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Vitamin D Receptor Gene Polymorphism and Bone Mineral Density in Iranian Menopausal and Postmenopausal Women

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Background: Osteoporosis is a systemic disease with a strong genetic component. Vitamin D receptor (VDR) gene is associated with inter-individual variance in bone mineral density (BMD).

Objectives: The aim of this study was to assess the association between BMD and the VDR gene polymorphisms.

Patients and Methods: A total of 233 women including 124 premenopausal and 109 postmenopausal women, who were older than 40 years old, were randomly selected from the participants of Tehran Lipid and Glucose Study (TLGS). Serum 25 (OH) vitamin D, calcium, and phosphorus levels were measured and body mass index (BMI), BMD of femoral neck, total femoral, trochanteric, and lumbar spine bones were calculated. The polymerase chain reaction (PCR) was used to determine the Bsm1 and Tru91 polymorphisms of VDR gene.

Results: According to PCR results, Bb genotype frequencies in premenopausal and postmenopausal woman were 39.5% and 31.2%, respectively, and Tt genotype frequencies were 41.9% and 33%, respectively. Moreover, b and T alleles were more frequent in these women, with a frequency of 0.57% and 0.18%, respectively. In premenopausal subjects, the mean BMD at all sites were higher in bb group than in the BB group ($P < 0.05$). After adjustment for age, BMI, and serum 25 (OH) D, the bb group had lower BMD at all these sites in postmenopausal women; however, the difference was insignificant. There was no association between Tru91 polymorphism and BMD in premenopausal and postmenopausal women.

Conclusions: It seems that there is a possible interaction between premenopausal status and the Bsm1 polymorphism of VDR gene polymorphism and the BMD levels in these regions.

Keywords: Vitamin D 3 Receptor; Bone Density; Menopausal

1. Background

Osteoporosis is a metabolic bone disease, characterized by low bone mass and deterioration of bone tissue, which leads to bone fragility and an increase in fracture risk. Osteoporosis is a disease with a complex etiology including environmental and genetic contributors (1, 2). Environmental factors that influence bone density include dietary factors such as calcium, alcohol, and caffeine intake and lifestyle factors such as exercise and smoking (3-5). Ethnic differences in the susceptibility to non-traumatic bone fracture suggest that genetic variations such as gene polymorphisms play an important role in this matter. Some examples of genetic markers that have been investigated in association studies of bone mineral density (BMD) or osteoporosis are as follows: the single nucleotide repeats (SNPs) in the vitamin D receptor (VDR) gene using Bsm1, Taq1, Apa1, Tru91, and Fok1 restriction enzymes; the estrogen receptor gene using Xba1 and Pvu1 restriction enzymes; and nucleotide repeat polymorphism in the Sp1 binding site in the collagen type 1 alpha 1 gene promoter (6). The majority of association studies of BMD and candidate gene markers have investigated markers for the VDR gene (7). Several studies have investigated the association between BMD and VDR gene polymorphism in different populations (8-12).

The gene encoding VDR by 14 exons is located on chromosome 12 (2) and approximately spans 75 kb of genomic DNA (4); some polymorphisms were studied in this gene and the Bsm1 polymorphism was the one variant, present in the intron, which demonstrated the association. This polymorphism leads to A/G substitution (13). Results of some studies showed that common allelic variations in the VDR gene were associated with BMD in racially diverse population groups, as well as in prepubertal girls, young adults, premenopausal, and postmenopausal women and men (14); however, other studies were not able to find any association between VDR polymorphisms and BMD (15). Comparing VDR genotype distributions found in other studies (16) with ours, Bsm1 polymorphisms were similar. The most detected VDR genotype was Bb among both premenopausal and postmenopausal women. In dif-

ferent European populations, the Bb genotype polymorphisms were frequent (17-19).

2. Objectives

We aimed to investigate the association between the Bsm1 and Tru91 polymorphisms of VDR gene and BMD (BMD) in a sample of premenopausal and postmenopausal women in Tehran City.

