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Association of Common Vitamin D Receptor Gene Variations with Fracture Risk and Bone Mineral Density in Postmenopausal Korean Population

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Abstract

Osteoporosis is characterized by impaired osteogenesis. BMD is a major determinant of bone strength. The role of the *VDR* gene in predisposition to primary osteoporosis has been recognized. However, population-based case-control studies have been reported controversial results for known candidate genes in an ethnically distinct group. To determine the genetic effects of *VDR* variants on osteoporosis and BMD, we directly sequenced the *VDR* gene in 24 unrelated Korean individuals and identified eighteen sequence variants. We investigated the potential involvement of eight SNPs in osteoporosis in postmenopausal women (n = 729). Two SNPs (LD) in intron 2, $-5294G>C$ (rs2238135) and $-4817G>A$ (rs17882443) showed the evidence of association with enhanced BMD of the femoral neck ($p_{additive}$=0.031 for rs2238135; $p_{additive}$=0.017 and $p_{dominant}$= 0.019 for 17882443). Moreover, *VDR* $-4817G>A$ was significantly associated with protective effect on all fracture risk ($p_{recessive}$=0.035, OR=0.2, 95% CI=0.05～0.89), and tended to be higher BMD values at various proximal femur sites. Therefore, we suggest that the $-4817G>A$ may be useful genetic marker for vitamin D-related metabolism and may have an important role in the increased BMD of the proximal femur in postmenopausal Korean women.

Keywords: BMD, fracture, osteoporosis, polymorphism, VDR

Introduction

Osteoporosis is called “the silent bone disease”, characterized by compromised bone strength, predisposes individuals to increased risk of fracture, especially of the hip and vertebra in postmenopausal women (Eisman, 1999; Hwang *et al*., 2006). Bone loss from an imbalanced remodeling between osteoclastic resorption and osteoblastic formation is observed at the time of menopause, mainly due to estrogen deficiency and calciotropic hormone insufficiency such as vitamin D (Boyle *et al*., 2003; Harada & Rodan, 2003; Khosla, 2001).

Vitamin D effects on bone are mediated through the vitamin D receptor (*VDR*), a nuclear transcription factor that regulates bone formation, bone resorption, and calcium homeostasis by interacting with vitamin D response elements (VDREs) within Vitamin D target genes. Allelic variation in the VDR gene explains 75% of the genetic variability in BMD used as a proxy measure (Eisman, 1999; Liu *et al*., 2003; Morrison *et al*., 1994).

Since the first association by Morrison *et al*., allelic variations in genetic regulation of BMD have been subsequently studied in candidate genes related to important elements of bone mineral homeostasis, bone remodeling and bone matrix composition. These approach were practically performed by restriction fragment length polymorphisms (RFLPs) on various populations: Caucasians (Deng *et al*., 1999; Langdahl *et al*., 2000; Quesada *et al*., 2004), African-Americans (Zmuda *et al*., 1999; Harris *et al*., 1997), Mexican-Americans (Kammerer *et al*., 2004; McClure *et al*., 1997), and Asians (Mitra *et al*., 2006; Morita *et al*., 2004; Yamada *et al*., 2003; Zhang *et al*., 2004). Ethnicity was shown to be one of the important factors affecting BMD (Liel *et al*., 1988; Wang *et al*., 1997). However, controversial results of interethnic differences in allele or genotype distributions for BMD variation have been evidently presented, and a con-

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sensus on the association with BMD on known candidate genes locus has not been reached yet (Gamero *et al.*, 1995; Peacock, 1995).

In this study, we have analyzed the statistical association of Vitamin D receptor (*VDR*) gene polymorphisms with intermediate phenotypes (BMD; major risk factor of osteoporotic fracture) and fracture (Fx) to address confounding differences in ethnic backgrounds involving sample size and stochastic variability. We also performed an analysis of haplotypes in order to identify interaction of haplogenotypes related with osteoporosis.

