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Sabah Subhi Ismael barani
University of Mosul/Collage of Pharmacy, Department of Clinical Laboratory Sciences, College of Pharmacy, University of Mosul, sabah.barani@uomosul.edu.iq

Mohammed Abdullah Ajeel
University of Mosul/Collage of Pharmacy, Department of Clinical Laboratory Sciences, College of Pharmacy, University of Mosul, mohammed91@uomosul.edu.iq

Safwan Jasim Sultan
Alnoor University, College of Dentistry, Ninavah, safwan.jassim@alnoor.edu.iq

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ORIGINAL STUDY

The Impact of VDR Gene and Biochemical Indicators in Osteoporosis

Sabah Subhi Ismael Barani [a,*], **Mohammed Abdullah Ajeel** [a], **Safwan Jasim Sultan** [b]

[a] University of Mosul/Collage of Pharmacy, Department of Clinical Laboratory Sciences, College of Pharmacy, University of Mosul
[b] Alnoor University, College of Dentistry, Ninavah

ABSTRACT

Osteoporosis is a disorder caused by bone remodeling imbalance in which bone resorption outpaces bone production, resulting in weaker bone structure. Factors such as heredity and insufficient calcium and vitamin D consumption.

This study included an examination of 80 female patients of different ages (40–58 years) in Mosul. The participants were divided into two groups: 20 individuals served as the control group, while the rest 60 were osteoporotic women based on clinical cases and taking X ray DEXA. The blood samples are divided into two parts serum for biochemical tests and blood for genetic diversity in genes associated to Vitamin D Receptor (*VDR*) was then examined.

The results indicated the presence of mutations in the *VDR* gene at location (rs7975232). Furthermore, an observed variance in numbers of nucleotide after sequencing. there was also a significant decrease in vitamin D and Calcium levels in the blood serum of osteoporotic woman as compared to the control group.

Keywords: Osteoporosis, VDR gene, Calcium, Sequencing and biochemical test

1. Introduction

Osteoporosis, a common illness mostly detected by bone density examination, is associated with a greater susceptibility to fractures attributed to bone fragility (Voulgaridou et al., 2023). In 2019, around 25.5 million cases aged over 50 years old in the EU27 were affected by osteoporosis, including over 22 million women and 3.5 million men (Kanis et al., 2021). Furthermore, the number of osteoporotic fractures documented in 2019 was 4.28 million, with forecasts predicting an increase to 5.34 million in 2034 (Kanis et al., 2021). A global systematic review and meta-analysis indicated that the worldwide prevalence of osteopenia was 39.5% (95% CI: 22.3% to 59.7%), The greatest incidence was recorded in Europe at 41.9%, whereas Africa exhibited the lowest at 29.7% (Salari et al., 2021).

One of the most important vitamins for bone metabolism and mineralization is vitamin D. Circulating 25-hydroxyvitamin D levels below 20 ng/mL (50 nmol/L) are considered deficient, and this condition is becoming increasingly common across the world (Hadi, Ouda, and Alboaklah, 2022).

Vitamin D's metabolic functions are exerted through engagement of the active vitamin D receptor (*VDR*), known as 1,25-dihydroxy-vitamin D (1,25 (OH)). 2D) followed by the retinoic acid receptor (RXR) in binding to a compound, forming heterodimers that link specific gene promoter regions through vitamin D response elements This sequence is due to transcriptional regulation strains that down-regulate target genes. *VDR*s are present in nearly all tissues of human encompassing adipose tissue as well, which are important mediators of vitamin D function and consequently have a significant impact in the epigenome and the expression of more than 1000 genes (Carlberg, 2019).

Vitamin D and calcium play crucial roles in maintaining bone health, and insufficient levels of either

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* Corresponding author.
E-mail addresses: sabah.barani@uomosul.edu.iq (S. S. I. Barani), mohammed91@uomosul.edu.iq (M. A. Ajeel), safwan.jassim@alnoor.edu.iq (S. J. Sultan).

