Physiol. Res. 51: 501-509, 2002

Vitamin D Receptor Gene Polymorphisms, Bone Mineral Density and Bone Turnover: *Fok*I Genotype is Related to Postmenopausal Bone Mass

K. ZAJÍČKOVÁ[1], I. ŽOFKOVÁ[1], R. BAHBOUH[2] , A. KŘEPELOVÁ[3]

[1]Institute of Endocrinology, [2]Faculty of Philosophy and Department of [3]Biology and Genetics, First Faculty of Medicine, Charles University, Prague, Czech Republic

Received November 5, 2001
Accepted January 28, 2002

Summary

The relationship between vitamin D receptor (VDR) intragenic polymorphisms *Fok*I, *Bsm*I, *Apa*I and *Taq*I and bone mineral density (BMD) or biochemical markers of bone remodeling were investigated in 114 Czech postmenopausal women, on the average 62.5±8.9 years of age. Restriction fragment length polymorphisms in the VDR gene were assessed by PCR amplification and digestion with restriction enzymes *Fok*I, *Bsm*I, *Apa*I, and *Taq*I recognizing polymorphic sites in the VDR locus. Bone mineral density was measured at the lumbar spine and at the hip by dual-energy X-ray absorptiometry (DEXA, g/cm^2). After adjusting for age and the body mass index (BMI), subjects with the ff genotype had 9.4 % lower BMD at the hip than those with the Ff genotype (p=0.0459, Tukey´s test). FF individuals had an intermediate BMD at the hip. A similar pattern of lower lumbar spine BMD was also found in ff individuals, but it did not reach statistical significance. There was no relationship between *Bsm*I, *Apa*I and *Taq*I VDR polymorphisms and BMD at any skeletal site. Subjects with Aa (*Apa*I) genotypes had higher levels of propeptide of type I collagen (PICP) than homozygous AA (p=0.0459, Tukey´s test). In *Fok*I, *Bsm*I and *Taq*I restriction sites the biochemical markers of bone remodeling did not differ by genotype. In addition, no significant difference was observed in VDR genotypic distribution between osteoporotic women and non-osteoporotic controls in the study group. To conclude, the *Fok*I genotype of the vitamin D receptor gene is related to bone mass at the hip in Czech postmenopausal women, whereas the importance of remaining VDR genotypes was not evident.

Key words

Vitamin D receptor gene • *Fok*I polymorphism • Bone mineral density • Bone remodeling • Postmenopausal women

Introduction

Twin and family studies have suggested that bone mineral density and age-related bone loss have strong genetic deteminants (Pocock *et al*. 1987, Seeman *et al*. 1989, Spector *et al*. 1995).

The vitamin D receptor gene has been implicated as a factor affecting bone mass (Morrison *et al.* 1994). However, associations of VDR genotypes to bone mineral density in different populations have brought contradictory results (Morrison *et al*. 1994, Fleet *et al*. 1995, Kröger *et al*. 1995, Riggs *et al*. 1995, Jørgensen *et*

Fax+4202 24920590
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

al. 1996, Garnero *et al*. 1996, Uitterlinden *et al*. 1996, Hansen *et al*. 1998).

The aim of this study was to analyze relations between *Fok*I, *Bsm*I, *Apa*I, and *Taq*I VDR polymorphisms and bone mass or biochemical markers of bone remodeling in a cohort of 114 Czech postmenopausal women of Caucasian origin.

Methods

Subjects

Hundred and fourteen postmenopausal women were included in the study. Of these, 33 were healthy volunteers (mean age ± S.D. 60.1±10.3 years), while 65 were osteoporotic (T score < –2.5, i.e. more than 2.5 S.D. below the peak bone mass value in the young adult population), but otherwise normal (mean age 63.6±7.8 years), and 16 women were osteopenic (T score < –2.0) (mean age 63.4±9.8 years). All three subgroups of women were homogeneous for BMI (27.3±3.5, 24.7±2.9 and 27.2±4.4) and for years since menopause (10.8±7.6, 14.7±8.6 and 13.6±7.3, respectively). None of the investigated women had a history of early or late menarche or premature menopause (before 45 years of age). Women with markedly irregular menstrual periods or with clearly undefined menopausal status were not included in the study. The current and prior menstrual history of healthy, osteoporotic and osteopenic subjects was regular (11-13 cycles/year). The study group did not include any women with endocrinopathy, other serious internal disease or psychosis, alcoholics or severe smokers. No women were markedly underweight or obese and they all had normal calcium, caloric, and protein intake. The daily life of the probands comprised usual physical activity. None of the women was treated with either calciotropic drugs or estrogen replacement therapy. Informed consent was obtained from all of the women, and all procedures were approved by the Ethical Commitee of the Institute of Endocrinology, Prague.

