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ApaI and *Fok1* Variants of Vitamin D Receptor Gene Associated with Metabolic Syndrome Among Jordanian Women

Manar Fayiz Atoum*

Department Medical Laboratory Sciences, Faculty Applied Health Sciences, The Hashemite University, Zarqa, Jordan

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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 women. ***Methods:*** This case-control study enrolled 100 women with MS and 100 age-matched women as control at Al-Hikma Modern Hospital in Jordan between January 2019 and January 2020. The levels of glycated hemoglobin, fasting glucose, triglyceride, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and 25-hydroxy 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 and restriction fragment length polymorphism. ***Results:*** There was a significant difference between MS patients and control in terms of body mass index (34.3±3.1 vs. 28.1±2.5), glycated hemoglobin (5.9±1.1 vs. 4.6±1.2), fasting blood glucose (6.4±1.6 vs. 5.2±1.4), and total cholesterol (6.5±1.2 vs. 5.3±1.8). The results also demonstrated a statistical difference in the number of MS patients and control with 25(OH)D deficiency (69.0 vs. 33.0), 25(OH)D insufficiency (25.0 vs. 42.0), and 25(OH)D sufficiency (6.0 vs. 25.0) ($p < 0.001$). MS was significantly associated with VDR polymorphisms among *Apa1* and *Fok1* genes. The genotype distribution for CC (47.0% vs. 53.0%; $p = 0.002$) and CA (37.0% vs. 45.0%; $p = 0.001$) genotypes among Apa1 VDR polymorphism, as well as among TT genotype (38.0% vs. 20.0%; $p = 0.025$) 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 may increase the risk of metabolic syndrome among Jordanian women.

Many studies have shown the relationship between vitamin D deficiency and various chronic diseases. Vitamin D deficiency may impact 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 (MS).[3]

The term MS represents 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 cholesterol.[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]

Several studies have linked vitamin D deficiency with the development of metabolic diseases such as dyslipidemia, hypertension, obesity, insulin resistance, and hyperglycemia.[7] Other studies demonstrated an inverse association between serum 25-hydroxy vitamin D (25(OH) D) level and MS,[8–10] while some did not find such a correlation.[11] A 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 (OR) of developing MS (OR = 0.85; 95% CI: 0.80–0.91).[12]

*Corresponding author: ✉manar@hu.edu.jo

Vitamin D receptor (VDR), which is part of the steroid/thyroid hormone receptor superfamily, 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 possible genetic contributors 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.[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 MS and 25(OH)D levels as well as the association between VDR gene polymorphisms (*Apa1*, *Bsm1*, *Fok1*, and *Taq1*) and MS among Jordanian females.

METHODS

This case-control study enrolled 100 women with MS from Al-Hikma Modern Hospital in Jordan along with 100 age-matched healthy women, between January 2019 and January 2020. The entire procedure of the current research was confirmed by the Hashemite University Institutional Review Board (Ref. IRB 3/29/2019) and was conducted according to the Helsinki Declaration. Each participant's body mass index (BMI), fasting blood glucose, glycated hemoglobin (HbA_{1C}), triglyceride, HDL, low-density lipoprotein, total cholesterol, and 25(OH) were measured.

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

The VRD polymorphisms *Apa1*, *Taq1*, *Bsma1*, and *Fok1* were detected using polymerase chain reaction (PCR)-restriction fragment length polymorphism.[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 OD260/OD280 ratio was determined to confirm purity (reference range: 1.8–2.0). The 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 10 mM Tris-HCl, 200 mM dNTP, and 20 pmol DNA primer (Promega, USA).

A proportion of the whole blood sample was utilized for DNA extraction (QIAGEN, Germany) and genotyping. A serum level of 25(OH)D was determined according to the manufacturer's instructions via an Alinity 25-OH Vitamin D (Abbott, Ireland) assay kit and was classified into (a) deficient: 25 (OH)D ≤ 50 nmol/L, (b) insufficient: 25(OH)D level 50–75 nmol/L, and (c) optimal: 25(OH)D > 75 mol/L.[24]

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

The 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 conducted 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*, the 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

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

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

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; 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 conducted using MedCalc for windows, version 20.027 (MedCalc Software, Ostend, Belgium). Categorical data was analyzed by Chi-square. The association between independent variables was assessed using an adjusted OR at 95% CI. *P*-values < 0.05 were considered significant.

RESULTS

The demographic information for patients with MS and control group is summarized in Table 2. The MS group included 100 patients, and the control consisted of 100 healthy females bearing no criteria for MS. The mean age of the MS group was 48.9±4.3, and 47.9±6.7 for the control group. No statistically significant difference was found in terms of age between MS patients and control subjects ($p = 0.211$). A statistically significant difference was found in BMI, HbA_{1C}, fasting blood glucose, triglyceride, total cholesterol, and 25(OH)D between MS patients and the control group. Also, significant differences were detected between the MS patients and control group with vitamin D deficiency (69.0% vs. 33.0%), vitamin D insufficiency (25.0% vs. 42.0%), and vitamin D sufficiency (6.0% vs. 25.0%) ($p < 0.001$).

