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Study of Vitamin D Receptor Gene Polymorphisms in Egyptian Patients with Primary Osteoporosis

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A B S T R A C T

Osteoporosis is a common skeletal disease characterized by a generalized reduction in bone mineral density (BMD), architectural deterioration of bone tissue and increased risk of fracture. Primary osteoporosis is the most common type and is more frequent in postmenopausal women caused by severe decrease in serum estrogen levels after cessation of the ovarian function. A genetic contribution to the pathogenesis of osteoporosis has been recognized. One of the first genes to be studied for its association with osteoporosis is that for the Vitamin D receptor (VDR). To investigate the association of four polymorphisms (TaqI, ApaI, BsmI, FokI); characterized by the ability to distinguish between different polymorphisms by digestion with the restriction endonucleases; of VDR gene to BMD and to estimate the frequencies of those different polymorphic genotypes in a study population. In a pilot study, sixty five Egyptian women with primary osteoporosis were selected according to measurements of DEXA test. In addition, thirty women who did not suffer of any skeletal disorder were also included in the study as control subjects. Genomic DNA was extracted from peripheral blood leukocytes using the salting out procedure. Polymerase chain reaction –restriction fragment length polymorphism (PCR-RFLP) was performed to identify the VDR genotype of the four chosen polymorphic loci. We found a close association of specific genotypes of the four studied polymorphisms in the VDR gene with low BMD in Egyptian patients with primary osteoporosis. Based on the data obtained in this study as well as several other community-specific profiles, the four selected polymorphisms of the VDR gene were proved to be reliable, simple, cheap and non-invasive markers to detect susceptibility to osteoporosis, thus lead to better management of primary osteoporosis cases and reduce or delay the pathogenic consequences of the disease.

Introduction

Remodeling is a process that involves continuous removal of discrete packets of old bone from the skeleton (a sub process called bone resorption), replacement of these packets with newly synthesized proteinaceous matrix, and subsequent mineralization of the matrix to form new bone (a sub process called ossification or bone formation). Osteoporosis (OMIM: 166710) is a systemic skeletal disease in which bones become fragile and more likely to break [1]. It is a common disease that is characterized by low bone mass, disturbed microarchitecture and composition of bone tissue, and susceptibility to nontraumatic fracture [2,3].

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Osteoporotic fracture, especially those of the hip and spine, is a serious manifestation of osteoporosis and could affect the quality of life of the sufferer [4].

Primary osteoporosis is a disease of the elderly, particularly among older women, with most cases occurring in the sixth and later decades of life. It is estimated that osteoporosis can affect 30-50% of women and 15-30% of men [5]. Osteoporosis is a polygenic disorder, determined by the effects of several genes, each with relatively modest effects on bone mass and other determinants of fracture risk [5].

In last years, candidate gene association studies (CGAS) have explored the association between osteoporosis and polymorphisms in candidate genes. Several genes have been characterized to be involved in bone mineral home

ostasis, bone remodeling and bone matrix composition, e.g. vitamin D receptor (VDR) gene [6], estrogen receptor [7-9], collagen type 1A1 (COL1A1) [10], and transforming growth factor B1(TGFB1) [11].

The VDR gene is located on chromosome 12q13.11 and is made up of 5.6 kb. In response to hormone binding (1, 25$(OH)_2$ D_3), VDR regulates the transcriptional activity of vitamin D3-responsive genes by complexing with a vitamin-D response element located in the promoter region of target genes. Allelic variation in VDR causes significant alteration in the activity of the receptor and thus the subsequent downstream vitamin-D mediated effects such as calcium absorption, excretion and modulation of cellular proliferation and differentiation. In fact, VDR was the first candidate gene to be studied in relation to osteoporosis, and most attention has focused on polymorphisms situated near the 3′flank of VDR recognized by the restriction enzymes *Bsm*I, *Apa*I, *Taq*I [12], and FokI in the translation initiation codon of exon 2 [13].

Allelic variants of the gene encoding VDR, recognized by *Apa*I (allele A/a), *Bsm*I (allele B/b), *Fok*I (allele F/f) and *Taq*I (allele T/t) restriction endonucleases have been associated with Bone Mass Density (BMD) in many studies. These Allelic variants are used in defining the community specific haplotype of osteoporosis, which is a set of DNA variations, or polymorphisms, that tend to be inherited together [14].

