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The Role of rs4626 and rs7221352 Polymorphisms on the *TOB1* Gene in Turkish Relapsing-Remitting Multiple Sclerosis Patients

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ABSTRACT

Objective: Multiple sclerosis often causes neurological disability and reduced quality of life. Genetic biomarkers are important tools for the diagnosis and prognoses of diseases. This study has been conducted to explore the haplotype frequencies formed by rs4626 and rs7221352 single-nucleotide polymorphisms (SNPs) in the coding region variant (rs4626) and 5' upstream region intron variant (rs7221352) of the transducer of the *ERBB2.1* (*TOB1*) gene in individuals with relapsing-remitting multiple sclerosis.

Materials and Methods: Thirty patients with an Expanded Disability Status Scale (EDSS) score<3, 30 patients with EDSS≥5, and 30 healthy controls participated in the study. The *TOB1* rs4626 T/C and rs7221352 G/A single-base variations were applied using the quantitative real-time polymerase chain reaction method in accordance with the TaqMan SNP Genotyping Assays instructions.

Results: The genotype frequencies of *TOB1* rs4626 TT/TC/CC were respectively 3.3%, 53.3%, and 43.3% in the EDSS<3 cases and 10%, 53.3%, and 36.7% in the EDSS≥5 cases. The genotype frequencies of *TOB1* rs7221352 GG/AG/AA were respectively 3.3%, 86.7%, and 10% in the EDSS<3 cases and 10%, 70%, and 20% in the EDSS≥5 cases. With respect to the estimated values in the study cohort, allelic variant frequency was higher in the patient group for both SNP variants (p<0.001).

Conclusion: The presence of variant alleles in the rs4626 and rs7221352 polymorphisms in *TOB1* may have a role in the disease immunopathogenesis. Further investigations involving larger groups are required to understand the effects of *TOB1*.

Keywords: *TOB1*, multiple sclerosis, single-nucleotide polymorphisms, allelic variation, quantitative real-time PCR

INTRODUCTION

Multiple sclerosis (MS) is mainly defined by the disruption of myelin sheaths and inflammation of the central nervous system (CNS) neurons. The most frequent clinical subtype is called relapsing-remitting MS (RRMS). Patients with RRMS experience distinct attacks of disease resulting either in recovery or neurological disability. MS is considered to be an autoimmune disease, and recent data have shown genetic, immunological, and environmental factors to be able to contribute to disease complexity, causing

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a different onset and course for each individual (1). Therefore, discovering reliable biomarkers is important for identifying susceptible individuals, diagnosing the disease, and predicting prognosis. Although no ideal marker has been defined so far, genetic biomarkers would potentially be invaluable as they are not strongly affected by environmental conditions and may be studied via blood samples instead of more invasive procedures.

TOB1 is considered a promising biomarker of MS (2) and occurs in the anti-proliferative Tob/Btg-1 protein family, which possesses a regulatory role in cellular growth and proliferation (3). *TOB1* is located on chromosome 17 at the 17q21 cytogenetic location. It has two exons, with transcription occurring in the 5'-3' direction on the reverse strand of DNA. Promoter and enhancer sequences regulate transcription on the upstream side of the coding exon (4). The synonymous variant rs4626 is located in the Chr 17: 50863061 (forward strand) within the promoter region, providing an initial binding site for transcription factors and RNA polymerase. Additionally, the intron variant (non-coding transcript variant) rs7221352 (Chr 17: 50869864) is found within the promoter flanking sequences in the 5' upstream sequence region of *TOB1*. Although many transcription binding sites reside in the upstream region of the rs7221352 variant, the rs4626 single-nucleotide polymorphism (SNP) is positioned in the CCCTC-binding factor site (5,6).

The transcriptional CCCTC-binding factor is involved in many cellular processes and plays a repressor role in cellular pathways regarding chromatin architecture regulation, V(D)J recombination, dielectric activity, and transcriptional regulation. The V(D)J somatic recombination mechanism that contributes to the formation of various macromolecules such as antibodies, immunoglobulins, and T cell receptors in B and T cells has been observed in the early stages of lymphocytes (6,7). Active or inactive forms of *TOB*1 in T cells and brain tissue have been explored using the Genes and Regulation Database (7).

