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FokI and BsmI Polymorphisms of the Vitamin D Receptor Gene and Bone Mineral Density in a Random Bulgarian Population Sample

Jivka Ivanova,[1] Polet Doukova,[1] Mihail Boyanov,[2] and Plamen Popivanov[2]

[1]Department of Clinical Laboratory and Clinical Immunology; and [2]Endocrinology Clinic, Alexandrovska Hospital, Medical University Sofia, Sofia, Bulgaria

Numerous studies on vitamin D receptor (*VDR*) gene polymorphisms differ with conflicting data in various populations. We studied the association of FokI and BsmI polymorphisms in the gene encoding the vitamin D receptor with bone mineral density (BMD) in 219 persons of Bulgarian nationality. The calculated relative risk (RR) for low bone mineral density is higher for FokI marker (3.14) compared to BsmI marker (2.44). The etiological factor (EF), which shows association between polymorphisms investigated and illness on populational level, is defined as EF = 0.51 for FokI marker and EF = 0.42 for BsmI marker. Because of this we conclude that FokI and BsmI polymorphisms are closely related to low BMD at the forearm and lumbar spine. Both polymorphisms are useful genetic markers in determining BMD and osteoporosis risk. Further studies of larger cohorts and in ethnically diverse subgroups are necessary.

Key Words: Osteoporosis; vitamin D receptor; bone mineral density; polymorphisms; FokI and BsmI.

Introduction

BMD is a major determinant of fracture risk and has an important genetic background. The first gene of great interest was the *VDR* gene *(1,2)*. An area of discussion over the last few years is the potential implication of several *VDR* polymorphisms not only with BMD determination, pathophysiology of osteoporosis, primary and secondary hyperparathyroidism, and breast *(3,4)* and prostate carcinomas *(5–7)*, but also with some immunologically based diseases such as diabetes mellitus *(8,9)* and osteoarthritis *(10–12)*.

The *VDR* gene is localized on chromosome 12q13-14 in a region that contains other genes of interest to bone molecular biology *(13)*. The collagen type2α1 (*COL2A1*) gene is localized very close to the *VDR* gene and the 1α-hydroxylase gene is localized proximal to *VDR* and *COL2A1 (14)*.

The *VDR* gene has at least 11 exons and spans 60 kb *(15, 16)*. Several polymorphic variants described so far include a cluster of linked sites near or in exon 9 (BsmI, ApaI, TaqI, and Long/Short polyA track) and a FokI site in exon 2, containing the initiation codon *(16)*.

In 1992 Morrison et al. found an association between bone turnover and several polymorphisms at the 3' end of the VDR gene defined by the restriction enzymes BsmI, ApaI, and TaqI *(17)*. Two years later they reported a close association between *VDR*-BsmI genotype BB and low BMD in a twin study *(2)*.

In 1996 another locus of the *VDR* gene was reportedly associated with BMD. Gross et al. identified an initiation start codon polymorphism at the 5' end of the *VDR* gene associated with an individual's risk for osteoporosis *(18)*. These polymorphisms defined by the restriction enzyme FokI showed a translation product of *VDR* with a difference in length of three amino acids depending on the allelic variants *(19)*.

Because of the initial data for an association of these *VDR* polymorphisms with BMD, many additional studies on different populations have shown controversial conclusions and some investigators have found an association of these polymorphisms with BMD *(2,17–36)* while others have not *(37–40)*.

The first described polymorphic region is located between exon VIII and 3' noncoding region and was found for the restriction enzyme BsmI in intron VIII at 280 bp from the 5' start of the intron without amino acid change but disappearance of the restriction site for BsmI. BsmI cuts the b allele of the *VDR* gene but not the B allele. In 1992 Morrison et al. first described an important influence of this *VDR* polymorphism on mean serum osteocalcin levels in a group of unrelated Australians with the highest osteocalcin levels in the homozygous BB genotype *(17)*.

The human VDR DNA presents two potential translation initiation (ATG) codons in exon II *(18)*. A T/C polymorphism (ATG to ACG) has been shown at the first ATG,

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Author to whom all correspondence and reprint requests should be addressed: M. Boyanov, MD, PhD, Endocrinology Clinic, Alexandrovska Hospital, 1 G. Sofiiski str., Sofia 1431, Bulgaria (Europe). E-mail: boyanov@sun.medun.acad.bg

