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ORIGINAL ARTICLE

Evidence of association of Vitamin D receptor *Apa* I gene polymorphism with bone mineral density in postmenopausal women with osteoporosis

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Abstract The vitamin D receptor (VDR) was the first candidate gene to be studied in relation to osteoporosis, and most attention has focused on polymorphisms situated near the 3′ flank of VDR. The aim of this study was to investigate the association about VDR gene *Apa* I polymorphism with bone mineral density (BMD) in postmenopausal women with osteoporosis. We studied a total of 136 postmenopausal women with a mean age of 56.36± 10.29 years. Among them, a total of 75 had osteoporosis, 37 had osteopenia, and 24 had normal BMD. Venous blood samples were obtained for evaluation of bone metabolism and genotyping. The VDR *Apa* I genotype was determined by polymerase chain reaction-restriction fragment length polymorphism. BMDs at the lumbar spine and hip were measured by dual-energy X-ray absorptiometry. Postmenopausal women with aa genotype had significantly lower BMD values (grams per centimeter square) at lumbar spines compared to persons with AA genotype. Also, postmenopausal women with AA genotype had significantly higher serum Ca level than the subjects with aa genotype. In conclusion, our result may indicate that VDR *Apa* I gene polymorphism may be responsible for a important part of the heritable component of lumbar spine BMD in postmenopausal women, possibly related to impaired calcium absorption from the bowel.

Keywords Bone mineral density · Osteoporosis · Vitamin D receptor *Apa* I gene polymorphism

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Introduction

Osteoporosis is a common disease characterized by low bone mass, disturbed microarchitecture of bone tissue, and increased fracture risk. Osteoporosis is defined to exist when bone mineral density (BMD) values at the spine or hip fall 2.5 standard deviations (SD; T-score values) or more below the population average in young adults [1]. Peak bone mass is attained in early adult life but declines in postmenopausal women due to a reduction in estrogen production, with effects on bone and intestinal and renal calcium handling [2].

Previous studies in twins and families show that genetic factors play an important role in the formation of BMD. The heritability of BMD has been estimated to be between 50% and 85% in twin studies, with the strongest effects in the axial skeleton [3–6]. It also shows that 27–68% of the variance in osteoporotic fracture is heritable [7].

Osteoporosis is a polygenic disorder, determined by the effects of several genes, each with relatively modest effects on bone mass and other determinants of fracture risk. Population-based studies and case–control studies have similarly identified polymorphisms in several candidate genes that have been associated with bone mass or

osteoporotic fracture, including the vitamin D receptor (VDR), estrogen receptor, and collagen type IαI gene [8].

Vitamin D, through its principal bioactive form 1,25-dihydroxyvitamin D3(1,25-(OH)2D3) plays a crucial role in bone metabolism. The action of 1,25-(OH)2D3 is mediated through a specific hormone-receptor [9]. Mutations in VDR cause the syndrome of vitamin D-resistant rickets, which is a recessive condition characterized by alopecia, hypocalcaemia, hypophosphatemia, and severe rickets, and is resistant to treatment with vitamin D and its active metabolites [9, 10]. The VDR was the first candidate gene to be studied in relation to osteoporosis, and most attention has focused on polymorphisms situated near the 3′ flank of VDR recognized by the restriction enzymes *Bsm* I, *Apa* I, and *Taq* I [9]. However, few studies were performed to identify the relationship of candidate gene polymorphism underlying peak bone mass variation.

The objective of this study was to investigate the association about VDR gene *Apa* I polymorphism with BMD in postmenopausal women with osteoporosis.

Materials and methods

We studied a total of 136 postmenopausal women with a mean age of 56.36±10.29 years. Among them, a total of 75 had osteoporosis (fulfilled criteria for osteoporosis according to WHO guidelines and had a BMD T-score of lesser than −2.5 SD), 37 had osteopenia (T-score between −1.0 and −2.5 SD), and 24 had normal BMD (T-score greater than −1 SD) [1].

