RESEARCH ARTICLE

Investigation of the Role of VDR C>A rs7975232 SNP in the Incidence of Osteoporosis in Iraqi Women

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ABSTRACT

Background: Osteoporosis (OST) effects on both men and women but women usually have a higher risk of osteoporosis than men due to women having lower bone mass, pregnancy consuming a large amount of calcium and other nutrients, and mainly the cease of estrogen secretion by ovaries after menopause which precipitates rapid bone loss. This ovarian aging is accompanied by a gradual decline in follicular pool and a further decline in estrogen production.

Aim: To the evaluation of the role of VDR C>A rs7975232 SNP in the incidence of OST Iraqi women.

Methods: Vitamin D concentrations were estimated by high performance liquid chromatography (HPLC) and genetic analysis were assessed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Results: The results showing highly significant ($p < 0.05$) of VD levels (ng/mL) on VDR SNP C>A rs7975232 in different genotypes of OST and control groups, the mean ± SD of VD in were compared depending on AA, Aa, and aa genotypes The results of amplification and digestion by restriction enzyme (*ApaI*) of intron 8/exon 9 of VDR gene by PCR-RFLP assay were of two alleles AA (C/C) allele with two bands and has molecular size of 532 and 214 bp and aa (A/A) allele has single band 746 bp, and Aa (A/C) was digested by *ApaI* (RE) into three bands 746, 532, and 214 bp**.**

Conclusion: The results of the present study give additional evidence that vitamin D deficiency is significantly associated with *ApaI* SNPs of VDR and may be associated with susceptibility of opioid substitution therapy (OST) incidence in Iraqi women.

Keywords: Osteoporosis, Restriction Enzyme ApaI, Single Nucleotide polymorphism, Vitamin D, Vitamin D receptor,

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INTRODUCTION

Osteoporosis (OST) is a chronic metabolic bone disease and globally the most common age-related skeletal disease.[1] OST was defined by World Health Organization (WHO) as a "progressive systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility leading to a serious fracture even after trivial physical strain".[2] OST affects both men and lower bone mass in women which precipitates rapid bone loss. This ovarian aging is accompanied by a gradual decline in follicular pool and a further decline in estrogen production.[3] Bone breakdown after the age of thirty five outpaces bone build-up, resulting in a gradual decline in bone mineral density bone mineral density (BMD) and bone strength. Once this loss of bone reaches a certain point, a person has osteoporosis.[4] Opioid substitution therapy (OST) is a condition of skeletal fragility characterized by reduced bone density attributed to multiple pathogenic mechanisms. It is a consequence of the excessive breakdown of bone structure not compensated by adequate bone formation, thus an understanding of bone formation and remodeling is essential.[5] The fat-soluble vitamin D ("calciferol") is steroid hormone plays a key role in calcium and phosphorus metabolism and maintaining a healthy mineralized skeleton.[6] Humans obtain vitamin D from either sunlight exposure or dietary foods and artificial sources such as supplements and fortified food.[7] Skin synthesis is the major source of vitamin D, contributing to more than 90% of vitamin D serum concentration. Vitamin D is acting as a hormone in autocrine and paracrine manners. Therefore there are grounds to classify it as a hormone rather than a vitamin.[8] Vitamin D receptor is an intracellular receptor belonging to the steroid/thyroid nuclear receptor family, is expressed on various cells such as skeletal, intestine, bone marrow, brain, colon, breast, malignant cells, and immune cells and has a key role in calcium homeostasis.[9] The human *VDR* gene encodes a ligand-activated transcription factor which is localized on the short arm of chromosome 12q13.11 and contains 11 exons and spans approximately 75 kilo-bases

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of genomic DNA with numerous SNPs. Some of the SNPs play a key role in the modification of the uptake of 1,25(OH)2D.[10] this work aims to assess the role of VDR C>A rs7975232 SNP in the incidence of OST Iraqi Women.

MATERIALS AND METHODS

This study was designed as case-control study. The Patient group recruited in this study with 50 Iraqi women whose ages range from 35 to 55 years, the mean ± SD was (43.98 ± 5.562 years). Categorized into two groups: OST premenopausal women (n=25), mean age of (39.23 ± 3.09 years) and osteoporosis postmenopausal women (n=25) mean age (48.37 ± 2.59 years). Blood-containing EDTA tubes were stored at temperature (-30˚C) in deep freeze and later used in the genetic measurement of the current study. VD levels were estimated by HPLC as described by Elham *et al*., (2021).[11] The genomic DNA was isolated from the peripheral blood of subjects using Favorgene® kit Genomic DNA Purification Kit. Until analysis, DNA was stored at -20°C. The kit contents shows in Table 1.

