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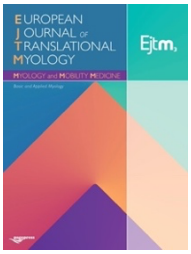

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Evaluation relationship between vitamin D Receptor and clinical and inflammatory factors in patients with relapsing-remitting multiple sclerosis

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Abstract

Adipocyte levels, including leptin and FABS-4 levels, adiponectin, obesity and vitamin D, may be associated with the occurrence and exacerbation of multiple sclerosis. This research aimed at determining the relationship between Vitamin D Receptor changes and clinical and inflammatory factors in patients with relapsing-remitting multiple sclerosis (RRMS). Present case/control study was conducted based on the Helsinki Ethical Principles. RRMS disease was confirmed based on history, clinical symptoms, radiological signs and neurologist diagnosis. The research population consisted of healthy people and patients with RRMS who were referred to Hazrat Rasool Akram Hospital between 2021 and 2023 and met the criteria for participation in the research. The FokI polymorphism is associated with a significant increase in risk with an odds ratio of 7.28 for individuals with the FF genotype and RRMS compared to healthy individuals (OR = 7.28: 95% CI; 1.86, 28.41). The presence of a FokI polymorphism significantly increases the likelihood of developing RRMS in individuals with the FF genotype compared to healthy individuals, with an odds ratio of 28.7. RRMS patients with genotypes did not show a significantly increased risk of FokI, ApaI, TaqI and BsmI polymorphisms compared to controls.None of the polymorphisms

examined showed a significant risk in obese patients with different genotypes compared to obese people. Further research, including additional cases, is needed to avoid results that could be inflated by small samples or low frequencies of minor alleles.

Key words: vitamin D receptor, gene changes, clinical and inflammatory factors, relapsing-remitting multiple sclerosis

Introduction

Although the causes of Multiple Sclerosis (MS) are still unknown, some studies have shown that environmental factors, oxidative stress, immune system defects, and inflammatory factors may play a role in this disease.1 Some evidence suggests that some people with inadequate vitamin D levels do not develop MS.[2-4] Vitamin D is a nutrient that the body needs to build and maintain healthy bones. Only when vitamin D is present can the body absorb calcium, the main component of bones. Vitamin D also regulates many other cellular functions in the body.[5] Its anti-inflammatory, antioxidant and neuroprotective properties support immune system health, muscle function and brain cell activity. Vitamin D doesn't occur naturally in many foods, but you can get it from fortified milk, fortified cereals, and fatty fish like salmon, mackerel, and sardines.[6]

According to the studies carried out, adipokine levels, including leptin and FABS-4 levels, adiponectin, obesity and vitamin D, may be associated with the onset and exacerbation of MS disease[5-7] and, on the other hand, with vitamin D deficiency A deficiency of Vitamin D Receptor (VDR) and its responsiveness can therefore influence the occurrence of MS.[8-10]

According to reports, some people with insufficient levels of vitamin D did not develop MS, and some even with sufficient or even high levels of vitamin D developed this disease, indicating that attention should be paid to the possibility of a connection between changes in the VDR must be associated with inflammatory and clinical factors such as obesity, three major Short Chain Fatty Acids (SCFA), including Propionate (PA), Butyrate (BA) and Acetate (AA), adipokine (leptin and adiponectin), occurs in patients with multiple sclerosis type RRMS before.[11,12]

Therefore, the aim of present study was evaluate the association between VDR changes and clinical and inflammatory factors in patients with Relapsing-Remitting Multiple Sclerosis (RRMS).

Materials and Methods

The present study was a case/control study conducted based on the Helsinki ethical principles[13] and was approved by the Department of Medical Ethics, Iranian University of Medical Sciences with no. IR.IUMS.FMD.REC.1401.429. RRMS disease was confirmed based on history, clinical symptoms, radiological signs and neurologist's diagnosis. The research population consisted of healthy people and patients with RRMS who visited Hazrat Rasool Akram Hospital between 2021 to 2023 and met the inclusion criteria.

Inclusion criteria

The study included: i) Patients with RRMS diagnosed using the 2017 McDonald's criteria;[14] ii) Both female and male gender; iii) Age group from 18 to 65 years; iv) EDSS between 1 and 3; v) Patients who have not taken vitamin D in the last 6 months.

