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RELATIONSHIP BETWEEN POSTMENOPAUSAL WOMEN'S BONE MINERAL DENSITY AND POLYMORPHISMS OF VITAMIN D RECEPTOR GENE (*TAQI* 731236)

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Abstract:

Background: Approximately 200 million women worldwide are affected with osteoporosis, which is a significant source of morbidity and mortality and has complications that are related to it. The most widely used diagnostic method is now dual-energy X-ray absorptiometry (DEXA) BMD scanning because of the disease's high correlation with bone mineral density (BMD). Women after menopause are at a heightened risk for illness. The correlation between vitamin D receptor gene polymorphism with different human diseases had been searched. It was reported that VDR gene polymorphism correlated to hyperparathyroidism, infectious diseases, inflammatory bowel disease (IBD), and prostatic tumor.

Objective: The presented work aimed to studying the genetic variation of the VDR gene (rs731236) in Iraqi postmenopausal women with osteoporosis and then investigates its function in the pathophysiology of the disease.

Materials and Methods: A case-control study was performed on 60 postmenopausal women with osteoporosis. Their age was ranged between 55-75 years; the second group includes 50 apparently healthy postmenopausal women. Patients with osteoporosis were randomly selected from the fragility examination unit of Al-Hussein Medical Hospital, Kerbala health directorates / Kerbala - Iraq between Nov., 2021 to June, 2022. Five milliliters of venous whole blood were deposited in gel tubes and centrifuged at 3000 x g for ten minutes from each sample in order to calculate the body mass index (BMI). The genotypes and allele frequencies of VDR variants were determined by polymerase chain reaction-allele specific using DNA isolated from peripheral blood. Dual-energy X-ray absorptiometry BMD measurement was used to confirm the presence of osteoporosis. The Hardy-Weinberg equilibrium assumption was used to interpret the results, and P 0.05 was taken into account as significant.

Result: The amplification of the VDR gene (rs 731236) SNP which were classified into three genotypes the major genotype group (TT) homozygous for the allele T, the minor genotype group (CC) homozygous for the allele C Heterozygous (TC). Genotype frequencies of the (rs 731236) polymorphism were consistent with Hardy–Weinberg equilibrium (HWE). Allele frequencies were 62%, 18% and 20% for TT, TC and CC, respectively, in control group. The Heterozygous (TC) and homozygous genotype (CC) significantly increased the risk.
Conclusion: Polymorphisms in VDR in the community of Iraqi postmenopausal women, *TaqI* was revealed to be a significant determining risk factor for osteoporosis advancement.
Keyword: Genotyping, Osteoporosis, Postmenopausal, 25(OH)D3, Receptor Polymorphisms, *TaqI* , Variant.

Introduction:

Approximately 200 million women worldwide are affected with osteoporosis, which is a significant source of morbidity and mortality and has complications that are related to it **(Melton III, 1995)**. Reduced BMD (2.5 standard deviations) is a multifactorial illness characteristic of osteoporosis **(Wu *et al.*, 2019)** . The most widely used diagnostic method is now dual-energy X-ray absorptiometry (DEXA) BMD scanning because of the disease's high correlation with bone mineral density (BMD) **(Burge *et al.*, 2007)** . Postmenopausal women are at high risk of disease. This is due to decreased levels of estradiol, produced mainly in the ovaries, leading to reduced bone mineral density (BMD). Through the action of its receptor VDR, vitamin D and its active metabolites play a role in maintaining calcium homeostasis, mineralizing bone tissue, and remodeling bone. The VDR receptor plays a significant role in calcium homeostasis and is expressed on the cell surfaces of the thyroid, kidney, and gut **(Ahn *et al.*, 2009)**. Osteocytes, osteoclasts, and osteoblasts all contain vitamin D receptors (VDR), which regulate bone remodeling. Through autocrine and paracrine pathways, the vitamin D metabolite 25(OH)D3 also regulates the osteoblast genesis of bone marrow stromal cells **(Abbas, Alaaraji and Alâ, 2020)**. Osteoclasts are differentiated from macrophages, osteoclast differentiation is controlled by vitamin D, either directly or indirectly **(Hou *et al.,* 2018)**. By inducing the production of hormones such fibroblast growth factor 23 (FGF23) and osteogenic hormones, vitamin D controls the remodeling of bones (prostaglandin E2, nitric oxide, and ATP) **(St. John *et al.,* 2014)**. The active form of vitamin D [1,25$(OH)_2$D3] play an important roles in bone growth and maturation while also inhibiting the generation of osteoclasts. As a result, vitamin D influences the activity of osteoblasts, osteoclasts, and osteocytes, as well as bone formation, resorption, and quality **(Hou *et al., 2018)***. Based on the complexity of vitamin D's active role in bones, osteoblasts, osteoclasts, and chondrocytes can all convert vitamin D into its active form. Since bone formation increases but bone resorption stays constant when CYP27B1 is overexpressed in mature osteoblasts, trabecular bone thickening results, increasing bone mass in both men and women. **(Abdel Aziz *et al.*, 2012).** Vitamin D insufficiency is linked to insufficient bone mass or insufficient bone remodeling, which can lead to bone fragility and an increased risk of fractures because vitamin D influences the

interaction between osteoblasts, osteoclasts, and osteocytes **(Jean, Souberbielle and Chazot, 2017**).

