Effects of ApaI, FokI, and BsmI gene polymorphisms of the vitamin D receptor on serum vitamin D level in Turkish MS patients with different types and severities of the disease

Tugba Turker Cetinel[1], Seyda Figul Gokce[2], Malik Ejder Yildirim[3], Asli Bolayir[2]

[1]Clinic of Neurology, Mersin Erdemli Hospital, Mersin, Turkey
[2]Department of Neurology, Faculty of Medicine, Sivas Cumhuriyet University, Sivas, Turkey
[3]Department of Medical Genetic, Faculty of Medicine, Sivas Cumhuriyet University, Sivas, Turkey

Abstract

Aim: Adequate vitamin D levels may reduce the risk of multiple sclerosis (MS) development and progress. It is believed that a number of genes cause the disease, including the genes that function on vitamin D metabolism, although information about the role of the said genes is quite contradictory. We aimed to investigate the status of serum vitamin D and ApaI, FokI, and BsmI gene polymorphisms of the vitamin D receptor in Turkish MS patients with different types and severities of the disease.

Materials and Methods: The patients were divided into three groups in accordance with the types of MS: relapsing-remitting MS (RRMS), secondary progressive MS (SPMS), and primary progressive MS (PPMS). Serum D vitamin level was studied, and polymerase chain reaction (PCR) applications were performed by designing primers suitable for the vitamin D receptor (VDR) gene regions in which FokI (rs2228570), BsmI (rs1544410), and ApaI (rs7975232) polymorphisms are localized. The Expanded Disability Status Scale (EDSS) was utilized to determine the disability status of MS patients.

Results: Although vitamin D levels were inadequate in MS patients and controls, the mean serum vitamin D level of MS patients was significantly higher than the controls (p<0.05). There was a statistically significant correlation with A/A, C/C, and A/C genotypes in SPMS patients and with ApaI A/A and A/C genotypes in PPMS patients. The rate of the EDSS score in the range of 0-6 was significantly higher in MS patients with BsmI G/G genotype in comparison with the other genotypes (p<0.05).

Conclusion: In the Turkish population, while all alleles of the ApaI gene are associated with SPMS, the A/A and A/C alleles of ApaI are related to PPMS, and the BsmI G/G genotype may be associated with less disability in MS patients.

Keywords: ApaI; bsmI; fokI; gene polymorphism; multiple sclerosis; Vitamin D

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease that influences the white and gray matter with multifocal involvement of the central nervous system (1). It is the second disease causing disability after traumas in young adults (2). Therefore, it is an intensively studied issue with a significant social and economic burden. Although its etiology is unknown, genetic predisposition and environmental factors are considered responsible for it (3). Within the scope of its etiopathogenesis, vitamin D level and the role of the vitamin D receptor (VDR) in MS and various neurodegenerative diseases have become the target of studies (4).

It is known that vitamin D affects cell proliferation, differentiation, neurotransmission, and neuroplasticity in the nervous system and plays a neurotrophic and neuroprotective role. Moreover, vitamin D has been recently classified as a neurosteroid, and accordingly, its functions in the central nervous system have been investigated (5). Vitamin D is mainly taken directly from foods or 7-dehydrocholesterol in the case of exposure to sunlight. 25(OH)D represents the major circulatory form with a half-life of 2-3 weeks. It shows both vitamin D intake and endogenous production. The 25(OH)D level must be measured to reveal whether vitamin D level is normal, deficient, or high. 1,25-Dihydroxyvitamin (D3), which is its active form, is activated by binding to VDRs in the cell nuclei (6). The presence of VDR was detected in the cerebellum, thalamus and hypothalamus, basal ganglions, hippocampus, olfactory system, temporal and orbital regions in the central nervous system (7).

It is an intracellular receptor, which regulates the genomic effects of vitamin D (8). VDR gene polymorphisms have

been studied in many diseases. They have been shown to be correlated to malignant or autoimmune diseases, such as type 1 diabetes mellitus, Graves' disease, breast cancer, malignant melanoma, and psoriasis vulgaris (9-11).

