Evaluation of mRNA Expression Levels of IL-10, IL-12, TGF-β, FOXP3, IFN in Multiple Sclerosis Patients

Ayşe CEYLAN[1], Nuray BİLGE[2], Eda BALKAN[1*]

[1] *Department of Medical Biology, Faculty of Medicine, Atatürk University, Erzurum, Turkey*

aysealbayrak13@hotmail.com, edadiyarbakir@atauni.edu.tr

[2] *Department of Neurology, Faculty of Medicine, Atatürk University, Erzurum, Turkey*

nuray.bilge@atauni.edu.tr

Abstract

Although great advances have been made in the treatment of Multiple Sclerosis (MS), a neurodegenerative disease due to autoimmune inflammation, the etiopathogenesis of the disease has not yet been fully understood. Therefore, current treatment strategies may be insufficient. This study, it was aimed to quantitatively measure the expression levels of some cytokines determined in patients receiving MS treatment. This study was carried out on patients diagnosed with MS and healthy volunteers followed in Atatürk University Health Research and Application Center Neurology Department, Erzurum, Turkey. mRNA expression changes of *IL-10*, *IL-12*, *TGF-β, FOXP3,* and *IFN* genes in blood samples taken from both groups were determined by quantitative Real-Time PCR. It was determined that there was no statistically significant difference between the patient and control group in the mRNA expression levels of the *IL-10* and *FOXP3* genes. A statistically significant difference was observed between the patient and control group in *TGF-β, IL-12*, and *IFN* mRNA levels. In this study, it was determined that important information about the course of the disease can be obtained by evaluating the expression levels of regulator genes in MS, together.

Keywords: Biomarker, Cytokines, Gene expression, Multiple sclerosis.

Received:22.03.2022
Accepted:22.06.2022
Published:30.06.2022
[*]*Corresponding author:* Eda BALKAN, *PhD*
Department of Medical Biology, Faculty of Medicine, Atatürk University
E-mail: edadiyarbakir@atauni.edu.tr
Cite this article as: E. Balkan, Evaluation of mRNA Expression Levels of IL-10, IL-12, TGF-β, FOXP3, IFN in Multiple Sclerosis Patients, *Eastern Anatolian Journal of Science, Vol. 8, Issue 1, 9-14, 2022.*

1. Introduction

Multiple sclerosis (MS) is common and a chronic neurological disease characterized by damage to axons and myelin in the central nervous system, leading to various manifestations such as cognitive problems, motor control, depression, and fatigue (Ascherio et al., 2012; Dobson & Giovannoni, 2019). Although genetic predisposition contributes to MS pathology, environmental factors such as ultraviolet exposure, obesity, and smoking are also known to play a role in the onset and progression of the disease (Ascherio, 2013). However, the etiology of MS remains unclear, as it is a multifactorial, heterogeneous, and immune-mediated neurodegenerative disease (Filippi et al., 2018).

Genome-wide association (GWAS) studies have identified more than 200 risk variants, most of which are associated with genes that control immune cell function and contribute to genetic susceptibility to disease risk (Baranzini & Oksenberg, 2017). The results show that the disease is mostly due to the dysregulation of pro and anti-inflammatory cytokines. Pro-inflammatory cytokines cause an increase in the permeability of the blood-brain barrier (BBB), leading to neurodegeneration and demyelination of the central nervous system, while anti-inflammatory cytokines can suppress the secretion of pro-inflammatory cytokines (Hashemi et al., 2018). Therefore, it is emphasized that inflammation may play a crucial roles in axon and neuron degeneration,

which is prominent in MS pathology (Hashemi et al., 2020).

Autoreactive CD4+ T cells differentiate into pathogenic helper T (Th) cells (Th1 and Th17) in secondary lymphoid organs, causing the production of proinflammatory cytokines and increased subpial blood vessel permeability, resulting in the escape of circulating autoreactive T cells and effector myeloid cells. Cytokines produced by these subtype cells such as Th1 and Th17 are dynamic players of the inflammatory process, which is considered important for the development of MS (Arellano et al., 2017). Cytokines produced by infiltrating immune cells are implicated throughout the entire course of the disease, from T-cell differentiation to tissue inflammation and damage in the CNS (Palle et al., 2017). Thus, previous studies have proven that cytokine availability and/or signaling management can be an effective approach for the therapy of MS as in different autoimmune diseases (Mirshafiey & Mohsenzadegan, 2009; Williams et al., 2014).