3. Patients and Method

3.1. Subjects

This study was conducted within the framework of Tehran Lipid and Glucose Study (TLGS), with the aim of assessing the association between the VDR gene polymorphism and BMD. The TLGS was designed to determine the risk factors for major noncommunicable disorders such as atherosclerosis, occurring in the urban population of Tehran, the capital city of Iran. It is an ongoing study involving about 15000 participants of all ages and has broad aims of developing population-based measures to alter the lifestyle of Tehran residents and prevent the incremental trend of diabetes mellitus, dietary disorders, and dyslipidemia (20, 21). Among TLGS participants, 124 premenopausal and 109 postmenopausal women were randomly selected for this study; detail of selection criteria are presented elsewhere (15). Menopause was defined according to the subject report of the date of last menstrual period (after a woman has stopped menstruating). Written informed consent was obtained from each subject and the research council of the Research Institute for Endocrine Science, Shahid Beheshti University of Medical Sciences, approved this study.

3.2. Clinical and Laboratory Assessments

A trained general physician obtained demographic data. Weight and height measured and body mass index (BMI) was calculated. Following 12-hour fasting, whole blood samples were collected and transferred to two tubes, one with and one without the anticoagulant EDTA. After ten-minute centrifugation at 3000 rpm, sera were separated (in the tube without anticoagulant) and stored at -70℃ in 1.5-mL aliquots. Serum 25-hydroxy vitamin D [25 (OH) D] was measured using the radioimmunoassay (RIA) method (Gamma BCTDPD; IDS, Boldon, UK coefficient of variation < 8.9%); Calcium and phosphorus levels were measured using colorimetric methods (Pars Azmuon, Tehran, Iran). Coefficients of variation for Calcium and phosphorous measurements were below 5%.

3.3. Densitometry Assessment

BMD (g/cm^2) was measured by a trained operator at the lumbar spine and total hip with dual X-ray absorptiometry (DXA) using a Lunar DPXMD densitometer (Lunar 7164, GE, Madison, WI, USA), according to manufacturer's instruction. Precision error for BMD measurement was 1% to 1.5% in the lumbar and 2% to 3% in the femoral regions. The device normative data of US population for spine BMD and third national Health and Nutrition Examination Survey (NHANES III) for femur BMD were used as reference values. According to the World Health Organization (WHO) classification system, T scores ≤ -2.5 were considered as osteoporosis and those between -1 to -2.5 as osteopenia.

3.4. Genotyping

For analysis of the Bsm1 polymorphism, buffy coats were separated from noncoagulated blood samples and stored at -70℃ until processing, when genomic DNA was extracted by the proteinase K using the salting-out method (22). The polymerase chain reaction (PCR) was used to amplify an 822-bp fragment in the VDR gene by using oligonucleotide forward (5'-AAC CAG CGG AAG AGG TCA AGG GTC-3') and revers primers (5'-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA-3') (23). Each amplification was performed using 100 ng of total genomic DNA in a final volume of 25 mL containing 40 pmoL of each primer, 0.2 mmol/L of each dNTP, 1.5 mmol/L of $MgCl_2$, 10 mmol/L of Tris at pH 8.4, and 0.25 units of Taq polymerase (Fermentase Co. Canada). The synthesis was performed in a DNA thermal cycler (Corbett co. Australia) with the following condition: denaturation of DNA templates at 94℃ for two minutes, amplification consisting of 35 cycles of 93℃ for 30 seconds, 62℃ for 90 seconds, and 72℃ for 120 second, with a final extension at 72℃ for ten minutes. PCR products were subjected to restriction enzyme analysis by digestion at 65℃ overnight with two units of Bsm1 restriction endonuclease in each 10 mL of PCR sample in the recommended buffer by the manufacturer of the endonuclease (Roche Co. City, Germany). The fragments were separated by electrophoresis on a 2% agarose gel and electrophoreses for one hour under constant voltage (200 V). After electrophoresis, the gel was treated with 1 μg/dl of ethidium bromide solution for ten minutes and the DNA fragments were visualized by gel documentation (Optigo Co. City, Holland). BB genotype with 822-bp fragments reflected the absence of restriction sites, Bb contained the 822-bp, 643-bp, and 179-bp fragment and finally, bb genotype contained 643-bp and 179-bp fragments (23).