The VDR gene is encoded on the 12q13 chromosome. The biological impacts of vitamin D are modified by some genetic factors, such as single nucleotide polymorphisms (SNPs) in the VDR gene (12). Four VDR gene polymorphisms have been identified: ApaI, BsmI, FokI, and TaqI. Apa-I (rs7975232), Bsm-I (rs1544410), Fok-I (rs10735810), and Taq-I (rs731236) are restriction endonuclease enzymes, which specifically recognize specific short DNA sequences and cut DNA from regions close to these sequences or from specific regions within the said sequences. While A/C replacement occurs in ApaI (rs-7975232) polymorphism, A/G replacement occurs in BsmI (rs-1544410) polymorphism, T/C replacement occurs in TaqI (rs-731236) polymorphism, and C/T replacement occurs in FokI (rs- 2227580) polymorphism. The mentioned polymorphisms do not cause structural changes in the VDR protein (13).

The main pathological mechanisms causing clinical symptoms in MS are inflammation, demyelination, and axonal degeneration (14). The disease may progress in various subtypes of disease such as relapsing-remitting (RRMS), secondary progressive (SPMS), and primary progressive (PPMS). Most of the patients start with RRMS. The process proceeding with clinical attacks and improvements progresses to SPMS with little or no improvement after a while. PPMS represents a group with poor prognosis with severe disability in a very short time of one year from the beginning (15-17).

The Expanded Disability Scale Score (EDSS) is commonly utilized to express disability in MS. It is scored between zero and 10, based on progressive grading from a clinical picture without disability to death (18).

The present research aimed to determine serum vitamin D levels and ApaI, FokI, and BsmI gene polymorphisms in MS patients and healthy controls in our city and genotype ratios and to reveal their effects, if any, on the clinical picture, progression, and disability of patients with RRMS, SPMS, and PPMS.

MATERIALS and METHODS

Patient population
After obtaining approval from Cumhuriyet University Human Research Ethics Committee (dated 25.07.2018 and numbered 2018-07/06) and the informed consent from the participants, a total of 92 MS patients (89 patients receiving MS-modifying therapy) who were followed up in the Neurology Clinic of Cumhuriyet University Hospital between August 2018 and December 2019 and were diagnosed with MS in accordance with the Mcdonald 2010 and 2017 criteria and 65 healthy volunteers without any disease and with similar age and sex as our patient group were included in the study. There was no restriction based on age and sex among the patients. All volunteers included in our study were of Turkish origin.

Our exclusion criteria were being under the age of 18, being pregnant, having any disease that would affect vitamin D metabolism (kidney diseases, parathyroid gland diseases, etc.) and having received vitamin D replacement therapy in the last three months. The neurological disability of the patients was clinically assessed by the Expanded Disability Status Scale (EDSS) during the first admission and follow-ups.

Collection of Blood Samples
While the volunteers from the patient and control groups were in a sitting position, 5 ml of blood samples were drawn from the right antecubital veins into EDTA tubes and biochemistry tubes once.

Measurement of Serum 25(OH)D Levels
Serum 25(OH)D levels in the blood samples collected from the patient and control groups were evaluated at Cumhuriyet University Hospital, Department of Biochemistry. The patient and control blood samples collected into biochemistry tubes were centrifuged at 4000 cycles for 15 minutes. Then, the sera obtained were placed in Eppendorf tubes and kept at -20 degrees. According to the kit's operating procedure, all reagents, standard solutions, and samples were prepared under appropriate conditions, and the reagents were brought to room temperature and studied at room temperature before use. Finally, the concentration was calculated in nanograms/milliliter over the values obtained. The samples were analyzed using Bioassay Technology Laboratory branded Human 25(OH)D Vitamin D Elisa kits and a GF-M3000 Microplate Reader Elisa reader.

DNA Isolation
Total genomic DNA isolation was performed by making modifications in the standard phenol-chloroform protocol described by Sambrook and W. Russell (19).

