Vitamin D Receptor gene polymorphism may predict response to vitamin D intake and bone turnover

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ABSTRACT

Background and the purpose of the study: The molecular and functional basis of the VDR polymorphisms is fundamental to appreciate their potential clinical implications. The rationale of this study was to determine the level of serum vitamin D response to vitamin D intake in different genotypes of VDR (FokI) polymorphism and its effect on the bone turnover in postmenopausal women.

Methods: The subjects for the study were 312 pre and post-menopausal women aged between 20-75 year randomly selected from the participants of Iranian multicenter osteoporosis study. After an overnight fast, 4ml of peripheral blood was taken and centrifuged to separate serum for measurement of serum parathyroid hormone, 25 hydroxyvitamin D, osteocalcin and cross laps. The *Fok*I polymorphism in exon 2 of the VDR gene was detected by the polymerase chain reaction-restriction fragment length polymorphism**.**

Results and major conclusion: FOKI genotype predicted serum cross laps after adjustment for age, menopausal status, serum vitamin D ($p<0.001$) but did not find significant prediction regarding serum osteocalcin ($p=0.3$).Also in this model FOKI genotype predicted serum vitamin D after adjustment for age, menopausal status, calcium and vitamin D intake ($p<0.001$).VDR gene polymorphism may modifies response to vitamin D intake and predicts bone turnover.

Keywords: Vitamin D receptor, Polymorphism, FokI, Bone turnover, Osteocalcin, Cross laps

INTRODUCTION

Osteoporosis is a common complex genetic disease, characterized by decreased bone strength predisposing to increased bone fragility and fracture risk (1). Bone strength depends to bone mineral density (BMD), geometry, bone microarchitecture structure, bone matrix and its turnover rate (2).

Genetic factors elucidate up to 80% of peak bone mass different in the population especially before puberty (3, 4). However influence of genetic factors on bone turnover in aging is less understood (5).

Bone turnover can be indirectly evaluated by a series of serum and urine biochemical markers that reflect bone formation (osteocalcin, bone specific alkaline phosphatase) and resorption (collagen degradation products, such as pyridinoline and C-terminal cross linking of type I collagen, CTx) (6).

Vitamin D is essential for calcium and phosphate homeostasis and bone metabolism. The vitamin D receptor gene (VDR) mediates the action of its ligand; therefore, VDR has been considered as an important candidate gene of osteoporosis (7).

The *VDR* gene is located on the long arm of chromosome 12 (12q12–14) and the first exon is not transcribed (8). The *Fok* I *VDR* polymorphism described by diallelic (ATG/ACG) variant in exon 2 of the gene and a RFLP using the restriction endonuclease *Fok* I can detect this variation (9). This variation is located on the translation initiation codon thus changing ATG to ACG can initiates translation at a second downstream ATG. This polymorphism describes a three amino acid difference in VDR length between *two* alleles that may alters the function of the VDR protein. The presence of the restriction site is designated as *f allele.*

Since 1994 when Morrison et al. (10) reported the relationship between VDR polymorphisms and BMD, several studies have been carried out on the association between VDR polymorphisms and diverse outcomes of osteoporosis, including BMD and fracture (7), but there are few studies on the influence of genetic variation of VDR on bone turnover.

A most challenging and demanding area is the assessment of consequences of polymorphisms for the VDR function.

The molecular and functional basis of the VDR polymorphisms is fundamental to appreciate their potential clinical implications. The rationale of this study was to determine the level of serum vitamin D

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response to vitamin D intake in different genotypes of VDR (FokI) polymorphism and its effects on bone turnover in postmenopausal women.

MATERIALS AND METHODS

Study population

The subjects for the study were 312 women aged between 20-75 years randomly selected (independent of BMD and genotype status) from the participants of IMOS (11). Detailed information on age, medical history, family history and life style was obtained from all the subjects. Exclusion criteria included endocrinological disorders (such as hyperthyroidism, hypo- and hyper- parathyroidism, diabetes mellitus), chronic disorders of liver and kidney, other skeletal diseases (Paget's disease, osteogenesis imperfecta and rheumatoid arthritis), use of medications that are known to affect bone density and metabolism (such as calcium supplements, corticosteroids, anticonvulsants and heparin), or unusual gynecological history like bilateral oophorectomy, early or late menarche or irregular cycles or premature menopause before the age of 40 years.