TOB1 encodes a 45-kDa protein (Tob1) with modulatory roles in mitosis and cytokine production (4). Its expression decreases during the G1/S phase to proceed to mitosis. Furthermore, it binds to erbB2 receptors and acts as a negative regulator of erbB2-related pathways (3). These findings and other data from the past two decades imply that *TOB1* acts as a tumor suppressor gene (3,8-13).

TOB1 also interferes with T-cell activation and cytokine production in lymphocytes. Increased expression of Tob1 in T cells has been demonstrated to inhibit $CD3^+$ and anti-CD28-related proliferation (14). Removal of endogenous Tob1 decreases the T-cell activation threshold and enhances the T cell receptor engagement response (14). Furthermore, the Tob1 protein inhibits T cell proliferation by decreasing the levels of pro-inflammatory cytokines such as interleukin-2, interleukin-4, interferon gamma, cyclins A, and cyclin E (14-16).

Previous studies have observed the Tob1 protein in the cytoplasm of T cells and implicated this in the mRNA stability and translational degradation of interleukin-2 (14-16). *TOB1* was later revealed to also be effective in post-transcriptional modifications. These observations suggest Tob1 to be involved in actively maintaining lymphocyte quiescence and to maybe even set a threshold for T-cell activation. Therefore, its role in autoimmune diseases and potential as a biomarker should be evaluated (17).

Experimental studies on humans suggest a possible relationship to exist between the downregulation of *TOB1* expression and the progression of MS. Corvol et al. (18) investigated a biomarker to foresee which clinically isolated syndrome (CIS) patients were more prone to progress to clinical definite MS and observed that the patients who had decreased expression of *TOB1* RNA and therefore decreased protein levels had significantly elevated rates of progressing to clinical definite MS within 1 year. Corvol et al. performed SNP analyses for five selected regions (rs11079937, rs9905480, rs9303568, rs4626, and rs7221352) within and near the gene in clinically mild MS patients (Expanded Disability Status Scale, EDSS<3, n=62) and severe MS patients (EDSS>6, *n*=74) to evaluate the role of *TOB1* in disease progression. Their results identified two markers: rs4626 in the coding and rs7221352 in the non-coding areas of *TOB1*, indicating these markers to possibly be related to the progression of MS (18).

Thus, changes in the structure of *TOB1* can cause inadequate functioning and create a tendency toward inflammation and autoimmunity. Therefore, its clinical effects should be evaluated in terms of disease initiation and progression. The current study examines two previously identified SNPs in *TOB1* with regard to patients diagnosed with mild (EDSS<3) and severe (EDSS≥5) MS in comparison to healthy individuals.

The study also evaluates the following patient information: age at disease onset, localization at onset, number of attacks in the first year of diagnosis, cerebrospinal fluid immunoglobulin G (IgG) index, and oligoclonal band (OCB) results (where available). The study additionally investigates whether a possible relationship exists between genotype status and these parameters. The study's aim is to investigate the possible genetic associations between rs4626 and rs7221352 polymorphisms in *TOB1* in terms of RRMS susceptibility and/or severity.

MATERIALS AND METHODS

Ethical Approval

This study was approved by the Ethics Committee of Bakirkoy Dr. Sadi Konuk Training and Research Hospital (Istanbul, Turkey; Project no: 2016-152). The study was carried out in accordance with the Declaration of Helsinki and the review board-approved protocols of Istanbul University. Study participants provided written informed consent.

Study Population

Two SNPs within (rs4626) and 5' upstream (rs7221352) of *TOB1* were designated for genotyping in 60 individuals with MS and 30 healthy control subjects. The patient subgroups are composed of 30 patients with EDSS<3.0 (mild RRMS) and 30

patients with EDSS≥5 (severe RRMS) and at least 5 years since disease onset. Of the patients in the EDSS≥5 group, 25 had reached an EDSS=5 within 5 years of disease onset, while the remaining five patients had reached an EDSS=3 within 5 years and reached or exceeded an EDSS=5 within 6–10 years after disease onset. These were considered to experience a more severe course of the disease. The patient groups had no history of cancer or autoimmune disease other than MS. All subjects were followed up at the outpatient clinics of the Bakirkoy Research and Training Hospital for Psychiatry, Neurology and Neurosurgery (Istanbul, Turkiye). The study also included 30 healthy volunteers matched for age and sex who were biologically unrelated to the patients and had no history of autoimmune diseases or cancer as the control group.

