ApaI and *Fok1* Variants of Vitamin D receptor Gene Associated with Metabolic Syndrome

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Abstract

Objectives: The association between vitamin D receptor (VDR) polymorphisms and metabolic syndrome (MS) remains debatable. The current study aimed to determine the correlation of VDR gene polymorphisms with MS among Jordanian females.

Methods: This case-control study enrolled 100 MS females and 100 age-matched control. HbA1C, fasting glucose, triglyceride, LDL, HDL, total cholesterol, and vitamin D (25(OH)D) were determined from serum samples of all participants. DNA was extracted from whole blood samples, and VDR gene polymorphisms *Apa1*, *Taq1*, *Bsm1*, and *Fok1* were analyzed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (PCR-RFLP).

Results: The results indicated a statistically significant difference between MS patients and control subjects in terms of (Body mass index) BMI (34.3±3.1; 28.1±2.5), HbA1C (5.9±.1.1; 4.6±.1.2), fasting blood glucose (6.4±1.6; 5.2±1.4), and total cholesterol (6.5±1.2; 5.3±1.8). The results also demonstrated a statistical difference in the number of individuals with 25(OH)D deficiency (69, 33), 25(OH)D insufficiency (25, 42), and 25(OH)D sufficiency (6, 25) between MS patients and control subjects ($p< 0.001$). MS was significantly associated with VDR polymorphisms among *Apa1* and *Fok1* genes. In fact, the genotype distribution for CC (47%; 53%, $p = 0.002$) and CA (37%; 45%, $p = 0.001$) genotypes among *Apa1* VDR polymorphism, as well as among TT genotype (38%; 20%, $p=0.025$) among *Fok1*VDR gene polymorphism significantly differed between MS patients and healthy individuals. However, no associations were detected among *Taq1* and Bsm1 VDR genotypes.

Conclusions: VDR gene polymorphism of *Apa1* and *Fok1* variants increase the risk of metabolic syndrome development among Jordanian MS females

Keywords: Lipid Profile; Vitamin D; Metabolic Syndrome; Polymorphism.

Introduction

Vitamin D deficiency is considered a health problem. Many studies have shown the relationship between vitamin D deficiency and various chronic diseases. Moreover, vitamin D deficiency is associated with energy homeostasis and the regulation of the immune and endocrine systems.[1] Vitamin D also has a significant effect on calcium and phosphorus homeostasis in skeletal muscles.[2] Vitamin D deficiency has been associated with many types of cancer, cardiovascular disorders, diabetes, and metabolic syndrome.[3]

Metabolic syndrome (MS) is a cluster of common abnormalities, including insulin resistance, impaired glucose tolerance, abdominal obesity, reduced high-density lipoprotein (HDL)-cholesterol levels, elevated triglycerides, and hypertension.[4] The International Diabetes Federation defines MS as abdominal obesity plus two of five cardiovascular

risk factors, namely hyperglycemia, diabetes, hypertension, hypertriglyceridemia, and low HDL-C.[5] The World Health Organization, on the other hand, has a different definition that includes insulin resistance as a required criterion in defining MS.[6]

Nowadays, vitamin D association with MS has attracted great attention because it is linked to many abnormalities, such as obesity, hyperglycemia, hypertension, hyperlipidemia, cardiovascular disorders, diabetes, and dyslipidemia. Several studies have shown a link between vitamin D deficiency and the development of metabolic diseases such as dyslipidemia, hypertension, obesity, insulin resistance, and hyperglycemia.[7] Other studies demonstrated an inverse association between serum 25(OH) D level and MS,[8-10] while some did not have such a correlation.[11] A meta-analysis indicated a negative association between serum vitamin D concentration and the MS risk in a dose-response manner. This meta-analysis showed that every 25 nmol/L increase in serum vitamin D levels was significantly associated with a 15% reduction in the odds ratio of developing MS (OR: 0.85; 95% CI: 0.80-0.91).[12]

