotor region or intron) in terms of direct relation with the disease (19,20).

Single nucleotide polymorphisms are the most common DNA sequence variations in the human genome. SNPs are thought to be old and stable mutations found in the whole of genome. These properties make them good targets for genetic studies. Although these mutations are mostly neutral, some may play a role in predisposition to morbidity or resistance to morbidity (21,22).

CD4+CD25+Treg cells which are thought to be important in the immune etiopathogenesis of autoimmune diseases realize

Table 1. The demographic data and distribution of clinical properties in the control and patient groups

	Male	Female	Total
MS group	48	100	148
Age	40.9±9	37.5±8	38.3±9
Disease duration (years)	11±9	9±5	9.5±6
EDSS (mean)	2.7±1	1.9±1	2.1±1
RR MS	34	84	118
SP MS	14	16	30
Control Group	34	68	102
Age	41.2±8	35.2±9	37.9±8

MS: Multiple Sclerosis, EDSS: Expanded Disease Status Scale, RR: Relapsing remitting, SP: Secondary Progressive

Table 2. Distribution of FOXP3 +2710 (C/T) polymorphism in the MS and healthy control group

+2710 (C/T)	MS n=148	%	Control n=102	%
Allele frequency				
C	296	100	198	97
T	0	0	6	3
Genotype frequency				
CC	148	100	99	97
TT	0	0	3	3
TC	0	0	0	0

MS: Multiple Sclerosis, C: Cytosine, T: Thymine

central tolerance in the thymus and show regulatory functions in the periphery. Disruption in the immunological self-tolerance leads to occurrence of autoimmune diseases (7). Although treg cells in MS patients are not different in the peripheral blood and cerebrospinal fluid, there is important evidence showing that these cells have problems in suppressing autoimmunity (13).

It has been shown that CD25+ cell group can prevent increase in CD4+ pathogenic cells and cytokine release. Recently, the view that CD4+ CD25+ Treg cells prevent autoimmunity by way of FoxP3 has been widely accepted (6). FoxP3 is the key factor in development and function of Treg cells which express IL2 receptor α chain (CD25) and is effective in regulation of both natural and acquired immunity. FOXP3 gene is located on the X-chromosome. The FOXP3 transcription factor is basically produced by T cell sequence and is the main regulator in inhibition of T cell activation (14,15,16). Deletion in the gene coding FOXP3 results in increased proliferation of active T cells and development of both autoimmune and allergic diseases. It has been shown that mutation in the FOXP3 gene causes to IPEX

syndrome which is a rare autoimmune genetic diseases in humans. IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked inheritance) is an immune system disease characterized with hyperreactivity of T cells (23).

The number of studies investigating the role of FOXP3 in the etiopathogenesis of MS has increased in recent years. In 2003, Putheti et al. reported that CD4+ CD25+ T cells increased (11). In 2004, they reported that these cells did not change (12). However, in 2004, Viglietta showed that the suppressive functions of CD4+ CD25+ T cells decreased, but did not give information if this decrease was related with the level of expression of the FOXP3 gene (13). In 2005, Huan et al. showed that the reduction in the functionally suppressive ability of peripheral CD4+ CD25+ T cells was related with the reduction in the level of FOXP3 expression (17). Similarly, Venken reported that both the suppressive functions of CD4+ CD25+ T cells and the amount of FOXP3 protein decreased in RRMS patients in a study published in 2008 (24). Studies conducted in this area in recent years have mostly aimed to demonstrate the relation of Treg cells with therapeutic agents. A few studies investigating the effect of interferon beta (IFN B) (one of the first line immunomodulator drugs used in treatment of multiple sclerosis) treatment on Treg cells have been published. In one of these studies, Namdar et al. found that IFN B increased Treg frequency and suppressive function significantly, but no change was observed in FOXP3 gene expression at the 6th month after treatment in 18 RRMS patients and stated that this treatment regulated the function of regulatory T cells in some RRMS patients and suppressed the immune activity which had an uncontrolled course (25). In another study, the relation of CD4+ CD 25 (high) T cell phenotype with FOXP3, ITCH and CBLB (molecules which provide regulation of FOXP3 transcription factors) gene expression was studied in patients who did and did not receive IFN B treatment; it was found that the level of cytotoxic T lymphocyte antigen 4 (CTLA 4) expressed on the CD4+ CD 25 (high) cellular surface which shows correlation with FOXP3 mRNA was low in the patients who did not receive treatment and increased significantly after IFN B injection. In addition, correlation of FOXP3 mRNA expression with CBLB, ITCH and T helper 2 cytokine level was shown (26). As observed in the literature, the initial studies related with FOXP3 function showed controversial results. Later studies supported the role of FOXP3. In a significant study conducted to demonstrate the effect of Treg cells in treatment, CD4+ T cells were transformed to Treg cells by using lentiviral vector system and these cells were given intranasally for treatment of mice in which EAE was induced. After application, it was found that Treg cells reached various regions of the brain and EAE symptoms regressed clinically. In addition, it was observed that mRNA levels of inflammatory cytokines including IL-12 and IFN-gamma were decreased in the brain tissue (27).