Methods

Subjects

The study population was composed of apparently healthy postmenopausal Korean women (n=729) who visited the Asan Medical Center (AMC) in Seoul, Korea. Menopause was defined as the absence of menstruation for at least 6 months and was confirmed by measurement of serum follicle-stimulating hormone levels. Women who were prematurely menopausal (aged <40 years) were excluded. Those who had taken drugs that might affect bone metabolism for >6 months, or within the previous 12 months, were also excluded. Additionally, women were excluded if they had any disease that might affect bone metabolism. The mean age was 58.7 ± 7.5 years and the mean number of YSM was 9.4 ± 7.8 (range $1\sim35$) years.

BMD measurement

Areal BMD (g/cm^2) at the lumbar spine (L2-L4) and femoral neck was measured using dual-energy X-ray absorptiometry (Lunar equipment, Expert XL, Madison, WIS) in 476 women. In the other 253 women, BMD was measured using Hologic equipment (Hologic, QDR 4500-A, Waltham, MA). The precisions of the Lunar and Hologic equipment, presented as coefficient of variations, were 0.82% and 0.85% for the lumbar spine and 1.12% and 1.20% for the femoral neck, respectively. These values were obtained by scanning 17 volunteers who were not part of the study; each volunteer underwent five scans on the same day, getting on and off the table between examinations. To derive cross-calibration equations between the two systems, BMD values were measured by the two machines in 109 healthy Korean women (mean age 55 ± 11, range $31\sim75$ years), and cross-calibration equations were calculated as follows (Oldroyd et al., 2003):

L2-L4 BMD (g/cm^2): Lunar = $1.1287\times$Hologic -0.0027

Femoral neck BMD (g/cm^2): Lunar=$1.1556\times$Hologic $-$ 0.0182

We also obtained BMD values at other proximal femoral sites, all taken after January 2001. The Hologic machine did not measure BMD at the femoral shaft. BMD values at the femoral shaft and at other proximal femur sites (trochanter, total femur, Ward' s triangle) were available for 228 and 319 participants, respectively. Associations between these BMD values and *VDR* genetic variations were determined using statistical adjustments with the machine as a covariate, because the cross-calibration data are not yet available for Korean women at these sites.

Sequencing of the VDR gene

We sequenced all *VDR* exons, including exon-intron boundaries, and the promoter region (ca. 1.5 kb), to detect SNPs. We sequenced 24 Korean DNA samples using the ABI PRISM 3730 DNA analyzer (Applied Biosystems, Foster City, CA). Sixteen primer sets for amplification and sequencing analyses were designed based on GenBank sequences (Ref. Seq. of *VDR* mRNA: NM_001017535.1, and contig: NT_029419.11). Sequence variants were verified by automated sequencing chromatograms. SNPs were detected by multiple alignments of sequences using the Phred/Phrap/Consed package and polyphred (Ewing *et al.*, 1998; Gordon *et al.*, 1998; Nickerson *et al.*, 1997).

Golden gate assay for genotyping

Genotyping was performed at a multiplex level using the Illumina Golden Gate genotyping system (Oilphant *et al.*, 2002), and data quality was assessed using duplicate DNAs (n=10). The genotype quality score for data retention was set at 0.25. SNPs that could not satisfy the following criteria were excluded: (i) a minimum call rate of 90%; (ii) no duplicate error; (iii) Hardy-Weinberg equilibrium greater than $p>0.001$.

Statistics

The χ^2 test was used to determine whether individual variants were in equilibrium at each locus in the population (Hardy-Weinberg equilibrium). We examined Lewontin' s D' (ID'l), and the linkage disequilibrium (LD) coefficient γ^2, between all pairs of biallelic loci. Genotypes were given codes of 0, 1, and 2 for the additive model; 0, 1, and 1 for the dominant model; and 0, 0, and 1 for the recessive model. Multiple regression analyses of BMD at the lumbar spine and femoral neck

were performed using age, YSM, weight, and height, as covariates. Associations of BMD values at other proximal femur sites, such as the total femur, the trochanter, Ward's triangle, and the shaft, with *VDR* genotypes, were determined after further statistical adjustments made necessary by the choice of bone densitometer, because cross-calibration data are not available, to date, for these values in Korean women. The genotype distributions between participants with or without fracture were analyzed using a logistic regression model controlling for age, YSM, weight, and height.

