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Vitamin D Receptor Alleles, Bone Mineral Density and Turnover in Postmenopausal Osteoporotic and Healthy Women

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Key Words
Bone mineral density · Vitamin D receptor gene polymorphism · Osteoporosis · Menopause

Abstract
Objective: Vitamin D receptor (VDR) gene polymorphisms and bone metabolic markers were investigated as potential genetic markers for osteoporosis in postmenopausal Turkish women. The relationship between their VDR gene polymorphisms and bone states was determined. ***Materials and Methods:*** Restriction fragment length polymorphisms at the VDR gene locus (i.e., for *Bsm*I, *Apa*I, and *Taq*I) was investigated in 75 postmenopausal osteoporotic (53.16 ± 1.31 years) and 66 healthy (52.62 ± 1.69 years) Turkish women and the genotypes were related to bone mineral density (BMD) at femoral neck (FN), lumbar spine (L1–4), trochanter, Ward's triangle (Ward's) and metabolic parameters of bone turnover. ***Results:*** In osteoporotic women, *Taq*I genotype-related differences of the VDR gene were found to be significant at all BMD sites; TT genotype had higher L1–4 BMD values than Tt and tt ($p < 0.05$); tt genotype had significantly lower BMD at FN ($p < 0.05$), trochanter ($p < 0.01$), and Ward's ($p < 0.05$) compared to TT genotype. The tt genotype was found to be associated with higher ($p < 0.05$) serum osteocalcin levels compared to Tt and TT genotypes in the osteoporotic women, whereas no such association was found for the healthy women. ***Conclusion:*** Our data showed an association between *VDR Taq*I genotype and BMD at the FN, L1–4, trochanter and Ward's triangle in nonobese postmenopausal osteoporotic women. Thus the VDR gene *Taq*I polymorphism modulates differences in BMD in the postmenopausal osteoporotic women.

Introduction

Osteoporosis is characterized by low bone mineral density (BMD) and microarchitectural deterioration of bone leading to increased bone fragility and a high risk of fracture. BMD is influenced by genetic factors [1] and several twin studies suggest that genetic factors account for as much as 80% of total variance of BMD, the major predictor of osteoporosis and fragility fractures [1–7].

Although studies performed by Morrison et al. [8, 9] have focused attention on the possibility that polymorphisms in the vitamin D receptor (VDR) gene may

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account for a significant part of this variation, the distribution of BMD in the population strongly suggests a polygenic inheritance [10, 11]. Several groups have studied the relationship between VDR and bone mass as well as bone turnover in different populations, and obtained discordant results [8, 12–23]. In Australian women of English-Irish descent, after adjustment for age and environmental factors, subjects with BB or the tt genotype were found to have higher serum osteocalcin levels and lower BMD than those with the bb or TT genotypes. Heterozygotes (Bb/Tt) had intermediate values [8]. Furthermore, the BB genotype is associated with a higher rate of bone loss in premenopausal Japanese women [12] and in postmenopausal Caucasian women [13, 14]. These findings show an active role of the VDR genotype in determining bone density in postmenopausal women. Other studies have found a correlation between the BMD and VDR genotype [15–17], while others still have failed to detect such a correlation [18–23]. In general, the studies that have detected lower BMD found smaller differences between groups than that reported by Morrison et al. [9]. The ethnic differences among the study populations may explain this variability to some extent but only a few studies have focused on this issue [22, 24, 25].

No data have yet been published examining the prevalence and relevance of the various VDR polymorphisms in Turkish women. We therefore studied VDR genotypes in Turkish postmenopausal osteoporotic women and examined the relationship between the genotypes defined by the polymorphisms *Apa*I, *Bsm*I, and *Taq*I at the L1–4, the femoral neck (FN), trochanter and Ward's triangle in healthy and osteoporotic women. The major aim of this study was to define the possible associations of VDR gene alleles and bone turnover markers with BMD.