Considering this complex and heterogeneous mechanism of MS, detecting the change in immune response may help identify validated biomarkers that can be used in disease diagnosis and diagnosis. Defining the alterations in the expression levels of critical genes involved in the disease is considered very valuable in terms of contributing to the determination of the effective treatment approach to be applied to individuals who have been treated for MS, as well as elucidating the critical molecular mechanisms for the disease (Hendrickx et al., 2017). This study, it was aimed to contribute to the elucidation of the etiology of the disease through relevant molecular factors by detecting the changes in the mRNA expression levels of some cytokines in patients diagnosed with MS and healthy control groups.

2. Materials and Methods

2.1. Sample collection and ethics statement

This study was carried out in a patient group consisting of 30 patients (18 females - 12 males, newly diagnosed with MS and not taking any medication) who were followed in Erzurum Atatürk University Health Research and Application Center Directorate Neurology Department Polyclinic and diagnosed with MS (according to 2010 McDonald Diagnostic Criteria) and 20 healthy (13 females - 7 males) without any systemic disease carried out on a control group of individuals. Those who were in the MS attack period and received cortisone treatment in the last three months were excluded from the study. The patient and control group who agreed to participate in the study signed the informed consent form, and the clinical and laboratory parameters of the patient group and the drugs used were recorded in the Neurology Patient Anamnesis Forms. This study was approved by the Atatürk University Faculty of Medicine Clinical Research Ethics Committee (05/01-07.06.2018). Detailed sampling informations are summarized in Table 1.

Table 1. Demographic and clinical characteristics of the samples. EDSS: Extended Disability Status Scale, RRMS: Relapsing remitting multipl skleroz

	MS (30)	**Control (20)**
Age (mean ± SD)	38.43 ± 7.62	35.37 ± 8.81
Sex (% female/male)	60/40	65/35
EDSS (mean ± SD)	1.72 ± 0.87	1.82 ± 1.24
MS Type	RRMS	-
Sampling Date(s)	01/09/2018-01/01/2019	

2.2. Gene expression analysis

Total RNA extraction from the blood samples collected from the patient and control groups was performed using a commercial isolation kit (NucleoSpin® RNA Blood, Macherey-Nagel, Germany). The procedure and chemicals recommended by the manufacturer were used for isolation. The concentration and purity of the obtained RNAs were determined using a NanoDrop spectrophotometer (MaestroNano - USA). The obtained products were stored at -80 °C until use. cDNA was synthesized using the ProtoScript® II First Strand cDNA Synthesis Kit (NEB, USA) according to the manufacturer's protocol. All cDNAs were stored at –20°C until use.

mRNA expression profiles of interleukin 10 (*IL-10*), interleukin 12 (*IL-12*), transforming growth factor-beta (*TGF-β*), interferon (*IFN*), and forkhead box P3 (*FOXP3*) genes were measured using the Taqman (GoTaq® Probe qPCR Master Mix, Promega) based qPCR method. qPCR reactions were

performed in a Roche LightCycler 480 (Roche Diagnostics). The amounts of the components that make up the reaction mixture in each tube and the amplification temperature conditions were determined following the protocol recommended by the manufacturer. All measurements were analyzed in triplicate for each sample. Beta-actin (ACTB) was used as a reference gene (housekeeping) for the normalization of target genes.

2.3. Statistical analysis

SPSS 20.0 (Statistical Packages for the Social Sciences for Windows XP Release 20.0 version) program was used to evaluate all statistical data related to the study. A Chi-square test was performed on the patient and control groups. Statistically significant differences are presented as follows: $p > 0.05$ (not significant, ns) and $p < 0.05$ (significant).