Results

To investigate the genetic effects of *VDR* variants on BMD and osteoporosis, we sequenced all the *VDR* exons and their boundaries including 1.5 kb of the 5' flanking region and identified eighteen polymorphisms three were located in the promoter, three in coding regions of exons (two synonymous and one non-syn.), nine in the 3'-untranslated regions (UTRs), and three in the introns. We genotyped the eight selected SNPs for association studies in postmenopausal women (n=729). Clinical profiles and the correlations between BMD and age, weight, height, and years since menopause (YSM) are listed in Table 1. The mean age of the participants was 58.7±7.5 years (range 43～82 years), and the mean YSM was 9.5±7.8 years (range 1～32 years). As expected, age and YSM correlated negatively with BMD at both the lumbar spine and femoral neck. Weight and height correlated positively with BMD at both sites. In these participants, the genotype frequency of each of

Table 1. Clinical profiles and multiple regression analyses of bone mineral density (BMD) (g/cm^2) in postmenopausal women (n=729)

Clinical profiles		Lumbar spine BMD			Femoral neck BMD		
Variables	Mean (±SD)	β	SE	p-value	β	SE	p-value
Age (years)	58.69 (±7.5)	−0.004	0.002	0.018	−0.002	0.001	0.077
Weight (kg)	56.24 (±7.1)	0.006	0.001	0.000	0.004	0.001	0..000
Height (cm)	155.04 (±5.3)	0.001	0.001	0.103	0.001	0.001	0.560
YSM (years)	9.44 (±7.8)	−0.005	0.002	0.005	−0.004	0.001	0.006
		Adjusted R^2=0.225			Adjusted R^2=0.198		

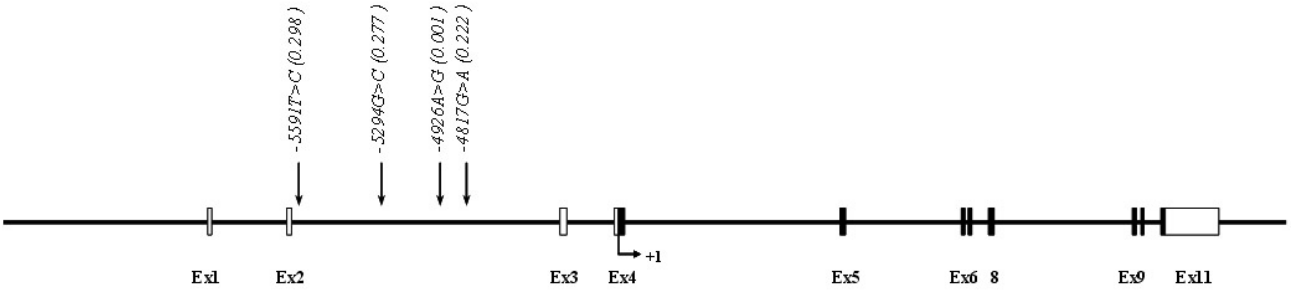
*Values are presented as means (±SDs) unless otherwise specified. YSM: years since menopause; β: regression coefficient; SE: standard error.

Table 2. Frequencies of VDR polymorphisms in postmenopausal women

Loci	Position (Amino acid change)	rs ID	Genotype				MAF	Heterozygosity	HWE[a]
−5591T>C	Intron2	rs2853564	T	CT	C	N	0.297	0.417	0.623
			356	308	61	725			
−5402G>A	Intron2	rs11574034	G	GA	A	N	0.000	-	-
			729	0	0	729			
−5294G>C	Intron2	rs2238135	G	CG	C	N	0.279	0.402	0.913
			379	291	57	727			
−4926A>G	Intron2	rs11574035	A	AG	G	N	0.001	0.001	0.985
			728	1	0	729			
−4817G>A	Intron2	rs17882443	G	AG	A	N	0.222	0.346	0.668
			439	256	34	729			
−4257G>A	Intron2	rs11574038	G	GA	A	N	0.000	-	-
			729	0	0	729			
−3949C>T	Intron2	rs11574039	C	CT	T	N	0.000	-	-
			729	0	0	729			
+34169C>T	Exon11 (T362I)	rs11574115	C	CT	T	N	0.000	-	-
			729	0	0	729			

[a]p-values of deviation from Hardy-Weinberg Equilibrium among all subjects. MAF: minor allele frequency.