Materials and Methods

Subjects and Bone Density Measurements

Seventy-five postmenopausal osteoporotic (53.16 ± 1.31 years) and 66 healthy (52.62 ± 1.69 years) women were included in the study. All osteoporotic women had a BMD of at least 2.5 SD below the mean value of healthy premenopausal women in either the hip or spine. The control group consisted of healthy, age- and risk factor-matched subjects with BMD ± 1 SD T score. A detailed medical history was obtained and physical examination was performed by one of the investigators (R.T.). Patients were excluded from the study on the basis of the following criteria: presence of concomitant disease (disorders of calcium metabolism, renal, thyroid, hepatic dysfunction, Paget's disease, Cushing's syndrome, sarcoidosis, rheumatoid arthritis, malignancy, malabsorption, and malnutrition), previous use of oral/transdermal hormone replacement therapy or any other osteoporosis treatment, thyroid hormone replacement, glucocorticoid or anticonvulsant drug, biochemical evidence of osteomalacia and severe osteoarthritis. Osteoporosis risk factors, such as cigarette smoking, premenstrual irregularity of menses, insufficient sun exposure and calcium intake as well as sodium, protein, coffee and alcohol consumption, and physical activity, were assessed by a questionnaire. Lateral radiographs of the spine were examined, and the compression fracture was defined as >20% reduction in anterior height. BMD of the lumbar spine and proximal femur in grams per square centimeter was measured by dual energy X-ray absorptiometry (Hologic QDR 1000 Plus, USA).

Measurement of Bone Turnover Markers

Following an overnight fast, urine samples for the measurement of 24-hour calcium and hydroxyproline [26] were collected and measured. Serum calcium and osteocalcin were also measured. Body mass index was calculated as an estimate of obesity [weight (kg)/height (m^2)].

Genotype Assignment

Genomic DNA was prepared from 10 ml of EDTA-treated blood with a simple salting-out procedure [27]. The DNA sequences were amplified by polymerase chain reaction (PCR) [28]. Detection of the *Bsm*I site in intron 8 was performed by PCR amplification of a region carrying the *Bsm*I site with primers originating in exon 7 (primer 1: 5′-CAA CCAAGACTACAAGTACCGCGTCAGTGA-3′) and intron 8 (primer 2: 5′-AACCAGCGGGAAGAGGTCAAGGG-3′) producing an 825-basepair (bp) fragment [9]. To amplify the VDR DNA sequence carrying *Apa*I and *Taq*I sites, primers in intron 8 (5′-CAGAGCATGGACAGGGAGCAAG-3′) and exon 9 (5′-GCAACTCCTCATGGCTGAGGTCTCA-3′) were used which produced a 740-bp fragment [8]. PCR products were generated in a 60-µl reaction volume containing 100–200 ng of DNA, 0.5 µ*M* of each primer, 200 µ*M* of dNTPs, 50 m*M* KCl, 10 m*M* Tris-HCl (pH = 9.0), 1.5 m*M* $MgCl_2$, 0.1% Triton X-100, and 0.8 U of Taq DNA polymerase.

To determine the presence of a restriction site within an amplified product, a 10-µl aliquot was digested with 5 U of endonuclease *Bsm*I at 65°C, *Apa*I at 37°C, or *Taq*I at 65°C for 1 h. Digestion products were electrophoresed in a 2% agarose gel containing ethidium bromide (50 µg/ml). DNA fragments were visualized by ultraviolet illumination and fragment sizes estimated by comparison to the 50-bp ladder run on the same gel. The genotype detection protocol was repeated 2 times for the heterozygous samples to eliminate the probability of partial digestion. The presence of the *Bsm*I restriction site generates 175- and 650-bp fragments, whereas the absence of this site yields a 825-bp fragment. The homozygous absence of the *Taq*I site yields bands of 245 and 495 bp, whereas the homozygous presence of this site yields fragments of 205, 245 and 290 bp. Heterozygotes for the *Taq*I site exhibit fragments of 490, 290, 245 and 205 bp. Digestion of the 740-bp PCR product with *Apa*I gives fragments of 220 and 520 bp for the presence of the restriction site, whereas the absence of the restriction site leaves the PCR product undigested. The presence of the restriction enzyme site is indicated by a lowercase letter and the absence of the site by an uppercase letter.