Vitamin D receptor (VDR) is a member of the steroid/thyroid hormone receptor that creates a complex with vitamin D and acts as a transcriptional activator.[13] It regulates gene transcription by binding to vitamin D-responsive elements that are evolutionarily preserved in the promoter region of many targeted genes.[13] VDR has pleiotropic functions resulting from VDR activation of many genes in the human genome.[14] VDR polymorphisms are a possible genetic contributor to many metabolic conditions.[15] Several polymorphisms have been reported for the VDR gene, such as rs7975232 (*Apa1*), rs1544410 (*Bsm1*), rs2228570 (*Fok1*), and rs731236 (*Taq1*).[16] Studies that investigated the role of VDR polymorphisms in the pathogenesis of MS were inconclusive. VDR genetic variants have been associated with MS among different ethnic groups.[17-19] A meta-analysis showed a significant correlation between VDR polymorphisms and MS susceptibility. *Bsm1* polymorphisms have been linked with an increased risk of MS, whereas *Apa1* polymorphisms have been related to decreased risk of MS.[20,21] Other studies showed no association between VDR polymorphisms and MS.[22]

There are insufficient studies on the relationship between vitamin D levels among MS, and there is an inconclusive relationship between VDR gene polymorphisms and MS. Therefore, the current study aimed to examine the association between 25(OH)D levels among MS Jordanian females. Also, the association between VDR gene polymorphisms (*Apa1*, *Bsm1*, *Fok1*, and *Taq1*) and MS among Jordanian females.

Materials and methods

This case control study enrolled one hundred women with MS, along with 100 age-matched healthy women, were enrolled in this case-control study from Al-Hikma Modern Hospital (Jordan) between 2019 and 2020. The entire procedure of the current research was confirmed by Hashemite University Institutional Review Board and was according to the 1964 Helsinki Declaration (IRB 3/29/2019). BMI was measured, by dividing the weight of an individual in kilograms by the square of the height in meters (kg/m2). Fasting blood samples were collected and biochemical parameters, including HbA1C, fasting blood glucose, triglyceride, HDL, LDL, total cholesterol, and 25(OH) vitamin D were measured.

MS inclusion criteria include the presence of at least three or more of the five criteria: waist circumferences ≥ 88 cm; triglyceride ≥ 150 mg/dL (1.7mmol/L) or lipid lowering agents; HDL < 50 mg/dL (1.3 mmol/L); high blood pressure and on hypertensive lowering agents; fasting glucose ≥ 100 mg/dL or on anti-diabetic drugs.

Apa1, *Taq1*, *Bsma1*, and *Fok1* VRD polymorphisms and were detected using PCR-RFLP.[23] DNA was extracted from whole blood samples using the Qiagen kit according to the manufacturer's instructions (QIAGEN, Germany). A Nano drop analyzer (Thermo Fisher, Waltham, MA, USA) was used to assess the quality and quantity of the extracted DNA, and the ratio of OD260/OD280 was determined to confirm the purity (1.8–2.0). Samples were then stored at −20°C until analysis. PCR was carried out using Master Mix (Promega, USA). PCR amplification was conducted in a final volume of 25 µl containing 10mM Tris-HCl, 200mM dNTP, and 20pmol DNA primer (Promega, USA).

A proportion of the whole blood sample was utilized for DNA extraction (QIAGEN, Germany) and genotyping. Serum level of 25(OH)D was determined according to vitamin D standardization program using manufacturer's instructions via Alinity 25-OH Vitamin D (ABBOTT, IRELAND) assay kit Serum levels for 25(OH)D were classified

into deficient (25 (OH)D level ≤ 50nmol/L), insufficient 25(OH)D level between 50-75nmol/L and optimal 25(OHD level more than 75nmol/L.[24]

The sequences of the specific primers used to amplify *Apa1*, *Bsm1*, *Fok1*, and *Taq1* VDR genotypes are listed in Table 1.