Although the number of studies related with Treg cells and their functions has increased, there is no study investigating the association of FOXP3 gene polymorphism with MS disease. When the distribution of this polymorphism which has not been

screened in any other population before was examined with this objective, polymorphic allele was found in 3% of the control group, while it was not found in the patients (Table 2).

In 2003, Bassuny et al. compared 199 type 1 diabetes patients and 289 healthy controls in terms of predisposition to disease in the Japanese population and screened single nucleotide polymorphisms in the protein coding region of microsatellite and FOXP3 gene in the intron and promotor areas. They found a significant relation between FOXP3 gene and predisposition to type 1 diabetes (28). On the other hand, Zavattari et al. showed that there was no relation between FOXP3 gene and type 1 diabetes in the population who lived in Sardinia island (29). In another recent study, it was reported that there was no association with FOXP3 gene polymorphism in autoimmune thyroiditis and Addison disease (30).

When examined in terms of functionality, it can be thought that Treg cells and FOXP3 molecule may have important immune functions in terms of autoimmune diseases and thus predisposition to MS. However, controversial results have been obtained in few genetic studies conducted in this area. The reason for this may be inappropriateness of the genetic site selected for screening, differences which may be created by the method which is substantially sophisticated and inconvenient and insufficient number of subjects which is a very important issue in genetic studies. All these factors were also limitations of our study.

We could find no evidence to demonstrate the relation between SNP in the exon +2710(C/T) region of the FOXP3 gene and predisposition to MS. However, we thought that it was worth to publish our results, because there was no other study where FOXP3 gene polymorphism was screened in MS and we wanted to share the information that polymorphism in this region should not be screened in future studies. We think that identification of chromosomal regions and specific target genes will be helpful in development of new and more efficient methods in terms of treatment of the disease by helping in understanding the pathogenesis of MS.