Exclusion criteria

The study excluded: i) Patients in other phases of RRMS except relapse; ii) Patients with diabetes, liver disease (cirrhosis and types of hepatitis), kidney disease, congestive heart failure, high blood pressure, and cancer; iii) Taking other drugmedications.

In the present case/control study, 25 patients (female: 17; male: 7; mean age: 32.16 ± 5.87 years) with RRMS (test group) and 12 healthy individuals (female: 5; male: 7; mean age: 31 .58 ± 8.14 years; control group) were evaluated between 2021 and 2023. All study participants gave written informed consent and stated that there would be no additional costs, threats or problems imposed on them by the study. If they were unable to cooperate and continue to participate, they were excluded from the study without negligence.

Data collection: methods, process, and tools

In the present study, a questionnaire was used to collect demographic and clinical information, including age of onset (years), duration of MS disease (months), Comprehensive Disability

Status Scale (EDSS/1-3) score, and the severity of the multiple sclerosis scores (MSSS), the annual recurrence rate since the beginning of the year of this disease, the number of recurrences two years ago and doctors at the time of conducting the research, gender, disease and smoking history, vitamin D consumption in the last 6 months.[15]

MSSS is a useful measure of MS severity that includes EDSS and disease duration. Achieving an MSSS score ≥ 4.8 indicates a severe phase of the disease, and an MSSS < 4.8 indicates a mild phase of the disease.[15]

Peripheral blood (5 mL) samples with EDTA were collected from RRMS patients and healthy volunteers (from 8 am to 11 am). Two blood collection tubes containing sodium citrate were used for cell and plasma separation.

Peripheral Blood Mononuclear Cells (PBMC) were isolated using gradient centrifugation (920 g, 30 min). They were frozen in serum (FBS) with DMSO (10%) and stored in liquid nitrogen (−196 °C). The serum was separated into serum separation tubes by centrifugation (920 g, 15 min, room temperature). After centrifugation, the plasma and serum samples were divided into individual parts and stored at a temperature of −80°C.

The Redirection Fragment Length Polymorphism (PCR-RFLP) technique was used, which is one of the standard methods for identifying polymorphisms. For this purpose, the PCR product was mixed with restriction enzymes according to the manufacturer's instructions and the best temperature for cutting was chosen according to the cutting time of the enzyme in the instructions. After the product was incubated, it was electrophoresed on a 2% agarose gel. Based on the pattern of restriction enzyme cleavage, the different polymorphisms of the VDR examined in each participant were separated using the gel device and photographed. Documentation has been created. The adipocytes were quantified using ELISA. The samples were diluted 1:5,000 to 1:10,000 for adiponectin and 1:40 for leptin (Human ABTS Standard Development Kit, PeproTech, London, UK) and randomly distributed among the plates. Samples from each participant were analyzed in duplicate in one run, and each plate contained a sample from a control donor to control within-assay variability.

The inter- and intraassay variability was 29% and 21%, respectively, for leptin and 12% and 11%, respectively, for adiponectin. Serum levels of Interleukin-6 (IL-6) were measured using the single molecule array method (SIMOA). Whole blood was collected into PAXgene tubes and total RNA was extracted using the PAXgene Blood miRNA kit. One microgram of RNA was

then reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies Europe B.V.). Using TaqMan technology, duplicate qPCR was performed on cDNA diluted 1:1 (IL10), 1:10, or 1:50 with TaqMan Universal FAST PCR Master. Serum and plasma samples were used to evaluate adipokine and adiponectin.

First SCFA concentrations were examined and metabolic pathways associated with inflammatory biomarkers and clinical variables were examined in two cohorts: a group of individuals without health problems and a group of individuals diagnosed with RRMS.

Statistical analysis was performed using SPSS software version 23. the p-value less than 0.05, judged as "significant, To check the normality of the data, the Kolmogorov-Smirnov test was used. Two study groups were examined for demographic variables.

To measure the relationship between the factors and MS risk, odds ratio and 95% confidence limits were calculated. The relationship between MS patients and clinical factors was evaluated by the chi-square test and Fisher's exact test. An odds ratio and 95% confidence limits are calculated. SNP Analyzer software was used to analyze the frequency of alleles associated with each polymorphism in healthy and diseased people and compare the values.