Questionnaire was filled for all participants included information about general characteristics, dietary habits, life style, past medical history, sunlight exposure, menopausal status, age at menarche, age at menopause and other factors affecting bone metabolism. Calcium and vitamin D intake estimated from a detailed food recall interview for the previous month.

BMD measurements All subjects underwent BMD measurements (T/Z scores) by dual energy X-ray absorptiometery (DEXA) at lumbar spine (vertebrae L2–L4) and hip (femur neck). The BMD (g/cm2) was measured by dual energy X-ray absorptiometery (Lunar-DPX).

The coefficient of variation for longitudinal BMD measurements in the DEXA machine averaged at 1.04%. Normal bone mass was defined as BMD measurements at or above -1 standard deviation (S.D.) from the optimal peak bone density (T-score) of healthy young adult of the same sex. BMD measurement at or below -2.5 S.D. from the optimal peak bone density of healthy young adult of the same sex was osteoporotic, as World Health Organization standard definitions.

Genotyping About 4ml aliquots of peripheral blood samples were collected from the subjects and stored in EDTA coated vacutainers. Genomic DNA was isolated from peripheral blood leukocytes according to the standard methods of the QIAamp DNA blood maxi kit, according to the manufacturer's protocol (QIAGEN). The FokI polymorphism in exon 2 of the VDR gene was detected by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method previously described (9).

Polymerase chain reaction (PCR) was performed. Primers right: 5′- AGCTGGCCCTGGCACTGACTCT-GCTCT-3′and left:5′- ATGGAAACACCTTGCTTCTT-CTCCCTC-3′ were used to amplify the Fok1 polymorphic restriction site In summary, 2 μL genomic DNA was added to 47.6 μL PCR nucleotide mix containing 1 μL dNTP, 2 μL MgCl2, and 5 μL 10% dimethyl sulfoxide (Gibco, CA).

The region of interest was amplified by an initial denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds, and concluded with a final extension at 72°C for 10 minutes. Then, 5 μl of the PCR product (265 base pairs) was digested in 15-μl of reaction volume containing 1 U of Fok I with the buffer supplied by the vender. The digested PCR products were resolved on 2.0% agarose gels. Fok I digested the first ATG and yielded two products, 69 and 196 base pairs (f allele), while the T to C transition destroyed the Fok I site (F allele) . Figure 1 shows gel electrophoresis of this PCR-RFLP products .Validity of this PCR-RFLP for Fok I genotyping was confirmed by sequencing of 15% of total PCR products.

Laboratory measurements

After an overnight fasting, 6ml of peripheral blood was taken and centrifuged to separate serum for measurement of serum parathyroid hormone, 25 hydroxyvitamin D, and PTH, osteocalcin and cross laps.

Serum concentration of 25-hydroxy vitamin D_3 was measured by radioimmunoassay (RIA) method using a Biosource kit (Biosource Europe S.A, Belgium). Serum PTH was also detected using a Biosource kit (Biosource Europe S.A, normal range: 13-66 pg/ml). Serum calcium and phosphorous were measured both by colorimetery using the Kavoshyar enzyme kit (Kavoshyar, Iran) and Sheem enzyme kit (Sheem enzyme, Iran), respectively.

Markers of bone formation including osteocalcin was measured by immunoassay (ELISA) using a Bioscience kit (Nordic Bioscience Diagnostic A/S, Denmark). Marker of bone resorption including the serum Cross Laps was measured by immunoassay (ELISA) using a Bioscience kit (Nordic Bioscience Diagnostic A/S, Denmark). Daily intake of dietary calcium and vitamin D was calculated from a food frequency questionnaire that was approved by the nutrition group of EMRC.

Data were analyzed using SPSS software, version 11.5. The student's t-test and analysis of variance were used to compare the differences between the means of variables. The chi-square test was used to compare the frequency of variables. Pearson correlation was used to investigate correlation between two variables. In all tests, the level of significance was 0.05.