Table 1
Distribution of Genotypes and Alleles by FokI

FokI	Controls			Cases		
Genotype	FF	Ff	ff	FF	Ff	ff
Number	53	46	3	30	50	37
Frequency	0.52	0.45	0.03	0.25	0.43	0.32
H_0		0.38			0.50	
χ^2			34.57			
p			$p < 0.05$ df = 2			
PIC			0.31			
Alleles	F		f	F		f
Number	129		29	85		99
Frequency	0.75		0.25	0.47		0.53
χ^2			34.29			
p			$p < 0.05$ df = 1			

which is referred as a start codon polymorphism (SCP). The *VDR* allele with the T variant encodes two potential ATG initiation codons and while the translation of the *VDR* mRNA from this allele can initiate from the first AUG, the translation of mRNA from the allele with the C variant must initiate from the second AUG. Initiation of translation from the second AUG shortens the *VDR* by three amino acids *(33)*. Such a difference in *VDR* primary structure might contribute to altered receptor function in contrast to the silent polymorphism in intron VIII and exon IX. In the study of Arai et al., they report that the shorter form of the *VDR* showed a 1.7-fold greater transcriptional activation in HeLa cells than the longer form *(32)*. The restriction endonuclease FokI can detect the SCP *(18,19,36)*. *VDR* alleles with the first ATG are denoted by f (longer form of 427aa, named M1) and *VDR* alleles without the first ATG by F (shorter form of 424aa, named M4). The shorter form F or M4 is present in 65% of VDR alleles in humans that probably gives an evolutional advantage.

In this study we examined the relation of these VDR gene polymorphisms with BMD in a random population sample of 219 Bulgarians.

Results

FokI Marker

The distribution of genotypes and alleles by FokI in the subgroups of cases and controls is shown in Table 1. Allelic and genotype frequencies were calculated in cases (with low BMD and/or osteoporosis) and in controls (normal BMD), and in the homo- and heterozygotes (see Table 1). The results are statistically significant after χ^2-test ($p < 0.05$).

When compared with genotype frequencies in cases with low BMD and in controls, more common are ff in cases (32%) than in controls (3%). Less common FF are in cases (25%) compared with controls (52%). The frequency of heterozygotes is higher in controls (45%) than in cases (43%).

Table 2
Forearm and Lumbar Spine
BMD in the Different Genotypes by FokI

		Cases		
Genotype		FF	Ff	ff
Age (yr)		48.7 ± 1.0	51.3 ± 1.2	49.3 ± 0.9
Years since menopause		2.3	2.2	1.8
BMD	Distal site	0.451	0.434	0.381
(g/cm^2)	Ultradistal	0.375	0.335	0.300
Z %		86.0	81.9	80.1
T %		85.1	78.2	75.0
BMD L_1–L_4 (g/cm^2)		0.760	0.754	0.703
Z %		80.0	77.0	75.0
T %		73.0	71.0	67.0
		Controls		
Age (yr)		47.7 ± 1.0	49.3 ± 1.2	49.8 ± 1.0
Years since menopause		2.5	1.2	1.5
BMD	Distal site	0.542	0.527	0.516
(g/cm^2)	Ultradistal	0.471	0.413	0.399
Z %		103.4	101.8	100.5
T %		100.5	98.9	98.1
BMD L_1–L_4 (g/cm^2)		0.876	0.868	0.861
Z %		99	97	96
T %		92	91	90

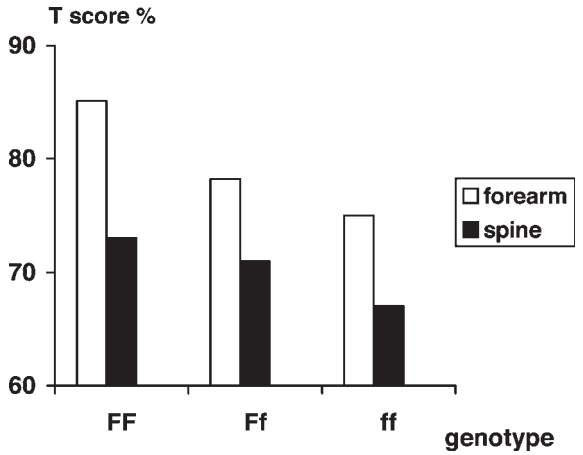

Fig. 1. Comparison of *T*-scores in percentage of the mean BMD in young healthy premenopausal adults at the distal forearm and the lumbar spine (L1–L4) in cases for different genotypes by FokI.

The statistical significance of these results was defined after χ^2-test (Table 1). p was in the limits of statistical significance ($p < 0.05$).

The correlation between the FokI genotype and BMD at the forearm site is shown in Table 2 and Fig. 1. Higher BMD was found in FF individuals and lower in ff individuals. The correlation remained unchanged when introducing *T*-scores (see Fig. 1).