The *Fok*I polymorphism is a T/C transition polymorphism (ATG to ACG) at the first of two potential translation initiation sites in exon 2 which is referred as a start codon polymorphism (SCP) has been detected by using the *Fok*I restriction endonuclease. On the other hand, the three adjacent RFLPs for *Bsm*I, *Apa*I and *Taq*I, respectively, in intron 8/ exon 9 at the 3′end of the Vitamin D receptor gene, have been most frequently studied. The *Taq*I polymorphism is a T/C nucleotide substitution (ATT to ATC) leading to a synonymous change at codon 352 (isoleucine) in exon 9. *Bsm*I (T/G) and *Apa*I (G/A) restriction site polymorphisms occur in the intron separating exons 8 and 9 [15]. These polymorphisms can be distinguished by digestion with the respective restriction enzymes. The presence or absence of restriction site defines the specific allele.

The aim of this study was to investigate the association of four selected polymorphisms (*Fok*I, *Taq*I, *Apa*I, *Bsm*I) of VDR gene with BMD in a study of Egyptian osteoporotic women and to detect the incidence of any specific genotype with the development of osteoporosis.

Subjects and Methods

Subjects

This study included 65 of female Egyptian patients (30-64 years) who were diagnosed with primary osteoporosis (cases with osteoporosis secondarily to other disorders such as endocrine, congenital, metabolic disorders or malignancies were excluded) with mean age of 51.9±6.31 and 30 healthy Egyptian women who were included as controls (33-60 years), with mean age of 46.6±7.4. Patients were chosen from National Research Center (NRC) clinical center. All subjects signed consent forms according to the guidelines of the ethical committee of the NRC prior to clinical evaluation.

Methods

DEXA measurement

All patients were subjected to bone mineral density (BMD) measurement using the Dual Energy X-ray Absorptiometry (DEXA, Hologic QDR 4,500 W) both at the lumbar spine (anterio-posterior projection of L1-L4) and the proximal femur (total score). In DEXA analysis, BMD data is expressed as g/cm^2 and SD scores compares individual BMD determinations to those of 35-year old healthy individual [16].

Polymerase chain reaction

Peripheral blood samples were collected for DNA extraction in EDTA-containing vacutainers. Genomic DNA was extracted using a standard salting out procedure as previously described by **Miller *et al.* [17]**. Genotyping of the four VDR polymorphisms was carried out by PCR amplification followed by RFLP analysis of the PCR products (PCR-RFLP). The VDR genotype of each subject was identified according to the digestion pattern and alleles according to the presence (f, t, b, and a) or the absence (F, T, B, and A) of the *Fok*I, *Taq*I, *Bsm*I and *Apa*I, restriction enzyme recognition sites, respectively **.**

VDR variants were amplified from each sample as follows: Four polymorphic sites in the VDR gene (Accession number: AY342401.1) were chosen to be amplified and subsequently analyzed with the restriction enzymes *Taq*I, *Fok*I, *Bsm*I and *Apa*I. The PCR reactions were set up in 25-µl reaction volume containing 0.5 µg genomic DNA, 10x PCR buffer, 0.25 mM dNTPs, 2.5 pmol of each primer (MWG-Biotech, Germany) and 2 units of Taq polymerase. PCR was carried out on Perkin Elmer thermal cycler (Applied Biosystem 2720) using an initial denaturation step for 5 min, followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at the respective temperature (**Table 1**) for 30 sec, and extension at 72ºC for 1 min followed by a final extension at 72ºC for 7 min.

VDR genotyping

PCR-amplified products were purified using PCR purification kit (Promega, USA), then 10µl of purified PCR products were digested with 10U of the respective restriction enzyme (Fermentas, Germany). Enzyme reactions were incubated at 37°C (except for *Taq*I reactions, which were incubated at 65°C), for 2 hours. Digestion products were then separated by electrophoresis onto 2% agarose gel and stained with ethidium bromide, where separation of uncut PCR product indicated the absence of the recognition sequence of the used restriction enzyme, while presence of the cut site resulted in separation of the restriction fragments specific for each enzyme (**Table 2**). In either case, presence or absence of the particular cut site, indicated presence of homozygote genotype, while in

case of heterozygote genotype, both the uncut PCR product and the specific restriction fragments were present and separated on the gel.