RESULTS

Total of 330 women (211 postmenopausal and 119 pre menopausal) participated in the study. The background characteristics of the study population are shown in table 1. Postmenopausal women had higher

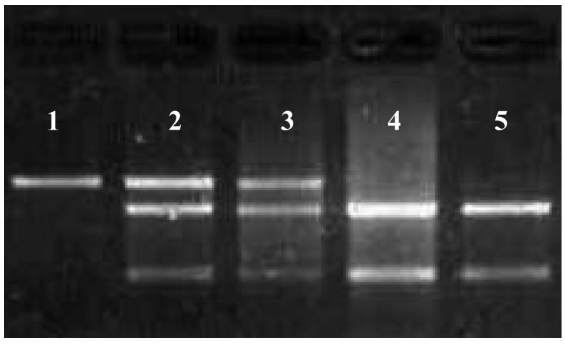

Figure 1. Gel electrophoresis related to Fok I polymorphism shows different genotype groups. Lane 1 shows FF genotype and lane 2, 3 shows Ff genotype and lane 4, 5 shows ff genotype.

bone turnover and lower BMD comparing to pre menopausal group (Table1). Intra- and inter-assay coefficients of variation (CV) of serum vitamin D measurement were 5.2% and 7.5%, respectively (normal range: 2.5-75 ng/ml) and intra- and inter-assay CV of serum PTH evaluation were 6.3% and 5.7%, respectively. Also intra- and inter-assay CV for serum osteocalcin were 2.6% and 4.7%, and for serum cross laps were 5.1% and 6.6%, respectively.

The frequency of FF, Ff and ff genotypes were 51.21%, 37.87% and 10.91% in all participants 52.94%, 35.29% and 11.76% in the pre menopausal group, and 50.24%, 39.33% and 10.43% in the postmenopausal group respectively. The observed proportions of FokI genotypes were not significantly different in pre and postmenopausal women (p=0.9). The prevalence of FokI genotypes did not differ significantly from Hardy– Weinberg equilibrium, although the number of Ff genotypes was 5% lower than expected. The genotype grouping of pre and postmenopausal women separately did not differ significantly by age, body mass index(BMI) and calcium and vitamin D intake (Table 2, 3).

Menopause has been associated with an increase in osteocalcin and cross laps values, Therefore the role of menopausal status was assessed by regression and covariance including age, menopausal status, and FOKI genotype analyses. Menopausal status was a weaker determinant of serum cross laps concentrations than FOKI polymorphism.

Analyzing pre-menopausal and post-menopausal women separately did not alter the results, and genotype was a stronger predictor than menopausal status (Table 2, 3).

Using analysis of covariance, it was found that subjects with ff genotype exhibited a significantly ($P < 0.001$) lower lumbar spine bone mass, by dual-energy X-ray absorptiometry relative to those with Ff and FF genotypes in pre- and post- menopausal women. Conversely, Serum osteocalcin and cross laps were significantly higher in ff and Ff compared to FF genotype.

Finally, FOKI and predicted serum cross laps after adjustment for age, menopause status, serum vitamin D ($p<0.001$). However this genotype did not have relationship with serum osteocalcin (p=0.3). Also this genotype after adjustment for age, menopausal status, calcium and vitamin D intake predicted serum vitamin D ($p<0.001$).

DISCUSSION

The association of VDR *Fok* I polymorphism with BMD is controversial. Several studies have shown lower bone mineral densities for *ff* genotype (9, 12-19), which are confirmed by results of this study. Our data suggest that FokI VDR polymorphism may contributes to the determination of bone mass and turnover in both pre- and postmenopausal women in this geographically isolated population,

There have been numerous studies relating Fok1 polymorphism to bone density and bone loss. In Mexican women there was lower BMD and greater bone loss in ff as compared to FF (9), and lower BMD was also found in ff women as compared to FF women (20). However, in another report VDR-Fok1 genotype was unrelated to BMD or bone loss in 100 black women over age 65 (21) .The discrepancies between different studies could be explained by several reasons.

The influence of the genetic components of BMD may change with age. If this is the case, some polymorphisms may be associated with peak bone mass and others with bone loss. Therefore, related to target population different result may be reported.

