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Vitamin D receptor gene polymorphisms and cardio-metabolic profile in women with hypovitaminosis D

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Background: The possible role of vitamin D receptor (VDR) variants in determining cardiovascular risk factors has been suggested previously. The purpose of the present study was to evaluate the associations of variants of vitamin D receptor (VDR) gene with metabolic profile in Iranian overweight women with hypovitaminosis D.

Methods: Variants in VDR gene including FokI (rs2228570), BsmI (rs1544410) and rs757343 were detected using PCR-sequencing. Metabolic Profile including fasting plasma glucose, total cholesterol, LDL-C, HDL-C, and triacylglycerol was assessed using commercial kits. Circulating 25 (OH) D was measured using ELISA kits. Fat mass and visceral fat were measured using Bio Impedance Analysis.

Results: A sample of 123 overweight women with hypovitaminosis D (25 (OH) D<75nmol/L) was studied. The mean 25 (OH) D level was 28.7±17.0nmol/L. The FokI polymorphism was significantly associated with total cholesterol (p=0.03) and LDL-C (p=0.01). Carriers of the CC genotype of the FokI polymorphism had lower total cholesterol (162.9±30.4 vs. 176.1±32.4, p=0. 02) and LDL-C (98.0±26.0 vs. 110.6±28.5, p=0. 01) compared with carriers of CT+TT genotype. Regression analysis revealed that the FokI variant and fat mass explained 18% of the variance in total cholesterol. Regarding LDL-C, FokI variant explained 21% of the variance in LDL-C. None of the studied polymorphisms were significantly associated with adiposity phenotypes (p>0.05). Also, adiposity phenotype measures were not significantly different in the genotype groups of the studied SNPs (p>0.5).

Conclusion: The present results indicate that the FokI polymorphism is significantly associated with total cholesterol and LDL-C in Iranian overweight women with hypovitaminosis D.

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Introduction

Vitamin D deficiency which is mostly due to insufficient sun exposure and inadequate intake or absorption of vitamin D, has become a major health problem. Approximately, one billion people have hypovitaminosis D (deficiency or insufficiency), worldwide [1]. Inadequate serum

level of 25(OH)D in Iran is as high as other middle east countries and according to different reports, 70 -75% of Iranian women have vitamin D insufficiency or deficiency [2-4]. The risk of several diseases including cardiovascular diseases, diabetes mellitus, obesity, multiple sclerosis, certain types of cancers, autoimmunity, allergy, cognitive decline, depression and even pregnancy complications is increased in vitamin D deficiency state [5, 6].

Vitamin D metabolism, storage, and action both influence and are influenced by adiposity [7]. Observational studies have shown that circulating 25(OH)D is lower in obese subjects [8, 9] and it is negatively linked to whole body fat mass, visceral fat and adipocyte size [10-12]. Besides obesity, vitamin D deficiency is linked to higher incidence of cardiometabolic risk factors including hyperglycemia and hyperlipidemia. [13]. The proper function of the active form of vitamin D (1,25 dihydroxyvitamin D3 ($1,25(OH)_2D3$)) involves interaction with its nuclear receptor called vitamin D receptor (VDR). The VDR gene is a large gene (>100 kb) on the chromosome 12 at position 13.11. The VDR gene is expressed in several tissues including adipose tissue and influence the action of vitamin D [14]. High level of VDR was detected early in adipogenesis and regulates the transition of pre-adipocyte to the mature adipocyte. Body fat mass, as well as plasma triglyceride and cholesterol, were lower in VDR-knockout mice compared with wild-type mice [15, 16]. VDR gene variations alter the proper action of the gene product and have been linked to susceptibility to several diseases including obesity and metabolic syndrome [17-20].

Regarding the high prevalence of vitamin D deficiency in Iran and the possible role of VDR variants in determining adiposity phenotypes and metabolic profile we undertook these analyses to investigate associations of single nucleotide polymorphisms (SNPs) of the VDR gene with adiposity phenotypes and metabolic profile in overweight women with low levels of 25(OH) D.