Exclusion criteria for this study were diseases well known to affect bone metabolism (thyroid disease, parathyroid disease, renal disease, liver disease, and malignancy), any medication known to affect bone turnover such as glucocorticoids, and anticonvulsant drugs. Patients were also excluded from study if they had taken anti-osteoporotic treatments including calcium supplementation >1,000 mg/day, vitamin D supplementation >400 IU/day, parathyroid hormone (PTH), calcitonin, estrogen, bisphosphonates, or strontium ranelate within 12 months previous to study entry.

Bone mineral density measurements

BMD was measured in all patients and controls by dual-energy X-ray absorptiometry (Hologic QDR 4,500 W) both at the lumbar spine (antero-posterior projection of L1-L4) and the proximal femur (total score). The instrument was calibrated daily according to the manufacturer's instructions. BMD data were expressed as grams per centimeter square and SD scores which compare individual BMD determinations to those of young (T) and age/sex-matched (Z) normal populations [1].

Biochemical analyses

Following on overnight fasting (at least 8 h), venous blood samples were obtained for evaluation of bone metabolism. Bone metabolism was evaluated by serum calcium (Ca), phosphorus, alkaline phosphatase, PTH, 25 (OH) vitamin D, osteocalcin (OC), and serum C-telopeptide cross-linked collagen type I (CTX) levels. Serum PTH was measured by immunoradiometric assay (Allegro Intact PTH, Nichols Institute, San Juan, Capistrano, CA, USA). Serum CTX was measured by electrochemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany). Serum OC was measured with a radioimmunoassay (RIA) technique (DiaSorin, Saluggia, Italy). The level of 25-(OH) vitamin D, was measured by RIA (Nichols Institute Diagnostics). The level of alkaline phosphatase was measured by standardized colorimetric method. The level of phosphorus was measured by ammonium phosphomolybdate colorimetric method. The level of serum calcium was measured by o-cresolphthalein endpoint colorimetric method.

Genotyping

Genomic DNA was extracted and purified from ethylenediaminetetraacetic acid blood samples using routine procedure. Genotypic analysis of VDR gene *Apa* I polymorphisms was determined by polymerase chain reaction (PCR)-restriction fragment length polymorphism. VDR gene fragment including the *Apa* I polymorphism site was amplified using primers 5′-CAG AGC ATG GAC AGG GAG CAA-3′ and 5′-GCA ACT CCT CAT GGC TGA GGT CTC-3′ [11]. The PCR was carried out in 30 μL of a buffer solution: Tris–HCl 10 mmol/L, KCl 50 mmol/L, MgCl2 1.5 mmol/L, 200 μmol/L each of the four deoxyribonucleotides, 2.5 U of *Taq* polymerase, and 0.25 μmol/L of each primer. PCR was performed with the following steps: at 94 C for 3 min and then at 94 C for 1 min, at 63 C for 20 s, at 72 C for 20 s, for 35 cycles, and at 72 C for 7 min. After amplification, VDR gene fragment was digested with *Apa* I restriction endonuclease and electrophoresed in 2.0% agarose gel. The *Apa* I genotype was named as follows: AA (absence of the restriction), aa (presence of the restriction site), Aa (heterozygous for the restriction site).

Statistical analyses

All parametric results were expressed as mean±SD. Differences were considered significant if the p values were less or equal to a level of 5%. Chi-square (χ^2) test was used for comparison of frequencies distribution of genotypes for VDR *Apa* I between postmenopausal women with osteoporosis, osteopenia, and normal BMD. The association

Table 1 Comparison of frequencies distribution of genotypes for VDR *Apa* I between postmenopausal women with osteoporosis, osteopenia, and normal BMD

	VDR *Apa* I gene genotype			*P* value
	AA	Aa	aa	
Postmenopausal osteoporotic women (*n*=75)	17 (22.7%)	44 (58.7%)	14 (18.7%)	0.278
Postmenopausal osteopenic women (*n*=37)	9 (24.3%)	17 (45.9%)	11 (29.7%)	
Postmenopausal women with normal BMD (*n*=24)	8 (33.3%)	14 (58.3%)	2 (8.4%)	
Total	34 (25%)	75 (55.1%)	27 (19.9%)	

VDR vitamin D receptor, *BMD* bone mineral density

between VDR *Apa* I genotype and demographic features, bone metabolism markers, and BMD values of postmenopausal women were tested using analysis of covariance.