Table 3
Distribution of Genotypes and Alleles by BsmI

BsmI	Controls			Cases		
Genotype	BB	Bb	bb	BB	Bb	bb
Number	6	45	51	39	44	34
Frequency	0.06	0.44	0.50	0.33	0.38	0.29
H_0		0.40			0.5	
χ^2			26.71			
p			$p < 0.05$ df = 2			
PIC			0.32			
Alleles	B		b	B		b
Number	34		125	100		90
Frequency	0.28		0.72	0.52		0.48
χ^2			26.40			
p			$p < 0.05$ df = 1			

The relationship between the different genotypes FokI and BMD at the lumbar spine is shown in Table 2. The relationship between lumbar spine *T*-scores and the genotype FokI is similar as that with forearm *T*-scores (see Fig. 1).

BsmI Marker

The distribution of genotypes and alleles by BsmI in the subgroups of cases and controls is shown in Table 3. Table 3 summarizes allelic and genotype frequencies by BsmI as defined in cases (with low BMD and/or osteoporosis) and in controls, and in the homo- and heterozygotes. The results were statistically significant after χ^2-test ($p < 0.05$).

The BB genotype was more common in cases with low BMD and/or osteoporosis (33%) vs 6% in controls with normal BMD and inversely the bb genotype was less common in cases (29%) than in controls (50%) (Table 3). The heterozygotes Bb were more common in controls (44%) compared with cases (38%). Statistical significance of these results as defined from χ^2-test was within the limits ($p < 0.05$).

The correlation between the type of genotype and BMD at the distal forearm is shown in Table 4. The introduction of *T*-scores did not change the correlation (Fig. 2).

The relationship between these genotypes and lumbar spine BMD is shown in Table 4. The relationship between lumbar spine BMD and the genotype is similar as that with BMD measured at forearm sites. Lumbar spine *T*-scores are higher in bb and lower in BB individuals (Fig. 2).

The relative risk (RR) for low BMD and/or osteoporosis in presence or absence of a given marker was calculated as follows: for the FokI marker, RR = 3.14; for the BsmI marker, RR = 2.44. The RR for low BMD and osteoporosis is higher for FokI vs BsmI. The risk of low BMD is higher for f carriers than for B carriers.

The EF showing what part of the illness is attributable to the associated factor on a population level was calculated as follows: for the FokI marker EF = 0.51; for the BsmI marker EF = 0.42. The EF for both markers shows that most of the illness is associated with FokI (51%) than with BsmI (42%). This represents the FokI marker as more informative.

Table 4
Forearm and Lumbar Spine
BMD in the Different Genotypes by BsmI

		Cases		
Genotype		BB	Bb	bb
Age (yr)		50.7 ± 0.9	50.2 ± 1.0	49.3 ± 1.3
Years since menopause		3.5	3.8	2.0
BMD	Distal	0.410	0.416	0.427
(g/cm^2)	Ultra	0.309	0.315	0.322
Z %		76.5	81.5	85.2
T %		72	79.5	81.1
BMD L_1–L_4 (g/cm^2)		0.790	0.822	0.839
Z %		83.0	86.0	88.0
T %		75.0	77.0	81.0
		Controls		
Age (yr)		49.7 ± 0.9	48.2 ± 0.8	48.3 ± 1.2
Years since menopause		2.5	2.4	1.5
BMD	Distal site	0.469	0.557	0.565
(g/cm^2)	Ultradistal	0.354	0.424	0.436
Z %		98	101.5	103.6
T %		92.5	97.4	99.9
BMD L_1–L_4 (g/cm^2)		0.877	0.879	0.883
Z %		96.5	96.5	98.5
T %		92.5	92.5	93.0

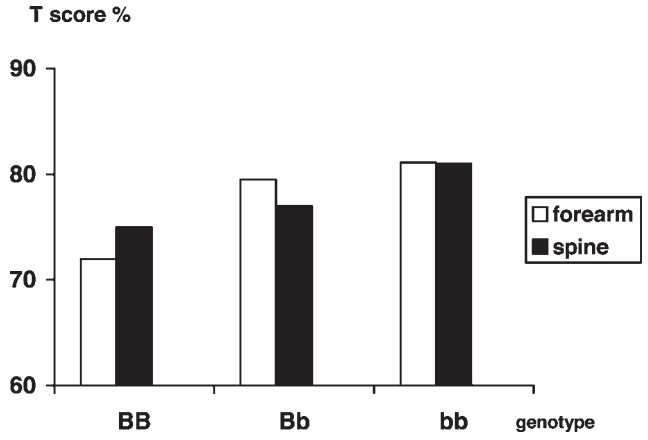

Fig. 2. Comparison of *T*-scores in percentage of the mean BMD in young healthy premenopausal adults at the distal forearm and the lumbar spine (L1–L4) in cases for different genotypes by BsmI.