Genotyping the rs4626 and rs7221352 Polymorphisms

Peripheral blood samples were drawn from all the subjects and collected in EDTA tubes. Genomic DNA were isolated from the peripheral blood lymphocytes using the Quick Blood Genomic DNA Extraction Kit (Hibrigen Biotechnology, Istanbul, Türkiye). The amount and quality of the samples were measured by the NanoPhotometer P330 (Implen, GmbH, Munchen, Germany). TaqMan® SNP Genotyping Assays, human (Applied Biosystems, Foster City, CA, USA) were used for genotyping the *TOB1* rs4626 and rs7221352 polymorphisms on the Stratagene Mx3005P™ Multiplex Quantitative PCR System (Agilent Technologies, Santa Clara, CA, USA). FAM™ and VIC™ dye-labeled TaqMan minor groove binder (MGB) probes were used for allele discrimination. The presence of two probes in each reaction allowed the genotyping of two possible variant alleles at the polymorphic site of the *TOB1* DNA sequence (Table 1). Genotyping assays were performed by adding approximately 20–25 ng of purified genomic DNA for a total volume of 25 µL. Quantitative real-time polymerase chain reactions (qPCR) were carried out under the following conditions: 95°C for 10 min (denaturation cycle), followed by 40 cycles at 95°C for 15 s, then at 60°C for 60 s (annealing/extension). The results were analyzed using the software Stratagene MxPro™ QPCR (v. 4.10). For quality control, qPCR analysis for genotyping was repeated using randomly selected samples.

Statistical Analysis

The programs SPSS (v. 20.0; SPSS, Inc., Chicago, IL, USA) and RGui in R-3.4.1 (i386, 32-bit, R Computing Group, Vienna, Austria) were used to conduct the statistical analyses. The direct gene counting method was implemented to calculate the genotype and allele frequencies. The chi-square test of independence was used to evaluate the significance of the distributions for each subgroup. All *p* values are two-sided, with estimated values of $p<0.05$ being accepted as statistically significant.

Pearson's chi-square (χ^2) test was applied to estimate and compare both the allele and genotype frequencies of the groups, with Fisher's exact test and Yates's correction for continuity being used where appropriate. Odds ratios (ORs) at a 95% confidence interval (CI) were assessed using logistic regression analyses (19). Haplotype analyses were carried out by calculating the co-occurrence of each allele for the rs4626 (T/C) and rs7221352 (G/A) polymorphisms. Individuals carrying the T-G, C-A, T-A, C-G genotypes were identified in each study group (EDSS<3, EDSS≥5, control), with the haplotype frequencies and *p* values being calculated according to Pearson's chi-square test.

Hardy-Weinberg Equilibrium (HWE) was applied to assess genotype frequencies among the patient and healthy control groups. The observed and expected number of genotypes and their compliance to the Hardy-Weinberg principle were calculated using the Gene-Calc portal website (20). In addition, expression quantitative trait loci (eQTLs) values for rs4626 and rs7221352 were obtained from the Genotype-Tissue Expression (GTEx) Analysis Portal (dbGaP Accession phs000424.v8.p2) (21).

RESULTS

Patient Demographics and Clinical Parameters

The groups' mean ages were 44.1 ± 9.45 years for the EDSS<3 patient group, 47.2 ± 9.84 years for the EDSS≥5 patient group, and 48.13 ± 12.51 years for the healthy control group. The female-to-male ratios are 19:11 for the EDSS<3 group, 20:10 for the EDSS≥5, and 20:10 for the healthy controls. No statistically significant difference was found in terms of the distributions of age or gender ($p>0.05$). Table 2 summarizes the laboratory

Table 1. TaqMan SNP Genotyping Assay design details for *TOB1* rs4626 and rs7221352 polymorphisms.