Informed consent was obtained before the examination, and approval for the study was granted by the local ethical committee of the university.

Results

The distribution of VDR *Apa* I genotype of the 136 postmenopausal women evaluated were as follows: AA 34 (25%), Aa 75 (55.1%), and aa 27 (19.9%), respectively. Comparison of frequencies distribution of genotypes for VDR *Apa* I between postmenopausal women with osteoporosis, osteopenia, and normal BMD did not show any significant differences (Table 1).

Comparison of bone metabolism markers and BMD values of 136 postmenopausal women on the basis of VDR *Apa* I genotype showed that postmenopausal women with aa genotype had significantly lower BMD values (grams per centimeter square) at lumbar spines compared to persons with AA genotype. Also, postmenopausal women with AA genotype had significantly higher serum Ca level than the subjects with aa genotype. However, comparison of BMD values at proximal femur and other bone metabolism markers did not show any significant differences between postmenopausal women with AA, Aa, and aa genotype (Table 2).

Discussion

Several studies have since been carried out on the relationships between VDR genotype, bone density, and other aspects of calcium metabolism. However, the results of the previous studies are conflicting. Zhang et al. studied the frequencies distribution of VDR gene *Fok* I, *Apa* I, *Bsm* I, and *Taq* I polymorphisms and their association with BMD in postmenopausal women, and they found that cross-genotyping *Apa* I and *Bsm* I or *Taq* I polymorphisms was not associated with BMD in postmenopausal women [12]. In another study, Qin et al. studied association of VDR *apa* I and estrogen receptor-α gene polymorphisms with peak bone mass in premenopausal Chinese women. They did not find any significant association with BMD at lumbar spine and proximal femur [13]. However, Gennari et al. showed that there was some evidence of 13% higher lumbar BMD

Table 2 Comparison of demographic features, bone metabolism markers, and BMD values of 136 postmenopausal women on the basis of VDR *Apa* I genotype

n=136	AA (*n*=34)	Aa (*n*=75)	aa (*n*=27)	*P* value
Age (years)	54.28±10.71	57.17±9.75	56.61±11.30	0.412
Body mass index (kg/m^2)	28.17±3.1	27.98±4.2	28.35±3.3	0.542
Lumbar spine BMD (g/cm^2)	0.950±0.16[a]	0.904±0.18	0.828±0.15[a]	0.026
Lumbar spine BMD (T-score)	−1.93±1.53	−2.35±1.46	−2.81±1.12	0.060
Lumbar spine BMD (Z-score)	−1.23±1.55	−1.50±1.40	−2.07±1.08	0.063
Proximal femur BMD (g/cm^2)	0.858±0.13	0.818±0.13	0.804±0.14	0.190
Proximal femur BMD (T-score)	−1.54±0.86	−1.81±1.12	−1.90±1.15	0.355
Proximal femur BMD (Z-score)	−0.52±0.91	−0.56±0.86	−0.75±1.09	0.594
Osteocalcin (ng/ml)	18.04±7.39	20.39±9.07	21.40±12.12	0.455
ALP (U/l)	202.99±83.9	185.53±72.89	167.19±70.40	0.228
CTX (ng/ml)	0.50±0.22	0.55±0.32	0.57±0.29	0.576
Calcium (mg/dl)	9.64±0.47[a]	9.47±0.46	9.21±0.47[a]	0.005
Phosphorus (mg/dl)	3.81±0.61	3.63±0.55	3.67±0.59	0.340
PTH (pg/ml)	40.45±15.5	42.62±24.1	45.09±16.06	0.171
25-OH-vitamin D (ng/ml)	33.25±19.5	29.17±11.3	32.50±15.9	0.375