Statistical analysis

Data was expressed descriptively as percentages for qualitative values and mean ± standard deviation (SD) for quantitative parametric data. The complied data was computerized and analyzed by SPSS software package, version 16. The following tests of significance were used: analysis of variance (ANOVA) test between more than 2 means, t-test between means was used to analyze mean difference. Comparison of qualitative data was done using chi square test and cross tabs. A level of significance with $p \leq 0.05$ was considered significant, $p \leq 0.01$was considered of high significance and $p > 0.05$ insignificant.

Table 1: The primers used in the PCR amplification of the four different VDR polymorphic loci.

Polymorphism	Primer sequence	Annealing temperature	References
***Fok*I**	**F; 5'-AGCTGGCCCTGGCACTGACTCTG-3'** (23) Location: 10416238-10416260 **R; 5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3'** (27) Location: 10415994-10416020	**70ºC***	[18]
***Taq*I**	**F; 5'-CAGAGCATGGACAGGGAGCAA-3'** (21) Location: 10382336-10382356 **R; 5'-CACTTCGAGCACAAGGGGCGTTAGC-3'** (25) Location: 10381856-10381880	**61ºC**	[19]
***Apa*I**	**F; 5'-CAGAGCATGGACAGGGAGCAAG-3'** (22) Location: 10382335-10382356 **R; 5'-GCAACTCCTCATGGCTGAGGTCTCA-3'** (25) Location: 10381612-10381636	**65ºC**	[20]
***Bsm*I**	**F; 5'-CAACCAAGACTCAAGTACCGCGTCAGTGA-3'** (29) Location: 10383757-10383786 **R; 5'-AACCAGCGGAAGAGGTCAAGGG-3'** (22) Location: 10382965-10382986	**70ºC***	[21]

*In these reactions, the annealing step was omitted and the extension step was carried out at 70ºC instead of 72ºC.

Table 2: Restriction fragment patterns resulted from digestion with the used restriction endonucleases.

Polymorphism	Location	Size of undigested PCR products (bp)	Length of restriction fragments produced (bp)
***Fok*I**	Exon 2	267	196, 71
***Taq*I**	Intron 8/Exon 9	501	296, 205
***Apa*I**	Intron 8/Exon 9	745	525, 220
***Bsm*I**	Exon 7/Intron 8	822	650, 172

Results

DEXA results

BMD was measured in all patients and controls by DEXA scan in both the lumbar spine and the proximal femur (**Table 3**). DEXA results showed that 33 females had osteoporosis while 32 females had osteopenia. On the other hand, no osteoporosis was found in the 30 control females established by their DEXA examination.

Molecular studies

The distributions of *Taq*I, *Fok*I, *Bsm*I and *Apa*I polymorphisms in osteoporotic women and controls are shown in **Figures (1-4)** and **Table (4)**.

From these results, the most significant VDR variants among patients were Ff (p=0.007), tt (p= 0.002), Aa (p=0.002) and BB (p=0.002) in *Fok*I, *Taq*I, *Apa*I *and Bsm*I loci, respectively.

Table 3: Demographic data of BMD and DEXA analysis.

Groups \ DEXA results		Age	Weight	Height	Mean of BMD of Femur	Mean of BMD of Spine	T-Score of Femur	T-Score of Spine
Patients N=(65)	**Mean**	51.87	75.02	157.64	0.64	0.91	-2.20	-1.26
	SD	6.32	11.19	5.67	0.07	0.12	0.65	0.78
Controls N=(30)	**Mean**	46.60	79.42	160.87	0.84	1.14	-0.32	0.18
	SD	7.37	9.20	5.76	0.09	0.13	0.87	0.70