A. Map of *VDR* (vitamin D (1,25- dihydroxyvitamin D3) receptor) on chromosome 12q13.11 (41.2 kb) NM_001017535.1



B. Haplotypes in *VDR*

Hap.	-5591T>C	-5294G>C	-4926A>G	-4817G>A	Freq.
ht1	T	G	A	G	0.430
ht2	C	G	A	G	0.290
ht3	T	C	A	A	0.219
ht4	T	C	A	G	0.053
ht5	C	C	A	G	0.005
ht6	T	G	A	A	0.002
ht7	C	G	G	G	0.001
ht8	C	G	A	A	0.001

C. LDs among *VDR* polymorphisms

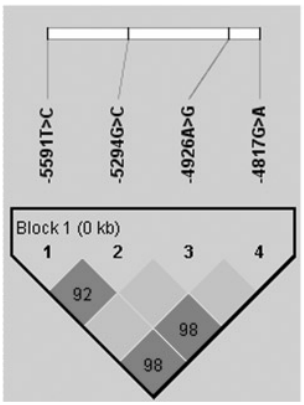


Fig. 1. Gene maps, haplotypes and LD coefficients of *VDR*. Coding exons are marked by black blocks, and 5'- and 3'-UTRs with white blocks. The first base of the translation start site is denoted nucleotide '+1'. Polymorphisms genotyped in a larger population (n=729). The frequencies of polymorphisms not subject to larger-scale genotyping were based on sequence data (n=24). (A) Polymorphisms identified in *VDR* on chromosome 12q13.11 (Ref. Genome Seq. NM_001017535.1). (B) Haplotypes of *VDR*. (C) Linkage disequilibrium coefficients LD and LD blocks among *VDR* polymorphisms.

the eight SNPs studied is shown in Table 2; genotype distributions were in Hardy-Weinberg equilibrium ($p > 0.001$). The *VDR* $-5402G>A$, $-4926A>G$, $-4257G>A$, $-3949C>T$ and $+34169C>T$ SNPs were extremely rare or monomorphic in our subjects. Four common haplotypes (freq. >0.05) in LD block accounted for 43.0% (ht-1), 29.0% (ht-2), 21.9% (ht-3) and 5.3% (ht-4) of the distribution, and were used for further analysis (Fig. 1).

We found that $-4817G>A$ was significantly associated with the risk of all fracture (p=0.035). The minor allele (A) of $-4817G>A$ in intron region showed susceptibility to the risk of fracture in the recessive (p=0.035, OR=0.2, 95% CI=0.05~0.89). Consistent with fracture result, VDR-ht3 (TCAA) (Fig. 1B) comprising risk allele also showed the evidence of association with fracture risk by protective effect (p=0.05, OR=0.23, 95% CI =0.05-1), although the statistical significance was marginal (Table 3).

In logistic regression analysis adjusted for confounding variables, we found that the *VDR* $-4817G>A$ and *VDR-ht3* were significantly associated with the BMD of various proximal femur sites as well as femoral neck. *VDR* $-4817G>A$ (rs17882443) showed a significant association with BMD at the femoral neck (p=0.017 and 0.019), trochanter (p=0.035 and 0.014), shaft (p=0.012 and 0.020), and total femur (p=0.030 and 0.016) in the additive and dominant models, respectively (Table 4). In addition, $-5591T>C$ in LD block was also associated with higher BMD of femoral neck ($p_{additive}$=0.031) and shaft ($p_{additive}$=0.021 and $p_{dominant}$=0.018), respectively (data not shown). VDR-ht3 was associated with increased BMD values of the femoral neck ($p_{additive}$=0.028 and $p_{dominant}$=0.034), trochanter ($p_{additive}$=0.042 and $p_{dominant}$=0.017), shaft ($p_{additive}$=0.014, $p_{dominant}$=0.024 and $p_{recessive}$=0.039), and total femur ($p_{additive}$=0.034 and $p_{dominant}$ =0.019), respectively (Table 4).