3. Results

In this study, the mRNA expression levels of IL-10, IL-12, TGF-β, IFN, and FOXP3 genes were investigated from peripheral blood samples collected from 30 patients with MS and 20 healthy individuals.

According to the findings; It was determined that there was a significant difference in the mRNA expression levels of *IL-12, IFN, TGF-β* genes in patients diagnosed with MS compared to the control group. There was no significant difference between the patient and control groups in *IL-10* and *FOXP3* expression levels. The averages and significance levels of the expression levels of the relevant genes are summarized in Figure 1 and Table 1.

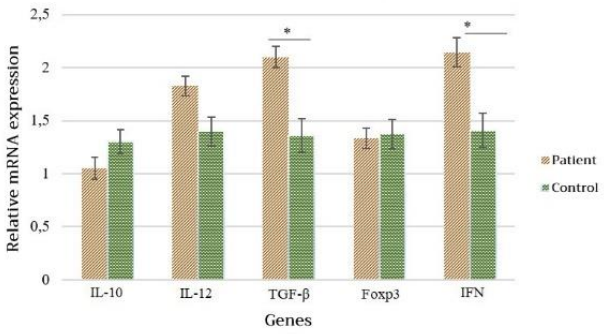

Figure 1. Comparison of expression levels of all genes (*: p<0.005)

Table 1. Patient and control group gene expression levels. MS: Multiple sclerosis, n: total number of subjects sampled

Gene symbol	MS (30) median	Control (20) median	*p* value
IL-10	7.41	10.183	>0.05
IL-12	15.54	10.056	<0.05
TGF-β	13.9	9.736	<0.005
FOXP3	10.02	10.179	>0.05
IFN	18.05	12.087	<0.005

4. Discussion

The incidence of MS, a common non-traumatic disease that mostly affects young adults, is increasing worldwide, due to the socioeconomic impact of the disease (Balkan & Bilge, 2021; Kobelt et al., 2017). Although the mechanisms underlying the disease are not fully understood, gene-environment interactions are thought to play an prominent role (Browne et al., 2014). Today, advances in the diagnostic methods and criteria used for MS, together with the developing technology, enable the diagnosis of the disease to be made at earlier stages. For this reason, the hypothesis that key genes identified in the light of available information and determined to be associated with the disease can be used as biomarkers gains more importance (Monforte & McPhail, 2005). Identification of confirmed biomarkers based on gene expression approach increases the chance of early diagnosis/diagnosis for the related disease, as well as in terms of determining an effective treatment approach (van't Veer et al., 2005; Wei et al., 2004). Therefore, in this study, the most important members of many different major gene groups known to be associated with MS (I*L-10, IL-12, TGF-β, IFN,* and *FOXP3*) were selected and their mRNA expression levels were evaluated together.

It is known that IL-12 production is very important systemically, as it can negatively affect the Th1 response and increase susceptibility to intercellular pathogens (Trinchieri, 1995). It has been determined by previous studies that the amount of IFN also increases by means of T cells that can be activated depending on the increased IL-12 (Balashov et al., 1997). Accordingly, in a study by Reiche et al., IL-12 and serum IFN levels were found to be higher in RR-MS (Relapsing-remitting MS) patients compared to controls (Kallaur et al., 2013). Different

studies have also shown that IL-12 and IFN levels are increased in the brain, cerebrospinal fluid (CSF), and peripheral blood of the patients (Huang et al., 2004). The results obtained in the present study are also in line with previous research.

TGF-β is a pleiotropic cytokine involved in the differentiation and function of T cells. Therefore, it is considered that it may have important functions in an immune system-related disease such as MS (Lee et al., 2017). Its immunosuppressive nature supports the hypothesis that a therapeutic approach to suppressing autoimmunity of TGF-β may be effective. In this direction, studies with rodent models suggest that the administration of TGF-β1 and TGF-β2 during the induction and progression stages of the disease shows positive results in terms of MS. It has been stated that relapse formation is prevented in mice injected with TGF-β1 and that TGF-β1 can be used as an anti-inflammatory due to its immunosuppressive feature against proinflammatory cytokines such as IL-1 (Kuruvilla et al., 1991). In addition, many studies have shown that fewer CNS lesions occur by reducing neurological damage with TGF-β administration (Johns et al., 1991). In the current study, results were obtained in parallel with previous studies, and it was observed that the expression level of *TGF-β* in MS patients increased significantly compared to the control as a result of drug treatment.