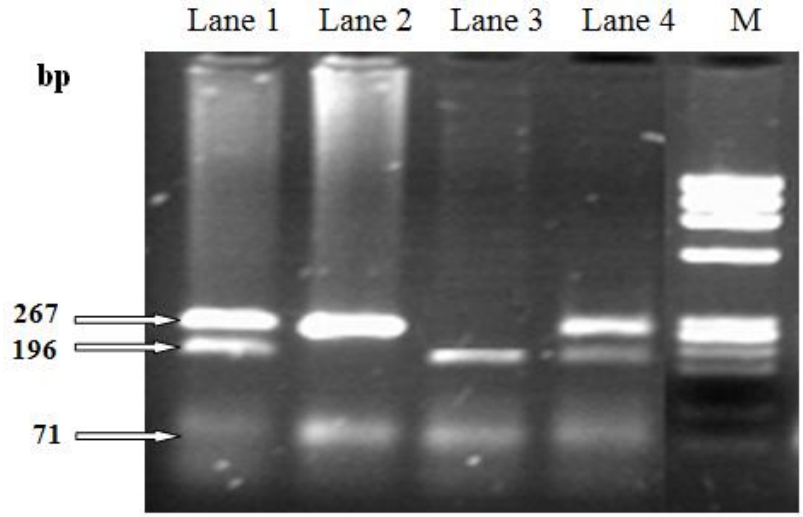

Fig. 1: A 2% agarose gel stained with ethidium bromide illustrating the digestion of PCR products of *Fok*I locus of the VDR gene with *Fok*I enzyme. **Lanes 1and 4,** two patients with **Ff** genotype (267 bp, 196 bp and 71 bp); **Lane 2**, one patient with **FF** genotype (267 bp); **Lane 3**, one patient with **ff** genotype (196 bp and 71 bp); **M,** Molecular size marker (PhiX174 DNA/*Hae*III; 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72 bps).

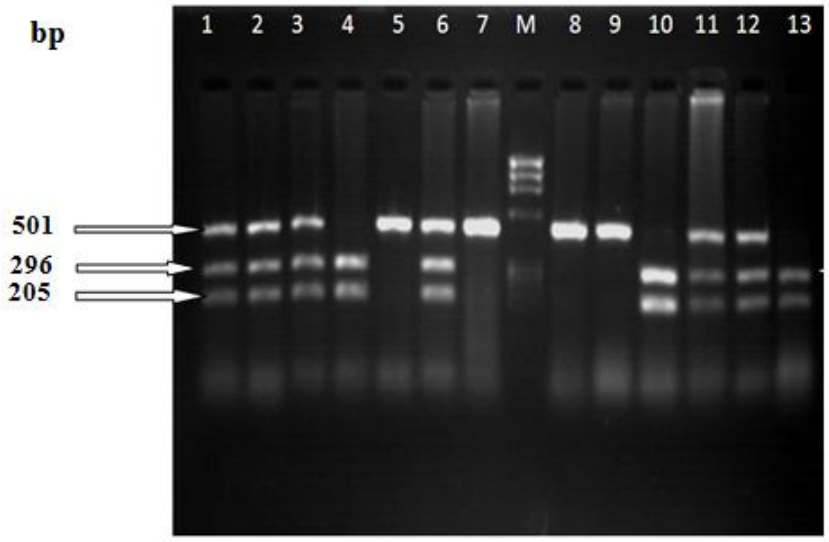

Fig. 2: A 2% agarose gel stained with ethidium bromide illustrating the digestion of PCR products of *Taq*I locus of the VDR gene with *Taq*I enzyme. **Lanes 1, 2, 3, 6, 11 and 12,** six patients with Tt genotype (501, 296, 205 bp); **Lanes 4, 10 and 13,** three patients with tt genotype (205, 296 bp); **Lanes 5, 7, 8 and 9,** four patients with TT genotype (501bp); **M,** Molecular size marker (PhiX174 DNA/*Hae*III; 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72 bps).

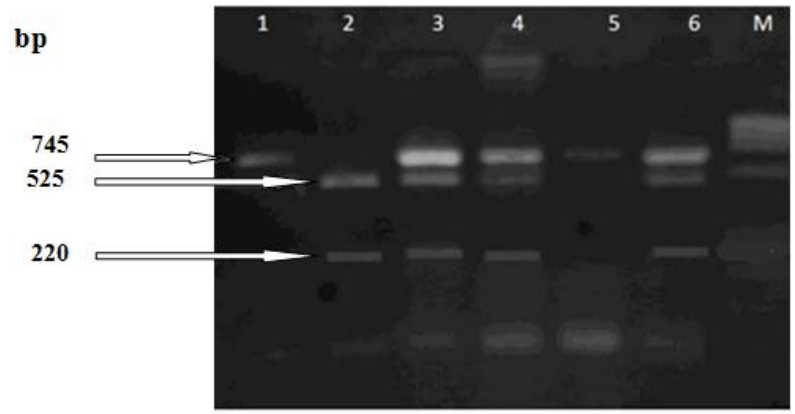

Fig. 3: A 2% agarose gel stained with ethidium bromide illustrating the digestion of PCR products of *Apa*I locus of the VDR gene with *Apa*I enzyme. **Lanes 1, 3, 4 and 6,** four patients with Aa genotype (745, 525, 220 bp); **Lane 2,** one patient with aa genotype (525, 220 bp); **Lane 5,** one patient with AA genotype (745 bp); **M,** Molecular size marker (PhiX174 DNA/*Hae*III; 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72 bps).