Protocol

Blood for the measurement of alkaline phosphatase (ALP), osteocalcin, carboxy-terminal propeptide of type I procollagen (PICP) and β_2 microglobulin levels was withdrawn in the morning, after overnight fasting. Samples were stored at –80 °C until analyzed. A fasting sample of the second morning urine was collected from all women for the measurement of free deoxypyridinoline (DPYD) concentrations. The values of urinary DPYD were expressed in nmol/mmol of urinary creatinine.

Analytical methods

Serum concentrations of ALP were assessed enzymatically using a Merck vitaLab-Eclipse (Germany) semi-automatic analyzer and Merck kit, osteocalcin by radioimmunoassay (RIA) using a CIS (France) kit, PICP by RIA kit from Orion Diagnostica (Finland) and β_2 microglobulin levels were estimated by RIA using a kit from Immunotech (Czech Republic). DPYD in urine was determined by Elisa kits (Metra Pyrilinks, USA). Urinary creatinine was estimated photometrically using an automatic analyzer (Merck VitaLab-Eclipse). Duplicate measurements were used for obtaining the mean values. The interassay coefficients of variation were as follows: 5.5 % for ALP, 14.0 % for osteocalcin, 6.6 % for PICP, 5.5 % for β_2 microglobulin, 4.0 % for DPYD and 6.0 % for creatinine.

VDR genotyping

Whole blood samples were collected in tubes containing EDTA and frozen at –20 °C before extraction. Genomic DNA was isolated from peripheral blood leukocytes by a standard chloroform:isoamylalcohol extraction procedure (Miler *et al.* 1988).

The *Fok*I polymorphic region of exon 2 was amplified by oligonucleotide primers that have been previously described (Gross *et al.* 1996, Harris *et al.* 1997). PCR amplification was carried out with *Taq* DNA Polymerase (Fermentas, Lithuania). PCR conditions were as follows: initial denaturation at 94 °C for 5 min, then 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 60 °C, and extension for 30 s at 72 °C. The reaction was terminated by 7 min elongation at 72 °C. PCR products were digested with restriction enzyme *BseG*I (Fermentas, Lithuania), an isoschizomer of the *Fok*I enzyme, at 55 °C for 120 min. Fragments were analyzed by electrophoresis through a 2 % agarose gel containing ethidium bromide, visualized and photographed. The presence of the *Fok*I restriction site on both alleles (defined as ff) generates 196 and 69 bp fragments, whereas the absence (FF) yields one undigested 265 bp fragment. Heterozygous Ff exhibits fragments of 265, 196, and 69 bp.

A single amplification was performed with primers spanning the *Apa*I and *Taq*I intragenic

polymorphisms. Sequences of primers were published previously (Riggs *et al.* 1995). After the first denaturation at 94 °C for 5 min, 30 cycles followed: 94 °C 1 min, 68 °C 1 min, 72 °C 1 min 30 s. The reaction was terminated with final elongation at 72 °C for 7 min.

PCR products (740 bp) were digested with both *Taq*I (Fermentas, Lithuania) at 65 °C for 90 min or *Bsp*120I (Fermentas, Lithuania), an isoschizomer of *Apa*I, at 37 °C for 120 min. Fragments were separated electrophoretically on 2 % agarose. According to the presence or absence of restriction site genotypes were identified as AA (one undigested PCR fragment of 740 bp), aa (520, 220 bp) and heterozygous Aa. *Taq*I digestion reveals one obligatory restriction site, the homozygous TT (absence of the specific *Taq*I restriction site) yields bands of 245 bp and 495 bp. The homozygous tt exhibits 205, 245, 290 bp and the heterozygous Tt provides 495, 205, 245, 290 bp fragments.

PCR amplification of the polymorphic *Bsm*I site was performed with primers producing an 825 bp fragment (Morrison *et al.* 1994). PCR conditions were identical as for *Apa*I and *Taq*I polymorphisms. PCR products were digested with *Mva1269*I (Fermentas, Lithuania), an isoschizomere of the restriction enzyme *Bsm*I at 37 °C overnight. Fragments were separated on 2 % agarose gel electrophoresis and genotypes BB (825 bp), bb (650, 175 bp) and Bb were identified.

