Al-Kindy College Medical Journal (KCMJ)

Research Article

Correlation of Osteocalcin with Vitamin D Level in Postmenopausal Women Concerning CYP24A1 and VDR Gene Polymorphisms

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Article history:
Received 1 October 2023
Accepted 11 January 2024
Available online 1 August 2024

https://doi.org/10.47723/sdfrzk14

Keywords: postmenopausal osteoporosis; vitamin D; CYP24A1; osteocalcin; single nucleotide polymorphism

ABSTRACT

Background: Vitamin D is an essential agent in regulating body enzymes due to the wide distribution of vitamin D receptors throughout the body, the site where vitamin D acts. Vitamin D deficiency could be related to many factors. Polymorphism in receptor(s) and enzyme(s) responsible for vitamin D metabolism, like CYP24A1, could be liable for vitamin D deficiency.

Objective: This study aims to determine the impact of polymorphism in Vitamin D Receptor TaqI (rs731236) and CYP24A1 (rs2762934 and rs4809957) on vitamin D level and osteocalcin in postmenopausal women.

Subjects and Methods: Forty postmenopausal osteoporotic women (patients' group) according to WHO osteoporosis diagnostic criteria were enrolled in the study. This group received 50000 IU/week of vitamin D for eight weeks. In addition, 30 postmenopausal non-osteoporotic women were set to be a control group. Genetic and biochemical analyses were applied for both groups.

Results: Serum levels of osteocalcin in the patients' group were 9±6.19 and 4±6.6 in groups with vitamin D levels below 20nmole/L and above 20nmole/L, respectively, with a non-significant difference between them (p=0.49). Related to allele frequency, the A/G genotype of rs731236 of the vitamin D receptor, G/G genotype of rs2762934, and A/A genotype of rs4809957 of CYP24A1 showed non-significant differences among the study patients' group.

Conclusions: Our data indicates that rs731236 with the A/G genotype, rs2762934 with the G/G genotype, and rs4809957 with the A/A genotype were more prevalent than the other genotypes and could be responsible for being osteoporotic through their effect on vitamin D level and resistance to vitamin D supplementations.

Introduction

Vitamin D is a natural steroid (1) essential for sustaining the health of several organs, including the kidneys, liver, respiratory system, and even the immune system (2). Serum levels of vitamin D can impact bone mineral density in different demographic groups (3). The daily requirements of vitamin D depend on the population's age, skin tone, skin type, and whether they live in a region with high levels of sun exposure, or the presence of endocrine or central nervous system disorders. (4), and the deficiency in vitamin D increases the likelihood of developing acute or chronic illnesses (5). The prevalence of vitamin D deficiency was studied in Iraq and concluded that about 60% of the participants suffered from a deficiency with vitamin D levels between 10-30 ng/ml (6).

The synthesis and metabolism of vitamin D goes through several steps, first being synthesized in the skin through ultraviolet activation (7), then metabolically activation through the liver enzyme

CYP27A1, metabolically activated in the renal system by CYP27B1(8), and finally ending with inactivation through CYP24A1 to releases vitamin D to avoid toxic or harmful concentrations (9).

Specific receptors enable vitamin D to carry its supposed function in the body. As mentioned earlier, vitamin D is crucial since VDR is widely distributed. It can be found in the body's immune, digestive, and brain. Besides, it aids in the release of hormones like insulin, and prompts heart support, blood vessel, and motor function (10).

In recent years, responsibility of single nucleotide polymorphisms (SNPs) on disease progression or low response to treatment were assess, and these characteristics have been studied in pharmacogenetics (11). It has been recognized that when SNPs responsible for a disease progression were detected, this helps in avoiding progression factors, early disease diagnosis, select the most effective treatment, and preventing resistant therapies (12-14).

Vitamin D levels affected by one or more SNPs that enter in vitamin D metabolism or affecting receptor (VDR) function (15, 16). To date, four types of VDR polymorphism have been studied (17, 18). For CYP24A1 gene, many SNPs have been studied and thought to be responsible for high susceptibility to colorectal cancer (19), respiratory diseases and lung cancer (20), cardiovascular events (21), osteoporosis (22), and even autoimmune diseases (23).