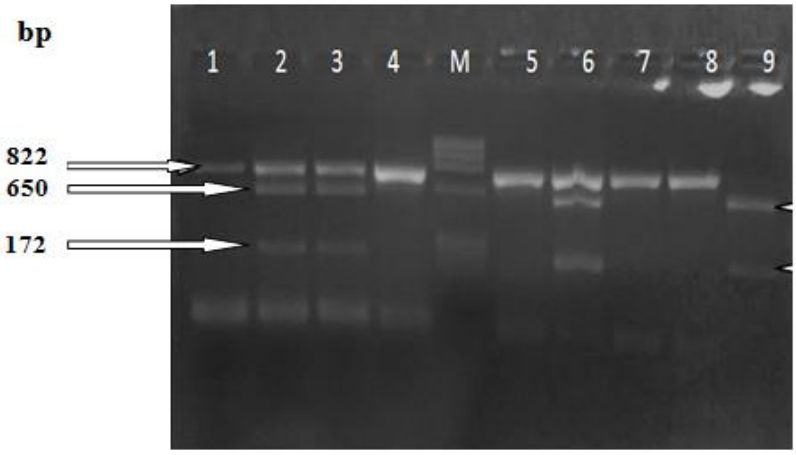

Fig. 4: A 2% agarose gel stained with ethidium bromide illustrating the digestion of PCR products of *Bsm*I locus of the VDR gene with *Bsm*I enzyme. **Lanes 1, 2, 3 and 6**, four patients with Bb genotype (822, 650, 172 bp); **Lane 9**, one patient with bb genotype (650, 172 bp); **Lanes 4, 5, 7 and 8**, four patients with BB genotype (822bp); **M,** Molecular size marker (PhiX174 DNA/*Hae*III; 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72 bps).

Correlation of the VDR gene polymorphism and BMD

The statistical correlation tests between the *Fok*I, *Bsm*I, *Apa*I and *Taq*I VDR gene polymorphisms and BMD of patients and control groups revealed a significant relationship between specific genotypes of VDR polymorphisms and BMD at the measured skeletal sites (both the lumbar spine and the proximal femur) (**Table 5**).

As for the correlation between VDR *Fok*I genotypes and mean of BMD of patient and control groups, individuals with heterozygous wild genotype Ff showed a highly significant difference association with femoral hip BMD relative to control where it had the lowest hip BMD. On the contrary, individuals with homozygous wild FF genotype showed a highly significant association with lumbar spine BMD where it had the lowest spine BMD. Meanwhile, carriers of the FF and Ff genotypes had the highest hip and spine BMD, respectively.

In case of VDR *Taq*I genotype, individuals with the **TT** genotype had the lowest hip BMD relative to control while individuals with the homogeneous mutant **tt** genotype had the highest hip BMD. Conversely, in case of lumbar spine, individuals with the **tt** genotype had the lowest spine BMD while the **Tt** genotype had the highest spine BMD.

With respect to the *Apa*I genotype, individuals with the heterogeneous wild **Aa** genotype had the lowest mean of hip and spine BMD; furthermore, individuals with the **aa** genotype had both the highest hip and spine BMD values.

Regarding the *Bsm*I polymorphism and its correlation to femoral hip BMD, the **bb** genotype was associated with lowest hip BMD while individuals with the **BB** genotype had the highest hip BMD. On the other hand, the **BB** genotype was associated with the lowest lumbar spine BMD while individuals with the **Bb** genotype had the highest spine BMD.

Table 4: Distribution of different genotypes of the four polymorphisms and alleles frequency in all groups.