Results

The mean age of participants in the case and control groups was 32.16 ± 5.87 years and 31.58 ± 8.14 years, respectively ($p = 0.829$). According to the Endocrine Society clinical guidelines, 25-hydroxyvitamin D levels are divided into deficiency (less than 20 ng/mL), inadequate vitamin D (21-29 ng/mL) and optimal vitamin D (30-20 ng/mL) groups (85 ng/mL) were divided.[16] In the test group, 88% of participants had 25-hydroxyvitamin D deficiency and in the control group, 66.7% of participants had vitamin D deficiency. According to the results of the Spearman test, there was a statistically significant difference in 25-hydroxyvitamin D levels between both groups ($p<0.001$).

The mean serum level of 25-hydroxyvitamin D was significantly lower in patients with diabetes than in healthy volunteers ($p<0.001$). In the univariate conditional logistic regression analysis, an Odds Ratio (OR) of 1.55 (95% CI 1.187–2.040; $p < 0.001$) was obtained, indicating that low 25-hydroxyvitamin D levels are associated with up to 551/increase the risk of developing MS. This association was statistically significant and a strong association was observed ($p < 0.001$). The mean and standard deviation of 25 hydroxyvitamin D levels in the patients enrolled in this study

in the mild and severe groups were 18.42 ± 3.64 ng/mL and 15.33 ± 3.89 ng/mL, respectively. According to the Mann-Whitney statistical test, a statistically significant difference was observed in 25-hydroxyvitamin D levels between the two light and heavy groups ($p<0.001$), so the vitamin D level was significantly lower in the heavy group as the mild group.

Genotype of polymorphisms and allelic distribution

According to Table 1, the FokI genotype distribution in RRMS patients was FF (wild) = 72%, Ff (heterozygous) = 28% and ff (mutant) = 0%. A statistically significant difference was observed between the percentage of genotypes in test and control groups ($p<0.05$). Statistical analyzes of FokI polymorphism showed a significant increase in risk in patients with FF genotype compared to controls (OR=7.28: 95% CI; 1.86, 28.41). Statistical analysis of the ApaI polymorphism showed no significant increase in risk in patients with the AA genotype compared to the control group (OR=1.28: 95% CI; 0.45, 3.62; $p>0.05$). Statistical analysis of TaqI polymorphism showed no significant increase in risk in patients with TT genotype compared to a control group (OR = 1.89: 95% CI; 0.74, 4.84; $p=0.294$). Statistical analysis of BsmI polymorphism showed no significant increase in risk in patients with TT genotype compared to controls (OR = 0.94: 95% CI; 0.42, 2.08; $p = 0.987$).

Obesity and VDR changes

According to Table 2, Statistical analysis of FokI, ApaI, TaqI and BsmI genotypes showed no significant increase in risk in RRMS patients with higher BMI compared to the control group (obese subjects) ($p>0.05$).

Adipokine and VDR changes

The mean adiponectin level in patients with RRMS was 11995.99 ± 620.34 and in the control group was 10466.7 ± 160.25 ($P < 0.01$). The mean leptin level in patients with RRMS was 42441.5 ± 571.5 and in the control group was 32461.03 ± 446.36 ($P < 0.01$). Adiponectin and leptin levels were higher in patients with RRMS than in the control group. Based on Table 3, statistical analysis of FokI polymorphism showed a significant increase in risk in RRMS patients with FF genotype compared to the control group (OR = 7.28: 95% CI; 1.86, 28.41). The odds ratio was 28.7. Statistical analyzes of ApaI, TaqI, and BsmI polymorphisms showed no

significantly increased risk in RRMS patients with genotypes compared to controls; The odds ratio was almost 1.00.

The concentration of SCFAs in RRMS patients (386.44 ± 37.64) was significantly lower compared to healthy subjects (436.08 ± 24.82) ($p<0.001$). The amount of acetate was significantly lower in RRMS patients compared to healthy subjects (364.72 ± 37.56 μmol/L vs. 414.58 ± 25.26 μmol/L; $p<0.001$). While there was no statistically significant difference in propionate and butyrate levels ($p>0.05$). According to Table 4, statistical analysis of FokI polymorphism showed a significant increase in risk in RRMS patients with FF genotype compared to the control group (OR = 0.1: 95% CI; 0.22, 0.49). Statistical analyzes of ApaI, TaqI and BsmI polymorphisms showed no significant increase in risk in RRMS patients with genotypes compared to controls.

Discussion

Based on the results of the present study, it was observed that low serum levels of 25-hydroxyvitamin D were observed in patients with MS. Patients with MS had significantly lower mean serum levels of 25-hydroxyvitamin D than healthy individuals, which was considered statistically significant. The present study observed a statistically significant association between low serum levels of 25-hydroxyvitamin D and MS progression.