Bone mineral densitometry

BMD (g/cm^2) at the hip and at the spine (L_1-L_4) were measured by dual-energy X-ray absorptiometry (DXA, Hologic ´QDR-2000´, USA). The precision of the method was 1 %.

Statistical analysis

The frequency distribution of VDR genotypes in the population was determined and evaluated using the χ^2-test. The relationship between various VDR genotypes and age- and BMI-adjusted BMD or biochemical markers of bone turnover were evaluated by ANOVA. If ANOVA was significant, differences in BMD and biochemical markers among the three genotype subgroups were tested by Tukey´s test. $p < 0.05$ was accepted as the value of significance.

Table 1. Distribution of VDR genotypes in osteoporotic and nonosteoporotic subjects. Osteopenic women (n=16) were excluded.

	Subjects		
VDR genotypes	**Osteoporotic (n=65)**	**Nonosteoporotic (n= 33)**	**All (n=98)**
FF	26 (40 %)	7 (21 %)	33 (34 %)
Ff	28 (43 %)	21 (64 %)	49 (50 %)
ff	11 (17 %)	5 (15 %)	16 (16 %)
BB	21 (32 %)	10 (30 %)	31 (32 %)
Bb	24 (37 %)	13 (39 %)	37 (38 %)
bb	20 (31 %)	10 (30 %)	30 (30 %)
AA	23 (35 %)	10 (30 %)	33 (34 %)
Aa	33 (51 %)	17 (52 %)	50 (51 %)
aa	9 (14 %)	6 (18 %)	15 (15 %)
TT	23 (35 %)	11 (33 %)	34 (35 %)
Tt	31 (48 %)	14 (42 %)	45 (46 %)
tt	11 (17 %)	8 (24 %)	19 (19 %)

*Fok*I *: Pearson´s* $\chi^2 = 4.187$ *df = 2 p = 0.1233, M-L* $\chi^2 = 4.308$ *df = 2 p = 0.116,*
*Bsm*I *: Pearson´s* $\chi^2 = 0.065$ *df = 2 p = 0.9810, M-L* $\chi^2 = 0.065$ *df = 2 p = 0.968*
*Apa*I *: Pearson´s* $\chi^2 = 0.439$ *df = 2 p = 0.8029, M-L* $\chi^2 = 0.435$ *df = 2 p = 0.805*
*Taq*I *: Pearson´s* $\chi^2 = 0.764$ *df = 2 p = 0.6826, M-L* $\chi^2 = 0.745$ *df = 2 p = 0.689*

Results

The distributions of *Fok*I, *Bsm*I, *Apa*I and *Taq*I restriction site genotypes in osteoporotic women and controls are shown in Table 1. To evaluate genotype distribution between both groups, 16 osteopenic women were excluded. The distributions of genotypes are similar to those previously described in Caucasian women in the *Fok*I, *Apa*I and *Taq*I restriction sites (Riggs *et al*. 1995, Gross *et al,* 1996, Jørgensen *et al*. 1996, Vandevyver *et al*. 1997*)*. After *Bsm*I digestion, we found a higher number of both homozygous genotypes (BB, bb) with a relative lack of heterozygous subjects (Bb) in comparison with other Caucasian populations (Morrison *et al*. 1992, Garnero *et al*. 1996, Hansen *et al*. 1998). The allele frequencies of *Bsm*I were marginally in Hardy-Weinberg equilibrium (p=0.053) (Khoury *et al*. 1993).

Table 2. Bone mineral density (BMD) and biochemical markers of bone turnover in relation to *Fok*I genotypes in the total studied population (n=114).

	***Fok*I**			
	FF (n=39)	**Ff (n=56)**	**ff (n=19)**	**p (ANOVA)**
BMD (g/cm^2)				
Lumbar spine L_1-L_4	0.739± 0.129	0.778± 0.164	0.660± 0.218	0.028
Total hip	0.856± 0.096	0.907± 0.126	0.822± 0.07	0.007
ALP (μkat/l)	0.97± 0.55	0.98± 0.53	1.30± 0.71	0.097
Osteocalcin (ng/ml)	20.7± 9.81	18.9± 6.21	20.4± 8.24	0.542
PICP (μg/l)	107.0± 31.23	97.2±41.98	93.5± 32.13	0.482
DPYD (nmol/mmol creatinine)	10.39± 1.96	11.49±5.13	10.31± 1.63	0.465
β_2 microglobulin (μg/ml)	1.10± 0.61	0.87± 0.87	0.9± 0.49	0.365

Data are expressed as means ± S.D.