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are major risk factors for osteoporosis (Fischer et al., 2018). Vitamin D is synthesized in human skin by exposure to sunlight. However, modern lifestyles often limit adequate sun exposure which is essential for the synthesis of this vitamin (Mithal et al., 2014). As a result, levels of 25-hydroxyvitamin D3 (25(OH)D3) are significantly reduced, which is especially common during certain seasons, especially winter and spring (Wyskida, Wieczorowska-Tobis, and Chudek, 2017). Establishing a standard serum vitamin D level that distinguishes between deficiency, insufficiency and sufficiency continues to be a topic of persistent discussion. Although it is widely accepted that a 25(OH)D level below 10 ng/mL (= to 25 nmol/l) indicates a major deficiency (Makris, Sempos, and Cavalier, 2020).

The human VDR gene is located on chromosome 12 at position q13.1. It spans roughly 75 kilobases of genomic DNA and is composed of 9 exons (exons 1a/1b, 1c, 2–9) (Etten and Mathieu, 2005). Several well-documented polymorphisms, including ApaI and TaqI, in the intron 8 of the human VDR gene, are widely researched genetic markers associated with changes in bone mineral density (BMD) in adult women. These polymorphisms influence the stability of mRNA and the expression of the VDR gene (Nasiri et al., 2005). ApaI and TaqI polymorphisms, located in intron 8 lies within the genomic region encoding the ligand-binding domain (LBD) of the VDR (exons 4–9), this interionic region do not result in amino acid change in the VDR structural protein however, the may effect regulatory and potential gene expression or processing of mRNA which may influence overall receptor activity that can alter vitamin D-binding specificity (Banjabi et al., 2020). the current study aimed to show the impact of VDR gene and biochemical indicators in osteoporosis patient women in Mosul city.

2. Material and methods

2.1. Study design

This research analysis obtained formal approval from the Ethics Committee of the University of Mosul. The investigation included 80 participants, categorized into two cohorts: 60 individuals diagnosed with osteoporosis and 20 individuals without the condition. All participants were 40 years of age or older and had been residing in Mosul during the study period from November 2023 to February 2024.

Participants were chosen through a two-stage selection process. Initially, a Discovery-W fan-beam densitometer (produced by Hologic Inc., Bedford, MA, USA) was utilized to conduct DEXA scans. Individuals with a confirmed bone mineral density (BMD) diagnosis and a T-score below –2.5 were included in the study. Healthy participants were considered only if their results indicated no osteoporosis and had T-score values above –1. Furthermore, individuals who were breastfeeding, pregnant, or had any organ failure or chronic illness were excluded from the study.

2.2. Collection of blood samples and DNA extraction

Blood samples were collected from all participants using EDTA tubes. DNA was extracted from the whole blood samples using a Whole Blood Genomic DNA Extraction Kit (add bio, Korea) in accordance with the manufacturer's guidelines. The quality of the extracted DNA was evaluated by measuring absorbance at two wavelengths (260 and 280) using a nanodrop spectrophotometer at the College of Science, University of Mosul. All extracted DNA samples were stored at −20 °C for subsequent analysis.

2.3. Molecular tests

DNA was isolated from the blood of all 80 samples included in the study, based on the method modified by May, 2010.

2.4. Tetra–ARMS-PCR reactions

The DNA concentration in each sample was adjusted with TE buffer solution to 25 ng/microliter for PCR amplification. For primer reactions, four primers were used, including F-outer and R-outer throughout the gene were employed for primer reactions. For the mutant allele, forward outer-reverse inner is used instead of the normal allele's forward outer-reverse inner. Nucleic acid from each sample was mixed with appropriate primers for the targeted mutations and the master mix components to form the PCR reaction blend in 0.2-ml PCR tubes. This mixture was quickly centrifuged to achieve optimal component. Following that, the PCR tubes were cycled in a thermocycler using customized procedures for particular mutations. The reaction product (at 2% concentration) was placed into the wells of a prepared agarose gel after a DNA ladder from Biolaps Company was injected into specified wells. After 40 minutes of electrophoresis to migrate the samples, the bands were imaged using a gel-electrophorese apparatus. Tetra-ARMS-PCR was used to assess the genetic variation of the *VDR* gene at locus (rs7975232). The primer sequences used in this study are provided in Table 1.