Osteoporosis (OP) is a condition with a multichemical pathophysiology that may be related to immune origin (24) or severe deficiency in vitamin D (25). These mechanisms affect bone resorption and formation, making bones fragile and susceptible to fractures (26-28). The bone turnover marker, osteocalcin (OC), is one of the bone formation markers (29, 30), which provides an impression about bone health and can be used to monitor the treatment and follow-up of osteoporosis (31, 32), and can relate Polymorphism in VDR and CYP24A1 thought to have an impact on OP prognosis through poor response to vitamin D doses, as seen in a study on specific SNP of VDR in Iraqi postmenopausal women, which revealed its effect on increased risk of incidence of OP and poor benefit from vitamin D dose(33). Thus, we tried in present study to find the role of VDR gene polymorphism (rs731236) and CYP24A1 gene polymorphisms (rs2762934 and rs4809957) on the serum levels of vitamin D in postmenopausal osteoporotic women with related to Bone turnover marker, osteocalcin.

Subjects and Methods

This prospective cohort study randomly selected 40 postmenopausal women who met the criteria of being osteoporotic according WHO guidelines based on Bone Mineral density (BMD) measurement (patients with T-score ≤ -2.5 were diagnosed to be osteoporotic, and those with T-score between -1 and -2.5 were diagnosed to have osteopenia, and patients with T-score >-1 were considered normal) (34) whom their age over 50 years old with at least two years of cut-off menstrual cycle. These patients set as patients' groups and assigned to receive 50000 IU/week of vitamin D for eight weeks. Besides, 30 postmenopausal non-osteoporotic women were considered as a control group. BMD is measured by dual-x-ray absorptiometry (DXA-scan) (manufactured by Lunar Prodigy Advance, Belgium) and this study was applied in Basra Teaching Hospital/ Consulting Clinics/ Orthopedic Unit from March 2022 to January 2023.

Clinical and laboratory investigations were performed for both groups Participants data were reported in a specific format that was prepared for this study. Ethical approval from the College of Pharmacy/ University of Baghdad was registered and carried no. RECAUBCP24112021B

Blood specimens from each participant were collected to perform genetic and biochemical analysis, as mentioned below.

Diagnostic Kits:

Diagnostic kits with their providers are presented in Table (1)

Table 1: Diagnostic kits with their providers

Diagnostic kits	Providers
Serum Osteocalcin ELISA kit	**Shanghai, China**
Serum vitamin D	**Cobas, Switzerland**
Serum parathyroid hormone	**Roche, Switzerland**
Serum Calcium	**bioMérieux, France**
Serum Phosphate	**bioMérieux, France**

Biomarkers analysis:

1- Vitamin D:

Vitamin D is measured using a specific ELISA test kit (Elecsys® Vitamin D total II by Cobas, Roche Diagnostics, Belgium) (35).

2- Osteocalcin:

Measured by ELISA test kit (Shanghai, China)

Principle of ELISA kit: This ELISA kit uses the Sandwich-ELISA principle. The micro-ELISA plate provided in this kit has been pre-coated with an antibody specific to osteocalcin and vitamin D. Standards or samples are added to the micro-ELISA plate wells and combined with the specific antibody. Then, a biotinylated detection antibody specific for OC and vitamin D is used. Free components are washed away. The substrate solution is added to each well. Plates that contain serum of OC or vitamin D will conjugate and appear in a new color. Adding a stop solution terminates the enzyme-substrate reaction; absorbance is measured at 450 nm (36).

3- Parathyroid Hormone:

Measured by (Cusabio, China). The antibody-antigen reaction is the principle of this kit, adding substrates to make reactions and changes of color. Color intensity is directly proportional to the concentration of intact PTH

4- Serum Ca:

It is measured by (bioMérieux, France). Serum calcium levels can be evaluated using a ready-made kit according to the colorimetric method. This method depends on the specific binding of calcium resulting in the complex, and then the absorbance of the complex is proportional to calcium concentration (37).

5- Serum PO4:

It is measured by (bioMérieux, France). Colorimetric determination, without deproteinization, of serum phosphorous using a single reagent, which forms a specific complex in the presence of a reducing agent, and then colorimetry measured the PO4 concentration (38).

DNA extraction:

The ABIO pure Extraction procedure was used to extract genomic DNA from a blood sample; DNA Extraction Kit, Promega, USA was used, which involved the following steps:

1. For protein digestion and cell lysis, 20µl of Proteinase K solution (20 mg/ml) and 200µl of Buffer lysine (BL) were added to 200µl of blood sample, then the tube was mixed using vortex and incubated at 60˚C for 5 minutes. 200µl of GSB buffer is added, mixed with vortex, and set for 5 minutes in a 60˚C water bath. 200µl of Absolute ethanol was added to the sample, pulse-vortex, to combine the sample thoroughly.