SNP ID / Assay ID	SNP Type / Cytogenetic location	Reporter / Quencher	Forward and Reverse Primer Concentration (µM)	Context Sequence
rs4626 C-159174_10	Silent Mutation 17q21.33	Allele 1: VIC/MGB-NFQ Allele 2: FAM/MGB-NFQ	Forward primer: 36 µM Reverse primer: 36 µM	TAAAATTCAAGCCATCTACAAAAGA **[C/T]** TTCTCATTGAGGCCTCCATAGGCTG
rs7221352 C-31810760_10	NA 17q21.33	Allele 1: VIC/MGB-NFQ Allele 2: FAM/MGB-NFQ	Forward primer: 36 µM Reverse primer: 36 µM	TTACTTTGGAATTGTGCAGGGGAGT **[A/G]** GAGGGCTAACTGCTAATTGTGCAGG

NA: Not applicable or not available, MGB: Minor-Groove Binder, NFQ: Non-Fluorescent Quencher, Ftable FAM and VIC: Dye-labeled probe (FAM Emission spectra: ~517 nm, VIC Emission spectra: ~551 nm). A: Adenine, T: Thymine, C: Cytosine, G: Guanine.

Table 2. Clinical and CSF parameters of patient groups.

	EDSS<3	EDSS≥5
Age of onset (years)	32.07 ± 10,14	32.83 ± 11.77
Disease duration (years)	12.7± 6.4	14.9 ± 5.5
Last EDSS	0.65 ± 0.82	6.48 ± 1.01
Number of attacks at the first year:		
1	24	19
2	4	9
3	1	1
4	1	1
IgG Index (mean)	1.01 ± 0.76	1.075 ± 0.95
CSF OCB positive (n/N)	18/21	14/16

EDSS: Extended Disability Status Scale, CSF: Cerebrospinal Fluid, n: Number of patients with positive results for CSF parameters, N: Number of patients with available CSF results, IgG: Immunoglobulin G, OCB: Oligoclonal Band.

results and clinical features. Statistically significant differences were not present between the patient groups in terms of age of onset, mean disease duration, number of attacks in the first year, mean IgG index values, or OCB positivity (p>0.05).

The sensory system onset ratio was higher in the EDSS<3 group, whereas the spinal and cerebellar localizations ratios were higher in the EDSS≥5 group. However, the differences in these distributions are not statistically significant (p>0.05). Optic neuritis, brainstem, and pyramidal system onsets were similar between the two groups. Compared to the EDSS<3 group, the EDSS≥5 group had a higher IgG positivity (IgG index >0.6) (P=0.023).

The distributions regarding rs4626 and rs7221352 SNPs for each subgroup were calculated and found statistically significant (Table 3).

Allelic Frequencies of rs4626 and rs7221352 in MS Patients with EDSS<3, EDSS≥5 and Healthy Controls

When considering the genotype TT as a reference for the rs4626 polymorphism, the genotype TC prevalence was found to be equal for the patients with MS in the EDSS<3 and EDSS≥5 subgroups. When compared to the healthy subjects, the patient groups showed a higher prevalence for the TC genotype (p=0.001 for EDSS<3, p=0.030 for EDSS≥5; Table 4). Additionally, the frequency of the rs4626 genotype CC was found to be higher in the patient groups than in the control group (p=0.005 for EDSS<3, p=0.018 for EDSS≥5; Table 4).

No significant difference was found between the EDSS<3 (30%) and the EDSS≥5 subgroups (36.7%) for the allele T of rs4626, which was more prevalent in the healthy control group (51.7%) than in patient groups (30% for EDSS<3, 36.7% for EDSS≥5; Table 4). However, the rs4626 allele C was more frequent in the patient groups than in the healthy control group, although this was solely significant for the EDSS<3 patients (p=0.016), not for the EDSS≥5 (p=0.099; Table 4).