Statistical Analysis

Statistical analyses were conducted using Unistat 5.1 software. Rare genotypes (n < 10 in either control or osteoporotic women) were excluded from the analysis. Data were considered significant at $p < 0.05$. The frequency distributions of VDR genotypes in osteoporotic

Table 1. Main clinical and biochemical characteristics, BMD, and bone turnover parameters in osteoporotic and control patients

	Osteoporosis (n = 75)	Control (n = 66)
Age, years	53.16 ± 1.31	52.62 ± 1.69
Menopause age, years	42.09 ± 0.72	44.42 ± 0.85
Weight, kg	62.28 ± 1.06**	67.56 ± 1.18
Height, m	1.55 ± 0.01**	1.59 ± 0.01
Body mass index, kg/m^2	25.67 ± 0.44	26.66 ± 0.59
Serum calcium, mg/dl	9.41 ± 0.17	9.69 ± 0.06
Urinary calcium mg/day	131.49 ± 7.49*	166.32 ± 13.98
Osteocalcin, ng/ml	5.03 ± 0.56***	2.61 ± 0.55
Hydroxyproline, mmol/mol Cr	15.87 ± 1.34	14.75 ± 1.53
L1–4 BMD, g/cm^2	0.80 ± 0.02***	0.93 ± 0.02
FN BMD, g/cm^2	0.68 ± 0.01*	0.76 ± 0.02
Trochanter BMD, g/cm^2	0.57 ± 0.01*	0.66 ± 0.02
Ward's BMD, g/cm^2	0.53 ± 0.01***	0.65 ± 0.03
Total BMD, g/cm^2	0.76 ± 0.03*	0.89 ± 0.09

Values are means ± SE.
* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Table 2. Allele and genotype frequencies of the *Bsm*I, *Apa*I, and *Taq*I polymorphisms of the VDR gene

VDR genotype	Osteoporotic women		Control women	
	observed n	frequency %	observed n	frequency %
*Bsm*I				
Bb	54	72	42	63.6
BB	18	24	17	25.8
bb	3	4	7	10.6
*Apa*I				
Aa	56	74.8	45	68.2
AA	13	17.2	15	22.7
aa	6	8	6	9.1
Taq				
Tt	42	56	28	42.4
TT	23	30.8	23	34.9
tt	10	13.2	15	22.7

and normal groups were compared using the χ^2 test. Both for the osteoporotic and control subjects, BMD levels and bone turnover parameters for the three genotype classes were compared with analysis of variance (ANOVA). Student's t test and the Mann-Whitney test were applied for statistical analysis between the two groups. All data are shown as mean ± standard error.

Results

The demographic and major clinical and biochemical values for both osteoporotic and control subjects are given in table 1. The osteoporotic women's height, weight, urinary calcium and all BMD values were significantly lower than those of the control women ($p \leq 0.01$ and 0.05). Although not significant, the bone resorption marker, hydroxyproline, was higher in the osteoporotic than the control group. Osteoporotic women had significantly higher ($p < 0.001$) serum osteocalcin levels compared to the control group. After amplification of genomic DNA with VDR-specific primers, followed by digestion with *Apa*I, *Bsm*I, or *Taq*I and agarose gel electrophoresis, restriction fragment length polymorphisms (RFLPs) were determined. No different result was obtained from partial digestion testing. The genotypic frequencies observed for these polymorphisms both in osteoporotic patients and control subjects are shown in table 2. The genotype frequencies in this population were 24% for BB, 72% for Bb, 4% for bb, 17.2% for AA, 74.8% for Aa, 8% for aa, 30.8% for TT, 56% for Tt, 13.2% for tt in the osteoporotic women, and 25.8% for BB, 63.6% for Bb, 10.6% for bb, 22.7% for AA, 68.2% for Aa, 9.1% for aa, 34.9% for TT, 42.4% for Tt and 22.7% for tt in the control women. The combination of genotypes defined by all three RFLPs is shown in table 3. The BbAaTt, BbAatt, and bbAaTT define the most frequently combined ones (69.3% in osteoporotic women, 56.1% in control women). The most common

Table 3. Frequency of VDR-combined genotypes

Combined genotype	Osteoporotic women (n = 75)		Control women (n = 66)	
	n	%	n	%
BbAaTt	31	41.3	17	25.8
BbAatt	15	20	10	15.15
bbAaTT	6	8	10	15.15
bbaaTt	5	6.76	–	–
BbaaTt	3	4	5	7.57
BBAAtt	3	4	3	4.54
Bbaatt	2	2.66	5	7.57
bbAaTt	2	2.66	5	7.57