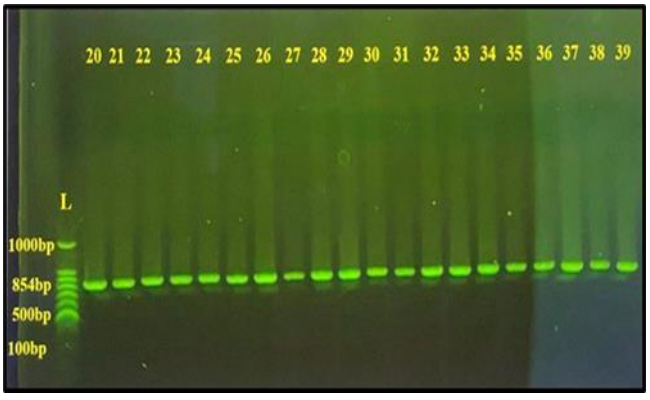

Figure 1: CYP24A1 gene amplification was fractionated using 1.5% agarose gel electrophoresis stained with green gel dye, L: ladder marker. Lanes20-39 resemble 854bp PCR products.

2. All of the mixtures were transferred to the mini-column carefully (GS tube), then centrifuged for 1 min at 6,000 x g above (>8,000 rpm), and the collection tube was replaced with a new one.
3. From washing buffer (BW1), 400µl was Added to the mini-column, then centrifuged for 30 seconds at (>8,000 rpm). From wash Buffer 2(BW1 with ethanol) (BW2), 600µl was applied. Centrifuged for 30 seconds at 6,000 x g above (>8,000 rpm). The passthrough was discarded, and the mini-column was reinserted into the collection tube.
4. The mini-column was centrifuged at full speed (>13,000 x g) for 3 minutes to remove residual wash buffer, and then the mini-column was placed into a fresh 1.5 ml tube.
5. From Acetate –EDTA buffer (AE), 100µl was added and incubated for 3 min at room temperature, then centrifuged at 5,000 rpm for 30 seconds.

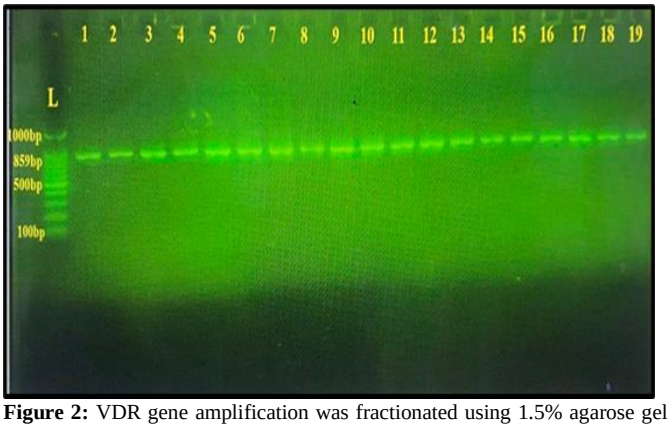

Figure 2: VDR gene amplification was fractionated using 1.5% agarose gel electrophoresis stained with green gel dye, L: ladder marker. Lanes1-19 resemble 859bp PCR products

Primers:

From the National Center of Biotechnology Information (NCBI) database, the information on three SNPs in CYP24A1 and VDR gene polymorphism was obtained, and specific primers were designed using program primer 3 to make a genotyping assay. Table 2 lists the primers used in this study and their suppliers.

Table 2: Primers Manufacturer and Supplier

Primer Name	Sequencing	Supplier	Product Size (bp)
CYP24A1	F:5'-TTCATGGGAGGCCTGATAAC-3' R:5'-AGCATCCCAACCAACAGAAC-3'	Macrogen	854
VDR	F:5'-AGAATGGGCTGGGTGGATA-3' R:5'-ACGTGGTCTGGGCTACAGA-3'		859

The primers in this study were designed using Primer 3 software. PCR results were sent for Sanger sequencing using a DNA sequencer (ABI3730XL, Macrogen Corporation South Korea). The results of this work were sent by email and then analyzed using the software Geneious Prime.