Polymorphism		Controls (n=30)	Patients (n=65)	*p* value
***Fok*I**	FF genotype	20 (66.7%)	41 (63.1%)	0.317 (NS)
	Ff genotype	9 (30%)	18 (27.7%)	0.007**
	ff genotype	1 (3.3%)	6 (9.2%)	0.059 (NS)
	F allele (%)	49 (81.7%)	100 (76.9%)	-
	f allele (%)	11 (18.3%)	30 (23.1%)	-
***Taq*I**	TT genotype	10 (33.3%)	15 (23.1%)	0.317 (NS)
	Tt genotype	18 (60%)	35 (53.8%)	0.020*
	tt genotype	2 (6.7%)	15 (23.1%)	0.002**
	T allele (%)	38 (63.3%)	65 (50%)	-
	t allele (%)	22 (36.7%)	65 (50%)	-
***Apa*I**	AA genotype	10 (33.3%)	21(32.3%)	0.048*
	Aa genotype	16 (53.3%)	39 (60.0%)	0.002**
	aa genotype	4 (13.3%)	5 (7.7%)	0.739 (NS)
	A allele (%)	36 (60.0%)	81 (62.3%)	-
	a allele (%)	24 (40 %)	49 (37.7%)	-
***Bsm*I**	BB genotype	7 (23.3%)	24 (36.9%)	0.002**
	Bb genotype	16 (53.3%)	25 (38.5%)	0.160 (NS)
	bb genotype	7 (23.3%)	16 (24.6%)	0.061 (NS)
	B allele (%)	30 (50 %)	73 (56.2%)	-
	b allele (%)	30 (50 %)	57 (43.8%)	-

Where: NS indicates non significant, (*) indicates significant, and ** indicates highly significant.

Table 5: Correlation between the different VDR genotypes with BMD.

Polymorphism	Genotype	Femoral hip			Lumbar spine		
		Patient	Control	*p* value	Patient	Control	*p* value
***Fok*I**	**ff**	0.6358	0.7370	0.149 (NS)	0.9140	1.0360	0.439 (NS)
	Ff	0.6246	0.8596	0.000**	0.9286	1.1879	0.000**
	FF	0.6435	0.8296	0.000**	0.9035	1.1292	0.000**
***Taq*I**	**tt**	0.6474	0.7690	0.082 (NS)	0.8841	1.1325	0.004**
	Tt	0.6406	0.8459	0.000**	0.9295	1.1476	0.000**
	TT	0.6205	0.8301	0.000**	0.8967	1.1400	0.000**
***Apa*I**	**aa**	0.6540	0.8618	0.016*	0.9466	1.1832	0.007**
	Aa	0.6293	0.8271	0.000**	0.9021	1.1113	0.000**
	AA	0.6489	0.8382	0.000**	0.9203	1.1779	0.000**
***Bsm*I**	**bb**	0.6226	0.8410	0.000**	0.9094	1.2061	0.000**
	Bb	0.6358	0.8449	0.000**	0.9255	1.1278	0.000**
	BB	0.6493	0.8107	0.000**	0.8981	1.1174	0.000**

Where: NS indicates non significant, (*) indicates significant, and ** indicates highly significant.

Table 6: The most specific haplotypes associated with osteoporosis.

Genotype	Haplotype	*p* value
TtFFAa	TFA	0.05
ttFFAA	tFA	0.02
ttAABB	tAB	0.008
ttFFBB	tFB	0.007
FFAabb	FAb	0.02
ttFFAABB	tFAB	0.02

Haplotypes of VDR gene polymorphism related to osteoporosis

Combining the different genotypes showed that the combination TtFFAa, ttFFAA, ttAABB, ttFFBB, FFAabb and ttFFAABB were the most specific haplotypes which considered as risk factors to the susceptibility to osteoporosis **(Table 6)**.

Discussion

The VDR gene is the first gene studied in relation to osteoporosis. It is known to regulate the transcription of target genes (e.g., calcium-binding protein and osteocalcin) that are crucial for calcium absorption and bone formation. The mechanism by which the VDR gene affects BMD remains unknown. In addition, polymorphisms in the VDR gene and other genes and pathways, such as the estrogen signaling pathway, transforming growth factor-beta superfamily, and the activating receptor of the nuclear factor κB (RANK) signaling pathway, can affect BMD and regulate the effect of the VDR gene polymorphisms on BMD [22].

Genetic association studies in osteoporosis found associations between the VDR polymorphisms and osteoporosis. **Gong *et al.*** [23] concluded that BMD is associated with VDR genotype, especially in females before the menopause. Similarly, our study found such an association of single alleles of the VDR to osteoporosis and revealed that the single alleles F (76.9%), t (50%), A (62.3%), and B (56.2%) of *Fok*I, *Taq*I, *Apa*I, and *Bsm*I polymorphic loci, respectively, were overrepresented in patients compared to controls. On the other hand, a Dutch study on elderly women and men by **Uitterlinden *et al.*** [24] showed no effect of single polymorphism on BMD, but a small effect was detected employing haplotype pattern of the VDR gene. Such conflicting findings, which are not exclusive for the field of genetic association analysis of osteoporosis, could be due to the fact that the 3′*Bsm-Apa-Taq* polymorphic loci are non functional themselves, as the *Bsm*I and *Apa*I RFLPs are located in intron 8 and are not affecting any splicing site and/or transcription factor binding site, and the *Taq*I RFLP is a "synonymous" polymorphism meaning that it is present in the coding sequence (i.e., exon 9) but it is not changing the amino acid sequence of the encoded protein [25].