Table 4. Selected characteristics of unrelated osteoporotic and healthy postmenopausal women in relation to VDR gene alleles

Characteristics	*Bsm*I		*Apa*I		*Taq*I		
	Bb	BB	Aa	AA	Tt	TT	tt
Osteoporotic women							
Number	54	18	56	13	42	23	10
Age, years	52.52 ± 1.62	54.33 ± 2.48	52.73 ± 1.54	53.76 ± 2.99	54.66 ± 1.84	49.05 ± 1.87	53.20 ± 4.24
Height, m	1.55 ± 0.01	1.55 ± 0.01	1.55 ± 0.01	1.56 ± 0.02	1.56 ± 0.01	1.55 ± 0.02	1.56 ± 0.02
Weight, kg	61.57 ± 1.14	65.15 ± 2.69	62.13 ± 1.16	62.23 ± 3.39	63.4 ± 1.43	60.30 ± 1.83	60.33 ± 2.99
BMI, kg/m^2	25.37 ± 0.45	27.05 ± 1.19	25.62 ± 0.45	25.66 ± 1.42	26.19 ± 0.65	24.79 ± 0.63	24.92 ± 1.07
Menopause age, years	41.81 ± 0.86	41.77 ± 1.39	42.27 ± 0.79	39.69 ± 1.66	42.61 ± 0.86	40.51 ± 1.55	40.70 ± 1.79
Serum Ca, mg/dl	9.40 ± 0.21	9.49 ± 0.33	9.49 ± 0.16	9.06 ± 0.67	9.51 ± 0.22	9.29 ± 0.29	9.22 ± 0.58
Urinary Ca, mg/day	136.99 ± 8.94	114.12 ± 15.59*	127.33 ± 8.63	114.09 ± 16.88	128.29 ± 8.37	127.51 ± 14.67	143.72 ± 28.42
Control women							
Number	42	17	45	15	28	23	15
Age, years	50.11 ± 1.59	50.14 ± 2.59	50.22 ± 1.67	53.66 ± 5.20	50.30 ± 2.57	56.11 ± 3.09	67.9 ± 2.26
Height, m	1.60 ± 0.01	1.58 ± 0.02	1.59 ± 0.01	1.61 ± 0.01	1.60 ± 0.01	1.62 ± 0.01	1.58 ± 0.02
Weight, kg	66.38 ± 1.30	68.57 ± 2.64	66.53 ± 1.55	68.92 ± 0.92	66.51 ± 2.09	68.61 ± 1.25	65.41 ± 3.1
BMI, kg/m^2	25.92 ± 0.68	27.43 ± 1.10	26.37 ± 0.78	26.70 ± 0.36	26.12 ± 1.08	26.97 ± 0.74	26.50 ± 1.33
Menopause age, years	44.00 ± 1.06	44.85 ± 1.87	43.89 ± 0.99	45.0 ± 2.07	43.55 ± 1.48	46.33 ± 1.05	47.70 ± 1.81
Serum Ca, mg/dl	9.80 ± 0.08	9.54 ± 0.05*	9.63 ± 0.06	9.85 ± 0.14	9.67 ± 0.08	9.81 ± 0.14	9.55 ± 0.04
Urinary Ca, mg/day	178.9 ± 20.2	151.00 ± 8.39	159.03 ± 6.83	189.15 ± 60.08	186.15 ± 31.2	146.2 ± 11.90	149.70 ± 7.91

Values are means ± SE.

* $p < 0.05$. Student's t test was used to compare the characteristics between the alleles of the VDR genes (*Bsm*I, *Apa*I), whereas ANOVA was used to test for the difference across the *Taq*I genotypes. BMI = Body mass index.

genotype combination in postmenopausal osteoporotic and control women was BbAaTt.