Tru91 polymorphism was analyzed with the same protocol used for the Bsm1 polymorphism to extract genomic DNA (22) and using the following protocol for PCR: The primers for the region containing the original reverse primer binding site are OrF (5' AAT ACT CAG GCT CTG CTC TT 3') and OrR (5' CAT CTC CAT TCC TTG AGC CT 3'). With these primers, a 331-bp fragment was amplified. PCR conditions were initial denaturation at 94℃ for five minutes, and 35 cycles at 94℃ for denaturation, 56℃ for annealing, and 72℃ for extension, each step lasting 45 seconds. The fragments were separated by electrophoresis with the same protocol for Bsm1. TT genotype with 331-bp fragments reflected the absence of restriction sites; Tt contained the 331-bp, 153-bp, and 178-bp fragments and finally tt genotype

contained the 153-bp and 178-bp fragments. (16, 24)

3.5. Statistical Analysis

The Kolmogorov-Smirnov goodness-of-fit test was used to assess normal distribution of continuous data. All normal continuous data were expressed as mean ± SD and/or median and categorical variables were expressed as percentage. All subjects were tested for Hardy-Weinberg equilibrium and allele frequency was calculated by Power Marker software (25). Mean differences of variables between the two separate groups of premenopausal and postmenopausal women were assessed for statistical significance by Student's t test.

ANCOVA was used to compare the mean BMD among three groups of genotype adjusted for age, BMI and 25 (OH) D levels. The statistical calculation was performed using SPSS 15 (SPSS Inc., Chicago, IL, USA). All statistical tests were two sided, and the differences with P values < 0.05 were considered statistically significant at all sites.

4. Results

Basic characteristics of the studied women are shown in Table 1. In this study, 124 premenopausal women were compared to 109 postmenopausal and the median of 25 (OH) D was different between the two groups (17.0 and 31.4 nmol/L, respectively; P < 0.05). Moreover, 68.5% of women had 25 (0H) D level < 20 ng/mL, while 74.5%had levels of < 30 ng/mL. Among the 233 samples, 160 and 137 women were studied for the Bsm1 and Tru91 polymorphisms, respectively. Frequencies of the B and b alleles of the Bsm1 polymorphism were 0.43% and 0.57%, respectively, and those of the T and t alleles of the Tru91 polymorphism were 0.82% and 0.18%, respectively. The majority of participants were TT homozygous and Bb heterozygous (37.4% and 35.3%, respectively) and only one subjects had the tt genotype (Table 2).

Adjusted BMD showed that in the premenopausal women, the presence of the bb genotype was significantly associated with higher BMD levels in the femoral neck (BB, 0.81 ± 0.09; Bb, 0.86 ± 0.11; and bb, 0.93 ± 0.13 g/cm^2; P < 0.05), trochanteric (BB, 0.69 ± 0.07; Bb, 0.73 ± 0.09; and bb, 0.78 ± 0.13 g/cm^2; P < 0.05), total femur (BB, 0.89 ± 0.09; Bb, 0.91 ± 0.11; and bb:0.98 ± 0.14 g/cm^2, P < 0.05), and for lumbar spine (L1-L4) (BB, 1.01 ± 0.15; Bb, 1.04 ± 0.15; and bb, 1.17 ± 0.20 g/cm^2; P < 0.05).

Nonetheless, in the postmenopausal women, BMD levels at all sites were higher in those with the BB genotype than in those with the bb one; however, it was not significant. There was no association between the Tru91 polymorphism and adjusted mean levels of BMD in two groups studied.