Materials and methods

Participants

In total, 139 overweight women (BMI:25-30 kg/m^2) aged 20-50 years were recruited by short message service (SMS) advertisements and also from nutrition and diet therapy clinic of Shahid Beheshti University of Medical Sciences in Tehran, Iran during March 2013 to June 2014. Circulating 25(OH)D was measured for these women. 129 women had low levels of serum 25(OH) D (≤75 nmol/L) [1]. Finally, 123 overweight women who were apparently healthy and free from major chronic diseases including diabetes mellitus, gastrointestinal disease, cardiovascular disease, renal disease and thyroid or parathyroid disorders completed all measurements. A written informed consent was taken from all participants and the study was approved by the Ethics Committee of Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences.

Demographic characteristics

Information on sociodemographic factors, physical activity, smoking habit, and sunscreen use was obtained by trained interviewers during a visit to diet therapy clinic of Shahid Beheshti University of Medical Sciences. Physical activity was assessed as MET/h which is calculated by multiplying the time of the exercise by the respective metabolic equivalent task (MET) using the International Physical Activity Questionnaires (IPAQ) [21].

Measurement of adiposity phenotypes

All anthropometric measurements were obtained according to the WHO standard procedures by trained interviewers. Weight was measured using a balance scale (Seca, Hamburg, Germany), while women wearing light clothing and no shoes. Woman's height, while standing without shoes, with their shoulders in a normal position, was measured using a Seca stadiometer. Body mass index (BMI) was calculated as weight (kg) divided by the square of height (m). Waist circumference at the midpoint between the lowest rib and the iliac crest and hip circumference around the widest portion of the buttocks was measured using a flexible tape. All the measurements were taken by the same person to decrease the error. The studied women were categorized according to waist circumference on the basis of the clinical criterion proposed for Iranian women of (≤90 and >90 cm).

Bioelectrical impedance analysis method was applied to measure adiposity phenotypes including body fat mass and fat-free mass by Body Stat Quad Scan (model 4000, Douglas Isle of Man, British Isles) as well as visceral fat and trunk fat by Tanita AB-140 ViScan (Tanita Corporation, Tokyo, Japan).

Table1. Characteristics of study population according to waist circumference categories (n= 123)

	Waist circumference		
Variables	≤ 90 cm (n= 43)	>90 cm (n= 80)	***P*-value**
Age (y)	35.1±6.9	37.6±6.5	0.51
BMI (kg/m^2)	27.3±1.8	28.6±1.7	<0.001
Hip Circumference (cm)	108.7±4.7	108.8±5.4	0.04
Fat mass (%)	39.7±3.7	41.9±3.5	<0.001
Fat free mass (%)	62.2±3.8	60.0±2.8	0.002
Visceral fat (%)	7.8±1.9	10.8±2.3	<0.001
FPG (mmol/L)	4.6±0.6	4.8±0.7	0.09
TAG (mmol/L)	1.0±0.5	1.2±0.7	0.1
TC (mmol/L)	4.2±0.7	4.5±0.9	0.02
LDL-C (mmol/L)	2.6±0.7	2.8±0.8	0.1
HDL-C (mmol/L)	1.1±0.2	1.2±0.2	0.3
25(OH)D (nmol/L)	27.3±17.7	29.4±16.7	0.6
MET-h/d	34.2±2.7	34.4±2.8	0.7
Sunscreen use [n (%)]			
Yes	13(42%)	51(55%)	0.2
No	18(58%)	41(45%)	
Smoking status [n (%)]			
Smoker	0(0%)	7(8%)	0.3
Non-smoker	33(100%)	83(92%)	

BMI, Body Mass Index; TC, Total Cholesterol; LDL-C, Low Density Lipoprotein Cholesterol; HDL-C, High Density Lipoprotein Cholesterol; TAG, triacylglycerol; FPG, Fasting Plasma Glucose; MET, Metabolic Equivalent Of Task.