The relationship between VDR gene alleles and selected characteristics of osteoporotic and control subjects is shown in table 4. In osteoporotic and control women, there were no significant differences in age, age at menopause, height, weight or body mass index among the common genotypes in *Bsm*I, *Apa*I, and *Taq*I RFLPs. Urinary calcium concentration was to be found lower in the osteoporotic women with the BB genotype than in those carrying Bb ($p < 0.05$). Table 5 demonstrates BMD and bone turnover markers with respect to VDR gene alleles in osteoporotic and control subjects. Hydroxyproline levels were significantly lower in BB than in the Bb genotype in osteoporotic women ($p < 0.05$); in contrast to osteoporotic women, hydroxyproline levels in the control group were significantly different with respect to genotype, in which lower values were found in Bb than BB. Although serum osteocalcin levels were not significantly different according to *Bsm*I and *Apa*I genotypes, higher concentrations were observed in the BB and AA for osteoporotic women. For the control women, osteocalcin levels were found to differ according to the *Apa*I genotype, where AA carriers had higher levels compared to Aa ($p < 0.01$), but no such difference was observed between *Bsm*I genotypes. In osteoporotic women serum osteocalcin levels were found to be highest in the tt genotype and lowest in the Tt, and intermediate in the TT genotype ($p < 0.05$).

There is some evidence of a relationship with higher BMD levels associated with the T allele at the FN. Our data showed significant *Taq*I-related differences in the osteoporotic women in FN as well as L1–4, trochanter and Ward's BMD. The osteoporotic group with the TT genotype had significantly higher FN ($p < 0.05$), trochanter ($p < 0.01$), L1–4 ($p < 0.05$) and Ward's ($p < 0.05$) BMD values with respect to the tt genotype.

Discussion

In the last two decades, compelling data have been obtained indicating that BMD is, at least in part, genetically determined [15, 16, 18, 19]. In the present study, we analyzed *Apa*I, *Bsm*I, and *Taq*I VDR polymorphisms and addressed the question whether BMD was affected by the VDR gene polymorphisms in postmenopausal osteoporotic women as well as healthy control subjects. The observed VDR genotype distributions were similar to pre-

Table 5. BMD and bone turnover markers according to different VDR gene alleles in unrelated osteoporotic and healthy postmenopausal women

Characteristics	*Bsm*I		*Apa*I		*Taq*I		
	Bb	BB	Aa	AA	Tt	TT	tt
Osteoporotic women							
Number	54	18	56	13	42	23	10
Osteocalcin, ng/ml	4.92 ± 0.58	5.45 ± 1.54	4.92 ± 0.59	5.93 ± 1.26	4.27 ± 0.57	5.24 ± 0.79	8.69 ± 2.94*
Hydroxyproline, mmol/mol Cr	16.42 ± 1.60	14.47 ± 2.92*	17.03 ± 1.63	13.33 ± 2.94	16.16 ± 1.84	14.96 ± 1.79	20.00 ± 5.17
L1–4 BMD, g/cm^2	0.79 ± 0.02	0.84 ± 0.04	0.79 ± 0.02	0.83 ± 0.05	0.77 ± 0.02	0.87 ± 0.03*	0.80 ± 0.05
FN BMD, g/cm^2	0.69 ± 0.01	0.67 ± 0.02	0.69 ± 0.01	0.69 ± 0.02	0.68 ± 0.02	0.73 ± 0.02*	0.63 ± 0.03
Trochanter BMD, g/cm^2	0.58 ± 0.01	0.56 ± 0.02	0.57 ± 0.01	0.57 ± 0.02	0.56 ± 0.01	0.63 ± 0.02**	0.52 ± 0.02
Ward's BMD, g/cm^2	0.54 ± 0.02	0.51 ± 0.03	0.54 ± 0.02	0.54 ± 0.03	0.52 ± 0.02	0.61 ± 0.02*	0.47 ± 0.04
Control women							
Number	42	17	45	15	28	23	15
Osteocalcin, ng/ml	2.55 ± 0.73	2.77 ± 1.04	1.86 ± 0.44	5.50 ± 1.55**	2.75 ± 0.92	2.31 ± 0.89	2.82 ± 1.24
Hydroxyproline, mmol/mol Cr	13.27 ± 0.84	20.98 ± 4.41*	15.79 ± 2.00	11.64 ± 2.46	16.17 ± 2.65	12.68 ± 2.03	15.30 ± 3.64
L1–4 BMD, g/cm^2	0.93 ± 0.03	0.95 ± 0.01	0.96 ± 0.03	0.89 ± 0.03	0.95 ± 0.03	0.88 ± 0.05	0.90 ± 0.02
FN BMD, g/cm^2	0.79 ± 0.03	0.71 ± 0.03	0.80 ± 0.03	0.67 ± 0.01*	0.78 ± 0.04	0.74 ± 0.04	0.76 ± 0.04
Trochanter BMD, g/cm^2	0.69 ± 0.03	0.63 ± 0.03	0.69 ± 0.03	0.60 ± 0.02	0.65 ± 0.03	0.67 ± 0.04	0.68 ± 0.04
Ward's BMD, g/cm^2	0.68 ± 0.04	0.60 ± 0.04	0.69 ± 0.04	0.57 ± 0.02	0.65 ± 0.05	0.64 ± 0.06	0.68 ± 0.06

Values are means ± SE.