400 µl volume of the blood samples stored in EDTA tubes was transferred to 1.5 ml Eppendorf tubes and suspended with 400 µl of the STE homogenization buffer (0.1 M NaCl. 0.01 M EDTA, 0.05 M Tris HCl (pH 8), 10% SDS) added to it. Proteinase K was added to a final concentration of 50 µg/ml and allowed to incubate at 55°C for 2-3 hours to mix at regular intervals. After extraction, one volume of phenol and one volume of chloroform:isoamyl alcohol (25:24:1) were applied to the extracts two times, and after applying the chloroform:isoamyl alcohol (24:1) extraction once more, the genomic DNA obtained by alcohol precipitation was dissolved in 1xTE (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0) buffer and stored at -20°C for use in other studies.

The samples were analyzed twice to reduce errors, and these values were averaged for each sample.

Polymerase Chain Reaction (PCR) Applications
PCR applications were performed by designing primers suitable for the VDR gene regions in which FokI (rs2228570), BsmI (rs1544410), and ApaI (rs7975232) polymorphisms are localized.

Statistical Analysis

The data obtained as a result of the study were evaluated using the "SPSS 23.0 for Windows" package program. First, the Shapiro-Wilk test was conducted for the purpose of determining the normal distribution of continuous data. Due to the non-normal distribution of the age and vitamin D data used in the study, the results were presented as mean, standard deviation (SD), median, minimum and maximum values. The Mann-Whitney U-test was performed to compare the quantitative data of the two groups (patient and control), and the chi-square (χ2) test was conducted for the comparison of qualitative data. In the study group, the Kruskal-Wallis analysis of variance was performed to compare the quantitative data in 3 subgroups (missing (0-12 ng/mL), inadequate (12-20 ng/mL), adequate (20-50 ng/mL)) based on the EDSS values (2 groups, 0-6, 6-9.5) and vitamin D levels. The chi-square (χ2) test was performed to investigate the compatibility of the genotype distributions of VDR polymorphisms in the control and patient groups with the Hardy-Weinberg equation for other factors that might be associated with the disease, such as serum 25(OH)D vitamin D levels. All statistical analyses were examined and interpreted at a significance level of 5%.

Whether each allele was associated with the disease was investigated individually and at the haplotype level to evaluate FokI, ApaI, BsmI genotypes and allele frequencies of the VDR gene region. The population was determined to be in the Hardy-Weinberg equilibrium.

RESULTS

Eight patients were excluded from the follow-up, and five patients could not complete the study. The study was completed with ninety-two patients. The clinical and demographic data of the patient and control groups are presented in Table 1.

Table 1. Clinical and demographic data of the patient and control groups

|	Patient Group (n:92)	Control Group (n:65)	P
Sex			
Female	60	40	0.637
Male	32	25	
Age	38.0±10.7	39.5±11.6	0.522
EDSS	2.1±1.8		
<6	86		
≥6	6		
Treatment			
Yes	89		
No	3		
MS subtype			
RRMS	74		
SPMS	12		
PPMS	6		
Time after diagnosis (years)	7.1±5.8		

EDSS: Expanded disability scale score, MS: Multiple Sclerosis, RRMS: Relapsing-Remitting Multiple Sclerosis, SPMS: Secondary Progressive Multiple Sclerosis, PPMS: Primary Progressive Multiple Sclerosis

Table 2. Genotype ratios of ApaI, BsmI, and FokI genes in RRMS, SPMS, PPMS, and control groups