Measurement of metabolic profile parameters

Following overnight fasting, peripheral blood samples were collected from each subject in EDTA containing test tubes. The samples were separated into two tubes: one whole blood sample for DNA extraction and VDR genotyping, and the other plasma sample for measuring metabolic variables. Both samples were stored at -80 ºC until analysis. All measurements were performed in the laboratory of the Research Institute for Endocrine Sciences of Shahid Beheshti University of Medical Sciences. Analysis of metabolic profile variables was performed using the Selectra 2 auto-analyzer (Vital Scientific, Spankeren, The Netherlands). Fasting plasma glucose (FPG) and triacylglycerol (TAG) were assessed using a colorimetric enzymatic method with commercially available Parsazmun kits (Parsazmun Inc., Tehran, Iran). Total cholesterol (TC) and high density lipoprotein cholesterol (HDL-C) were assessed using an enzymatic photometric method with Parsazmun kits (Parsazmun Inc., Tehran, Iran). Low density lipoprotein cholesterol (LDL-C) was indirectly calculated using the Friedewald equation. 25(OH)D was assessed using ELISA with DIA source kits (DIAsource ImmunoAssays, Louvain-La-Neuve**,** Belgium). Intra-assay and inter-assay coefficients of variations were respectively 1.8% and 2.1% for FPG, 2.4% and 2.6% for TAG, 2.1% and 2.4% for TC, 4.3% and 4.4% for HDL-C, 6.4% and 6.5% for 25(OH) D.

VDR Genotyping

We selected two most frequently studied SNPs including *Fok*I (rs2228570) and *Bsm*I (rs1544410) in the VDR gene. Another SNP (rs757343) which is close to *Bsm*I position on the VDR gene was detected while sequencing the gene for *Bsm*I polymorphism. Genomic DNA was extracted from whole blood using standard salting out/proteinase K method [22] and stored at -20˚C until the time of use for polymerase chain reaction (PCR). The DNA was quantitated on Spectrophotometer and the 260/280 nm absorbance noted. For identification of the SNPs, DNA samples were amplified using the PCR. The PCR products were electrophoresed on 8% polyacrylamide gel. The amplimers were confirmed for the presence or absence of polymorphisms by direct DNA sequencing method (3730xl/Bioneer 3730xl). To detect the VDR polymorphisms, sequences were analyzed by Chromas 2.33 software.

Statistical Analysis

All Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) for windows version 16.0. Allelic frequencies were estimated by gene counting and genotype frequency of the SNPs examined against Hardy-Weinberg equilibrium by Chi-square analysis. Normality assumption for variables was assessed using Shapiro-Wilk statistic. Normally distributed data

are reported as mean±SDs. Associations of 25(OH)D and SNPs with adiposity phenotypes and metabolic profile parameters were assessed using linear regression analysis. For further analysis, rare genotype and heterozygote genotype were combined due to a small number of samples in the rare genotype of two studied SNPs. Adiposity and metabolic variables were compared between two VDR genotype groups using a two-tailed Student t-test or a Mann-Whitney U test as appropriate. All P values were two-sided, and a P-value less than 0.05 was considered statistically significant.

Results

Of 139 women, 123 overweight women with low serum 25(OH)D completed all measurements. The studied women had a mean age of 36.9±6.7 y with a mean BMI, waist circumference, total body fat mass, and visceral mass of 28.2±1.8 kg/m^2, 93.8±6.9 cm, 41.2±3.7%, and 9.8±2.6%, respectively. The metabolic variables were all in normal ranges except for the HDL-C which was lower than 1.3 mmol/L (recommended level for women to reduce the risk of heart disease) in 75% of the studied group. The mean 25(OH) D level was 28.7±17.0 nmol/L. Descriptive characteristics of the women stratified by waist circumference are summarized in Table 1. Women with higher waist circumference had higher BMI ($p<0.001$), hip circumference ($p=0.04$), fat mass ($p<0.001$), visceral fat ($p<0.001$) and TC ($p=0.02$). Serum 25(OH)D was not different between two groups ($p=0.6$).