Several life style factors such as calcium and vitamin D intake influence BMD which can modify the genetic regulation of BMD. In the present study these effects were modified by adjustment of analysis for background and life style factors.

The association between VDR genotypes and BMD might differ according to the site of the bone. Our results showed that the lumbar spin BMD had a stronger association with *Fok*I polymorphism than did the total of hip BMD. Cortical bone may be less influenced by genetic factors than trabecular bone rich sites such as the lumbar spine (22, 23).

The prevalence of *Fok*I genotypes in subject group of this study was not significantly different from Hardy–Weinberg equilibrium, although the frequencies of homozygotes were slightly higher than expected. Similar distribution of FokI polymorphism reported in other studies. The prevalence of the *VDR-Fok1* genotypes in American population were 51% *FF*, 42% *Ff*, and 8% *ff*. (24) respectively. In African-American women the distribution of Fok1 polymorphisms were FF 60%, Ff 37% and ff 2% (25). In Finnish adolescent the observed proportions of FokI genotypes were 40% for FF, 43% for Ff, and 17% for ff (26). Results of this study suggest that the F allele could be more advantageous for bone health. This finding is confirmed by physiological mechanism that explains why this genetic variant is associated with higher BMD.

Whitfield and colleagues demonstrated functional significance of the translation initiation codon polymorphism (detected as *Fok*I RFLP) and the poly (A) stretch in the 3' UTR (27). In a series of 20

Table 1.Characteristics data of the population under study

Characteristic	Pre menopausal	Postmenopausal	P value
Age(years)	50.35 ±4.79	56.13 ±5.13	0.001
BMI(Kg/m^2)	27.76±2.25	28±1.71	0.01
Menarche age(years)	13.3 ±1.15	13.64±1.57	0.1
Calcium intake(mg/day)	853.85± 366.05	893.07 ± 247.7	0.36
Vitamin D intake(IU/day)	117.49 ± 55.81	113.64 ± 42.31	0.57
Hip BMD(g/cm^2)	0.97±0.1	0.92±0.1	0.002
Spine BMD(g/cm2)	1.15±0.14	1.1±0.21	0.05
Serum Vitamin D(nmol/L)	26.05 ± 8.22	29.02 ± 6.17	0.004
Serum PTH(pmol/L)	14.42 ± 8.98	14.07 ± 6.14	0.74
Serum Crosslaps (ng/mL)	0.18± 0.09	0.26± 0.14	0.001
Serum Osteocalcin (ng/mL)	6.44± 2.47	8.09±3.49	0.001

Values are expressed as mean ±SD, comparing of variables means in two groups performed by Student T test

Table 2. Background characteristics of the pre-menopausal women with respect to VDR genotypes

Characteristic	Genotype			P value ANOVA
	FF	Ff	ff	
N (%)	52.94))63	35.29))42	11.76))14	----
Age (years)	50.3±5.67	51.42± 1.2	48.41± 6.04	0.1
BMI(Kg/m2)	27.23±2.62	26.36±1.55	26.27± 2.17	0.1
Calcium intake (mg /day)	916.19±214.32	906.06± 268.09	805.29± 290.28	0.27
Vitamin D intake (IU /day)	117.73± 37.49	101.66± 36.58	125.82± 59.14	0.1
Serum 25-hydroxyvitamin D3 (nmol/dl)	31.86± 5.34	21.63± 4.56	18.88± 9.33	0.001
Serum PTH(pmol/L)	13.45± 6.9	17.96± 4.01	29.58± 3.12	0.001
Ostocalcin (ng/mL)	6.35± 2.29	5.27± 1.7	8.93± 3.32	0.001
Cross Laps(ng/mL)	0.14± 0.12	0.2± 0.13	0.25± 0.11	0.01
BMD (total hip, g/cm2)	1± 0.1	0.96± 0.05	0.90±0.12	0.001
BMD (L2-L4, g/cm2)	1.20± 0.17	1.14± 0.05	1.03± 0.13	0.001

Values are expressed as mean±SD
ANOVA, analysis of variance

fibroblast cell lines of different VDR genotype, the relative transcription efficiency was a measure of the endogenous VDR protein which was different by the genotype at the *Fok* I RFLP (F and f alleles) and the poly(A) stretch with long (L) and short (S) alleles which is acting as a transcription factor for a 1, 25-dihydroxyvitamin D3- responsive reporter gene. This study provided evidence for so-called high "FL" genotype and low "FS" genotype VDR activity. One of the possible explanations included differences in translational activity (rather than mRNA stability) of the different mRNA 3'UTR variants. Also in other studies, Allele *F* is suggested to function better in transactivation of the VDR responsive gene (12) owing to its better ability to dimerize with retinoid X receptors and bind to transcription factor IIB (28).