Discussion

The *VDR* gene is known as a candidate gene determining a part of the genetic basis of osteoporosis *(21–28)*. While the other studied polymorphisms do not alter the sequence of the *VDR* protein, the FokI polymorphism encodes alternate

proteins that differ in length by three amino acids *(26,36)*. That probably influences the function of *VDR* and contributes to the differences in BMD. The BsmI intronic polymorphism could be relevant for translational differences of the *VDR* (a "functional polymorphism," which is rather unlikely) or might act in strong linkage disequilibrium with a putative functional one in the *VDR* or a nearby gene *(38)*.

The genotype frequencies of controls ($n = 102$) from this study are 52% for FF, 45% for Ff, and 3% for ff. The genotype frequencies of cases ($n = 117$) are 25% for FF, 43% for Ff, and 32% for ff (see Table 1). The compared groups of Bulgarian and European and Asian populations differ by distribution of allelic frequencies. We compared these frequencies with published genotype frequencies for European populations *(21,28)*: 28–48% for FF, 41–58% for Ff, and 6–16% for ff; and with genotype frequencies for Asian populations *(32,34)*: 36–37% for FF, 49–51% for Ff, and 12–15% for ff (defined in menopausal women). Our data do not significantly differ from data published by Gross et al. regarding the association of the genotype with low BMD *(18)*. In their group ($n = 100$) genotype frequencies differed from that of our group and were 37% for FF, 48% for Ff, and 15% for ff and the differences are most significant for *ff (18)*.

The genotype frequencies of controls ($n = 102$) from this study are 6% for BB, 44% for Bb, and 50% for bb. The genotype frequencies of cases ($n = 117$) are 33% for BB, 38% for Bb, and 29% for bb (Table 3). The observed allelic frequencies in our study population did not differ significantly from data in European populations *(21,28)*. Comparing Bulgarian and Asiatic populations, the differences were significant *(32,34)*. The frequencies were compared with published genotype frequencies for European populations *(21,28)*: 12–25% for BB, 39–72% for Bb, and 16–48% for bb; and in Asian populations *(32,34)*: 0–1% for BB, 10–22% for Bb, and 77–90% for bb defined in groups of menopausal women). Our data did not differ from data published for European populations, but they differed significantly from those published for Asian populations.

Our BMD data confirm the results from other population studies. In line with other investigators we observed higher BMD in FF individuals and lower BMD in ff individuals *(26,28,29)*. The same trend was found with higher BMD in bb individuals and lower values in BB individuals.

In the group of 219 unrelated individuals, these with genotype ff (3% of the controls and 32% of the cases) have 10% lower BMD at the forearm than the subjects with FF (52% of the controls and 25% of the cases). The heterozygotes Ff (45% of the controls and 43% of the cases) have an intermediate BMD. The association between BMD and the genotype was also confirmed at the lumbar spine. In our population the reduction in lumbar spine BMD was more substantial than that in forearm BMD (–33% vs –25% at the forearm in ff and –27% vs –15% in FF).

In the group of 219 unrelated individuals, those with BB genotype (6% of the controls and 33% of the cases) have 9.0% lower BMD at the forearm than subjects with bb (50% of the controls and 29% of the cases). The heterozygotes Bb (44% of the controls and 38% of the cases) have an intermediate BMD. The association of BMD with the genotype was confirmed also for the lumbar spine and the reduction in BMD here is less than that at the distal forearm (–25% vs –28% at the forearm in BB and –19% vs –19% in bb).

Our study has some limitations: (1) the small number of individuals examined and (2) the restricted ethnicity of the group. Attempting to stress similarities between the ff genotype with low BMD and the findings of other authors must be viewed cautiously as there may be major differences in the populations being studied.

In conclusion, this is a pilot study examining the prevalence of two *VDR* genotype polymorphisms and their association with forearm and lumbar spine BMD. We were able to show that the prevalence of the polymorphisms under study was similar to that in the typical European population and different from that in an Asian population. The impact of the different genotypes on BMD was substantial both at the forearm and the lumbar spine. Our data underscore the potential benefit of screening subjects at risk for osteoporosis for their genetic predisposition.