For the rs7221352 polymorphism, the genotype AG prevalence in patients with EDSS<3 (86.7%) and EDSS≥5 (70.0%) was greater than in the control group (13.3%, p=0.000). Although the difference in prevalence of the rs7221352 genotype AA between the EDSS<3 patients and the control group was not significant (p=0.069), the EDSS≥5 patients showed a significantly higher frequency than in the control group (p=0.038; Table 4).

Table 3. Statistical significance of rs4626 and rs7221352 distributions among groups.

	rs4626			rs7221352		
	Value	**df**	**Asymp. Sig. (2-sided)**	**Value**	**df**	**Asymp.Sig. (2-sided)**
Pearson Chi-Square	19.361a	4	0.001	44.097a	4	0.000
Likelihood ratio	19.238	4	0.001	47.312	4	0.000
N of Valid cases		90		90		

[a] 0 cells (0.0%) have expected count less than 5. The minimum expected count is 5.00. Asymp. Sig.: Asymptotic Significance; df: degrees of freedom, N: Number

Table 4. Genotype and allele frequencies of rs4626 and rs7221352 polymorphisms in MS patients with EDSS<3, EDSS≥5 and healthy controls.

TOB1 polymorphism	Patients (EDSS<3) (n=30)	Patients (EDSS<3) Genotype Frequency	Patients (EDSS≥5) (n=30)	Patients (EDSS≥5) Genotype Frequency	Controls (n=30)	Controls Genotype Frequency	EDSS<3 Patients vs Controls			EDSS≥5 Patients vs Controls		
							OR (95%)	Test Values	P	OR (95%)	Test Values	P
rs4626 - Genotype												
TT	1	% 3.3	3	% 10	13	% 43.3	***Reference***			***Reference***		
TC	16	% 53.3	16	% 53.3	5	% 16.7	41.6 (1.036-13.383)	11.038**	0.001	13.866 (1.026-6.286)	4.693**	0.030
CC	13	% 43.3	11	% 36.7	12	% 40	14.083 (1.035-12.098)	7.721**	0.005	3.972 (1.024-5.548)	5.590**	0.018
rs4626- Allele												
T	18	% 30	22	% 36.7	31	% 51.7	***Reference***			***Reference***		
C	42	% 70	38	% 63.3	29	% 48.3	2.494 (1.012-2.355)	5.716*	0.016	1.846 (1.011-2.302)	2.71*	0.099
rs7221352- Genotype												
GG	1	3.3%	3	10%	20	66.7%	***Reference***			***Reference***		
AG	26	86.7%	21	70%	4	13.3%	130 (1.036-13.371)	30.056**	0.000	35 (1.026-6.357)	21.370**	0.000
AA	3	10%	6	20%	6	20%	10 (1.039-16.289)	-	0.069***	6.666 (1.026-6.669)	-	0.038***
rs7221352- Allele												
G	28	46.7%	27	45%	44	73.3%	***Reference***			***Reference***		
A	32	53.3%	33	55%	16	26.7%	3.142 (1.012-2.397)	8.602*	0.003	3.361 (1.012-2.40)	9.627*	0.001

A: adenine, T: thymine, C: cytosine, G: guanine, * $\chi 2$(Chi-square), **Continuity Correction (Yates Correction), *** Fisher Exact Significance

Table 5. Haplotype frequencies of TOB1 rs4626 and rs7221352 polymorphisms in patients with EDSS<3, EDSS≥5 and healthy controls.

TOB1 polymorphism	Patients (EDSS<3) (n=30)	Patients (EDSS<3) Genotype frequencies	Patients (EDSS≥5) (n=30)	Patients (EDSS≥5) Genotype frequencies	Controls (n=30)	Controls Genotype frequencies	EDSS<3 Patients vs Controls			EDSS≥5 Patients vs Controls		
							OR (95%CI)	χ^2	P	OR (95%CI)	χ^2	P
Haplotype (rs4626-rs7221352)												
T-G	16	18.4%	17	21.5%	17	42.5%	*Reference*			*Reference*		
C-A	28	32.2%	24	30.4%	7	17.5%	4.25 (1.017-3.412)	6.98	0.0082	3.43 (1.017-3.428)	5.02	0.025
T-A	17	19.5%	17	21.5%	4	10%	4.52 (1.02-4.349)	5.29	0.0214	4.25 (1.02-4.322)	4.90	0.026
C-G	26	29.9%	21	26.6%	12	30%	2.30 (1.015-3.019)	2.85	0.091	1.75 (1.015-3.057)	1.26	0.261