Table 3. Bone mineral density (BMD) and biochemical markers of bone turnover in relation to *Bsm*I genotypes in the total study population (n=114).

	***Bsm*I**			
	BB (n=34)	**Bb (n=42)**	**bb (n=38)**	**p (ANOVA)**
BMD (g/cm^2)				
Lumbar spine L_1-L_4	0.743± 0.160	0.764± 0.146	0.724± 0.197	0.583
Total hip	0.882± 0.127	0.871± 0.109	0.874± 0.105	0.911
ALP (μkat/l)	0.96± 0.58	1.18± 0.63	0.94± 0.50	0.156
Osteocalcin (ng/ml)	19.72± 7.57	19.84± 9.13	19.64± 6.93	0.994
PICP (μg/l)	85.72± 43.41	110.14± 29.14	103.37± 35.08	0.056
DPYD (nmol/mmol creatinine)	10.33± 2.66	10.98± 2.91	11.37± 5.36	0.657
β_2 microglobulin (μg/ml)	0.88± 0.96	0.94± 0.73	1.03± 0.48	0.714

Data are expressed as means ± S.D.

Fok I genotypes revealed a relationship to age and BMI-adjusted BMD at the hip (p=0.007, ANOVA) and at the lumbar spine (p=0.028, ANOVA) (Table 2). Particularly, subjects with the ff genotype had significantly lower BMD at the hip than those with the Ff genotype (p=0.0459, Tukey´s test), respectively. The values of the hip BMD in ff women were 9.4 % lower than in Ff. A similar trend was observed at the lumbar spine but the difference between ff and Ff did not reach statistical significance (p=0.07, Tukey´s test). At both measured sites Ff subjects reached the highest values of BMD whereas FF individuals had an intermediate BMD.

The difference between both homozygous genotypes (FF vs. ff) was not significant at either site. *Apa*I, *Bsm*I and *Taq*I polymorphisms were not associated with BMD at the hip or at the lumbar spine (Tables 3-5).

*Apa*I genotypes demonstrated a relationship to PICP (p=0.036, ANOVA) and alkaline phosphatase (p=0.045, ANOVA) (Table 4). Women with heterozygous genotype Aa had PICP levels higher than homozygous AA (P=0.0459, Tukey´s test). The differences in alkaline phosphatase were not significant in particular genotype subgroups. A similar tendency approaching statistical significance occurred between PICP and *Bsm*I genotypes (p=0.056, ANOVA) (Table 3). Biochemical markers of bone formation and bone resorption did not differ in *Fok*I and *Taq*I genotype subgroups (Tables 2 and 5).

Table 4. Bone mineral density (BMD) and biochemical markers of bone turnover in relation to *Apa*I genotypes in the total study population (n=114).

	***Apa*I**			
	AA (n=36)	**Aa (n=57)**	**aa (n=21)**	**p (ANOVA)**
BMD (g/cm²)				
Lumbar spine L_1-L_4	0.738± 0.157	0.760± 0.146	0.713± 0.233	0.533
Total hip	0.879± 0.124	0.870± 0.108	0.883± 0.109	0.873
ALP (µkat/l)	0.95± 0.56	1.16± 0.62	0.80± 0.37	0.045
Osteocalcin (ng/ml)	19.34± 7.50	19.85± 8.19	20.13± 8.24	0.931
PICP (µg/l)	87.00± 41.90	111.22± 30.44	96.48± 36.77	0.036
DPYD (nmol/mmol creatinine)	10.41± 2.56	11.61± 4.93	10.09± 2.21	0.334
β_2 microglobulin (µg/ml)	0.85± 0.94	0.99± 0.67	1.03± 0.48	0.603

Data are expressed as means ± S.D.

Table 5. Bone mineral density (BMD) and biochemical markers of bone turnover in relation to *Taq*I genotypes in the total study population (n=114).