| Genotypes		Control (65) n (%)	RRMS (74) n (%)	OR	95% CI	P	SPMS (12) n (%)	OR	95% CI	P	PPMS (6) n (%)	OR	95% CI	P
ApaI	A/A	31 (46.3)	29 (43.3)	0.95	0.44 2.06	0.89	4 (6)	0.17	0.04 0.74	0.00	3 (4.5)	0.07	0.01 0.47	0.01
	C/C	16 (41)	19 (48.7)	0.68	0.20 2.26	0.52	4 (10.3)	1.53	0.41 5.77	0.01	0	0.00	0.00 *	0.99
	A/C	18 (35.3)	26 (51)	1.45	0.65 3.21	0.36	4 (7.8)	0.18	0.04 0.86	0.00	3 (5.9)	0.16	0.03 0.94	0.04
BsmI	A/A	10 (58.8)	5 (29.4)	0.59	0.17 2.12	0.43	1 (5.9)	0.38	0.04 3.99	0.42	1 (5.9)	1.43	0.11 18.97	0.79
	G/G	26 (36.6)	39 (54.9)	2.09	0.79 5.57	0.14	4 (5.6)	0.00	0.00 0.00	-	2 (2.8)	2.59	0.33 2.33	0.37
	A/G	29 (42)	30 (43.5)	B	- -		7 (10.1)	-	- -	-	3 (4.3)	-	- -	-
FokI	C/C	36 (39.6)	44 (48.4)	0.87	0.40 1.87	0.71	8 (8.8)	1.89	0.43 8.37	0.39	3 (3.3)	0.70	0.09 5.11	0.73
	T/T	8 (57.1)	4 (28.6)	0.43	0.11 1.66	0.22	1 (7.1)	0.87	0.08 9.77	0.91	1 (7.1)	1.53	0.11 20.52	0.75
	C/T	21 (40.4)	26 (50)	B	- -		3 (5.8)	-	- -	-	2 (3.8)	-	- -	-

OR: Odds Ratio, CI: Confidence Interval, RRMS: Relapsing-Remitting Multiple Sclerosis, SPMS: Secondary Progressive Multiple Sclerosis, PPMS: Primary Progressive Multiple Sclerosis
*Analyzed by the chi-square test

The mean serum vitamin D level of MS patients was significantly higher than the controls (19.4 ± 9.3 ng/mL versus 14.9 ± 8.5 ng/mL, p <0.01). The mean serum vitamin D level of RRMS patients was significantly higher compared to the controls (20.0 ± 9.6 ng/mL versus 15.0 ± 8.5 ng/mL, p <0.05). No significant difference was revealed between the mean serum vitamin D levels of RRMS, SPMS, and PPMS patients (p> 0.05). The vitamin D levels of RRMS, SPMS, and PPMS patients are presented in Figure 1.
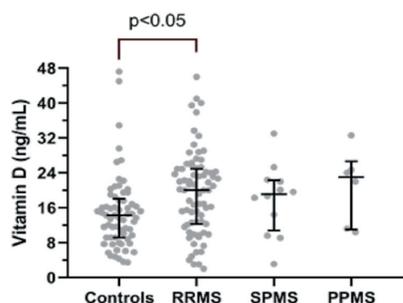
Data were analyzed with Kruskal-Wallis ANOVA with post hoc Dunn's test and were expressed as median with interquartile range

Figure 1. Vitamin D levels of RRMS, SPMS, PPMS patients and control
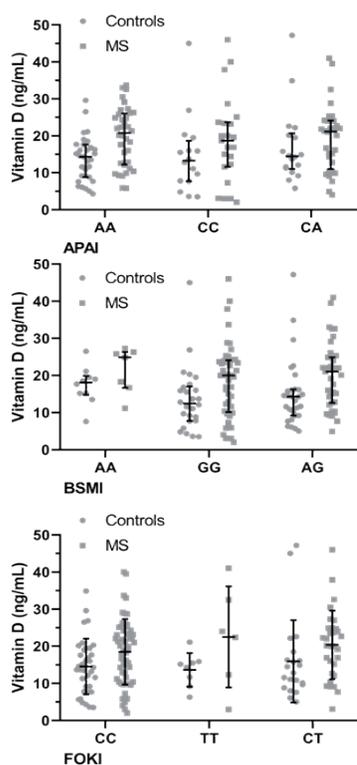
The data were analyzed by Kruskal-Wallis ANOVA and t-tests, and the interquartile interval was expressed as median.

Figure 2. Vitamin D levels of A/A, C/C, and C/A genotypes of the Apal, Bsml, and Fokl genes in MS patients and the control group

The mean serum vitamin D levels of MS patients according to the Apal, Bsml, and Fokl genotypes are presented in Figure 2. No significant difference was found between the

subgroups of MS patients according to the ApaI, BsmI, and FokI genotypes (p> 0.05).