Table 1: Forward and reverse primer sequences used to amplify *Apa1*, *Bsm1*, *Fok1*, and *Taq1* VDR genotypes.

Genes	Forward primer 5′ 3′	Reverse primer 5′ 3′
Fok1	AGCTGGCCCTGGCACTGACTCGCTCT	ATGGAAACACCTTGCTTCTTCTCCCTC
Bsm1	CAACCAAGACTACAAGTACCGCGTCAGTGA	AACCAGCGGGAAGAGGTCAAGGG
Taq1	CAGAGCATGGACAGGGAGCAA	CACTTCGAGCACAAG GGGCGTTAGC
Apa1	GGATCCTAAAGCACGGAGA	ACGTCTGCAGTGTGTTGGAC

PCR reaction mixture was prepared by mixing 25 μL consisting of 1.0 μL of the forward primer (10 ρmole/μL), 1.0 μL of the reverse primer (10 ρmole/μL), 12.5 μL of GoTaq® Green Master Mix ((Promega, USA). 4 μL of template DNA, and 6.5 μL of nuclease free water ((Promega, USA). PCR was carried out using the Bio-Rad iCycler (Bio-Rad, USA) The cycling process was initiated by DNA denaturation at 94 °C for 4 min, followed by 35 cycles at 94 °C for 1 min, 68 °C for 1 min, and 72 °C for 2 min with extra 10 min incubation at 72 °C for *Fok1*, *Bsm1*, and *Taq1* genes. For *Apa1*, thermos cycling program conditions consisted of DNA denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, and finally incubation at 72°C for 10 min. Genotyping was performed and categorized as homozygous and heterozygous according to the DNA band patterns.[25,26] For *Fok1* restriction polymorphism TT genotype yielded one band at 265 bp, CC genotype yielded two bands at 196 bp and 69 bp and TC yielded three bands at 265 bp, 196 bp and 69 bp. *Bsm1* restriction polymorphism, GG yielded a band at 820 bp, the AA yielded two bands at 650 and 170 bp and GA yielded three bands at 820, 650 and 170 bp. For *Taq1* restriction polymorphism, the homozygous TT yielded bands of 500 bp and 210 bp. The homozygous CC yielded band at 210 bp and the heterozygous TC yielded band at 290 bp. PCR products were electrophoresed on 2.5% agarose gel, stained with ethidium bromide and DNA visualized on an ultraviolet Trans illuminator (Thermo Fisher Scientific, USA).

Statistical analysis was carried out using MedCalc statistical software. Categorical data were analyzed by Chi-square. The association between independent variables was assessed using an adjusted odds ratio at a 95% confidence interval was calculated. The *p-value* of less than 0.05 was considered as statistically significant.

Results

Demographic information for patients with MS and control groups is summarized in Table 2. The MS group included 100 patients, and the healthy control consisted of 100 healthy females bearing no criteria for MS. The mean age of the MS group was 48.9±4.3, and the average age of the control subjects was 47.9 ±6.7. No statistically significant ($p = 0.211$) difference was found in terms of age between MS patients and control subjects. A statistically significant difference was found in BMI, HbA1C, fasting blood glucose, triglyceride, total cholesterol, and 25(OH) vitamin D between MS patients and the control group. Also, a significant ($p < 0.001$) differences were detected among individuals with vitamin D deficiency (69%, 33%), vitamin D insufficiency (25%.42%), and vitamin D sufficiency (6%, 25%) between the MS group and healthy subjects, respectively.

Table 2: Demographic and clinical characteristics of patients with metabolic syndrome and control subjects.