BMD bone mineral density, *VDR* vitamin D receptor, *ALP* alkaline phosphatase, *CTX* serum C-telopeptide cross-linked collagen type I, *PTH* parathyroid hormone

[a] <0.05

values in aabbTT genotype with respect to AABBtt genotype in postmenopausal women, but this difference of approximately 0.1 g/cm^2 did not reach statistical significance [14]. Also, Zambrano-Morales et al. studied the association of *Bsm* I, *Apa* I, and *Taq* I VDR gene polymorphism with osteoporosis in 147 postmenopausal women. They found that BBAAtt haplotype was a risk factor for osteoporosis and BbaaTT was a protection factor [15]. In contrast, we found that postmenopausal women with aa genotype had significantly lower BMD values (grams per centimeter square) at lumbar spines compared to persons with AA genotype. Consistent with our result, Huang et al. investigated association of *Apa* I polymorphism of VDR gene with bone mass in 388 healthy men. They found that AA genotype had higher bone mass [16].

Vitamin D interacts with its receptor to play an important role in calcium homeostasis by regulating bone cell growth and differentiation, intestinal calcium absorption, and PTH secretion [17]. VDR *Apa* I gene polymorphism may be responsible for some part of the heritable component of bone density in women, possibly related to impaired calcium absorption from the bowel. In our study, postmenopausal women with aa genotype had significantly lower serum Ca level than the women with AA genotype. This finding may show that persons with aa genotype for VDR *Apa* I gene may have a poor intestinal Ca absorption. This abnormality may lead to secondary hyperparathyroidism, which is characterized by high serum PTH and an increase in bone resorption. Also in our study, postmenopausal women with aa genotype had higher PTH level compared to AA and Aa genotype; however, this difference did not reach statistical significance. Yokoyama et al. showed that Japanese patients with end-stage renal disease with aa genotype for VDR *Apa* I had significantly higher concentration of blood PTH level than the patients with the AA and Aa genotypes [18]. Impaired intestinal Ca absorption and secondary hyperparathyroidism may explain the significantly low BMD values at lumbar spine in our postmenopausal women with aa genotype for VDR *Apa* I gene. However, the mechanisms by which this polymorphism modulates VDR function remain unclear: it may influence RNA stability, and isoforms of VDR encoded by different alleles may possess different functions [19].

It is interesting to observe that although the genotypic distribution was consistent with the Hardy–Weinberg's equilibrium law in most studies, the relative distribution of VDR *Apa* I genotypes varied remarkably between populations. For example, the AA genotype was 7.8% in Chinese women [20], 15.1% in Japanese women [21], 36% in Indian population [22], or 27% in Greek women [23]. In our study population, the AA genotype was 25%. It is not clear why there was such a major difference in the genotypic distributions; however, population stratification and/or mixed ethnicities could be the underlying responsible factors. Ethnicity may interact to the influence of VDR *Apa* I polymorphism in association to BMD. We also found that postmenopausal osteoporotic women had higher aa genotype and lesser AA genotype than the postmenopausal women with normal BMD. However, comparison of frequencies distribution of genotypes for VDR *Apa* I between postmenopausal women with osteoporosis, osteopenia, and normal BMD did not show any significant differences. Our study groups consisted of a small number of individuals, so our results may not reach statistical significance.

Our result also showed that *Apa* I genotype is not associated with BMD at proximal femur. *Apa* I polymorphism in VDR gene possibly influence loss of trabecular bone mass of the lumbar spine. Also, a recent meta-analysis showed that there was evidence of an association between spine BMD and VDR *Bsm* I polymorphism, and no association with femoral BMD was observed [24].

Low number of patients included is the main limitation of our study, which should be an argue that decreased the effect of statistical analysis.

In conclusion, our result showed that postmenopausal women with aa genotype had significantly lower BMD values (grams per centimeter square) at lumbar spines compared to subjects with AA genotype. VDR *Apa* I gene polymorphism may be responsible for a important part of the heritable component of BMD in women, possibly related to impaired calcium absorption from the bowel.

Disclosures None.

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