In Table 2, upon evaluating the genotypes and controls and disease subtypes (RRMS, SPMS, and PPMS), a statistically significant correlation was found with ApaI A/A, C/C, and A/C (p<0.01, p=0.01, p<0.01, respectively) in the SPMS group and with ApaI A/A and A/C genotypes (p=0.01 and p=0.04) in the PPMS group. Furthermore, the C/C allele was not detected in the PPMS group in the ApaI gene. The data were analyzed by the chi-square test.

Then, the EDSS range values and genotypes were compared by the chi-square test, and it was examined whether there was a change in the genotypes according to disability (Table 3). Accordingly, it was determined that patients with an EDSS score between 0-6 statistically significantly had the BsmI G/G genotype in comparison with other values (p<0.05).

Table 3. Genotype ratios of Apal, Bsml, and Fokl genes in MS patients with an EDSS score of 0-6 and 6-9.5

Genotypes		EDSS scores 0-6 n (%)	EDSS scores 6-9.5 n (%)	P
Apal	A/A	32(88.9)	4(11.1)	0.24
	C/C	23(100.0)	0(0)	
	A/C	31(93.9)	2(6.1)	
Bsml	A/A	6(85.7)	1(14.3)	<0.05
	G/G	45(100.0)	0(0)	
	A/G	35(87.5)	5(12.5)	
Fokl	C/C	52(94.5)	3(5.5)	0.57
	T/T	5(83.3)	1(16.7)	
	C/T	29(93.5)	2(6.5)	

EDSS: Expanded disability scale score
* Analyzed by the chi-square test

DISCUSSION

The results of this study suggest that having all APAI genotypes in SPMS patients and ApaI A/A and A/C genotypes in PPMS patients are significant risk factors for disease in Turkish population. In terms of the disease progression, it is more likely that the progression will be milder in MS patients with the Bsml G/G genotype. Moreover, having the ApaI A/A allele may worsen the prognosis, although it is not statistically significant.

MS is a chronic inflammatory disease that influences the white and gray matter with axonal degeneration, demyelination, and inflammation in the central nervous system. Etiology is uncertain. However, it is known to be triggered by genetic predisposition and environmental factors (1,3,20). The results of twin studies emphasize the importance of genetic factors in MS. The most significant genetic risk factor for MS is the presence of major histocompatibility complex (MHC) class II. Furthermore, the presence of human leucocyte antigen-DR (HLA-DR), human leucocyte antigen-DQ (HLA-DQ) and MHC class I

is also another important risk factor (21). More than 100 gene polymorphisms associated with MS were found in various studies (22). Some of these polymorphism studies were related to vitamin D, the reason for which is the available data showing that this vitamin plays a protective role in MS due to its immunological effects. It is known that vitamin D slows down neurodegeneration and affects the morbidity of the disease in later periods (23).

Vitamin D receptors are found in the gastrointestinal system, kidneys, bone, stomach, heart, pancreas, skin, ovary, breast, prostate tissue, peripheral monocytes, thymus tissue, and peripheral T cells, except for the central nervous system (8). The active form of vitamin D fulfills its function by binding to these receptors to regulate target gene expression, especially in the nervous system and immune system (24). The binding of VDR with the retinoid X receptor (RXR) results in heterodimerization, following which it binds to vitamin D response elements (VDRE) on DNA. After the intake of transcriptional regulatory proteins such as nuclear receptor coactivators, the VDR9/RXR complex begins to change gene expression, and many processes such as cell proliferation, differentiation, and immunomodulation take place (25). After vitamin D binds to VDR, it controls T helper 1 dominance, which is important in autoimmune diseases, by inhibiting the production of some interleukins, interferon (IFN) gamma, tumor necrosis factor (TNF) alpha and beta (26). As a result of a defect in this gene, the risk of autoimmune diseases characterized by increased inflammation, including MS, may increase (27).

The correlation between MS and the VDR gene ApaI, BsmI, and TaqI polymorphism differs in different populations. In some studies, these SNPs were associated with the risk of MS. However, significant results were found in terms of progression in some studies (28-30). Unlike these studies, no correlation was found concerning the risk of MS in the studies conducted in Canada, Holland, and Iran and concerning progression to disability in the studies conducted in Spain, Greece, and Japan (30).