Parameter	MS±SD (n=100)	Control ±SD (n=100)	*P*-value
Age	48.9±4.3	47.9 ±6.7	0.211
BMI(kg/m2)	34.3±3.1	28.1±2.5	< 0. 001
HbA1C	5.9±.1.1	4.6±.1.2	<0. 001
Fasting glucose (mmol/L)	6.4±1.6	5.2±1.4	<0. 001
Triglyceride(mmol/L)	2.7±0.9	1.7±0.7	< 0. 001
HDL(mmol/L)	1.3±1.1	1.7±2.3	0.118
LDL(mmol/L)	3.9±1.5	3.3±1.8	0.0112
Total cholesterol (mmol/L)	6.5±1.2	5.3±1.8	< 0.001

25(OH)D (nmol/L)	69	33	< 0.001
Vitamin D deficiency Vitamin	25	42	
D	6	25	
insufficiency			
Vitamin D sufficiency			

***BMI**: body mass index; **HDL**: high-density lipoprotein; **LDL**: low-density lipoprotein*

The genotype distribution of VDR polymorphisms between MS patients compared to controls showed a statistically significant difference among both *Apa1* and *Fok1* genotypes. Both *Apa1* CC and CA genotypes are negatively correlated with metabolic syndrome and showed a statistical difference with CC OR (95% CI) is 0.111(0.024 - 0.508) and CA OR (95%CI) is 0.102(0.022 - 0.476. In the contrary, TT *Fok1* genotype increased the risk of metabolic syndrome 2.47 folds (Table 3). No statistical differences were observed in the frequency of *Taq1* and *Bsma1* genotypes between the MS and control groups (Table 3).

Table 3: Genotype frequencies for *Apa1*, *Bsm1*, *Fok1*, and *Taq1* VDR genotypes among metabolic syndrome patients and control subjects

Genotype	Metabolic syndrome (n=100)	Control (n=100)	OR(95%CI)	P-value
Apa1				
CC	47(47%)	53(53%)	0.111(0.024 - 0.508)	0.002
CA	37(37%)	45(45%)	0.102(0.022 - 0.476)	0.001
AA	16(16%)	2(2%)		Reference
Taq1				
TT	91(91%)	84(84%)	3.25(0.638 - 16.547)	0.078
TC	7(7%)	10(10%)	2.10(0.324 - 13.614)	0.218
CC	2(2%)	6(6%)		Reference
Bsma1				
GG	90(90%)	92(92%)	0.978(0.135 - 7.095)	0.491
GA	8(8%)	6(6%)	1.333(10.144 - 12.369)	0.400
AA	2(2%)	2(2%)		Reference
Fok1				
TT	38(38%)	20(20%)	2.470(1.114 to 5.473)	0.025
TC	42(42%)	54(54%)	1.011(0.497 to 2.054)	0.976
CC	20(20%)	26(26%)		Reference

Discussion

The results of this study showed a statistically significant difference between MS patients and control regarding BMI, HbA1C, fasting blood glucose, triglyceride, total cholesterol (p< 0.001), and LDL (p=0.011). Previous reports demonstrated a relationship between vitamin D status, dyslipidemias, low 25(OH) vitamin D concentrations, increased triglycerides, and decreased HDL levels.[27] Vitamin D and cholesterol share the 7-dehydrocholesterol pathway, so the association between 25(OH)D and dyslipidemias may be related to a common pathway in the liver that shares lipoproteins and vitamin D precursor production. Vitamin D deficiency is considered a global health problem,[28] and vitamin D deficiency or insufficiency has been observed among females of all age groups and ethnicities.[29] Vitamin D has an essential role in maintaining calcium balance and bone formation, and its deficiency may lead to secondary hyperparathyroidism that results in the development of osteomalacia and osteoporosis, which are common in females.[30] In Jordan, the overall prevalence of low vitamin D status; 25(OH) D < 30 ng/ml, was 89.7%, with a higher prevalence in males (92.4%) compared to females (88.6%).[30] Among Jordanian females, the incidence of vitamin D insufficiency was reported to be 10.1%, while vitamin D deficiency was 78.5%.[31] Vitamin D has a para/autocrine metabolic activity whose receptors are highly expressed in most cells.[32] Recent studies have demonstrated an association between vitamin D deficiency and MS and diabetes among elderly Chinese individuals. Though, this association was significant only among elderly people with non-central obesity.[33]