Genotype and allele frequencies, as well as physical positions of the studied SNPs, are shown in Table 2. The distribution of none of the SNPs was in agreement with Hardy-Weinberg equilibrium by χ^2 testing ($\chi^2= 60.5$, $P<0.001$ for rs2228570, $\chi^2= 19.1$, $P<0.001$ for rs1544410, and $\chi^2= 11.6$, $P<0.001$ for rs757343).

Association of studied SNPs with adiposity phenotypes and metabolic profile are summarized in Table 3. No statistically significant association was observed for SNPs and adiposity phenotypes. Among metabolic profile variables, TC ($p=0.03$) and LDL-C ($p=0.01$) were significantly associated with *Fok*I polymorphism. Adiposity phenotype and metabolic profile values for the studied women according to different genotypes are shown in Table4. No significant difference was seen in adiposity phenotypes in the carriers of the different genotypes of the studied SNPs. Carriers of the CC genotype of the *Fok*I polymorphism had significantly lower TC ($p=0.02$) and LDL-C ($p=0.01$) compared to carriers of The CT+TT genotype.

Discussion

In the present study we determined the association of three SNPs of the VDR gene with adiposity phenotypes and metabolic profile in overweight women with low levels of 25(OH) D. Our findings showed that the *Fok*I polymorphism is associated with total cholesterol and LDL-C and carriers of CC genotype of the *Fok*I have lower total cholesterol and LDL-C compared to carriers of CT+TT genotypes. No association was found for the *Bsm*I and the rs757343 with studied adiposity and metabolic variables.

VDR is expressed in many tissues and regulates expression of various vitamin D responsive genes thus it is possible that allelic changes in the VDR gene may be a marker of genetic predisposition to the diseases including obesity [23, 24]. VDR is suggested to have a regulatory role in the proliferation and differentiation of preadipocytes and mature adipocytes. It is demonstrated that VDR inhibits initiation of preadipocyte differentiation to adipocyte through blocking the expression of adipogenic transcription factors including C/EBPb, PPARγ and SREBP1 as well as of the downstream adipocyte markers such as lipoprotein lipase, adipocyte lipid-binding protein 2 and fatty acid synthase [25].

FokI polymorphism (rs2228570) is a C/T transition polymorphism which lies in the VDR start codon in exon 2 [26]. It is known as a

Table 2. VDR single nucleotide polymorphisms (SNPs), locations, genotype and allele frequencies in the study population

SNP	Location	Genotypes%			Allele%	
*rs*2228570 (FokI:C/T)	12:47879112	CC (53%)	CT (43%)	TT (4%)	C (74%)	T (26%)
rs1544410 (BsmI:G/A)	12:47846052	GG (46%)	GA (61%)	AA (16%)	A (38%)	G (62%)
rs757343	12:47845892	GG (68%)	GA (31%)	AA (1%)	A (16%)	G (84%)

Table 3. Association of studied SNPs with adiposity phenotypes and metabolic profile