In terms of the ApaI genotype, in the study on VDR gene polymorphism in the Australian population conducted by Tajouri et al. (31), a significant difference was found between the groups, and it was determined that there was a predisposition to PPMS and SPMS in some frequencies. Our research has shown similar frequencies of higher APAI genotypes in the all SPMS groups and APAI A/A, A/C genotypes in PPMS groups. In another study conducted in the Slovak population, no correlation was revealed between the ApaI gene polymorphism and the development of MS and disease progression. Furthermore, in the current research, no significant difference was found between the mean values of vitamin D in the patient and control groups (12). Niino et al. (29) obtained significant results only in terms of A/A genotypes. In conclusion, our study revealed no significant difference between ApaI genotypes and the averages of vitamin D in both the patient and control groups. In the evaluation between the genotypes and

disease subtypes, ApaI A/A and A/C genotypes among MS patients in both SPMS and all and PPMS groups caused a statistically significant positive difference. Besides, because of the small number of patients with SPMS and PPMS, it was difficult to decide for these two subtypes. Besides, when the EDSS range values and genotypes were compared in our study, no associative feature was found. While the C/C allele increases the patient's probability of having RRMS due to the higher percentage of the C/C allele in the RRMS group in comparison with the control and other groups, although not statistically significant, the presence of this allele statistically reduces the probability of having PPMS.

The studies conducted with FokI polymorphism also yielded different results. Although it was stated in a British cohort study that the FokI ff (T/T allele) genotype was correlated to MS, other studies reported that MS and these gene polymorphisms were not related (32). Furthermore, a study showed that FokI SNPs negatively correlated with the probability of MS (6). Simon et al. (33) found a significant correlation between low vitamin D levels and the development of MS in patients with FokI polymorphism. Similar to our study, the study conducted by Bettencourt et al. (34) determined no significant difference between FokI genotypes and vitamin D levels in both the patient and control groups. In the same study, the FokI genotypes and alleles did not affect disease progression and disability, as in our study.

In a study conducted in Mexico, the T allele in the BsmI gene polymorphism was higher in the MS patient group. The study argued that there was a positive correlation between BsmI gene polymorphism and MS (35). In our study, no statistically significant difference was revealed between BsmI genotypes, the mean values of vitamin D, and disease subtypes. Our results indicated no significant correlation between the BsmI gene polymorphism and the risk of MS, as revealed by Agnello et al. (36). When the results of our study are evaluated in terms of morbidity, the patients with the G/G allele are not expected to have EDSS values above 6. Thus, it was concluded that having the G/G allele was a protective factor. There is also another study supporting our result in this regard (6).

In our study, vitamin D serum level was found to be statistically insufficient in both the patient and control groups. When these values were evaluated within themselves, they were determined to be significantly higher in the patient group than in the control group and conflicted with the theory considered in pathophysiology, which was considered to be related to the awareness and compliance of MS patients with the recommendations such as sunbathing and eating a diet rich in vitamin D although they did not receive vitamin D replacement in the last three months.

LIMITATIONS

The present study has several limitations. First, our number of patients was relatively small since it was a single-center study. Therefore, it was difficult to comment

on polymorphism in patients with PPMS and SPMS, which are less common subtypes. Besides, as stated previously, there were difficulties in evaluating the vitamin D levels of the patient group due to the compliance of the patients who did not receive vitamin D replacement to the recommendations of sunbathing as a lifestyle and eating a diet rich in vitamin D although they were included in the study, and increased awareness. In future studies, it would be more accurate to make a comparison of vitamin D levels in newly diagnosed naive patients with no lifestyle changes and to associate them with gene polymorphism. For these reasons, multi-center studies should be conducted in larger series in the future to emphasize genotype.

CONCLUSION

Furthermore, based on the results of our study, it can be argued that having all ApaI genotypes in the SPMS groups and Apal A/A and A/C genotypes in PPMS groups in the Turkish population represents a significant risk factor for the disease. In terms of the disease progression, pathologic features are more likely to appear milder in MS patients with the Bsml G/G genotype. Moreover, having the Apal A/A allele may worsen the prognosis. Our study is also important because it is the first study conducted in this regard in the Turkish population.

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