	***Taq*I**			
	TT (n=44)	**Tt (n=50)**	**tt (n=20)**	**p (ANOVA)**
BMD (g/cm²)				
Lumbar spine L_1-L_4	0.726± 0.188	0.749± 0.147	0.772± 0.173	0.583
Total hip	0.874± 0.101	0.867± 0.114	0.898± 0.132	0.588
ALP (µkat/l)	0.93± 0.48	1.14± 0.62	0.97± 0.65	0.231
Osteocalcin (ng/ml)	19.49± 7.06	20.23± 8.67	19.09± 8.03	0.838
PICP (µg/l)	104.87± 34.20	100.73± 33.48	90.64± 48.11	0.480
DPYD (nmol/mmol creatinine)	11.42± 5.17	11.20± 3.05	9.35± 1.73	0.234
β_2 microglobulin (µg/ml)	1.07± 0.50	0.94± 0.69	0.74± 1.14	0.276

Data are expressed as means ± S.D..

Discussion

In the present study, the *Bsm*I genotype distribution was different from that previously published in Caucasian women (Morrison *et al*. 1992, Kröger *et al*. 1995, Garnero *et al*. 1996, Jørgensen *et al*. 1996, Hansen *et al*. 1998). To avoid misclassification of genotypes due to technical problems, PCR amplification was carried out using the method of Morrison *et al*. (1994). For digestion we used *Mva1269*I, an isochizomer of *Bsm*I, and followed the manufacturer´s recommendations. The restriction pattern was verified several times. We failed to explain the distribution difference in *Bsm*I genotypes in the study population. In contrast to *Bsm*I, the genotype

distributions of *Fok*I, *Apa*I, and *Taq*I were comparable with other Caucasian women (Riggs *et al*. 1995, Gross *et al*. 1996, Jørgensen *et al*. 1996, Vandevyver *et al*. 1997).

*Fok*I polymorphism, resulting from the T/C transition in exon 2, creates a new start codon (ATG) at the VDR gene, thus changing protein sequence of vitamin D receptor in a length that may imply functional consequences (Baker *et al*. 1988). Arai *et al*. (1997) have reported that a shorter variant of VDR (F-VDR) exhibited greater transcriptional activity in transfected HeLa cells which may explain higher BMD in FF individuals. Conversely, Gross *et al*. (1998) did not find functional differences between F-VDR and f-VDR forms. Thus, the molecular mechanism of the start codon polymorphism role remains unclear. Nevertheless, the hypothesis that different allelic variants of VDR affect calcium homeostasis has led to considerable interest in the association between *Fok*I genotypes and BMD (Gross *et al*. 1996, Harris *et al*. 1997, Ferrari *et al*. 1998a, Gennari *et al*. 1999, Lucotte *et al*. 1999, Langdahl *et al*. 2000). However, a clear consensus has not been found.

After adjustment for age, weight and height in the studied population, we found that ff genotype is associated with significantly lower BMD at the hip. Our results support those of Lucotte *et al.* (1999) who demonstrated that ff genotype correlates with decreased BMD at the femoral neck in French postmenopausal women. Significantly lower femoral neck BMD was also found in ff premenopausal women (Harris *et al*. 1997). Although we found a weak association, our results are rather in agreement with Gross *et al.* (1996) and Gennari *et al.* (1999) who reported that ff genotype correlates with lower BMD at the lumbar spine in postmenopausal women. In contrast with the findings of Gennari *et al.* (1999), a higher prevalence of ff genotype in osteoporotic women than in non-osteoporotic controls was not observed in the present study. Our results are also in conflict with other findings (Eccleshall *et al*. 1998, Ferrari *et al*. 1998a, Langdahl *et al*. 2000). Ferrari *et al*. (1998a) reported that *Fok*I genotypes are related to BMD only in combinations with 3´-end polymorphisms – *Bsm*I, *Apa*I and *Taq*I. Although in the present study BMD at the lumbar spine and the hip differs significantly in *Fok*I genotypes, an allelic dose-response is missing. As FF subjects reached the intermediate levels of BMD at either site, we did not observe a progressive increase in BMD associated with the F allele. This finding, reported also in other studies (Fleet *et al*. 1995, Langdahl *et al*. 2000), makes the molecular explanation difficult.

We failed to demonstrate that *Bsm*I, *Apa*I and *Taq*I polymorphic variants in the VDR locus have a major impact on bone mass in our study group of postmenopausal women. This contrasts with previous reports showing that B and t alleles are associated with low BMD (Morrison *et al*. 1994, Fleet *et al*. 1995, Riggs *et al*. 1995, Gennari *et al*. 1998, Langdahl *et al*. 2000) or accelerated postmenopausal bone loss (Krall *et al*. 1995, Kikuchi *et al*. 1999). Our results are, however, consistent with other findings (Kröger *et al*. 1995, Garnero *et al*. 1996, Jørgensen *et al*. 1996, Uitterlinden *et al*. 1996, Hansen *et al*. 1998, Aerssens *et al*. 2000).