SNP	rs2228570		rs1544410		rs757343	
Variables	β ± SE	*P*	β ± SE	*P*	β ± SE	*P*
BMI (kg/m^2)	0.05± 0.33	0.9	0.04 ± 0.25	0.9	0.24 ± 0.36	0.5
WC (cm)	0.12±1.12	0.9	-0.08±0.94	0.9	0.25±1.40	0.9
Fat mass (%)	0.35±0.59	0.6	-0.09±0.53	0.9	-0.48±0.73	0.5
Visceral fat (%)	-0.18 ±0.41	0.7	-0.32±0.35	0.4	0.71±0.51	0.2
FPG (mmol/L)	0.36 ±1.91	0.8	0.31±1.66	0.9	-2.10±2.40	0.4
TAG (mmol/L)	1.24±8.64	0.9	0.52±7.46	0.9	-14.32±10.75	0.2
TC (mmol/L)	10.96±4.94	0.03	-2.55±4.34	0.6	-9.10±6.26	0.2
LDL-C (mmol/L)	11.10±4.28	0.01	-2.30±3.79	0.6	-7.88±5.47	0.2
HDL-C (mmol/L)	-0.54±1.37	0.7	-0.20±1.18	0.9	1.65±1.70	0.3
25(OH)D (nmol/L)	0.79±1.10	0.5	1.43±0.92	0.1	-0.87±1.27	0.5

BMI, body mass index; WC, waist circumference; FPG, fasting plasma glucose; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; TAG, triacylglycerol.

functional polymorphism because it changes the structure and function of the encoded protein. The allelic changes of this polymorphism result in two structurally different receptor proteins, from a 424 amino acids short protein to a 427 amino acids long protein. The short and long protein forms have different ability to induce transcription of vitamin D-dependent genes [27].

Previous studies have shown no association between the *Fok*I polymorphism and adiposity phenotypes. In a cross-sectional study on 191 Brazilian women aged 56-84 years, significant relationship of the *Fok*I or *Bsm*I polymorphisms with weight, BMI and fat-free mass percent was not found [28]. In another study on 176 polish men aged 25-65 years no significant association was found for the *Fok*I polymorphisms and body mass, BMI, WC as well as waist to hip ratio (WHR)[17]. Ochs-Balcom also evaluated the association of the *Fok*I variants with adiposity phenotypes in a large population of healthy American women. They showed no associations between *Fok*I variants and BMI, waist circumference and abdominal height [29]. Our findings regarding the *Fok*I polymorphism are in line with other studies in healthy different ethnic groups, which suggest *Fok*I may not be associated with adiposity phenotypes in Iranian overweight women.

*Bsm*I (rs1544410) polymorphism is a G/A transition located in intron 8. This polymorphism does not change the encoded protein but it affects the level of VDR gene transcription, mRNA stability, and posttranscriptional modifications [30]. Despite the *Fok*I, data regarding the *Bsm*I and adiposity phenotypes are controversial. In a cross-sectional study, AA variant of the *Bsm*I was associated with higher BMI and WC in Polish men [17]. In another cross-sectional study, AA variant of the *Bsm*I was positively and significantly related to higher hamstring muscle strength and fat mass but not to higher weight and BMI in 175 premenopausal Swedish women aged 20-39 years [31].

However, the *Bsm*I variants were not related to BMI in 351 healthy postmenopausal Polish women aged 50-60 years [32]. In a study on 617 Canadian women aged >50 years, WC and WHR

Table 4. Adiposity phenotypes and metabolic profile for genotype groups of studied SNPs