A: adenine, T: thymine, C: cytosine, G: guanine. EDSS: Extended Disability Status Scale

Allelic frequencies of the rs7221352 polymorphism were similar for the patients with EDSS<3 and EDSS≥5 (respectively, 46.7% vs. 45% for the rs7221352 allele G and 53.3% vs. 55% for the rs7221352 allele A). However, the allele G in the rs7221352 region was more frequent in the healthy control group (73.3%) than in the patient groups (46.7% for EDSS<3 and 45% for EDSS≥5), whereas the allele A frequency was significantly lower in the healthy control group compared to either the EDSS<3 group (p=0.003) or the EDSS≥5 group (p=0.001; Table 4).

Haplotype Association of the rs4626 and rs7221352 Polymorphisms in the EDSS<3 and EDSS≥5 MS Patient Groups and the Healthy Control Group

Table 5 shows the haplotype frequency results for the *TOB1* rs4626 and rs7221352 polymorphisms. With regard to the rs4626-rs7221352 haplotype analyses, haplotype T-G was considered as the reference, with no significant differences being observed for haplotype C-G between the control group and either the EDSS<3 (p=0.091) or EDSS≥5 (p=0.261) groups (Table 5). Haplotype C-A was significantly more frequent in individuals with MS than in the control group (p=0.0082 for the EDSS<3 comparison, and p=0.025 for the EDSS≥5 comparison; Table 5). Haplotype T-A was less frequent in the control group than in either the EDSS<3 (p=0.0.214) or EDSS≥5 (p=0.026) patient groups (Table 5). Comparing the haplotype frequency of the rs4626-rs7221352 polymorphisms between patients with mild (EDSS<3) and severe (EDSS≥5) RRMS showed no significant differences (p>0.05).

Genetic Association of rs4626 and rs7221352 Polymorphisms in the Subjects

The χ^2 test of independence for comparatively analyzing the presence of the rs4626 and rs7221352 polymorphisms between patients with EDSS<3 and those with EDSS≥5 showed no significant difference. However, a statistical significance was found in the relationship of the presence of rs4626 and rs7221352 polymorphisms between the RRMS patients and the healthy controls (Table 6).

Table 6. Chi-square (χ^2) test for independence results.

	χ^2 independence	df	p
rs4626			
EDSS≥5, EDSS<3, Control (n=90)	21.22	4	0.000285
EDSS≥5 vs. Control (n=60)	12.05	2	0.002417
EDSS<3 vs. Control (n=60)	11.8	2	0.002739
EDSS<3 vs. EDSS≥5 (n=60)	1.16	2	0.558221
rs7221352			
EDSS≥5, EDSS<3, Control (n=90)	44.09	4	6.14 e-9
EDSS≥5 vs. Control (n=60)	24.12	2	5.78 e-6
EDSS<3 vs. Control (n=60)	34.32	2	3.52 e-8
EDSS<3 vs. EDSS≥5 (n=60)	2.53	2	0.282

df: degrees of freedom, EDSS: Extended Disability Status Scale

Hardy-Weinberg Equilibrium Analysis

Table 7 shows the observed and expected genotype distributions according to the Hardy-Weinberg principle. The distributions of rs4626 in the EDSS<3 and EDSS≥5 compared to the distributions of rs7221352 in the EDSS≥5 groups were found to be consistent with the Hardy-Weinberg principle at a significance value of 0.05 (Table 8).

eQTL Analysis

eQTL analysis was obtained from the GTEx Analysis Portal (dbGaP Accession phs000424.v8.p2; https://www.gtexportal.org/). Different parts of brain tissue and whole blood (relevant immune cell type investigation) samples were analyzed separately for the eQTL identification of the rs4626 and rs7221352 SNP variants. The whole blood sample analysis revealed a significant eQTL p=0.00067 for the rs4626 and p=0.0000015 for the rs7221352 SNPs in the *TOB1* expression, with $p \leq 0.0045$ being accepted as statistically significant. Figure 1 presents the eQTL genotype-normalized *TOB1* expression plots.