Materials and Methods

Subjects

Two hundred and nineteen unrelated individuals of Bulgarian origin — 198 women and 21 men — were recruited for the study. Diseases and medications known to affect bone metabolism were used as exclusion criteria. All subjects were grouped according to their lumbar spine BMD. The 117 cases had low lumbar spine bone density (T-score ≤ -1.0) and the 102 controls had normal BMD (T-score > -1.0). The age of the participants ranged between 36 and 56 yr in cases and between 34 and 58 yr in controls. Mean age, years since menopause, and BMD data of the subgroups are shown in Tables 2 and 4. All participants gave their informed consent. This work has been approved by the responsible authorities at the Alexandrovska Hospital.

Bone Densitometry

BMD was measured at the distal forearm by single-energy X-ray absorptiometry (SXA) on a DTX-100 Unit (Osteometer Meditech, USA) and at the lumbar spine (L1–L4) by dual-energy X-ray absorptiometry (DXA) on a Hologic QDR 4500 A device (Hologic Inc., Bedford, MA, USA).

On the DTX-100 the distal region of interest begins at the 8 mm separation point between radius and ulna and then continues proximally for a distance of 24 mm. The ultra-distal site extends from the radial endplate proximally to the 8 mm point. BMD was measured according to the

manufacturer's instructions in g/cm^2 separately for the distal (including radius and ulna) and the ultra-distal site (including only the radius). *Z*-scores were calculated automatically based on the manufacturer's Danish database (issued 1994) *(41)*.

BMD of the lumbar spine in the posterior–anterior (PA) projection was measured on a Hologic QDR 4500 A densitometer, with software version 8.26:3 (Hologic, Inc., Waltham, MA, USA). BMD L1–L4 was expressed in g/cm^2 and additionally in terms of *T*- and age-matched *Z*-scores in percent. The manufacturer's American reference database was used (issued 1991) *(42)*.

Standardization was performed daily by scanning a Hologic anthropomorphic phantom (for DXA) and a manufacturer-supplied forearm phantom (for SXA).

Genotyping

DNA was isolated from whole blood. Primers and PCR conditions for amplifying exon 2 of the VDR gene were designed accordingly to Gross et al. *(18)*. The region of genomic DNA containing the BsmI polymorphic site in intron 8 was amplified as described by Ingles et al. *(6)*.

dATP, dCTP, dTTP, dGTP, 1.25 m*M* each, were used to amplify exon 2 and intron 8 with Taq DNA Polymerase. One hundred nanograms of the DNA were used as template in the PCR reactions.

Primers (2a and 2b) flanking exon 2 were used to amplify a 265 bp PCR product that is then digested with FokI. Digestion of the PCR product with FokI generates two fragments of 196 bp and 69 bp. Individuals homozygous for the FF genotype have a single uncut 265 bp fragment, while homozygous for the ff genotype have two fragments of 196 bp and 69 bp. The heterozygotes Ff have all three bands.

The polymorphic region was located in intron 8 at 280 bp from the 5' start of the intron without amino acid change but disappearance of the restriction site for BsmI. BsmI cuts the b allele of the *VDR* gene but not the B allele. The primers (U and L) were used to amplify 821 bp PCR product, which was then digested with BsmI. Digestion of the PCR product with BsmI generates two fragments of 650 bp and 175 bp. Individuals homozygous for the BB genotype have a single uncut 821 bp fragment, while homozygous for the bb genotype have two fragments of 650 bp and 175 bp. The heterozygotes Bb have all three bands.

PCR products were digested with FokI and BsmI for 4 h and electrophoresed through a 2% agarose gel. Individuals were scored as FF, Ff, ff and BB, Bb, bb according to the digestion pattern.

The observed allelic frequencies in controls and cases were compared with published allelic frequencies in European populations *(21,28)* as follows: 60–69% for F allele and 31–40% for f allele, 37–45% for B allele, and 52–67% for b allele; and with published allelic frequencies in Asian populations *(32,34)*: 62% for F allele and 38% for f allele, and 5–12% for B allele and 88–95% for b allele, normative for menopausal women.

Statistical Analysis

Data were evaluated by χ^2-test and presented as means ± SD. The relative risk (RR) to have low bone density if carrier of a specific marker was defined as:

$$RR = \frac{a \times d}{b \times c}$$

where a is the number of carriers among the cases, b is the number of not carriers among the cases, c is the number of carriers among the controls, d is the number of not carriers among the controls, as shown below:

Number	Carriers	Not carriers
Cases	a	b
Controls	c	d

The etiological factor (EF) showing what part of the illness is attributable to the associated factor on a population level was defined as:

$$EF = \frac{RR - 1}{RR} = \frac{a}{a + b}$$

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