Vickaryous *et al.* (2020) showed that serum 25(OH)D levels were higher in MS than in the control group (healthy subjects) (median 71 nmol/L vs. 49 nmol/L).[17] A population-based prospective cohort study of 145 people with RRMS living in southern Tasmania and Australia from 2002 to 2005 found that higher 25(OH)D levels were associated with a reduced risk of relapse. Every 10 nmol/L increase in 25(OH)D leads to a 12% reduction in the risk of recurrence. These clinical findings are also related to the radiological signs of MS disease activity.[18]

A prospective cohort study of 1482 participants with MS in the BEYOND study treated with interferon beta1b showed that a 50.0 nmol/L increase in 25(OH)D blood levels was associated with a 31% lower rate of new lesions was connected in the MRI. Is. Patients with 25(OH)D levels above 100.0 nmol/L had the lowest rate of new lesions on MRI. However, no significant association with recurrence rate was observed.[19]

In a large multicenter study, 1047 cases of Clinically Isolated Syndrome (CIS) from 17 different countries were observed over a period of 4.31 years. Clinical and biochemical variables were

evaluated to determine their value in predicting progression from CIS to Clinically Confirmed Multiple Sclerosis (CDMS). Patients with high 25(OH)D levels significantly reduced the risk of conversion in the univariate analysis. Multivariate analysis did not reproduce the same results, but at the same time reduced the risk of conversion and statistical significance.[20]

Yu *et al.* (2020) examined the association between VDR SNP and obesity (BMI ≥ 28 kg/m2) and reported that rs3847987 (AC vs. CC, adjusted OR: 1.938, 95% CI: 1.359–2.763, P = 0.000405) was associated with obesity. It was relevant. The C allele of rs3847987 was a risk factor for obesity.[21] In the present study, FokI polymorphism showed a significant increase in risk with an odds ratio of 28.7 in RRMS patients with FF genotype who had high adiponectin levels compared to the control group (OR = 7.28: 95% CI; 1.86, 28.41). Further studies are needed to clarify the contribution of VDR genetic SNPs on serum 25(OH)D in people with MS. The exact molecular mechanism explaining the association between VDR polymorphisms and serum 25(OH)D levels remains unknown. Levin *et al.* (2012) reported that greater VDR activity for a given amount of 25(OH)D can provide protection under low 25(OH)D substrate conditions.[22] However, no study was found that could accurately compare the results of the present study.

In the present study, the FokI polymorphism caused a significant increase in risk in RRMS patients with FF genotype compared to the control group with an odds ratio of 0.1 (OR = 0.1: 95% CI; 0.22, 0.49). However, ApaI, TaqI and BsmI polymorphisms in RRMS patients with genotypes did not show a significant increase in risk compared to the control group. A positive correlation between VDR levels and IL-6-encoding genes was observed in RRMS patients. In this regard, no study was found that could accurately compare the results of the present study.

Conclusions

The results of the current study suggest that the FokI polymorphism is associated with a significant increase in risk compared to healthy individuals, with an odds ratio of 7.28 for individuals with the FF genotype and RRMS (OR = 7.28: 95 % CI; 1.86, 28.41).). None of the polymorphisms examined showed a significant risk in obese patients with different genotypes compared to the control group (obese people). The presence of a FokI polymorphism significantly increases the likelihood of developing RRMS in individuals with the FF genotype compared to healthy individuals, with an odds ratio of 28.7. RRMS patients with genotypes did not show a significantly increased risk of FokI, ApaI, TaqI and BsmI polymorphisms compared

to controls. FokI polymorphisms demonstrated the association of VDR changes under the influence of inflammatory factors in RRMS patients. To understand how VDR works and to improve the chances of finding alleles associated with risk of common diseases, a study that examines the polymorphic change in the VDR gene is needed. Further research, including additional cases, is needed to avoid results that could be inflated by small samples or low frequencies of minor alleles.

List of abbreviations

MS, Multiple Sclerosis

CNS, Central nervous system

VDR, Vitamin D Receptor

Leptin, FABS Adoponectin

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Conflict of interest: the authors declare no potential conflict of interest, and all authors confirm accuracy.