Table 3. Logistic analysis of VDR polymorphisms in relation to fracture risk in postmenopausal women

Loci	Case (165)	Control (563)	Additive		Dominant		Recessive	
			OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
−5591T>C	0.299	0.296	0.98 (0.74~1.31)	0.909	1 (0.69~1.46)	0.995	0.92 (0.48~1.78)	0.804
−5294G>C	0.264	0.283	0.94 (0.69~1.26)	0.662	1.1 (0.76~1.61)	0.612	0.42 (0.18~1)	0.050
−4926A>G	0.000	0.001	-	0.984	-	0.984	-	-
−4817G>A	0.212	0.225	0.91 (0.65~1.26)	0.552	1.05 (0.72~1.54)	0.800	0.2 (0.05~0.89)	0.035
VDR_ht1	0.439	0.426	1.08 (0.83~1.41)	0.577	1.3 (0.86~1.96)	0.209	1.13 (0.64~2.02)	0.673
VDR_ht2	0.297	0.287	1 (0.74~1.33)	0.978	1 (0.69~1.46)	0.985	0.91 (0.45~1.84)	0.787
VDR_ht3	0.212	0.221	0.93 (0.67~1.29)	0.657	1.09 (0.74~1.6)	0.665	0.23 (0.05~1)	0.050
VDR_ht4	0.052	0.054	1.01 (0.57~1.79)	0.979	1.05 (0.55~1.99)	0.893	0.68 (0.07~6.93)	0.741

Genotype distributions and p-values for logistic analyses of three alternative models (additive, dominant and recessive models) controlling for age, weight, height, year since menopause, and type of evaluation machine, as covariates, are shown.

Table 4. Regression analysis of bone mineral density (BMD) (g/cm^2) of various sites in relation to VDR polymorphisms in postmenopausal women

Bone loci	Loci	C/C*	C/R	R/R	*Pa*†	*Pb*	*Pc*
Femoral neck	*−4817G>A*	439 (0.7±0.14)	256 (0.68±0.12)	34 (0.67±0.11)	0.017	0.019	0.242
	VDR_ht3	443 (0.7±0.14)	252 (0.68±0.12)	34 (0.67±0.11)	0.028	0.034	0.154
Trochanter	*−4817G>A*	189 (0.59±0.13)	119 (0.56±0.1)	12 (0.61±0.14)	0.035	0.014	0.970
	VDR_ht3	191 (0.59±0.13)	117 (0.56±0.1)	12 (0.61±0.14)	0.042	0.017	0.624
Shaft	*−4817G>A*	129 (1.01±0.19)	90 (0.96±0.16)	10 (0.98±0.21)	0.012	0.020	0.133
	VDR_ht3	130 (1.01±0.19)	89 (0.96±0.16)	10 (0.98±0.21)	0.014	0.024	0.039
Total femur	*−4817G>A*	189 (0.8±0.14)	119 (0.76±0.13)	12 (0.81±0.16)	0.030	0.016	0.783
	VDR_ht3	191 (0.8±0.14)	117 (0.77±0.13)	12 (0.81±0.16)	0.034	0.019	0.477

BMD values and p-values for regression analyses of three alternative models (additive, dominant, and recessive) controlling for age, weight, height, and years since menopause, as covariates, are shown.

*C/C, C/R, and R/R represent homozygotes for the common allele, and heterozygotes and homozygotes for the rare allele, respectively.

† *Pa, Pb* and *Pc* are p values of additive, dominant, and recessive models for multiple regression analysis, respectively.

‡ Means±standard deviations of BMD (g/cm^2).

Discussion

In the present study, we closely examined *VDR* gene region polymorphisms for the association of the genetic variations with fracture risk and quantitative traits (BMD values). In this regard, the *VDR* polymorphisms are expected to provide more extensive genetic information for osteoporosis or its related fracture, compared with previous standardized testing such as RFLP based on only four common endonuclease, *Apa* I (rs17879735), *Bsm* I (1544410), *Fok* I (17881966), and *Taq* I (rs1788009). *VDR*, an intracellular receptor that specifically binds the active form of vitamin D (1,25-dihydroxyvitamin D3 or calcitriol) and interacts with target-cell nuclei to produce a variety of biologic effects, contains 11 exons and spans over 41.2 kb on chromosome 12q13.11 (Baker *et al*., 1988).