Table 1. Participant Characteristics in Premenopausal and Postmenopausal Women [a,b]

Variables	Total (n = 233)	Premenopausal (n = 124)	Postmenopausal (n = 109)	P Values
Age, y	51.8 ± 8.9	46.6 ± 4.2	58.8 ± 7.4	< 0.001
BMI, Kg/m^2	29.7 ± 4.4	29.5 ± 4.7	29.9 ± 4.1	0.581
Serum 25 (OH) D, nmol/L	22.5 (12.7, 67.0)	17.0 (7.4, 35.3)	31.4 (20.0, 154.3)	< 0.001
Serum calcium, mg/dL	9.1 (8.8, 9.6)	9.0 (8.7, 9.5)	9.2 (8.9, 9.7)	0.180
Serum phosphorus, mg/dL	3.70 ± 0.59	3.61 ± 0.63	3.81 ± 0.52	0.009
BMD, g/cm^2				
Femoral neck	0.87 ± 0.14	0.87 ± 0.12	0.88 ± 0.15	0.619
Trochanteric	0.74 ± 0.12	0.73 ± 0.11	0.74 ± 0.13	0.637
Total femoral	0.93 ± 0.14	0.92 ± 0.13	0.94 ± 0.15	0.577
Lumbar spine (L1-L4)	1.06 ± 0.17	1.07 ± 0.18	1.05 ± 0.16	0.514

[a] Abbreviations: BMD, bone mineral density; BMI, body mass building.
[b] Data are presented as mean ± SD or median (interval quartile 25, 75).

Table 2. Genotypes and Alleles Frequency of Bsm1 and Tru91 Polymorphisms in the Study Population [a]

	Total	Premenopausal	Postmenopausal
Genotype frequency			
BB	27 (11.5)	13 (10.5)	14 (12.8)
Bb	83 (35.5)	49 (39.5)	34 (31.2)
bb	50 (21.3)	30 (24.2)	20 (18.3)
TT	88 (37.4)	52 (41.9)	36 (33)
Tt	48 (20.4)	28 (22.6)	20 (18.3)
tt	1 (0.4)	1 (0.8)	0 (0)
Allele frequency			
B	0.43	0.40	0.43
b	0.57	0.59	0.57
T	0.82	0.81	0.82
t	0.18	0.18	0.17

[a] Data are presented as No. (%) or percentage.

Table 3. Bone Mineral Density in Subjects With Different Vitamin D Receptor Genotype Groups in Premenopausal and Postmenopausal Women [a,b]

	Bone Mineral Density, g/cm^2			
	Femoral Neck	**Trochanteric Region**	**Total FemoralRegion**	**Lumbar Spines (L1-L4)**
		Premenopausal		
BB (n = 13)	0.81 ± 0.09	0.69 ± 0.07	0.89 ± 0.09	1.01 ± 0.15
Bb (n = 49)	0.86 ± 0.11	0.73 ± 0.09	0.91 ± 0.11	1.04 ± 0.15
bb (n = 30)	0.93 ± 0.13 [c]	0.78 ± 0.13 [c]	0.98 ± 0.14 [c]	1.17 ± 0.20 [c]
TT (n = 52)	0.89 ± 0.13	0.75 ± 0.12	0.94 ± 0.13	1.06 ± 0.19
Tt (n = 28)	0.85 ± 0.12	0.72 ± 0.09	0.92 ± 0.12	1.06 ± 0.17
tt (n = 1)	0.83	0.67	0.87	1.03
		Postmenopausal		
BB (n = 14)	0.91 ± 0.16	0.75 ± 0.13	0.97 ± 0.17	1.10 ± 0.10
Bb (n = 34)	0.90 ± 0.17	0.75 ± 0.15	0.94 ± 0.17	1.06 ± 0.20
bb (n = 20)	0.81 ± 0.15	0.70 ± 0.12	0.89 ± 0.15	0.99 ± 0.16
TT (n = 36)	0.86 ± 0.16	0.72 ± 0.13	0.92 ± 0.17	1.03 ± 0.18
Tt (n = 20)	0.88 ± 0.14	0.74 ± 0.12	0.93 ± 0.13	1.06 ± 0.17
tt	–	–	–	–

[a] Data are presented as adjusted mean ± SD.
[b] Adjusted for BMI, age, and Vitamin D by ANCOVA.
[c] $P < 0.05$.