Table 1. Shows the primers used to detect different genes at locus (rs7975232) using PCR.

rs7975232 for VDR	F-outer	5′CAAACACTTCGAGCACAAGGGGCGT3′	514 bp	67 °C
	R-outer	5′GGGATGGACAGAGCATGGACAGGG3′		
	F-inner	AGGCACAGGAGCTCTCAGCTGGACC3′	317 bp	
	R-inner	5′GGGGTGGTGGGATTGAGCAGTGAAGT3′	248 bp	

2.5. Genetic variation in the VDR gene (rs7975232) was determined using the Tetra–ARMS-PCR technique

Detection of the A→C mutation for VDR gene at the site (rs7975232), 4 μl of template DNA (100 nanograms) and 1 μl of each mutation-specific primer (10 picomoles) were added for VDR gene cloning the changes, which were provided by the Macrogen (Korean company), down to the master mix materials (Al-Mawlah et al., 2021).

The reaction tubes were then put inside the thermocycler to carry out the amplification process according to the specific program authorized for this reaction, as shown in Table 2.

In this reaction, the optimum temperature for primer binding was determined using the gradient setting of the thermocycler, which allowed a range of ±5 degrees after which the PCR reaction was analyzed by separation through a 2% agarose gel.

2.6. DNA sequencing

The rs7975232 loci were subsequently confirmed by Sanger sequencing, using an Applied Biosystems 3130xl Genetic Analyzer (Thermo Fisher Scientific).

2.7. Biochemical test

This study also includes various biochemical parameters considered as markers of osteoporosis, like:

2.7.1. Calcium level measurement

The calcium level was measured using the FUJIFILM device, which was prepared by the German company FUJIFILM Europe Gmbh.

2.7.2. Vitamin D level measurement

Vitamin D level was measured using the VIDAS device.

2.8. Statistical analysis

The statistical evaluations were executed utilizing SPSS version 20.0. The Mann–Whitney test, a non-parametric approach, was applied to compare the parameters across the groups. Allele frequencies were computed by dividing the count of the test allele by the total allele count in the population. The odds ratio (OR), 95% confidence intervals, and P values for genotype distributions and allele frequencies were derived using a Chi-square test, in accordance with the Hardy–Weinberg equilibrium principle. A P value of less than 0.05 was deemed statistically significant.

3. Results and discussion

3.1. Determination of genetic variation of the VDR gene in situ (rs7975232) using Tetra–ARMS-PCR technique

The results in Fig. 1 showed that there is a relationship between women who suffer from osteoporosis and the genetic variation in the VDR gene at the site (rs7975232) on chromosome 12, as it is clear from the PCR reaction that the genetic variation of the gene appears in the three genotypes AA, AC, CC.

Table 2. Shows the operating system used by the ARMS-PCR method to detect the mutation (rs7975232).