When haplotype LD was analyzed based on the individual genotype information of selected SNPs, *VDR-ht3* was significantly associated with all fracture risk (Table 3). Among SNPs of VDR-ht3, the minor allele of *−4817G>A* was also associated with protective effect of fracture. Interestingly, the effects of VDR *−4817G>A* on quantitative BMD of the femoral neck was allele-dose dependent; the highest BMD being found in homozygotes for the common allele (0.7±0.14), intermediate BMD in heterozygotes (0.68±0.12), and the lowest BMD in homozygotes for the rare allele (0.67±

0.11) (Table 4). In addition, both the *−4817G>A* and *VDR-ht3* comprising risk allele also showed the evidence of association with higher BMD values of various proximal femur sites.

In our results, the *VDR* polymorphisms are associated with higher BMD of proximal femur sites as well as femoral neck, but not with that of the lumbar spine. Since the femoral shaft, trochanter and total femur have higher cortical bone content that the lumbar spine, these polymorphisms may influence cortical bone strength. Accordingly, BMD values at femur neck predict fractures better than those at vertebral BMD. Because the mean age of our subjects was 58.7 years, these results may have been due to osteoarthritis, degenerative changes such as bony spurs, or aortic calcification which occur more often in the elderly individuals, and therefore may have falsely changed BMD measured by dual energy X-ray absorptiometry (Burger *et al*., 1996). Although multiple environmental factors are involved in the pathogenesis of osteoporosis, genetic factors are also largely responsible for bone mass, accounting for about 50-85% of the variance in BMD on the basis of twin and family studies (Arden and Spector, 1997; Gueguen *et al*., 1995; Ng *et al*., 2006). However, the heritability of fractures has been estimated to lie only between 25 and 35% (Deng *et al*., 2000; MacGregor *et al*., 2000), which is much lower than the heritability of BMD values, many fall-related factors other than BMD must contribute to fracture risk.

The genetic effects of VDR polymorphisms on BMD are not dramatic. Because the associated p-values did not retain statistical significance for multiple comparisons. If Bonferroni correction were strictly adopted, associated p-values could not retain all significances. However, although there is a chance of type I error due to multiple comparisons, when considering the facts that consistent positive signals at the same sites (*−4817G>A* and *VDR-ht* 3) with various bone-related phenotypes, the significance of associations might be reasonable. Nevertheless, it might be worthwhile to follow up on the effects of this important common gene with larger cohort studies, considering its vital role in bone mineral metabolism. Further biological and functional evidence would be needed to confirm the suggestive association of *VDR* variants with bone-related phenotypes.

After a multitude of studies, several standardized variations by RFLP have been investigated for associations with fracture and BMD, but study results are inconsistent (Dvomyk *et al*., 2004; Horst-Sikorska *et al*., 2007; Kiel *et al*., 2007; Long *et al*., 2004; Uitterlinden *et al*., 2006). have performed a large-scale meta-analysis of studies from prospective multicenter to investigate the effect on fracture incidence of four restriction-fragment-length polymorphisms (*Fok* I, *Bsm* I, *Apa* I, and *Taq* I). These polymorphisms were not associated with lumbar spine or femoral neck BMD. However, these results are inconsistent with our investigations carried out for Korean menopausal women. This is probably due to the fact that the study design did not examine all *VDR* polymorphisms, contribute more to identify genetic risk factors. Our results suggest that *VDR* polymorphisms and haplotypes provide more genetic information on osteoporosis risk.

In conclusion, we identified 18 polymorphisms of VDR in Korean population, found that *−4817G>A* (rs17882443) and *VDR-ht3* were significantly associated with enhanced BMD and fracture risk protection as genetic factor. Our observations suggest that these sequence-based variants are candidates for genetic determinants of BMD of the hip in postmenopausal women.

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