Several hypotheses have been reported to explain conflicting results between VDR genotypes and BMD in different populations, such as linkage disequilibrium with functionally relevant genetic variants and environmental factors modifying the genotype effect on BMD (Dawson-Hughes *et al*. 1995, Krall *et al*. 1995, Ferrari *et al*.1998b).

As BMD is under polygenic control, other candidate genes have been tested, as well as their gene-by-gene interactions, for their potential relation to bone mass. Deng *et al*. (1998) have found positive correlations between VDR and estrogen receptor (ER) genotypes and bone loss and bone response to hormone replacement therapy in postmenopausal women. Willing *et al*. (1998) have confirmed that *Bsm*I polymorphism is related to BMD only in combination with allelic variations in the ER locus. Interactions with other candidate genes and with the environment might bring further insights into the complex pathophysiology of a polygenic disease such as osteoporosis.

Although *Apa*I polymorphism was not related to BMD at any skeletal site, we found a relation between the heterozygous Aa genotype and PICP or alkaline phosphatase levels. This documents a regulatory role of this gene in bone formation. *Fok*I, *Bsm*I and *Taq*I genotypes were not related to markers of bone remodeling in postmenopausal women which contrasts with the report of Morrison *et al*. (1992), but it is in agreement with other authors (Garnero *et al*. 1996, Gross *et al*. 1996, Hansen *et al*. 1998).

The relation between VDR polymorphisms and biochemical markers of bone resorption was also investigated. Neither circulating β_2 microglobulin (Ripoll *et al*. 1996, Žofková *et al*. 1999) nor urinary DPYD differ in the VDR genotype subgroups.

The present study has several limitations. First, it was conducted on a limited sample of postmenopausal women of Caucasian origin , so that our findings cannot

be generalized to other races, gender or different age subgroups. Second, although the onset of menopause was comparable, the adjustment of bone mineral density according to the duration of menopause rather than to age would be more appropriate for ANOVA analysis.

To conclude, the current study found that *Fok*I polymorphism at the vitamin D receptor gene may contribute to decreased bone mass at the hip in postmenopausal women, whereas *Bsm*I, *Apa*I and *Taq*I polymorphisms were not related to bone mineral density at any skeletal site. Except for *Apa*I, in the group as a whole, we did not find a relevant relation of VDR genotypes to biochemical markers of bone remodeling.

Acknowledgements

This study was supported by grant NB 7065-3 from the Internal Grant Agency of the Ministry of Health of the Czech Republic.

References

AERSSENS J, DEQUEKER J, PEETERS J, BREEMANS S, BROOS P, BOONEN S: Polymorphisms of the VDR, ER and COLIA1 genes and osteoporotic hip fracture in elderly postmenopausal women. *Osteoporos Int* **11**: 583-591, 2000.

ARAI H, MIYAMOTO K, TAKETANI Y, YAMAMOTO H, LEMORI Y, MORITA K, TONAI T, NISHISHO T, MORI S, TAKEDA E: A vitamin D receptor gene polymorphism in the translation initiation codon: effect on protein activity and relation to bone mineral density in Japanese women. *J Bone Miner Res* **12**: 915-921, 1997.

BAKER AR, DONELL DP, HUGHES M, CRISP TM, MANGELSDORF DJ, HAUSSLER MR, PIKE JW, SHINE J, MALLEY BW: Cloning and expression of full-length cDNA encoding human vitamin D receptor. *Proc Natl Acad Sci USA* **85**: 3294-3298, 1988.

DAWSON-HUGHES B, HARRIS SS, FINNERAN S: Calcium absorption on high and low calcium intakes in relation to vitamin D receptor genotype. *J Clin Endocrinol* **80**: 3657-3661, 1995.

DENG HW, LI J, LI J-L, JOHNSON M, GONG G, DAVIS KM, RECKER RR: Change of bone mass in postmenopausal Caucasian women with and without hormone replacement therapy is associated with vitamin D receptor and estrogen receptor genotypes. *Hum Genet* **103**: 576-585, 1998.