No.	Stage	locus	Temperature	Time	Cycle number
1	Initial denaturation	for all sites	94 °C	5 min.	1
2	Denaturation	for all sites	94 °C	30 sec.	35
3	Annealing	(rs1107946)	59 °C	30 sec.	
		(rs412777)	69 °C		
		(rs7975232)	67 °C		
4	Extension	for all sites	72 °C	1 min.	
5	Final extension	for all sites	94 °C	7 min.	1
6	Stop reaction	for all sites	4 °C	5 min.	1

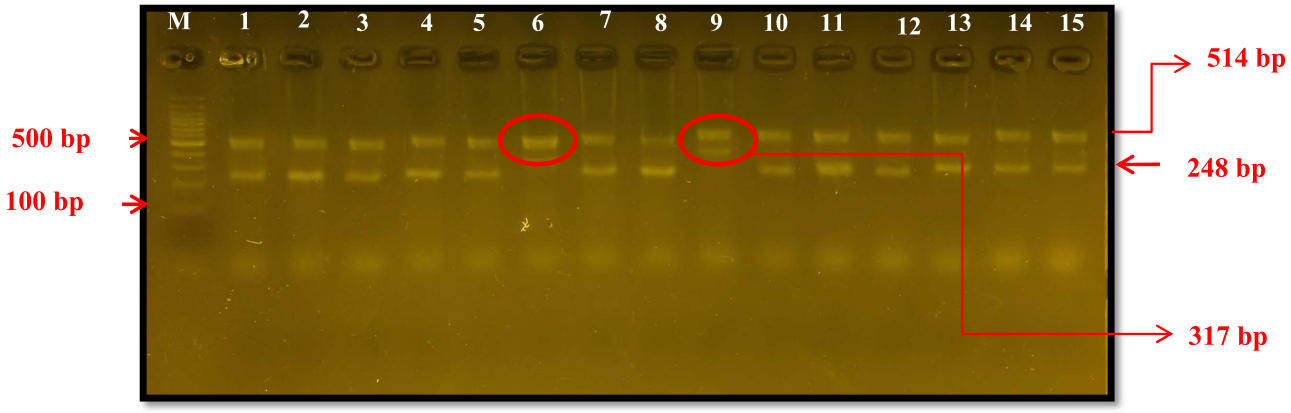

Fig. 1. The product of the PCR reaction of the genetic variation (rs7975232) of the VDR gene. The result was a reaction containing 3 bundles, the first with a size of 514 bp for the main gene, the second with a size of 248 bp for the natural allele, and the third bundle with a size of 317 bp For the mutant allele, M is the Ladder with a size of 100 bp, which was prepared by Biolabs and separated by 2% agarose gel.

3.2. Determination of nucleotide sequencing of amplified pieces using DNA sequencing technology for VDR gene

Sequencing experiments of the amplified *VDR* gene revealed variations in nucleotide levels. These observations can be seen in the figures that follow:

Reviewing Table 3, it shows a variety of genetic variants along with their positions along the *VDR* genes. These differences were identified after sequence analysis and subsequent comparisons of genes obtained from the NCBI database as shown in Fig. 2.

Table 4 illustrates the allelic and genotypic distributions of VDR ApaI polymorphisms within both

Fig. 2. Shows the alignment results between the nucleotide sequences derived from the *VDR* gene and the original genes of NCBI.

Table 3. Details the specific loci and variants identified in the VDRG gene in individuals affected by osteoporosis.

ID sequence	Nucleotide	Location	Mutation type	Identity	Gaps
AY342401.1	A → G	61967 + 61870 + 61798	Transition	90%	0%
AY342401.1	G → T	61936 + 61723 + 61675	Trans version	90%	0%
AY342401.1	C → A	61862 + 61872 + 61839 + 61776 + 61778	Trans version	90%	0%
AY342401.1	C → G	61864 + 61868 + 61871 + 61877 + 61879 + 61786	Trans version	90%	0%
AY342401.1	C → T	61865 + 61866 + 61869 + 61774	Transition	90%	0%
AY342401.1	A → T	61867 + 61875 + 61770 + 61781 + 61719	Trans version	90%	0%
AY342401.1	G → A	61837	Transition	90%	0%
AY342401.1	T → A	61764	Trans version	90%	0%
AY342401.1	A → -	61682	Deletion	90%	0%
AY342401.1	A → C	61727	Trans version	90%	0%
AY342401.1	G → C	61721	Trans version	90%	0%

Table 4. The allelic and genotypic frequencies of ApaI (rs7975232) *VDR* genetic variant in the studied subjects.