* $p < 0.05$; ** $p < 0.01$. Student's t test was used to compare the characteristics between the alleles of the VDR genes (*Bsm*I, *Apa*I), whereas ANOVA was used to test for the difference across the *Taq*I genotypes.

vious reports [16, 17]. It is expected that BMD is determined by genetic and environmental interacting influences. FN BMD was found to be decreasing continually with growing age, in both women and men even in the late decades of life [29]. The differential effects of VDR alleles on BMD among different populations could partly explain the differences between Italian and Irish studies [30, 31].

We found significant effects of VDR gene alleles on bone mass. *Taq*I genotypes were found to have a striking effect on all BMD values studied. The TT genotype-carrying osteoporotic women had higher BMD levels compared to either Tt or tt. The same effect of *Taq*I polymorphisms on BMD could not be observed in healthy women. Our observation is in good agreement with the finding of Spector et al. [15] who confirmed the link between the VDR genotype (*Taq*I poymorphism) and BMD in postmenopausal British twins. In their study, postmenopausal twins with the TT genotype had an about 10% higher bone density than women with the tt genotype.

Various studies have shown that obesity can mask the influence of the VDR genotypes. Dawson-Hughes et al. [32] showed that women with the BB genotype of the VDR gene had a reduced efficiency in calcium absorption and a low calcium intake compared to women of the bb genotype consistent with a functional defect in the intestinal VDR. The impact of this heritable difference is reduced at higher calcium intakes. This evidence is consistent with the fact that the relationship between the VDR genotypes and FN bone loss rate is enhanced at low calcium intakes in postmenopausal women. In obese postmenopausal women, estrogen concentrations appear to increase [33]. This estrogen increase in obese women may mask the relationship of VDR polymorphisms with BMD. For these reasons overtly obese women were not included in our study. Vandevyver et al. [34] concluded that the VDR gene polymorphism influences FN and BMD in nonobese postmenopausal women. They found that the link between VDR genotypes and FN and BMD is not seen in obese postmenopausal women, which suggests that factors related to obesity obscure such an effect in overweight women.

Although no significant differences were observed between the genotypic distribution of the VDR gene in the osteoporotic and control groups in our study, for each of the three loci tested, heterozygous classes had a higher frequency in osteoporotics than in controls, which implies a heterozygous disadvantage. Furthermore, the BMD val-

ues for the different sites were not found to associate with any of the *Bsm*I and *Apa*I genotypes in osteoporotic women, which means that no relationship between the VDR *Bsm*I polymorphism and osteoporosis was observed. We found a close relation between *Taq*I genotypes and BMD values at all sites studied in the osteoporotic women. The L1–4, FN, trochanter and Ward's BMD levels differed with respect to *Taq*I polymorphism and all were significantly higher in the osteoporotic women with TT genotype compared to Tt and tt, which demonstrates the relationship between VDR *Taq*I polymorphism and osteoporosis. Sheehan et al. [31] reported 29 and 40% higher osteocalcin levels of the tt genotype compared to the Tt and TT genotypes, respectively, in healthy adults. According to our results the tt VDR genotype was associated with significantly higher serum osteocalcin compared with the Tt and TT genotypes, 50.8 and 39%, respectively, in the osteoporotic women, whereas no such association was found in the healthy women. The effects we observed in our subjects are of genetic and biological significance.

Conclusion

This study shows an association between the VDR gene *Taq*I polymorphism and BMD at the FN, lumbar spine, trochanter and Ward's triangle in a group of non-obese, postmenopausal osteoporotic women. Thus, the VDR gene *Taq*I polymorphism modulates differences in BMD in the postmenopausal osteoporotic women, although the interaction of the gene and BMD and the role of VDR genotypes deserve further study with larger numbers of subjects.

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