SNPs	rs2228570	rs1544410	rs757343						
	CC (n=58)	CT+TT (n=65)	*P*	GG (n=46)	GA+AA (n=77)	*P*	GG (n=84)	GA+AA (n=39)	*P*
Variables									
BMI(kg/m^2)	28.1±1.9	28.2±1.8	0.9	28.2±1.5	28.1±2.0	0.9	28.1±1.9	28.3±1.7	0.5
WC(cm)	93.4±7.2	94.2±6.6	0.5	94.1±5.7	93.7±7.5	0.8	93.7±7.0	94.0±6.6	0.9
Fat mass(%)	40.8±3.6	41.5±3.8	0.3	41.5±3.6	41.0±3.8	0.5	41.3±3.8	40.9±3.5	0.6
Visceral fat(%)	9.8±2.7	9.8±2.5	0.9	10.0±2.3	9.7±2.7	0.4	9.6±2.4	10.3±2.9	0.2
FPG(mmol/L)	85.0±13.9	85.7±10.6	0.7	85.0±10.5	85.6±13.2	0.8	86.1±13.3	84.0±9.5	0.4
TAG(mmol/L)	100.0±64.6	105.7±45.0	0.6	103.4±38.7	102.8±62.9	0.9	107.4±59.7	93.1±42.6	0.2
TC(mmol/L)	162.9±30.4	176.1±32.4	0.02	173.5±35.4	167.8±29.9	0.3	172.8±33.6	163.7±28.2	0.2
LDL-C(mmol/L)	98.0±26.0	110.6±28.5	0.01	108.2±29.6	102.5±26.9	0.3	107.1±29.6	99.2±23.8`	0.2
HDL-C(mmol/L)	45.2±9.1	44.4±8.4	0.7	44.7±9.6	44.8±8.2	0.9	44.3±8.7	46.0±8.8	0.3
25(OH)D(nmol/L)	11.1±7.1	11.9±6.6	0.5	10.6±6.3	12.0±7.1	0.3	11.8±7.0	11.0±6.6	0.6

BMI, Body Mass Index; WC, Waist Circumference; FPG, Fasting Plasma Glucose; TC, Total Cholesterol; LDL-C, Low Density Lipoprotein Cholesterol; HDL-C, High Density Lipoprotein Cholesterol; TAG, Triacylglycerol.

as markers of central obesity were not related to the *Bsm*I variants [33]. In a large Study on 1773 healthy American women aged 35-80 years, the *Bsm*I variants were not related to adiposity markers including BMI, WC and abdominal height [29]. Our findings are similar to the aforementioned studies and do not support the role of the *Bsm*I variants in determining adiposity phenotypes in Iranian overweight women. The rs757343 has not been well studied before. Dorjgochoo et al., studied 198 vitamin D-related SNPs in a sample of 6922 women aged 25-70 years and found no significant relationship between the rs757343 and BMI or weight. We could not show any associations for the rs757343 in our sample of Iranian overweight women.

Variations in VDR gene can influence the susceptibility to cardiovascular diseases as other authors have mentioned previously. Mackawy et al. showed significantly higher total cholesterol, TAG, LDL-C and lower HDL levels in the CT and TT genotype carriers in diabetic patients without Metabolic syndrome [34]. Prabhakat et.al demonstrated that in patients with ischemic stroke, the TT genotype carriers significantly have higher cholesterol levels as compared to CC carriers [35]. Schuch et al. reported significant higher TAG and lower HDL-C levels in diabetic patients with the TT genotype of *Fok*I [18]. Our results are in line with the mentioned studies and indicate that carriers of CC genotype of the *Fok*I polymorphism have lower levels of total cholesterol and LDL-C compared to two other genotypes of the *Fok*I.

There are some possible mechanisms by which vitamin D-VDR axis could affect lipid profiles [34]. First: Vitamin D induced suppression of PTH secretion, and it has been reported that PTH could reduce lipolysis [36]. Second: Vitamin D increases intestinal calcium absorption and this can trigger a decrease in serum triacylglycerol levels by reducing the hepatic triacylglycerol formation and secretion [37], Third: Vitamin D might improve insulin secretion and insulin sensitivity, thereby indirectly influencing lipid metabolism [38] and Fourth: VDR activation increases expression of human cholesterol 7a-hydroxylase which in turn leads to a reduction in cholesterol levels [39].

Conclusion

Regarding the findings of our study, we conclude that VDR gene variants may not be related to adiposity phenotypes but the *Fok*I polymorphism may contribute to risk of cardiovascular diseases through altering total cholesterol and LDL-C levels in healthy Iranian overweight women with hypovitaminosis D. Although we studied a homogenous group of women with true vitamin D deficiency, the small study population limited interpretation of our results. Therefore, more genetic epidemiological studies in larger populations of both men and women are needed for better understanding of the relationship between VDR variations and adiposity phenotypes.

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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