VDR-SNP, *Apa*I (rs7975232) detection was done by PCR-RFLP technique with using unique primer sequences as showing in Table 2.

PCR Reaction Components and protocol for Amplification of *ApaI* (rs7975232) gene are listed in Table 3.

After amplification, the PCR products were separated by gel electrophoresis through 1% agarose with stained with loading dye. The PCR product length has 746 bp for *ApaI* (rs7975232). The restriction reaction carried out by mixing the following:

- 10 µL of the selected restriction enzyme, Restriction buffer 2 µL (each restriction enzyme has its restriction buffer supplied by the manufacturer). The reaction mixture then completed to 12 µL by molecular grad water, and the final reaction mixture incubated in 25°C water bath for 24 hours.

RESULTS

OST group were classified according to Clinical characteristics as shown in Table 4.

In the present study, osteoporosis women and control groups were classified into two groups depending on age, BMI, menopause status and family history to explain the effect of these risk factors, as shown in Figure 1.

VDR SNP C>A rs7975232, also known as the *ApaI* polymorphism, is a shown in Figure 2.

The current study showing the amplification and digestion by restriction enzyme (*ApaI*) of intron 8/exon 9 of VDR gene by PCR-RFLP analysis were of two alleles AA (C/C) allele with two bands and has molecular size of 532 and 214 bp and aa (A/A) allele has single band 746 bp, and Aa (A/C) was digested by *ApaI* (RE) into three bands 746, 532, and 214 bp, as shown in Figure 3.

All women (OST pre and post and CONT pre and post) were classified according to the number of genotypes

Table 1: Contents of DNA extraction kit

No.	*Item*	*Quantity*
1	FavorPerp Blood Genomic (FAPG) Buffer	40 mL
2	W1 Buffer	45 mL
3	FABG Colom	100 pcs
4	Wash Buffer (concentrated)	25 mL
5	Elution Buffer	30 mL
6	Proteinase K	10 mg, 3 vial

Table 2: The sequences of primers for PCR-RFLP

VDR SNP	*Primer5'→3'*	*length*	*Tm*	*GC%*
(rs7975232)	F: CAGAGCATGGACA GGGAGCAA R:G AACTCCTCATGG CTGAGGTCTC	21 24	63.3 68.7	57 58

Table 3: PCR program for amplification of ApaI (rs7975232) gene

No	*Stage*	*Temperature*	*Incubation time*	*Cycle Number*
1	Initial denaturation	95°C	7 minutes	1
2	Denaturation	95°C	40 seconds	
	Annealing	63°C	30 seconds	35 cycle
	Polymerization	72°C	50 seconds	
3	Final polymerization	73°C	5 minutes	1

Table 4: Characteristics of OST group

Clinic-pathological variables	*OST*	*Control*	X^2
Total number of women	*50*	*50*	*100*
Age			
<45	24	27	0.758
≥45	26	33	
MS			0.688
Pre	25	23	
Post	25	27	
BMI			0.001
<25	17	36	
≥25	33	14	
FH			
Yes	32	12	0.001
No	18	38	

Abbreviations: MS, Menopausal status: BMI, body mass index: FH, family history

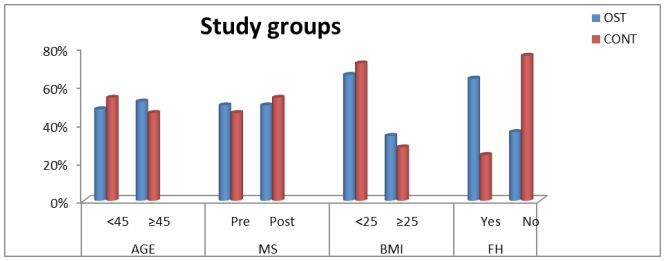

Figure 1: Classification percentage of study groups depending on age, MS,BMI, and FH

(AA, Aa, and aa) in pre and post of OST and control groups, as showing in Figure 4:

The frequencies of VDR SNP C>A rs7975232 in OST group and it correlation with age, BMI, MS, and FH were summarized in Table 5.