References

- O'Connor KC, Bar-Or A, Hafler DA. The neuroimmunology of multiple sclerosis: possible roles of T and B lymphocytes in immunopathogenesis. *J Clin Immunol* 2001; 21:81-92.
- Steinman L, Martin R, Bernard C, Conlon P, Oksenberg JR. Multiple sclerosis: deeper understanding of its pathogenesis reveals new targets for therapy. *Annu Rev Neurosci* 2002; 25:491-505.
- Scholz C, Patton KT, Anderson DE, Freeman GJ, Hafler DA. Expansion of autoreactive T cells in multiple sclerosis is independent of exogenous B7 costimulation. *J Immunol* 1998; 160:1532-1538.
- Baecher-Allan C, Vigieta V, Hafler DA. Human CD4+CD25+ regulatory T cells. *Seminars in Immunology* 2004; 16:89-97.
- Sakaguchi S. Regulatory T Cells: Minireview Key Controllers of Immunologic Self-Tolerance. *Cell* 2000; 101:455-458.
- Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; 299:1057-1061.
- Shevach EM. Regulatory T cells in autoimmunity. *Annu Rev Immunol* 2000; 18:423-449.
- Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of selftolerance causes various autoimmune diseases. *J Immunol* 1995; 155:1151-1164.
- Lovett-Racke AE, Trotter JL, Lauber J, Perrin PJ, June CH, Racke MK. Decreased dependence of myelin basic protein-reactive T cells on CD28-mediated costimulation in multiple sclerosis patients. A marker of activated/memory T cells. *J Clin Invest* 1998; 101:725-730.
- Vigieta V, Kent SC, Orban T, Hafler DA. GAD65-reactive T cells are activated in patients with autoimmune type 1a diabetes. *J Clin Invest* 2002; 109:895-903.
- Putheti P, Morris M, Stawiarz L, Teleshova N, Kivisakk P, Pashenkov M, Kouwenhoven M, Wiberg MK, Bronge L, Huang YM, Soderstrom M, Hillert J, Link H. Multiple sclerosis: a study of chemokine receptors and regulatory T cells in relation to MRI variables. *Eur J Neurol* 2003; 10:525-535.
- Putheti P, Pettersson A, Soderstrom M, Link H, Huang YM. 2004. Circulating CD4+ CD25+T regulatory cells are not altered in multiple sclerosis and unaffected by disease-modulating drugs. *J Clin Immunol* 2003; 24:155-161.
- Vigieta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by regulatory T cells in patients with multiple sclerosis. *J Exp Med* 2004; 199:971-979.
- Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+ CD25+ regulatory T cells. *Nat Immunol* 2003; 4:330-336.
- Ramsdell F. Foxp3 and natural regulatory T cells: key to a cell lineage? *Immunity* 2003; 19:165-168.
- Ochs HD, Ziegler SF, Torgerson TR. FOXP3 acts as a rheostat of the immune response. *Immunol. Rev* 2005; 203:156-164.
- Huan J, Culbertson N, Spencer L, Bartholomew R, Burrows GG, Chou YK, Bourdette D, Ziegler SF, Offner H, Vandenbark AA. Decreased FOXP3 Levels in Multiple Sclerosis Patients. *J Neurosci Res* 2005; 81:45-52.
- Polman CH, Reingold SC, Edan G, Filippi M, Hartung HP, Kappos L, Lublin FD, Metz LM, McFarland HF, O'Connor PW, Sandberg-Wollheim M, Thompson AJ, Weinshenker BG, Wolinsky JS. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". *Ann Neurol* 2005; 58:840-846.
- Dyment AD, Ebers GC, Sadovnick AD. Genetics of Multiple Sclerosis. *Lancet* 2004; 3:104-113.
- Oksenberg JR, Hauser SL. New insights into the immunogenetics of multiple sclerosis. *Curr Opin Neurol* 1997;10:181-185.
- Kruglyak L. The use of a genetic map of biallelic markers in linkage studies. *Nat Genet* 1997; 17:21-24.
- Weiss KM, Terwilliger JD. How many diseases does it take to map a gene with SNPs? *Nat Genet* 2000; 26:151-157.
- Owen CJ, Jennings CE, Imrie H, Lachaux A, Bridges NA, Cheetham TD, Pearce SH. Mutational analysis of the FOXP3 gene and evidence for genetic heterogeneity in the immunodysregulation, polyendocrinopathy, enteropathy syndrome. *J Clin Endocrinol Metab* 2003; 88:6034-6039.

24. Venken K, Hellings N, Thewissen M, Somers V, Karen Hensen K, Rummens JL, Medaer R, Hupperts R, Stinissen P. Compromised CD4+ CD25high regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level. *Immunology* 2008; 123:79-89.
25. Namdar A, Nikbin B, Ghabaee M, Bayati A, Izad M. Effect of IFNbeta therapy on the frequency and function of CD4(+)CD25(+) regulatory T cells and Foxp3 gene expression in relapsing-remitting multiple sclerosis (RRMS): a preliminary study. *J Neuroimmunol* 2010; 218:120-124.
26. Sellebjerg F, Krakauer M, Khademi M, Olsson T, Sørensen PS. FOXP3, CBLB and ITCH gene expression and cytotoxic T lymphocyte antigen 4 expression on CD4(+) CD25(high) T cells in multiple sclerosis. *Clin Exp Immunol* 2012;170:149-155.
27. Fransson M, Piras E, Burman J, Nilsson B, Essand M, Lu B, Harris RA, Magnusson PU, Brittebo E, Loskog AS. CAR/FoxP3-engineered T regulatory cells target the CNS and suppress EAE upon intranasal delivery. *J Neuroinflammation* 2012;9:112. doi:10.1186/1742-2094-9-112.
28. Bassuny WM, Ihara K, Sasaki Y, Kuromaru R, Kohno H, Matsuura N, Hara T. A functional polymorphism in the promoter/ enhancer region of the FOXP3/ Scurfin gene associated with type 1 diabetes. *Immunogenet* 2003; 55:149-156.
29. Zavattari P, Deidda E, Pitzalis M, Zoa B, Moi L, Lampis R, Con-tu D, Motzo C, Frongia P, Angius E, Maioli M, Todd JA, Cucca F. No association between variation of the FOXP3 gene and common type 1 diabetes in the Sardinian population. *Diabetes* 2004; 53:1911-1914.
30. Owen CJ, Eden JA, Jennings CE, Wilson V, Cheetham TD, Pearce SH. Genetic association studies of the FOXP3 gene in Graves' disease and autoimmune Addison's disease in the United Kingdom population. *J Mol Endoc* 2006; 37:97-104.