In fact, the alleles of the VDR gene are hypothesized to function differently and contribute to the physiologically diverse levels of osteocalcin (the most abundant noncollagenous bone protein) because the expression of osteocalcin is induced by calcitriol through VDR [26]. *Bsm*I polymorphism in intron 8 of the VDR gene could be correlated to serum osteocalcin concentration, and was subsequently found to be associated with differences in BMD in a twin study in postmenopausal women [27]. Allelic variants of the gene encoding VDR, recognized by *Fok*I (allele F/f), *Taq*I (allele T/t), *Apa*I (allele A/a), and *Bsm*I (allele B/b) restriction endonucleases, have been associated with Bone Mass Density (BMD) in many studies, as well as with bone loss in elderly subjects and gain after 1,25-dihydroxy Vitamin D_3 treatment. These allelic variants are used in defining the community specific haplotype of osteoporosis [14].

Additionally, these polymorphisms have also been associated with bone remodeling process [25]. In agreement with our results, **Moran *et al.*** [22] found that women with the BB genotype have reduced lumbar bone mass compared to those with the bb genotype. Women with the BB genotype experienced accelerated bone remodeling with increased bone resorption, higher observed degradation of collagen type I, and increased calcitriol and phosphate values. These observed phenomena produce long-term mineral loss from the skeleton and low BMD. The initial studies by **Morrison *et al.*** [26-28] suggested that the "B" allele of the *Bsm*I RFLP-site is the risk allele associated with low BMD, other studies either could confirm this [29], did not find any effect [30], or reported the opposite [31].

In the present study, our data showed the different frequencies of each genotype of the four polymorphisms of the VDR gene among the patient and control groups. Statistical analysis comparing the BMD to each genotype identified the degree of association of each polymorphism with osteoporosis. Also analysis of the different combination of genotypes with BMD revealed specific haplotypes which could be highly significant to identify disease susceptibility.

The analysis of the *Fok*I polymorphism of the VDR gene showed that **FF** genotype was found to be the most prevalent genotype in patients and control groups, and was associated with low lumbar spine BMD values, a finding that was in concordance with a study by **Kurt *et al.*** [3], who found that **FF** genotype was associated with low femoral neck and total hip BMD values. In the meantime, the **Ff** genotype was found to be associated with low femoral neck BMD values and was significantly prevalent in osteoporotic patients as compared to control group. On the contrary, **Zajíčková *et al.*** [32] and **Uitterlinden *et al.*** [33] found **FF** genotype was associated with higher BMD at femoral neck. Also, in Mexican-American postmenopausal women, the **ff** subjects were related with decreased BMD at the lumbar spine and increased rate of bone loss at the hip [22, 34-36]. Both lumbar and femoral BMD were observed to be highest in ''**FF**'' homozygous by **Vidal *et al.*** [37] in postmenopausal Maltase women. On the other hand, **MacDonald *et al.*** [38] and **Langdahl *et al.*** [39] could not find any relation between BMD values and VDR *Fok*I genotypes.

According to our study, the *Taq*I polymorphism of the VDR gene revealed that **Tt** genotype was significantly higher than **TT** and **tt** in both patient and control group. On the other hand, the **TT** and **tt** genotypes were found to be correlated with the lowest mean of BMD in the hip and the lumbar spine, respectively. Similarly, the average BMD of the subjects with **TT** and **Tt** genotypes was found to be significantly higher at the spine and hip than those with **tt** genotypes in postmenopausal Indian women [34]. Also, **Morita *et al.*** [40] approved the bone

loss at the lumbar spine in the premenopausal women from Japan with **tt** genotype which was significantly greater than that of subjects with **Tt** or **TT**. **Douroudis *et al.*** [41] reported similar results which showed that **TT** genotype had a high risk for osteoporosis in postmenopausal women of Hellenic origin. In contrast with **Langdahl *et al.*** [39] study, the BMD of the individuals with **TT** genotype was higher than **Tt** and **tt** genotypes. Also, **Duman *et al.*** [42] found that the osteoporotic group with the **TT** genotype had significantly higher femoral neck BMD values in respect to the **tt** genotype in Turkish postmenopausal women.