To investigate the effects of VDR SNP C>A rs7975232 on VD levels (ng/mL) in different genotypes of OST and control groups, the mean ± SD of VD in were compared depending on AA, Aa, and aa genotypes, as shown in Table 6.

DISCUSSION

The genotype frequencies analysis of VDR gene variants presented in Table 5, showed significant differences in their distribution between both groups. The present study suggests that among women with OST, those with had significantly genotyping differences in cases of higher ages (≥45) and no significant association in BMI, while pre MS and FH compared to showing significant differences with post MS, and without FH. In the current study, we found a significant difference in the allelic and genotypic distribution of VDR rs7975232 (*ApaI*) among pre and post MS with OST. Thus, Aa heterozygote of rs 7975232 (*ApaI*) was significantly associated with an increased risk of OST. Arai et al (2001) were reported that women with the rs7975232, rs1544410, and rs731236 minor homozygous genotypes had the lowest BMD level compared to bearers of other genotypes [12]. Qin *et al.* (2013) were showed that the restriction enzymes *BsmI* and *ApaI* were affected on body mass index. As polymorphisms at the *VDR* gene locus have been suggested to be related to bone mass, the VDR gene polymorphisms were considered important for increased risk of OST.[13] Morita et al investigating *Apa*I, *Taq*I, and *Fok*I found that the effect of the VDR genotype on bone mass was negligible in Japanese women.[14] Other studies reported that the *VDR* gene has no significant effect in BMD.[15] However, other studies have shown a relationship between the VDR genotype and OST.[16] Compared with the results of the present study, other studies mention that rs1544410, rs731236, and rs10735810 showed an almost similar genotype distribution in normal BMD, osteopenia, and OST group but rs7975232 genotype distribution showed a difference in OST group.[17]

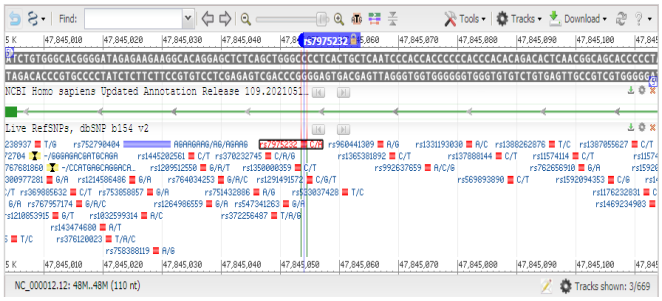

Figure 2: NCBI data base represent the location of VDR SNP C>A rs7975232 on chromosome 12

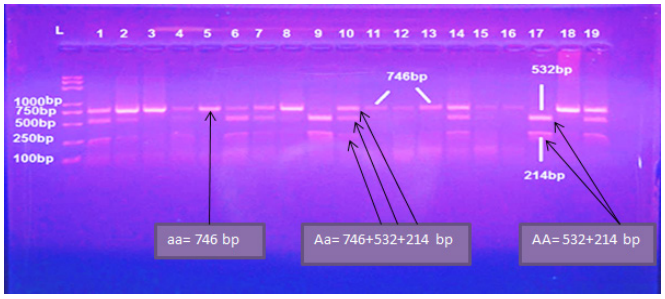

Figure 3: A- Site of restriction enzyme (ApaI) was cut of 746 bp into two bands. B- Electrophoretic picture represent VDR SNP, where lane L is 100 bp DNA ladder, lane (5,8,18) has one band at 746 bp representing the homozygous of (aa) allele, lanes (1,4,6, 7,10,14, 15,16,19) has three bands at 746,532,and 214 bp representing heterozygous (Aa) allele and lanes (9,17) has two bands at 532 and 214 bp as homozygous (AA) alleles

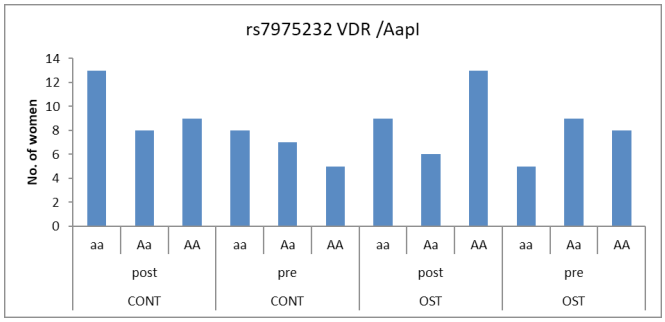

Figure 4: Genotyping distribution of VDR SNP C>A rs7975232 (ApaI) in study groups