	Genotypic frequencies (%)			Allelic frequencies (%)		
Groups	AA	Aa	aa	A	a	p-value
Cases	38 (47.5)	33 (41.25)	9 (11.25)	109 (54.5)	51 (25.5)	0.067
Control	9 (45)	8 (40)	3 (15)	26 (13)	14 (7)	0.321
Total	47 (47)	41 (41)	12 (12)	135 (67.5)	65 (32.5)	0.712
p-value	0.0021			0.0092		

cohorts. The ApaI genotypes were categorized as recessive homozygous (aa, tt), dominant homozygous (AA, TT), and heterozygous (Aa, Tt). The prevalence of the AA genotype was observed to be 47.5%, with a statistically significant *p* value of 0.021. When compared to the aa genotype, the Aa and AA genotypes emerged as potential risk factors for osteoporosis, evidenced by a *p* value of 0.029. Furthermore, the frequencies of the A and a alleles in the ApaI polymorphism demonstrated statistical significance between the two groups, with a *p* value of 0.0092 for the comparison of allele A versus allele a.

These findings underscore the relevance of VDR ApaI polymorphisms in the context of osteoporosis risk. The higher frequency of the AA genotype and its association with osteoporosis suggest a genetic predisposition that could be crucial for early diagnosis and targeted therapeutic strategies. The significant differences in allele frequencies further emphasize the potential role of genetic variations in influencing susceptibility to osteoporosis. Future studies should consider expanding the sample size and exploring additional polymorphisms to validate these findings and enhance our understanding of the genetic factors contributing to osteoporosis.

3.3. Biochemical test results

The results of the study, as shown in Fig. 3, showed that the serum levels of calcium and vitamin D in women with osteoporosis were significantly decreased at a probabilistic level of $p \leq 0.05$ compared with healthy group. The study showed that compared to healthy patients, the calcium level was (7.040 U/ml), and healthy was (8.716 U/ml), and the vitamin D level in patients and healthy group were (20.158 mg/dl), (57.190 mg/dl) respectively as in Table 5 is shown.

According to research, the *VDR* gene is also polymorphic, with single nucleotide polymorphisms (SNPs) occurring when one single nucleotide is changed by another and being the most prevalent sort of mutation in DNA (Doffe et al., 2021). This genes inactivation arises mostly through point mutations, however gene deletions or nucleotide insertions have also been observed, these alterations may impair *VDR* function, increasing the risk of osteoporosis (Kim and Lozano, 2018).

Another study established an association between *VDR* BsmI gene mutations and LS (posterior lumbar spine) BMD (bone mineral density) levels in pediatric patients Children carrying the bb gene tend to have lower BMD levels while compared with children with the B allele (Chen et al., 2020).

These genetic polymorphisms in the VDR gene sequence result in the production of less efficient receptor protein with reduced vitamin D binding affinity. This impaired binding capacity subsequently compromises vitamin D transport to target tissues, exacerbating health complications associated with vitamin D deficiency, including osteoporosis. Also, these genes have many genetic polymorphisms as a result because it contains more than 120 variables in the nucleotide sequences (Kim et al., 2017).