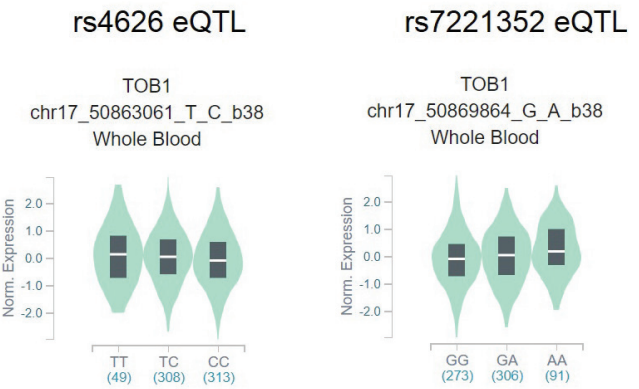

Figure 1: eQTL plots of rs4626 and rs7221352 SNP variants for whole blood.

DISCUSSION

MS is a multifactorial disease, and research on its genetic aspects has rapidly accelerated within the past two decades. MS's tendency to cluster in families and certain ethnic groups supports the genetic aspects of MS. First-degree relatives of MS patients have a 30-40 times greater risk of MS compared to the normal population (22). The HLA-DRB1*1501 locus is known to have the strongest relationship, with more than 200 MS-predisposed genes having so far been identified in genome wide association studies (GWAS). Still much remains to be achieved (23-25). Researchers hope to reveal all the responsible genes and individualize risk assessment. These data may ultimately help the early identification of susceptible individuals and develop individualized targeted therapies for MS in the future (24-26).

Table 7. Expected Hardy-Weinberg Equilibrium (HWE) distribution and observed results.

rs4626			**rs7221352**		
	HWE distribution	**Observed Results**		**HWE distribution**	**Observed Results**
TT	0.156	0.188	**GG**	0.302	0.267
CT	0.239	0.412	**AG**	0.247	0.167
CC	0.367	0.400	**AA**	0.202	0.567

A: adenine, T: thymine, C: cytosine, G: guanine, HWE: Hardy-Weinberg Equilibrium

Table 8. The compliance analysis between observed and expected number of genotypes based on Hardy-Weinberg law.

	p	**Chi-square**	**Yates's chi-square**	**Yates's p**	**Status**
rs4626, EDSS<3	0.335	2.184	1.298	0.522	*Distribution consistent
rs4626, EDSS≥5	0.718	0.660	0.270	0.873	*Distribution consistent
rs4626, Control	0.0012	13.318	-	-	**fis= 0.666
rs7221352, EDSS<3	0.0002	16.475	14.323	0.0007	**fis= -0.741
rs7221352, EDSS≥5	0.076	5.145	3.971	0.137	*Distribution consistent
rs7221352, Control	0.0014	13.032	10.474	0.0053	**fis= 0.659

*Distribution consistent with Hardy-Weinberg's principle at a level of significance of 0.05, **Distribution not consistent with Hardy-Weinberg's principle at a level of significance of 0.05, fis: inbreeding coefficient value

This study has assessed the rs4626 and rs7221352 SNPs of *TOB1* regarding their possible relationships to MS onset and/or prognosis. According to Corvol et al. (18), despite 975 differentially expressed transcripts being observed in $CD4^+$ T cells among CIS patients and healthy subjects, only 108 genes exhibited variable expression levels in higher-risk patients with MS. *TOB1* showed the largest expression difference (a 7-fold downregulation) compared to other downregulated and upregulated genes involved in pro-apoptotic processes and cellular cycle regulation. The majority of $CD4^+$ T cells from subjects with a high risk of disease activity were prone to progressing to a proliferative state and experiencing new clinical attacks. Researchers have also suggested *TOB1* to be an important biomarker of MS progression (18, 27) and also identified five SNPs positioned within or near *TOB1*, with the allelic frequencies of rs4626 (significant) and rs7221352 (not significant) among them showing differences when comparing mild and severe cases (18). The current research found no other studies in the literature to have evaluated these two SNPs together for each individual.