Another study suggested that the influence of VDR polymorphism on BMD may be related to interactions between calcium and vitamin D status and VDR genotypes and in, fact it has been suggested that there is a better response to calcium supplementation in FF genotype (29) .In terms of the biological rationale for a different response to vitamin D within each of the Fok1 polymorphisms and PTH levels it has been reported a variation with Fok1 polymorphisms (30) and the FF polymorphism was reported to be the more active form with a greater efficiency in exerting 1, 25(OH)2D effects (31). This report is consistent with our results which indicated that PTH level showed significant difference with respect to FOKI genotyping groups (Tables 2, 3).

At another level, the responses by VDR genotype have been analyzed as differences in serum bone markers. Several different serum markers are assumed to be vitamin D specific, such as osteocalcin, cross laps and PTH. In particular, osteocalcin and cross laps have been analyzed because this is a highly vitamin D responsive gene and it is frequently measured in clinical practice to monitor bone metabolism.

Our results show that FOKI genotype influence

Table 3. Background characteristics of the postmenopausal women with respect to VDR genotypes

Characteristic	Genotype			P value ANOVA
	FF	Ff	ff	
N (%)	50.24))106	39.33))83	10.43))22	----
Age (years)	56.54± 6.04	55.03± 4.15	57.37± 4.08	0.19
BMI(Kg/m2)	28.32±1.94	27.58±1.59	28.07± 1.09	0.12
Calcium intake (mg per day)	803.2± 207.56	916± 524.75	856.31± 272.68	0.35
Vitamin D intake (IU per day)	118.58± 62.53	121.75± 55.66	105.68± 33.82	0.58
Serum 25-hydroxyvitamin D3 (nmol/dl)	33.27± 4.16	22.77± 4.34	21.63± 9.77	0.01
Serum PTH(pmol/L)	10.58± 4.08	14.17± 4.67	23.05±3.82	0.01
Ostocalcin (ng/mL)	7.47± 2.86	10.12± 3.94	9.2± 3.21	0.01
Cross Laps (ng/mL)	0.19± 0.14	0.27± 0.13	0.40± 0.14	0.01
BMD (total hip, g/cm2)	0.93± 0.11	0.93±0.12	0.91±0.11	0.5
BMD (L2-L4, g/cm2)	1.15± 0.14	1.11± 0.13	1.04± 0.14	0.001

Values are expressed as mean±SD
ANOVA, analysis of variance

bone turnover by improving bone metabolism. In this mechanism FF genotype associated with PTH suppression and decreased cross laps. In the other study it was shown that In the FF group, there was greater PTH suppression and a significant decline in urinary FDPD with vitamin D as compared to placebo (25).

Also there is evidence that frequently occurring RFLP markers at the vitamin D receptor locus on chromosome 12 (32) are highly correlated with the serum levels of osteocalcin, a vitamin D- responsive product encoded on chromosome 1 (33).

The functional difference in the alleles is expressed in the degree to which the osteocalcin gene is induced by the trans acting transcriptional activation function of the vitamin D receptor.

It has been reported that (34) serum osteocalcin was under strong genetic influence in twins, with up to 80% of the variance in osteocalcin levels explained by genetic factors. Moreover, the difference in osteocalcin levels among dizygotic or nonidentical twin pairs predicted the difference in bone density, as measured by dual photon absorptiometry, in the same twins. This result supports the hypothesis that genetic effects on bone turnover relate to genetic effects on bone density (23).

CONCLUSION

Finding of this report indicated that VDR gene polymorphism may modify response to vitamin D intake and predict bone turnover. These finding may be useful for classification of osteoporotic patients based on VDR genotype for better management.

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