ECCLESHALL TR, GARNERO P, GROSS C, DELMAS PD, FELDMAN D: Lack of correlation between start codon polymorphism of the vitamin D receptor gene and bone mineral density in premenopausal French women: the OFELY study. *J Bone Miner Res* **13**: 31-35, 1998.

FERRARI S, RIZZOLI R, MANEN D, SLOSMAN D, BONJOUR J-P: Vitamin D receptor gene start codon polymorphisms (*Fok*I) and bone mineral density: interaction with age, dietary calcium and 3´-end region polymorphisms. *J Bone Miner Res* **13**: 925-930, 1998a.

FERRARI SL, RIZZOLI R, SLOSMAN DO, BONJOUR J-P: Do dietary calcium and age explain the controversy surrounding the relationship between bone mineral density and vitamin D receptor gene polymorphisms ? *J Bone Miner Res* **13**: 363-370, 1998b.

FLEET JC, HARRIS S, WOOD RJ, DAWSON-HUGHES B: The BsmI vitamin D receptor restriction fragment length polymorphism (BB) predicts low bone density in premenopausal black and white women. *J Bone Miner Res* **10**: 985-990, 1995.

GARNERO P, BOREL O, SORNAY-RENDU E, ARLOT ME, DELMAS PD: Vitamin D receptor gene polymorphisms are not related to bone turnover, rate of bone loss, and bone mass in postmenopausal women: The OFELY Study. *J Bone Miner Res* **11**: 827-834, 1996.

GENNARI L, BECHERINI L, MASI L, MANSANI R, GONELLI S, CEPOLLARO C, MARTINI S, MONTAGNANI A, LENTINI G, BECORPI AM, BRANDI ML: Vitamin D and estrogen receptor allelic variants in Italian postmenopausal women: evidence of multiple gene contribution to bone mineral density. *J Clin Endocrinol Metab* **83**: 939-944, 1998.

GENNARI L, BECHERINI L, MANSANI R, MASI L, FALCHETTI A, MORELLI A, COLLI E, GONNELLI S, CEPOLLARO C, BRANDI ML: FokI polymorphism at translation initiation site of the vitamin D receptor gene predicts bone mineral density and vertebral fracture in postmenopausal Italian women. *J Bone Miner Res* **14**: 1379-1386, 1999.

GROSS C, ECCLESHALL TR, MALLOY PJ, VILLA ML, MARCUS R, FELDMAN D: The presence of a polymorphism at the translation initiation site of the vitamin D receptor gene is associated with low bone mineral density in postmenopausal Mexican-American women. *J Bone Miner Res* **11**: 1850-1855, 1996.

GROSS C, KRISHNAN AV, MALLOY PJ, ECCLESHALL TR, ZHAO XY, FELDMAN D: The vitamin D receptor gene start codon polymorphism: a functional analysis of *Fok*I variants. *J Bone Miner Res* **13**: 1691-1699, 1998.

HANSEN TS, ABRAHAMSEN B, HENRIKSEN FL, HERMANN AP, JENSEN LB, HØRDER M, GRAM J: Vitamin D receptor alleles do not predict bone mineral density or bone loss in Danish perimenopausal women. *Bone* **22**: 571-575, 1998.

HARRIS S, ECCLESHALL TR, GROSS C, DAWSON-HUGHES B, FELDMAN D: The vitamin D receptor start codon polymorphism (FokI) and bone mineral density in premenopausal American black and white women. *J Bone Miner Res* **12**: 1043-1048, 1997.

JØRGENSEN HL, SCHØLLER J, SAND JC, BJURING M, HASSAGER C, CHRISTIANSEN C: Relation of common allelic variation at vitamin D receptor locus to bone mineral density and postmenopausal bone loss: cross-sectional and longitudinal population study. *Br Med J* **313**: 586-590, 1996.

KIKUCHI R, UEMURA T, GORAI I, OHNO S, MINAGUCHI H: Early and postmenopausal bone loss is associated with *Bsm*I vitamin D receptor gene polymorphism in Japanese women. *Calcif Tissue Int* **64**: 102-106, 1999.

KHOURY MJ, BEATY TH, COHEN BH: *Fundamental of Genetic Epidemiology*. Oxford University Press, New York, 1993, pp 49-54.

KRALL EA, PARRY P, LIGHTER JB, DAWSON-HUGHES B: Vitamin D receptor alleles and rates of bone loss: influences of years since menopause and calcium intake. *J Bone Miner Res* **10**: 978-984, 1995.