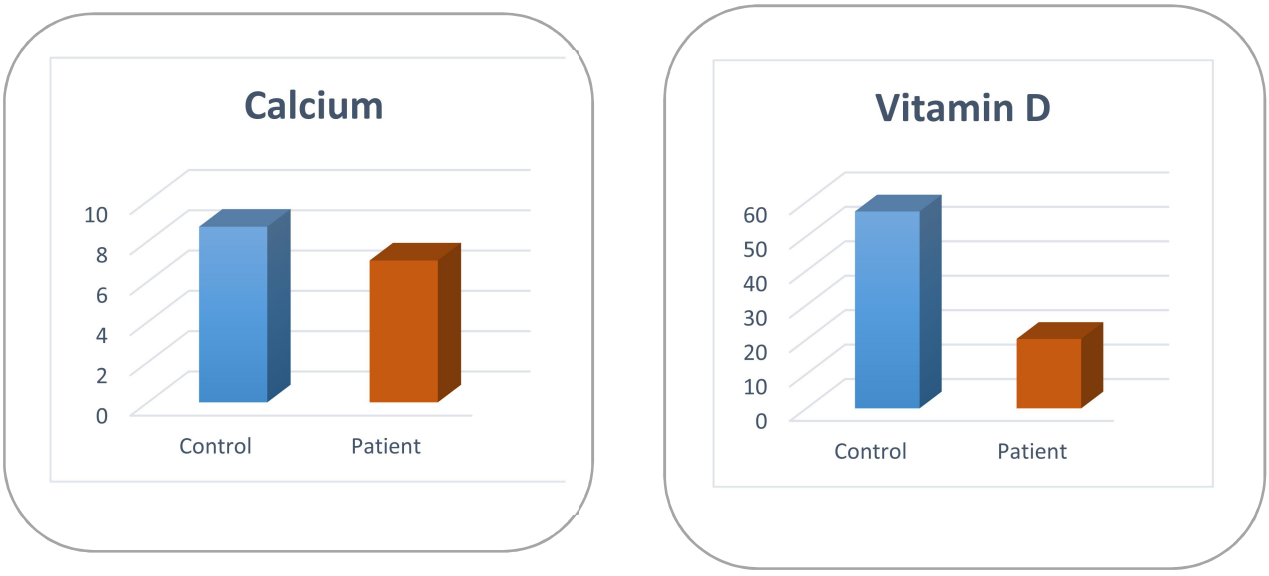

Fig. 3. Comparison of serum's Calcium and vitamin D level between osteoporosis patient and control groups.

Table 5. Shows the results of biochemical variables in the group of women with osteoporosis.

Sample	Ca test ± SE mg/dl	VD test ± SE U/ml
control	8.716 ± 0.266	57.190 ± 4.218
patient	7.040 ± 0.0873	20.158 ± 1.082

In recent studies, it was found that patients with osteoporosis had a low level of calcium and vitamin D. This result indicates the association between calcium and vitamin D and disease activity. In other studies, it was found that calcium and vitamin D deficiency increases inflammatory activity in patients with osteoporosis compared to the control group. Therefore, monitoring calcium and vitamin D levels will be very useful to identify patients at risk of developing osteoporosis (Reid, 2016).

Voulgaridou et al. suggest that vitamin D, either independently or in combination with calcium, increases circulating 25(OH)D levels. Vitamin D supplementation with calcium supplements was found to increase bone mineral density. However, no significant difference was observed between supplements containing vitamin D alone and calcium and vitamin D combination when considering absolute fracture risk reduction (Voulgaridou et al., 2023).

4. Conclusion

This study confirms the relationship between osteoporosis and the percentage of calcium and vitamin D in patient's serum is clearly lower compared to the control group. This indicates the association between calcium and vitamin D and disease activity. The mutation in VDR gene also have a strong relationship with osteoporosis because this study found mutations like transversion, transition and deletion of nucleotide in patients with osteoporosis when compared to healthy control group. Compared to genotype aa, Aa and AA was found as a candidate risk factor of osteoporosis. However, Further studies are needed to examine the potential effects of the vitamin D- and calcium-containing *VDR* gene on markers of bone turnover.

Author contribution

Sabah Subhi Ismael and Mohammed Abdulla designed the study. Safwan Jassim Sultan performed the experiments. Sabah Subhi Ismael and Mohammed Abdulla were involved in writing the draft of the manuscript. all authors confirm the authenticity of all the raw data and have read and approved the final manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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