For the *Apa*I polymorphism of the VDR gene, results approved that **Aa** genotype was found to be associated with low BMD values in both lumbar spine and femoral neck. It was also found to be high significantly associated to osteoporotic patients when compared to control. Similarly, **Mitra *et al.*** [34] showed that the subjects with **Aa** genotype had significantly lesser BMD than those with genotype **aa**. In disagreement with our study, **Li *et al.*** [43] showed that the mean BMD at the femoral neck was significantly higher in the subjects with **Aa** genotype than those with **aa** genotype. Similarly, **Dundar *et al.*** [2] found postmenopausal women with **aa** genotype, who had significantly lower BMD values at lumbar spines compared to persons with **AA** genotype, they also reported that *Apa*I genotype was not associated with BMD at proximal femur.

Regarding the fourth polymorphism studied, the *Bsm*I, the obtained results showed that there was no significant difference within the patient group genotypes. The comparison of patients to controls revealed that **BB** genotype was found to be highly significantly associated to osteoporotic patients. The **bb** genotype was found to be correlated with the lowest mean of BMD in the hip while **BB** genotype was correlated with the lumbar spine. Results of **Ivanova *et al.*** [44] were in agreement with our results documenting that **BB** genotype was found to be more common in cases with low BMD and/or osteoporosis and inversely, **bb** genotype was less common in the Bulgarian population. Similar findings were observed in another study which reported that the **BB** genotype was associated with a more than twofold increased risk of hip fracture compared with the bb genotype [45]. Their findings were also consistent with the results obtained by **Garnero *et al.*** [46]**. Jia *et al.*** [47] reported that the **bb** genotype was a protective factor in East Asians where they found that the **bb** genotype was associated with a significantly decreased risk of osteoporosis in overall comparisons. Subgroup analyses showed that the **bb** genotype had a decreased risk of developing osteoporosis in postmenopausal women in Africans rather than in Asians and Caucasians. These findings suggest that VDR *Bsm*I polymorphism may be involved in the pathogenesis of osteoporosis.

Some investigations have found no relation between VDR gene polymorphisms and BMD in different populations [48,49]. These controversies between different studies could be attributed to numerous reasons such as the environmental influence on bone mass [50]. However, different genotypes may also have diverse effects on different parts of the skeleton. This might explain why several studies did not find an association between VDR polymorphisms and BMD [51]. Other reasons for such inconsistencies between these studies can be attributed to various factors such as the sample size, study design, age, ethnic ancestry, and lifestyle factors (physical activity, obesity, calcium intake), all of which could affect gene regulation in different genotypes and subjects and result in BMD loss or gain [52].

Regarding the relationship between the calcium homeostasis and VDR gene expression, **Stathopoulou *et al.*** [53] revealed the increased risk of osteoporosis by 118% and 132% in presence of the **B** and **t** alleles, respectively, within the low calcium intake (< 680 mg/d). On the other hand, **Gennari *et al.*** [54] observed that the intestinal calcium absorption in postmenopausal Italian women was significantly lower in **BB** and **tt** genotypes than in **bb** and **TT** genotypes, respectively. Also, **Zambrano-Morales *et al.*** [55] found that **BBAAtt** genotype was a risk factor for osteoporosis while **BbaaTT** was a protection factor in postmenopausal Spanish women. On the other hand, **Li *et al.*** [43] showed that *Bsm*I and *Apa*I polymorphisms are weakly associated with BMD at some skeletal sites in the Chinese postmenopausal women, with heterozygous subjects had a higher BMD. **Zhang *et al.*** [56] found that cross genotyping of *Apa*I and *Bsm*I or *Taq*I polymorphisms was not associated with BMD in postmenopausal women.

In conclusion, this study proved that the four polymorphisms chosen were associated with osteoporosis and several community-specific profiles were found that may be used as indicators for susceptibility of osteoporosis. Also, subjects with the specific haplotype **ttFFAABB** are more susceptible to osteoporosis and should be advised to start early with preventive measures (supplementation of calcium and vitamin D, exposure to sun and weight bearing exercise) in order to minimize the risk of becoming osteoporotic.

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