Table 5: Association between VDR SNP C>A rs7975232 in OST group with clinic- pathological characteristics

Variables		*Genotypes/ OST n =50 (%)* *AA 21(42%)*	*Aa 15 (30%)*	*aa 14 (28%)*	*p-value*
Age	<45 24 (48%) ≥45 26 (52%)	10 (20) 11 (22)	6 (12) 9 (18)	8 (16) 6 (12)	0.041*
BMI	<25 33 (660%) ≥25 17 (34%)	14 (28) 7 (14)	10 (20) 5 (10)	9 (18) 5 (10)	0.079
MS	Pre 25 (50%) Post 25 (50%)	6 (12) 15 (30)	10 (20) 5 (10)	9 (18) 5 (10)	0.0000**
FH	YES 32 (64%) NO 18 (34%)	13 (26) 8 (16)	10 (20) 5 (10)	9 (18) 5 (10)	0.024*

Data is represented as number (%)

Table 6: The comparison of levels of VD (ng/mL) between all genotypes of study groups

Genotype (Co-dominant)	*(VD ng/mL) levels in CONT (Mean ± SD)*	*(VD ng/mL) levels in OST (Mean ± SD)*	*p-value*
AA	27.4 ± 1.5	19.6 ± 2.9	0.0001**
Aa	26.86 ± 2.0	18.7 ± 2.5	0.0001**
aa	25.02 ± 1.9	17.45 ± 2.8	0.0001**
AA vs. Aa+ aa (Dominant)	25.69 ± 1.9	17.54 ± 2. 7	0.0001**
AA+ Aa vs. aa (Recessive)	26.98 ± 2.3	18.41 ± 2.8	0.0001**
AA+ aa vs. Aa (over dominant)	26.56 ± 1.9	16.83 ± 2.6	0.0001**

The present study did not find significant associations of VDR ApaI with BMI and these findings have also been shown in the previous study in women.[18] Although several studies on different populations revealed an association of VDR gene variants with BMD and serum VD levels (19). The VDR *ApaI* (rs7975232 C>A), *BsmI* (rs1544410 C>T), and *TaqI* (rs731236 A>G) SNPs are located at the 3' un-translated end. They do not alter the amino acid sequence of the encoded protein but influence gene expression, regulating mRNA stability.[20,21] The main finding of single VDR gene variants association analysis is that the presence of VDR *ApaI* AA and *BsmI* BB homozygous genotypes is significantly associated with increased OST risk, and this data are following the data reported in meta-analysis.[22] where *ApaI* and *BsmI* were the most frequent markers, associated with OST risk. Unlike VDR *BsmI, ApaI*, and *TaqI* polymorphisms affect mRNA stability, which results in a change of biological levels and function of VD (23). These results suggested that *ApaI* VDR polymorphisms might directly or indirectly be involved in mechanisms related to VD levels declines in OST patients. On the other hand, the allele and genetic models of *ApaI* within intron 8 of the VDR in the Iraqi women might have regulatory effects on expression level and function of the VDR functions. Similar to results of this study, the protective effect of the homozygous dominant of AA has previously been shown for Asian groups[24] as well as the Caucasian population.[25] Li *et al.* (2017) conducted a study to evaluate the association of VDR gene polymorphisms and serum VD levels in patients with generalized vitiligo. They investigated four VDR polymorphisms (*FokI*, *BsmI*, *ApaI*, and *TaqI*) to determine whether they are associated with vitiligo susceptibility in the Chinese population.[26] The results of the present study give additional evidence that vitamin D deficiency significantly associated with *ApaI* SNPs of VDR and may be associated with susceptibility of OST incidence in Iraqi women.

CONCLUSION

The aa genotype and a allele of VDR gene *ApaI* (SNP C> A rs7975232) in intron 8 can be considered as independent risk factors for OST, hence the significant association between aa genotype and a allele with OST suggested by this study.

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