Materials and Methods:

A case-control study was performed on 60 postmenopausal women with osteoporosis with age ranged between 55-75 years; the second group includes 50 apparently healthy postmenopausal women. Patients with osteoporosis were randomly selected from the fragility examination unit of Al-Hussein Medical Hospital, Kerbala health directorates / Kerbala - Iraq between Nov., 2021 to June, 2022. From each sample the body mass index (BMI) was measured, 5 ml of venous whole blood was placed in gel tube and centrifuge at 3000 x g for 10 min. By using polymerase chain reaction-allele specific, DNA taken from peripheral blood was used to ascertain the genotypes and allele frequencies of VDR variations. Dual-energy X-ray absorptiometry BMD measurement was used to confirm the presence of osteoporosis. All individuals' samples withdrawn were collected in EDTA tubes following the manufacturer's instructions, the genomic DNA was extracted from whole blood samples using a ReliaprepTM blood gDNA miniprep system whole Blood Genomic DNA Purification Mini Kit (Promega USA). By calculating the absorbance at two wavelengths, the quality of each DNA sample that was extracted was evaluated (260 and 280). At a temperature of – 20 °C, all DNA samples were stored.

The different genotypes of the VDR gene (*TaqI*) were determined using a (polymerase chain reaction-Allele-specific technique). PCR was performed to amplify the regions of interest in the VDR gene. To perform PCR in a 25 µl reaction, 13 µl Thermo Scientific™ GoTaq G2 Green Master Mix (Promega USA), 1.5 µl of each forward and reverse primer, 6 µl of nuclease-free water (Promega USA), and 3 µl genomic DNA (100 ng/µl) were added into PCR tubes. Then, all tubes were centrifuged at 5000 rpm for 5 min before being placed in a PCR thermocycler. Thermocycling conditions consisted of an initial denaturation at 95 °C for 3 min, followed by 30 cycles of 30 sec. at 95 °C, 30 sec. at 63 °C and 30 sec. at 72 °C with a final extension at 72 °C for 5min.

Participants' data were entered into a computerized database, checked for mistakes or inconsistencies, and then maintained, processed, and analysed using IBM's SPSS version 28 for social sciences software. Scale variables presented in mean, standard deviation (SD), while descriptive statistics for nominal (categorical) variables represented as frequency (number of participants) and proportion (percentage). Scales variables like Age and weight that follow the statistical normal distribution, so parametric test was applied. Student's test for two independent samples was used to compare means between groups. To compare more than two means, one way analysis of variance (ANOVA) was performed. The connection between categorical variables was evaluated using chi square. Fisher's exact test was used as an alternative when the chi square was inapplicable.

Results:

Table-1 show the difference in biomarkers between patients and controls by performing a student t-test to compare the mean ± SD levels of each of age and body mass index, bone mineral density, T score, 25(OH)D3, total alkaline phosphatase activity, parathyroid hormone, estrogen

and progesterone. There was significant difference in mean levels of T-score, BMD, 25(OH)D3, progesterone between patients and controls $p < 0.05$. Genotype distribution and allel frequency of rs 731236 T/C as shown in table 2 . the odds ratios of the detected genotypes of the (rs 731236) of the patients with levels of BMD, D3, ALP, PTH, E2, Progesterone. The logistic analysis of the (rs 731236) SNP of the patients concluded that elevated BMD level was significantly related to the TT allele in comparison with CC and TC alleles (0.893, 0.893 $p < 0.05$). Also elevated D3 level was significantly related to the TT allele in comparison with CC and TC alleles (0.802, 0.89 $p < 0.05$). Also elevated E2 level was significantly related to the TT allele in comparison with TC alleles (0.925, $p < 0.05$). Also, lower Progesterone level was significantly related to the TT allele in comparison with CC alleles (1.359, $p < 0.05$) as shown in table 3 .