Ethics approval: the Ethics Committee of Iran university of medical sciences approved this study (IR.IUMS.FMD.REC.1401.429). The study is conformed with the Helsinki Declaration of 1964, as revised in 2013, concerning human and animal rights.

Informed consent: all patients participating in this study signed a written informed consent form for participating in this study.

Patient consent for publication: the present study was conducted based on the ethical principles of Helsinki and was approved by the Department of Medical Ethics of Iran University of Medical Sciences. Informed consent form was obtained from all patients and control group. Ethical consent form was obtained by the coordinator of the project before the patients entered the study at Rasoul Akram Hospital with a complete description of the study

Availability of data and materials: patient information has been collected and analyzed based on ethical principles and full satisfaction in Rasul Akram Hospital, Tehran

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Table 1. Association between polymorphisms and haplotypes of the VDR and the odds ratio of polymorphisms

SNPs		**Genotype**	**Genotypic frequency (%)**		***S*ignificance**	**Odds ratio (95% CI)**
			RRMS (n=25)	Control (n=12)		
FokI	rs2228570	FF	18 (72)	3 (25)	$\chi 2$= 11.96	7.28
		Ff	7 (28)	5 (41.7)	P= 0.003	(1.86, 28.41)
		ff	0	4(33.3)		
ApaI	rs7975232	AA	10 (40)	6 (50)	$\chi 2$=5.19	1.28
		Aa	14 (56)	3 (25)	P=0.075	(0.45, 3.62)
		aa	1 (4)	3 (25)		
TaqI	rs731236	TT	12 (48)	4 (33.3)	$\chi 2$=2.44	1.89
		Tt	10 (40)	4 (33.3)	P=0.294	(0.74, 4.84)
		tt	3 (12)	4 (33.3)		
BsmI	rs1544410	BB	10 (40)	5 (41.7)	$\chi 2$=0.025	0.94
		Bb	6 (24)	3 (25)	P=0.987	(0.42,2.08)
		bb	9 (36)	4 (33.3)		

χ^2 chi-square test; p: p-value; Significant at $P \leq 0.05$

Table 2. Association between obesity and VDR changes in patients with RRMS

SNPs		**Genotype**	**Genotypic frequency (%)**		***S*ignificance**	**Odds ratio (95% CI)**
			RRMS (n=21)	Control (n=6)		

FokI	rs2228570	FF	14 (66.7)	2 (33.3)	$\chi2$= 4.72	4.60
		Ff	7 (33.3)	3 (50)	P= 0.094	(0.82, 25.74)
		ff	0	1(16.7)		
ApaI	rs7975232	AA	8 (38.1)	3 (50)	$\chi2$=1.56	1.00
		Aa	12 (57.1)	2 (33.3)	P=0.457	(0.25, 4.44)
		aa	1 (4.8)	1 (16.7)		
TaqI	rs731236	TT	10 (47.6)	3 (50)	$\chi2$=2.66	1.49
		Tt	9 (42.9)	1 (16.7)	P=0.265	(0.43, 5.1)
		tt	2 (9.5)	2 (33.3)		
BsmI	rs1544410	BB	9 (42.9)	2 (33.3)	$\chi2$=0.175	1.22
		Bb	6 (28.6)	2 (33.3)	P=0.916	(0.41,3.64)
		bb	6 (28.6)	2 (33.3)		

Table 3. Association between adipocyte and VDR changes

SNPs		**Genotype**	**Odds ratio (95% CI)**	
FokI	rs2228570	FF		7.28
		Ff	(1.86, 28.41)	
		ff	P=0.04	
ApaI	rs7975232	AA	1.28	
		Aa	(0.45, 3.62)	
		aa	P=0.636	
TaqI	rs731236	TT	1.89	
		Tt	(0.74, 4.84)	
		tt	P=0.180	

BsmI	rs1544410	BB	0.94
		Bb	(0.42,2.08)
		bb	P=0.887

Table 4. Association between short-chain fatty acids and VDR changes.

SNPs		**Genotype**	**Odds ratio (95% CI)**
FokI	rs2228570	FF	0.10
		Ff	(0.22, 0.49)
		ff	P=0.004
ApaI	rs7975232	AA	0.77
		Aa	(0.186, 3.242)
		aa	P=0.729
TaqI	rs731236	TT	0.82
		Tt	(0.28, 2.42)
		tt	P=0.825
BsmI	rs1544410	BB	3.10
		Bb	(0.86,11.13)
		bb	P=0.0.83