5. Discussion

In this cross-sectional, population-based study of healthy menopausal women in Tehran, a significant association was found between the Bsm1 polymorphism of the VDR gene in determining BMD level. In fact, the presence of the bb genotype showed a higher BMD in premenopausal women, and vice versa in postmenopausal subjects, indicating a possible interaction between menopause and VDR genotype in determining BMD level. Bone mass and its mineral content are influenced by genetic (26). As reported by several twin studies, up to 80% of total variance of BMD in the general population can be attributed to genetic factors (27, 28). Some SNPs in the VDR gene, estrogen receptor, and nucleotide repeat polymorphisms in the collagen type I alpha-1 gene promoter have been investigated in association studies of BMD or osteoporosis (6). The VDR genes have been shown to be a major locus for genetic effects on BMD (26). A significant number of studies have found an association between the Bsm1 VDR genotype and BMD in various racially diverse populations (8, 29, 30). Two meta-analyses of available studies have documented associations of the Bsm1 or Bsm1-Apa1-Taq1 haplotype and BMD (31, 32). Moreover, recent meta-analysis of the effect of the VDR gene polymorphism on BMD in Caucasians has concluded that the B allele was related to the lower BMD at the spine in postmenopausal females and that it acts in relation to BMD, i.e. BB = Bb < bb (30). These findings indicate that the Bsm1 polymorphism contributes to bone mass acquisition in this adult female population and may confirm the prominent role of VDR in calcium metabolism, suggesting that this gene is a likely candidate gene for causing low BMD and osteoporosis (33, 34). Nonetheless, there are many studies that have not found any association between this VDR genotype and BMD (35-38). Recent meta-analysis reported that the Bsm1-Apa1-Taq1 haplotypes have no effect on either BMD or fracture risk (39). On the other hand, no association between bone fractures and VDR polymorphism gene were found in British, Spanish, and Italian populations (17, 36, 40, 41). This discrepancy might be explained by differences in ethnic background, sample size, frequency, and distribution of polymorphisms among races, allelic heterogeneity at the VDR locus, calcium intake, age, and other environmental factors (42). In present study, we found that premenopausal females with bb genotype had higher BMD than those with BB or Bb did. On the other hand, BMD was lower in postmenopausal females with bb than in those with BB genotype. This finding could be related to the presence of estrogen in premenopausal women, which might modulate the effect of b allele on BMD, leading to higher BMD levels in those with bb genotype. In a British study of postmenopausal twins (95 dizygotic pairs of twins and 87 monozygotic pairs of twins, aged 50 to 69 years) adjusted BMD was significantly lower in the BB group at the hip, lumbar spine, and the whole body (43), which was contrary to our findings. Nevertheless, some studies did not report high BMD in premenopausal women with BB (29, 44). In comparison with VDR genotype distribution

in other studies (16), Bsm1 polymorphisms in our study were similar to those in other studies (16). The most commonly detected VDR genotype among both premenopausal and postmenopausal women was the Bb. When we compared premenopausal with postmenopausal women, we found that women with the bb genotype had higher BMD at the femur neck, trochanteric, total femur, and lumbar spine regions in premenopausal women, whereas in postmenopausal women the opposite result was obtained, showing that BB genotype had higher BMD at all those sites.

There were some limitations to our study. First, we did not record and assess the intake of calcium, 25 (OH) D, and physical activity. Second, it would have been better to examine the entire VDR gene to find the best association. Third, as in most related studies, our study had a cross-sectional design. Finally, our study was limited by its sample size, which was not large enough for the determining association between menopausal statues and VDR gene polymorphism.

In summary, there were significant association between Bsm1 genotype and adjusted mean BMDs of femoral neck, trochanteric, total femoral, and lumbar spine (L1-L4) regions in the premenopausal women in Iran. We found that with bb genotype had higher risk of BMD in the femur neck, trochanteric, total femur, and lumbar spine regions in premenopausal women, whereas in postmenopausal women, the BB genotype had higher risk of BMD in all these regions. In fact, it seems that presence of estrogen in premenopausal women is important for modulating effect of the b allele on the bone mass.

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Authors' Contributions

Farhad Hosseinpanah gathered the patient and in separate project, analyzed the vitamin D concentration. Maryam Sadat Daneshpour supervised the genetic analysis and Nazanin Ahmadi and Sara P Behnami performed the genotyping, Fereidoin Azizi supervised the TLGS study and these samples were from TLGS.

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