KRÖGER H, MAHONEN A, RYHÄNEN, TURUNEN A-M, ALHAVA E, MÄENPÄÄ: Vitamin D receptor genotypes and bone mineral density. *Lancet* **345**: 1238, 1995.

LANGDAHL BL, GRAVHOLT CH, BRIXEN K, ERIKSEN EF: Polymorphisms in the vitamin D receptor gene and bone mass, bone turnover and osteoporotic fractures. *Eur J Clin Invest* **30**: 608-617, 2000.

LUCOTTE G, MERCIER G, BURCKEL A: The vitamin D receptor *Fok*I start codon polymorphisms and bone mineral density in osteoporotic postmenopausal French women. *Clin Genet* **56**: 221-224, 1999.

MILER SA, DYKES DD, POLESKY HF: A simple salting out procedure for extracting DNA from human nucleated cells. *Nucl Acid Res* **16**: 1215, 1988.

MORRISON NA, QI JC, TOKITA A, KELLY P, CROFTIS L, NGUYEN TV, SAMBROOK PN, EISMAN JA: Prediction of bone density by vitamin D receptor alleles. *Nature* **367**: 284-287, 1994.

MORRISON NA, YEOMAN R, KELLY PJ, EISMAN JA: Contribution of trans-activating factor alleles to normal physiological variability: vitamin D receptor gene polymorphisms and circulating osteocalcin. *Proc Natl Acad Sci USA* **89**: 6665-6669, 1992.

POCOCK NA, EISMAN JA, HOPPER JL, YEATES PN, SAMBROOK PN, EBERT S: Genetic determinants of bone mass in adults: a twin study. *J Clin Invest* **80**:706-710, 1987.

RIGGS BL, NGUYEN TV, MELTON LJ, MORRISON NA, O´FALLON WM, KELLY PJ, EGAN KS, SAMBROOK PN, MUHS JM, EISMAN JA: The contribution of vitamin D receptor gene alleles to the determination of bone mineral density in normal and osteoporotic women. *J Bone Miner Res* **10**: 991-996, 1995.

RIPOLL E, REVILLA M, HERNANDEZ ER, ARRIBAS I, VILLA LF, RICO H: New evidence that β_2 microglobulin behaves as a biological marker of bone remodelling in women. *Eur J Clin Invest* **26**: 681-685, 1996.

SEEMAN E, HOPPER JL, BACH LA, COOPER ME, PARKINSON E, MCKAY J, JERUMS G: Reduced bone mass in daughters of women with osteoporosis. *N Engl J Med* **320**: 554-558, 1989.

SPECTOR TD, KEEN RW, ARDEN NK, MORRISON NA, MAJOR PJ, NGUYEN TV, KELLY PJ, BAKER JR, SAMBROOK PN, KANCHBURY JS: Influence of vitamin D receptor genotype on bone mineral density in postmenopausal women: a twin study in Britain. *Br Med J* **310**: 1357-1360, 1995.

UITTERLINDEN AG, POLS HA, BURGER H, HUANG Q, VAN DAELE PL, VAN DUIJN CM, HOFMAN A, BIRKENHAGER JC, VAN LEEUWEN JP: A large-scale population-based study of the association of vitamin D receptor gene polymorphisms with bone mineral density. *J Bone Miner Res* **11**: 1241-1248, 1996.

VANDEVYVER C, WYLIN T, CASSIMAN J-J, RAUS J, GEUSENS P: Influence of the vitamin D receptor gene alleles on bone mineral density in postmenopausal and osteoporotic women. *J Bone Miner Res* **12**: 241-247, 1997.

WILLING M, SOWERS M, ARON D, CLARK MK, BURNS T, BUNTEN C, CRUTCHFIELD M, D´AGOSTINO D, JANNAUSCH M: Bone mineral density and its change in white women: estrogen and vitamin D receptor genotypes and their interaction. *J Bone Miner Res* **13**: 695-705, 1998.

ŽOFKOVÁ I, BAHBOUH R, BENDLOVÁ B, KANCHEVA RL Circulating β_2 microglobulin in relation to bone metabolism: implications for bone loss with aging. *Calcif Tissue Int* **65**: 442-446, 1999.

Reprint requests

K.Zajíčková, Institute of Endocrinology, Národní 8, Prague 1, 116 94, Czech Republic, fax: 420-2-24905325, e-mail: katka2222@volny.cz