This study has investigated the possible relationships between different haplotypes formed by the rs4626 and rs7221352 polymorphisms in EDSS<3 and EDSS≥5 RRMS patient groups, as well as in healthy subjects. The frequencies of the genotype TC, CC, and allele C of rs4626 were found to occur more frequently in MS patient groups compared to the healthy subject group (statistically significant for all except allele C in the EDSS≥5 vs. control group comparison). Additionally, genotype AG and allele A of rs7221352 occur more frequently in the patient groups than in the healthy controls. The rs7221352 genotype AA occurred significantly more frequently in the EDSS≥5 patient subgroup compared to in the healthy subjects. Healthy controls had higher frequencies of the reference genotypes (TT for rs4626 and GG for rs7221352) compared to the patients.

Haplotype T-G (reference) of the rs4626-rs7221352 polymorphisms was observed less frequently in both the EDSS<3 and EDSS≥5 patient groups compared to in the control group. Additionally, rs4626-rs7221352 haplotype C-A and haplotype T-A were significantly more frequent in patients with EDSS<3 and EDSS≥5 than in the controls. No significant differences were found in the rs4626-rs7221352 haplotype C-G frequencies between the MS patient groups and the healthy control group.

In summary, the study has found more variant genotypes (not significant for the EDSS<3 vs. controls for genotype AA of rs7221352) and variant alleles (not significant for EDSS≥5 for rs4626) in the MS patient groups than in the control group. The haplotype analyses showed the control group to have significantly more wild-type haplotypes. These findings suggest these two SNPs to be involved in MS immunopathogenesis.

No statistically significant differences were found among the patient subgroups regarding either of the SNP markers, which differs from the results from Corvol et al. (18).

In the current study, the variant allele (represented by C [cytosine] on the forward strand) frequency in all participants was approximately 48% with regard to rs4626. Population studies observed a 50% variant allele frequency in East Asians and a 70% in Europeans (28), while the current study found a variant allele (A) frequency for rs7221352 of 27%, which is between those found in European (41%) and East Asian (15%) populations (29). However, larger population studies will be required in order to obtain more accurate results for the Turkish population.

A number of SNPs were identified as potential risk factors for MS. A recent study by Gresle et al. described MS risk eQTL associations for more than a 100 genes (24,30). The current study could find no other study that had evaluated eQTL specifically for the *TOB1* gene with regard to MS. However, data from the GTEx Analysis Portal blood analysis showed a significant eQTL effect for rs4626 and rs7221352 SNPs in the *TOB1* gene (21), which could imply a possible relationship to MS pathogenesis. However, further research is still needed.

Treatment status may have been a possible intervening factor in this study. Two patients with EDSS<3 did not consent to therapy, while four had not initially consented but only did so a few years after disease onset. Because they showed good prognosis regardless of late treatment, the study results were not affected. One patient in the EDSS≥5 group never consented to therapy, while three patients from this group consented during their disease course rather than at the beginning. Although these patients underwent late or no treatment, they were included in the study based on their aggressive clinical course and radiological lesion load since disease onset. All the patients were followed up and treated by an MS specialist in accordance with the latest available guidelines. On limitation of the study involves the impossibility of following up on all the patients who do not receive treatment.

The sample sizes of the patient and control groups were small, which is another important limitation. However, this was a preliminary study analyzing polymorphisms in the *TOB1* region in the Turkish population. This is the first study in the published literature to have analyzed these two SNPs together with respect to both RRMS patients and healthy subjects.

CONCLUSION

The presence of variant alleles in the rs4626 and rs7221352 polymorphisms of the *TOB1* gene may play a role in MS immunopathogenesis. Further studies over larger populations will be required to clarify the role of *TOB1* polymorphisms in MS.

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Informed Consent: Written consent was obtained from the participants.

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