Table 1: Difference in mean levels of biomarkers studied between patients and controls

Lab. Parameters	Patients N = 60	Controls N = 50	P value
	Mean ± SD	Mean ± SD	
Age (years)	63.92 ± 7.69	56.48 ± 7.73	**< 0.001***
BMI (kg /m^2)	29.16 ± 4.49	29.99 ± 4.87	0.356
T- score	-3.12 ± 0.46	-1.90 ± 0.76	**<0.001***
BMD	0.71 ± 0.06	0.83 ± 0.08	**<0.001***
25(OH)D3 (ng /ml)	24.37 ± 12.27	19.89 ± 9.67	**0.039***
ALP (U/L)	223.05 ± 47.54	206.58 ± 39.90	0.054
PTH (pg /ml)	75.52 ± 29.42	85.19 ± 28.84	0.086
E2 (pg /ml)	26.42 ± 8.28	26.34 ± 4.92	0.953
Progesterone (ng /ml)	0.46 ± 0.18	0.55 ± 0.10	**0.003***

***Indicate significant**

Three genotypes were determined for the amplification of VDR gene rs731236 SNPs (TT) genotype homozygous wild type), (TC) (heterozygous type) and (CC) (mutant type) figure 1.

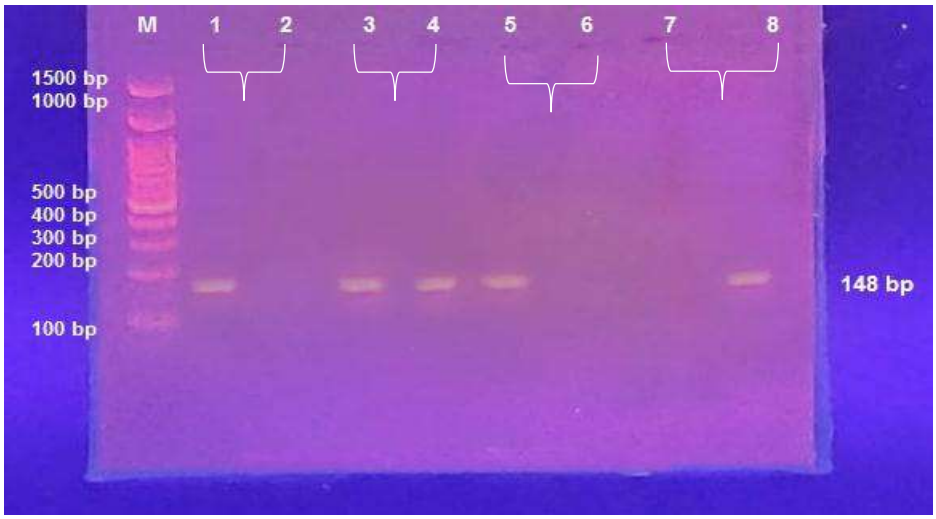

Fig. 1: Genotyping of gene VDR (rs 731236) in osteoporosis women

Table-2: Genotype distribution and allel frequency of rs 731236 T/C in patient and control groups.

Genotype T/C	Patient No. (%)	Control No. (%)	P value	Odd ratios	95% CI	P value
TT	15(25)	31 (62)	P <0.001	Reference		-
TC	21 (35)	9 (18)		0.433	0.237 – 0.792	**0.007**
CC	24 (40)	10(20)		0.366	0.202 – 0.662	**<0.001**
Total	60(100%)	50(100%)				
Allele	Frequency					
T	0.425	0.71	Reference			
C	0.575	0.29	2.66	0.372 – 0.982		0.157

Table -3: The odds ratios of VDR Polymorphism (rs 731236) with other biomarkers level

Variables	SNP	OR (95% CI)	p value
BMD	**TT**	Reference	-
	TC	0.893 (2.718-1.032)	**0.050 ***
	CC	0.893 (8.963-1.269)	**0.014 ***
25(OH)D3	**TT**	Reference	-
	TC	0890 (0.838-0.945)	**<0.001 ***
	CC	0.802 (0.739-0.870)	**<0.001 ***
ALP	**TT**	Reference	-
	TC	1.011 (0.999-1.024)	0.073
	CC	1.010 (0.997-1.024)	0.134
PTH	**TT**	Reference	-

	TC	0.981 (0.962-1.000)	0.052
	CC	0.994 (0.974-1.014)	0.561
E2	**TT**	Reference	-
	TC	0.925(0.857-0.997)	**0.042 ***
	CC	0.962 (0.897-1.033)	0.286
Progesterone	**TT**	Reference	-
	TC	23.87 (0.962-592.144)	0.053
	CC	1.359 (1.044-41.643)	**0.049 ***

***Indicate significant**

Discussion:

Osteoporosis is a genetically based illness with an unidentified origin, However, there is data showing the significance of factors including age, gene interaction, and environmental factors in the disease's development. **(Durusu Tanriover *et al.*, 2010)** . Serum alkaline phosphatase is the most commonly used markers of bone formation. ALP is a ubiquitous enzyme that plays an important role in osteoid formation and mineralization. Total serum alkaline phosphatase in postmenopausal women with osteoporosis was higher the postmenopausal women without osteoporosis was within normal range. The mean level of serum alkaline phosphatase in the patients group (217.33 ±49.28) U/l, while in the controls group (206.58 ± 39.9) U/l , the normal range for serum alkaline phosphatase is (98 – 279 U /L); Like that findings, several studies showed that the serum alkaline phosphatase level was significantly higher in postmenopausal women with osteoporosis than in postmenopausal women without osteoporosis **(Indumati, Patil and Jailkhani, 2007)**. As the enzyme function in the metabolism of bone tissues, it may be expected that its increased activity is a way to compensate for lost calcium and phosphate by recruiting more of these elements to build new bone tissue **(Indumati, Patil and Jailkhani, 2007)**. Rapid bone loss occurs in post-menopausal women due to hormonal factors that lead to an increased risk of fractures. Thus, the present study was undertaken to observe the serum calcium and alkaline phosphatase levels in post-menopausal women as these substances are biochemical markers of bone metabolism **(Adamczyk *et al.*, 2014)**. The results of current study show a significant increase in mean level of serum PTH in osteoporosis group. This finding was in accordance with results obtained by **(Agool, Mohammed and Hashim, 2015)** which indicated that when blood calcium decreases, the secretion of PTH increases stimulating osteoclast activity which results in an increased bone breakdown, therefore, serum calcium becomes normal **(Lai and Fyfe, 2000).** The primary results of this study were reduced BMD in the patient group, lower progesterone in PMOP

compared to healthy women. We know that bone loss begins in women well before menopause, while ovulation is still occurring and estrogen levels are essentially normal; there are a variety of ways by which progesterone can affect bone metabolism. For example, the hormone appears to stimulate new bone formation **(Sitruk-Ware and Utian, 1991)**. In addition, progesterone appears to increase levels of insulin- like growth factor-1, which promotes bone formation **(Sitruk-Ware and Utian, 1991)**. Amplification products were obtained to have a size of 148 bp for rs731236, and genotypes of VDR gene rs731236 SNPs were assessed using allele-specific PCR for quick polymorphism screening in patients.

Molecular product was electrophoresed and immediately seen on an agarose gel that was dyed with ethidium bromide and illuminated by UV light. SNP rs731236, located in exon 9 at the 30 end of the VDR gene, results in a synonymous change and has been proved to affect mRNA stability **(Banjabi *et al.*, 2020)**. The current study confirms that there is a significant association between VDR rs731236 and risks of PMO, which is in agreement with earlier studies **(Sassi *et al.*, 2015).** Vitamin D is regarded as a significant predictor of BMD since it is essential for intestinal calcium absorption and bone metabolism. The active form of vitamin D, 1,25 $(OH)_2D3$, binds to the cytosolic/nuclear VDR before joining forces with the retinoid X receptor (RXR).The resultant heterodimer controls the transcription of the target gene by attaching to vitamin D response elements (VDREs) **(Ansari *et al.*, 2021).** Regarding vitamin D's potential impact on PMO risk, numerous studies in diverse populations have been conducted on its impacts. The main factors contributing to vitamin D's enormous popularity are its role in bone production, calcium homeostasis, and bone mineral density modulation. The VDR gene codes for a particular steroid receptor that vitamin D interacts to VDR functions as a transcription factor and affects the expression of many target genes. Through careful examination of the VDR gene, polymorphic variations that could affect the structural or functional properties of proteins have been found. So, these variations could potentially be used as clinical and diagnostic indicators of bone and muscular disease. **(Urano and Inoue, 2014 ; Wang *et al.*, 2021 and Zhang *et al.*, 2014)**. The effects of *TaqI* CC genotypes on bone mass in Japanese women have been investigated extremely infrequently because they are relatively less common than those described in European nations. For instance, related allelic needs regarding the *TaqI* T. genotype Approximately 40% of Europeans have and C, compared to 10% of Japanese people **(Lurie *et al.*, 2007 ; Tokitan *et al.*, 1996 and Douroudis *et al.*, 2003)** found an association between the TT genotype and osteoporosis , **(Gursoy, Maier and Chi, 2008 and Marozik *et al.*, 2013)** found no association of osteoporosis for the *TaqI* polymorphism (rs731236), while other studies explained the association between CC genotype and osteoporosis **(Mitra, Desai and Khatkhatay, 2006 and Duman *et al.*, 2004)**. Some of these conflicting results are attributed to small sample sizes or genetic effects that may have been obscured by gene-gene-environment interactions.

Conclusion:

The observed data indicated a strong association between BMD, VDR gene variant, PMO risk level, and serum 25(OH)D3 levels and VDR *TaqI* polymorphisms have been found as significantly specific risk factors for the development of osteoporosis in postmenopausal Iraqi women.

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