Finally, mRNA expression levels of the *FOXP3* gene were investigated in our study. FOXP3 is a transcription factor involved in the production and normal functioning of T cells (Isik et al., 2014). recent studies have confirmed the involvement of FOXP3 in the regulation of CD4+, CD25+, and Treg cells (Li et al., 2015). In the study performed by Taheri et al., it was observed that single nucleotide polymorphisms (SNPs) in the promoter seciton of the gene in question can change the expression level of the gene and the disease susceptibility may increase, consequently (Eftekharian et al., 2016). Studies conducted by different groups also reveal that the insufficiency of FOXP3 and similar mechanisms that prevent the development of pathogenic T cells may be a significant factor in the formation of autoimmune diseases such as MS (Huan et al., 2005). The results of our study also show that *FOXP3* mRNA expression is decreased in the MS group compared to the control group.

5. Conclusion

Although the etiology of MS is unknown, previous studies strengthen the hypothesis that it is a T cell-mediated autoimmune disease. Major genes related to MS were evaluated together in the current study to help understand the pathogenesis of MS and improve the life quality of patients by mediating the development of new and more effective methods in the treatment of the disease. It is thought that the findings obtained have the potential to shed light and form a basis for studies to be carried out to elucidate the molecular etiology of MS. However, there are several limitations to this study that should also be noted. The first of these is the small sample size. Therefore, further works should consider sampling size. Secondly, the genes targeted in this study need to be further examined at the protein level to verify the analysis results.

6. Acknowledgement

This study was supported by Atatürk University Scientific Research Projects Coordination Unit (Project Number: TYL-2019-7045).

Conflict of Interests

The authors declare that there is no potential conflict of interest for the research, authorship, and/or publication of this article. All authors read and approved the final manuscript.

Author Contributions

Design of the study: EB, Sample collection: NB, Performed the experiments: AC, Data Collection and/or Processing: EB, NB, AC, Writing Original Manuscript: EB, NB, AC. EB contributed to revising the work and final approval of the final version of the manuscript.

Ethical Approval

This study was approved by the Atatürk University Faculty of Medicine Clinical Research Ethics Committee (05/01-07.06.2018).

References

ARELLANO, G., ACUNA, E., REYES, L. I., OTTUM, P. A., DE SARNO, P., VILLARROEL, L., CIAMPI, E., URIBE-SAN MARTIN, R., CARCAMO, C., and NAVES, R. (2017). Th1 and Th17 Cells and Associated Cytokines Discriminate among Clinically Isolated Syndrome and Multiple Sclerosis Phenotypes. Front Immunol, 8, 753. doi:10.3389/fimmu.2017.00753

ASCHERIO, A. (2013). Environmental factors in multiple sclerosis. Expert Rev Neurother, 13(12 Suppl), 3-9. doi:10.1586/14737175.2013.865866

ASCHERIO, A., MUNGER, K. L., and LUNEMANN, J. D. (2012). The initiation and prevention of multiple sclerosis. Nat Rev Neurol, 8(11), 602-612. doi:10.1038/nrneurol.2012.198

BALASHOV, K. E., SMITH, D. R., KHOURY, S. J., HAFLER, D. A., and WEINER, H. L. (1997). Increased interleukin 12 production in progressive multiple sclerosis: induction by activated CD4+ T cells via CD40 ligand. Proc Natl Acad Sci U S A, 94(2), 599-603. doi:10.1073/pnas.94.2.599

BALKAN, E., and BILGE, N. (2021). Expression levels of IL-17/IL-23 cytokine-targeting microRNAs 20, 21, 26, 155, and Let-7 in patients with relapsing-remitting multiple sclerosis. Neurol Res, 43(9), 778-783. doi:10.1080/01616412.2021.1935099