The genotype distribution of VDR polymorphisms between MS patients compared to the control showed statistically significant differences in both *Apa1* and *Fok1* genotypes. Both *Apa1* CC

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

Parameters	MS patients ± SD (n = 100)	Control ± SD (n = 100)	*p*-value
Age, years	48.9 ± 4.3	47.9 ± 6.7	0.211
BMI, kg/m^2	34.3 ± 3.1	28.1 ± 2.5	< 0.001*
HbA_{1C}	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	0.011*
Total cholesterol, mmol/L	6.5 ± 1.2	5.3 ± 1.8	< 0.001*
25(OH)D, nmol/L n (%)	69 (69.0)	33 (33.0)	< 0.001*
Vitamin D insufficiency, n %	25 (25.0)	42 (42.0)	< 0.001*
Vitamin D sufficiency, n %	6 (6.0)	25 (25.0)	< 0.001*

*HbA_{1C}: glycated hemoglobin; BMI: body mass index; HDL: high-density lipoprotein; LDL: low-density lipoprotein; 25(OH)D: 25-hydroxy vitamin D. *p-value is statistically significant.*

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

Genotype	MS patients (n = 100) n (%)	Control (n = 100) n (%)	OR (95% CI)	*p*-value
Apa1				
CC	47 (47.0)	53 (53.0)	0.111 (0.024–0.508)	0.002*
CA	37 (37.0)	45 (45.0)	0.102 (0.022–0.476)	0.001*
AA	16 (16.0)	2 (2.0)		Reference
Taq1				
TT	91 (91.0)	84 (84.0)	3.250 (0.638–16.547)	0.078
TC	7 (7.0)	10 (10.0)	2.100 (0.324–13.614)	0.218
CC	2 (2.0)	6 (6.0)		Reference
Bsm1				
GG	90 (90.0)	92 (92.0)	0.978 (0.135–7.095)	0.491
GA	8 (8.0)	6 (6.0)	1.333 (10.144–12.369)	0.400
AA	2 (2.0)	2 (2.0)		Reference
Fok1				
TT	38 (38.0)	20 (20.0)	2.470 (1.114–5.473)	0.025*
TC	42 (42.0)	54 (54.0)	1.011(0.497–2.054)	0.976
CC	20 (20.0)	26 (26.0)		Reference

*OR: odds ratio; VDR: vitamin D receptor; *p-value is statistically significant.*

and CA genotypes were negatively correlated with MS and showed significant differences between the patients and control. In contrast, TT *Fok1* genotype increased the risk of MS by 2.470 folds [Table 3]. No significant differences were observed in the frequencies of *Taq1* and *Bsm1* genotypes between the MS patients and control group [Table 3].

DISCUSSION

The results of this study showed statistically significant differences between MS patients and control regarding BMI, HbA_{1C}, fasting blood glucose, triglyceride, total cholesterol, and low-density lipoprotein. Previous reports on MS patients also demonstrated similar relationships between vitamin D status, dyslipidemias, low 25(OH) D concentrations, increased triglycerides, and decreased HDL levels.[27]

Vitamin D deficiency is considered a global health problem,[28] especially among females of all age groups and ethnicities.[29] 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 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]

A previous study in Jordan found 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%).[29] In another study 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. However, this association was significant only among elderly people with non-central obesity.[33]

Our findings indicated significantly higher mean BMI (34.3±3.1) in women with MS compared to the control group (28.1±2.5), as well as a significant difference in the number of individuals with deficient, sufficient, and insufficient amounts of 25(OH)D level between MS and control groups [Table 2]. Our results are consistent with a cross-sectional study in Saudi Arabia, which found the MS group had a significantly lower level of vitamin D than the control group.[34] They showed a negative

relationship between 25(OH)D levels and type-2 diabetes, glucose homeostasis, and MS. 25(OH)D level was also inversely associated with waist circumference, triglycerides, and HbA_{1C}, as well as cardiovascular risk. This association was also reported by a 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 resistance in the MS group.[35]

Several single nucleotide polymorphisms (SNPs) in the VDR gene 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, *Bsm1*, *Apa1*, and *Taq1* that influence mRNA stability and VDR expression.[36] Other SNPs, such as *Fok1*, are located in the proximity of the promoter region resulting in altered VDR protein activity.[37] Our study showed significant differences in VDR gene polymorphisms only in *Apa1* genotype distribution, namely CC and CA, as well as in the TT genotype frequency of *Fok1*. No statistical differences were observed in the *Taq1* and *Bsm1* 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. A Chinese study found that VDR *Apa1* polymorphism was associated with hypertriglyceridemia in MS patients, while the *BsmI* and *Taq1* polymorphisms affected HDL levels.[22] Another meta-analysis showed that *Bsm1* polymorphisms protect against MS; however, no association was observed between VDR SNPs and the risk of vitamin D deficiency.[16] Among Chinese children with MS, Wang et al,[38] found that VDR *Apa1* polymorphisms were correlated with overweight/obesity and glucose intolerance, while AA genotype of *Fok1* SNP was significantly associated with MS.

There were studies with contrary results as well. A cross-sectional study of 697 Russian women showed no association between VDR polymorphisms and the risk for MS development.[18] However, its results should be interpreted with caution as the participants were general clinic visitors to treat various minor ailments; in addition, the study excluded women who had kidney or gastrointestinal diseases, diabetes mellitus, regular insolation, and were taking vitamin D supplements. Erasmus et al,[7] showed no correlation between VDR *Fok1* and *Taq1* with glycemic status. They also reported that *Fok1* was not linked with 25(OH)D deficiency, while *Taq1* was linked with vitamin D insufficiency. Another meta-analysis study indicated that VDR *Apa1* and *Fok1* polymorphisms increased the susceptibility to gestational diabetes mellitus.[39] Therefore, VDR screening among different ethnic groups may be a good molecular marker to determine a population's susceptibility to MS.

The limitations of this research included the relatively small sample size and the fact that only four VDR polymorphisms (*Apa1*, *Bsm1*, *Fok1*, and *Taq1*) were analyzed.

CONCLUSION

Jordanian women with MS have a significant association with VDR gene polymorphisms among *Apa1* and *Fok1* genotypes. Genotype distribution for CC and CA among *Apa1* VDR polymorphism, and TT among *Fok1* VDR gene polymorphism significantly modify the risk of MS development in this demography.

Disclosure
The author declared no conflicts of interest.

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