Our findings indicated a statistically significant increase in BMI (34.3±3.1) in the MS group when compared to the control group (28.1±2.5), as well as a statistically significant difference (Table 2) in the number of individuals with deficient, sufficient, and insufficient amounts of 25(OH) D level ($p<0.001$) between MS and control groups. Our results were consistent with the earlier reports concerning such an association, a cross-sectional study conducted by Khoja and colleagues,[34] who showed the same correlation between MS and Vitamin D levels. Their results revealed that the MS group had a significantly low level of vitamin D compared to the control group ($p < 0.001$). They showed a negative relationship between 25(OH) vitamin D levels and type-2 diabetes, glucose homeostasis, and metabolic syndrome. Serum vitamin D level was also inversely associated with waist circumference, triglycerides, and HbA1C, as well as cardiovascular risk. This association was also reported by another systematic review, which found that four out of five observational studies reviewed showed that vitamin D levels were significantly associated with obesity, BMI, dyslipidemia, and insulin in the MS group.[35]

Many VDR gene SNPs have been recorded in metabolic disorders related to vitamin D deficiency and insufficiency.[18] Three SNPs are located at the 3′ untranslated regions of the VDR gene, namely rs1544410 (*Bsm1*), rs7975232 (*Apa1*), and rs731236 (*Taq1*), that influence mRNA stability and VDR expression.[36] Other SNPs, such as rs2228570 (*Fok1*), are located in the proximity of the promoter region resulting in altered VDR protein activity.[37] This study showed statistically significant differences in VDR gene polymorphisms only in *Apa1* genotype distribution, namely CC ($p = 0.002$) and CA ($p = 0.001$), as well as in the TT genotype frequency of *Fok1* ($p=0.025$). No statistical differences were observed in the *Taq1* and *Bsma1* genotypes.

Genetic polymorphisms in the VDR gene have been reported to be associated with anthropometric parameters related to obesity, lipid profile abnormalities, and MS among different populations. In their study on the association between VDR polymorphisms and MS indices, Jin and colleagues[22] showed that the VDR *Apa1* polymorphism was associated with hypertriglyceridemia, while the *BsmI* and *Taq1* polymorphisms affected HDL in MS patients. Another meta-analysis 16 showed that *Bsm1* (rs1544410) polymorphisms protect against MS; however, no association was observed between VDR SNPs and the risk of vitamin D deficiency. Wang et al.[38] investigated a link between VDR polymorphisms and vitamin D deficiency, overweight, obesity, and MS. They found that VDR *Apa1* polymorphisms were correlated with overweight/obesity and glucose intolerance, while AA genotype of *Fok1* SNP was significantly associated with MS.

In contrast, other reports showed no association between VDR polymorphisms and the risk for MS development 18. Erasmus et al.[7] also showed no correlation between VDR *Fok1* and *Taq1* with glycemic status. They also reported that *Fok1* was not linked with 25(OH) vitamin D deficiency, while *Taq1* was linked with vitamin D insufficiency. Liu et al.[39] indicated in their meta-analysis that VDR *Apa1* (rs7975232) and *Fok1* (rs2228570) polymorphisms increased the susceptibility to gestational diabetes mellitus. Therefore, VDR screening among different ethnic groups may be a good molecular marker to determine susceptibility to MS.

The limitations of this research include the sample size and the number VDR polymorphisms that we analyzed in this study was only four (*Apa1*, *Bsm1*, *Fok1*, and *Taq1*).

Conclusion

MS patients were significantly associated with VDR gene polymorphisms among *Apa1* and *Fok1* genotypes. Genotype distribution for CC and CA among *Apa1* VDR polymorphism, and among TT *Fok1* VDR gene polymorphism significantly modify the risk of MS development among Jordanian MS females

Author contribution

Design of the idea, conducting experiments, interpretation of data, analysis, and writing the manuscript drafting the article and approved it

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Competing interests

The authors declare no competing interests with anybody

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