BARANZINI, S. E., and OKSENBERG, J. R. (2017). The Genetics of Multiple Sclerosis: From 0 to 200 in 50 Years. Trends Genet, 33(12), 960-970. doi:10.1016/j.tig.2017.09.004

BROWNE, P., CHANDRARATNA, D., ANGOOD, C., TREMLETT, H., BAKER, C., TAYLOR, B. V., and THOMPSON, A. J. (2014). Atlas of Multiple Sclerosis 2013: A growing global problem with widespread inequity. Neurology, 83(11), 1022-1024. doi:10.1212/WNL.0000000000000768

DOBSON, R., and GIOVANNONI, G. (2019). Multiple sclerosis - a review. Eur J Neurol, 26(1), 27-40. doi:10.1111/ene.13819

EFTEKHARIAN, M. M., SAYAD, A., OMRANI, M. D., GHANNAD, M. S., NOROOZI, R., MAZDEH, M., MIRFAKHRAIE, R., MOVAFAGH, A., ROSHANAEI, G., AZIMI, T., INOKO, H., and TAHERI, M. (2016). Single nucleotide polymorphisms in the FOXP3 gene are associated with increased risk of relapsing-remitting multiple sclerosis. Hum Antibodies, 24(3-4), 85-90. doi:10.3233/HAB-160299

FILIPPI, M., BAR-OR, A., PIEHL, F., PREZIOSA, P., SOLARI, A., VUKUSIC, S., and ROCCA, M. A. (2018). Multiple sclerosis. Nat Rev Dis Primers, 4(1), 43. doi:10.1038/s41572-018-0041-4

HASHEMI, R., HOSSEINI-ASL, S. S., AREFHOSSEINI, S. R., and MORSHEDI, M. (2020). The impact of vitamin D3 intake on inflammatory markers in multiple sclerosis patients and their first-degree relatives. PLoS One, 15(4), e0231145. doi:10.1371/journal.pone.0231145

HASHEMI, R., MORSHEDI, M., ASGHARI JAFARABADI, M., ALTAFI, D., SAEED HOSSEINI-ASL, S., and RAFIE-AREFHOSSEINI, S. (2018). Anti-inflammatory effects of dietary vitamin D3 in patients with multiple sclerosis. Neurol Genet, 4(6), e278. doi:10.1212/NXG.0000000000000278

HENDRICKX, D. A. E., VAN SCHEPPINGEN, J., VAN DER POEL, M., BOSSERS, K., SCHUURMAN, K. G., VAN EDEN, C. G., HOL, E. M., HAMANN, J., and HUITINGA, I. (2017). Gene Expression Profiling of Multiple Sclerosis Pathology Identifies Early Patterns of Demyelination Surrounding Chronic Active Lesions. Front Immunol, 8, 1810. doi:10.3389/fimmu.2017.01810

HUAN, J., CULBERTSON, N., SPENCER, L., BARTHOLOMEW, R., BURROWS, G. G., CHOU, Y. K., BOURDETTE, D., ZIEGLER, S. F., OFFNER, H., and VANDENBARK, A. A. (2005). Decreased FOXP3 levels in multiple sclerosis patients. J Neurosci Res, 81(1), 45-52. doi:10.1002/jnr.20522

HUANG, W. X., HUANG, P., and HILLERT, J. (2004). Increased expression of caspase-1 and interleukin-18 in peripheral blood mononuclear cells in patients with multiple sclerosis. Mult Scler, 10(5), 482-487. doi:10.1191/1352458504ms1071oa

ISIK, N., YILDIZ MANUKYAN, N., AYDIN CANTURK, I., CANDAN, F., UNSAL CAKMAK, A., and SARU HAN DIRESKENELI, G. (2014). Genetic Susceptibility to Multiple Sclerosis: The Role of FOXP3 Gene Polymorphism. Noro Psikiyatr Ars, 51(1), 69-73. doi:10.4274/npa.y7098

JOHNS, L. D., FLANDERS, K. C., RANGES, G. E., and SRIRAM, S. (1991). Successful treatment of experimental allergic encephalomyelitis with transforming growth factor-beta 1. J

Immunol, 147(6), 1792-1796. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/17162779

KALLAUR, A. P., OLIVEIRA, S. R., COLADO SIMAO, A. N., DELICATO DE ALMEIDA, E. R., KAMINAMI MORIMOTO, H., LOPES, J., DE CARVALHO JENNINGS PEREIRA, W. L., MARQUES ANDRADE, R., MULITERNO PELEGRINO, L., DONIZETE BORELLI, S., KAIMEN-MACIEL, D. R., and REICHE, E. M. (2013). Cytokine profile in relapsingremitting multiple sclerosis patients and the association between progression and activity of the disease. Mol Med Rep, 7(3), 1010-1020. doi:10.3892/mmr.2013.1256

KOBELT, G., THOMPSON, A., BERG, J., GANNEDAHL, M., ERIKSSON, J., GROUP, M. S., and EUROPEAN MULTIPLE SCLEROSIS, P. (2017). New insights into the burden and costs of multiple sclerosis in Europe. Mult Scler, 23(8), 1123-1136. doi:10.1177/1352458517694432

KURUVILLA, A. P., SHAH, R., HOCHWALD, G. M., LIGGITT, H. D., PALLADINO, M. A., and THORBECKE, G. J. (1991). Protective effect of transforming growth factor beta 1 on experimental autoimmune diseases in mice. Proc Natl Acad Sci U S A, 88(7), 2918-2921. doi:10.1073/pnas.88.7.2918

LEE, P. W., SEVERIN, M. E., and LOVETT-RACKE, A. E. (2017). TGF-beta regulation of encephalitogenic and regulatory T cells in multiple sclerosis. Eur J Immunol, 47(3), 446-453. doi:10.1002/eji.201646716

LI, Z., LI, D., TSUN, A., and LI, B. (2015). FOXP3+ regulatory T cells and their functional regulation. Cell Mol Immunol, 12(5), 558-565. doi:10.1038/cmi.2015.10

MIRSHAFIEY, A., and MOHSENZADEGAN, M. (2009). TGF-beta as a promising option in the treatment of multiple sclerosis. Neuropharmacology, 56(6-7), 929-936. doi:10.1016/j.neuropharm.2009.02.007

MONFORTE, J., and MCPHAIL, S. (2005). Strategy for gene expression-based biomarker discovery. Biotechniques, Suppl, 25-29. doi:10.2144/05384su05

PALLE, P., MONAGHAN, K. L., MILNE, S. M., and WAN, E. C. K. (2017). Cytokine Signaling in Multiple Sclerosis and Its Therapeutic Applications. Med Sci (Basel), 5(4). doi:10.3390/medsci5040023

TRINCHIERI, G. (1995). Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu Rev Immunol, 13, 251-276. doi:10.1146/annurev.iy.13.040195.001343

VAN'T VEER, L. J., PAIK, S., and HAYES, D. F. (2005). Gene expression profiling of breast cancer: a new tumor marker. J Clin Oncol, 23(8), 1631-1635. doi:10.1200/JCO.2005.12.005

WEI, J. S., GREER, B. T., WESTERMANN, F., STEINBERG, S. M., SON, C. G., CHEN, Q. R., WHITEFORD, C. C., BILKE, S., KRASNOSELSKY, A. L., CENACCHI, N., CATCHPOOLE, D., BERTHOLD, F., SCHWAB, M., and KHAN, J. (2004). Prediction of clinical outcome using gene expression profiling and artificial neural networks for patients with neuroblastoma. Cancer Res, 64(19), 6883-6891. doi:10.1158/0008-5472.CAN-04-0695

WILLIAMS, S. K., MAIER, O., FISCHER, R., FAIRLESS, R., HOCHMEISTER, S., STOJIC, A., PICK, L., HAAR, D., MUSIOL, S., STORCH, M. K., PFIZENMAIER, K., and DIEM, R. (2014). Antibody-mediated inhibition of TNFR1 attenuates disease in a mouse model of multiple sclerosis. PLoS One, 9(2), e90117. doi:10.1371/journal.pone.0090117