PCR Steps:

Conventional PCR analysis was applied to amplify the isolated genomic DNA of human samples. The PCR process includes three fundamental steps: denaturation, annealing, and extension. In the first step, denaturation of the DNA occurs at high temperatures (from 90 - 97o C). In the next step, primers are annealed with the strands of the DNA template to prime extension. Finally, an extension is carried out at the terminal of the annealed primers to produce a complementary DNA copy strand, and this step is followed by a final extension step, which is carried out as a validated step. The temperature, time, and the total number of cycles for the PCR program are listed in Table 2

Table 3: The PCR Program Temperature, Duration, and Number of Rounds

Steps	**Temperature (°C)**	**Duration**	**Rounds**
Initial Denaturation	**95**	**5minutes**	**1**
Denaturation	**95**	**30 seconds**	**30**
Annealing	**55**	**30seconds**	
Extension	**72**	**30seconds**	
Final extension	**72**	**7minutes**	**1**
Hold	**10**	**10minutes**	

Sequencing

The ABI 3730XL, an automated DNA sequencer from Macrogen Corporation, Korea, performed Sanger sequencing on polymerase chain reaction products. Data was received by email and analyzed by using Geneious Prime software.

Sanger Sequences Data Analysis

1- Analysis of CYP24A1 (rs2762934) SNP

Analysis of rs2762934 SNP of CYP24A1 gene Utilizing Sanger sequence explained in figure 3. If a single "A" peak emerges, it indicates that the individual is homozygous for the A allele. A single

"G" peak indicates the presence of a G homozygous allele. Furthermore, the "A" and "G" peaks indicate the A/G heterozygous allele.

2-Analysis of CYP24A1 (rs4809957) SNP

Analysis of rs4809957 SNP of CYP24A1 gene Utilizing Sanger sequence explained in figure 4. If a single "A" peak emerges, it indicates that the individual is homozygous for the A allele. A single "G" peak indicates the presence of a G homozygous allele. Furthermore, the "A" and "G" peaks together indicate the A/G heterozygous allele.

3- Analysis of VDR (rs731236) SNP

Analysis of rs731236 SNP of VDR gene using Sanger sequencing as presented in figure 5. If a single "A" appeared, indicate A homozygous allele. If a "G" peak occurred, meaning A homozygous allele. The presence of "A" and "G" peaks concomitantly indicates an A/G heterozygous allele.

Statistical Analysis:

Using IBM SPSS Statistics (Version 26, IBM Corp., 2019), the data that was gathered was statistically examined. Mean and standard deviation (SD) were used to express continuous variables. Discrete variables were presented as counts and percentages. The frequencies and percentages of alleles and genotypes were calculated using direct count. P=0.05 was used to determine significance. The chi-square test was used for non-parametric data(39).

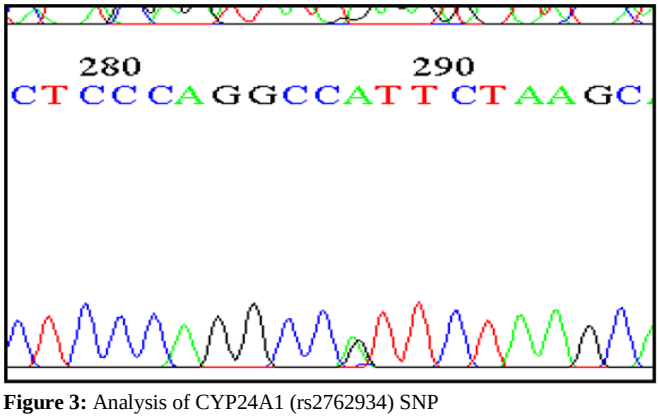

Figure 3: Analysis of CYP24A1 (rs2762934) SNP

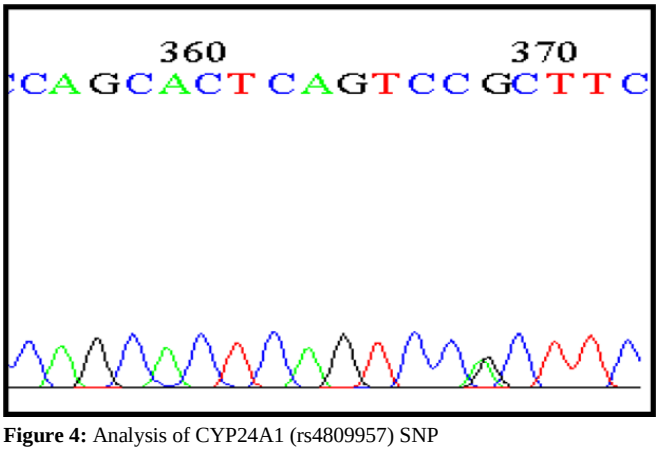

Figure 4: Analysis of CYP24A1 (rs4809957) SNP

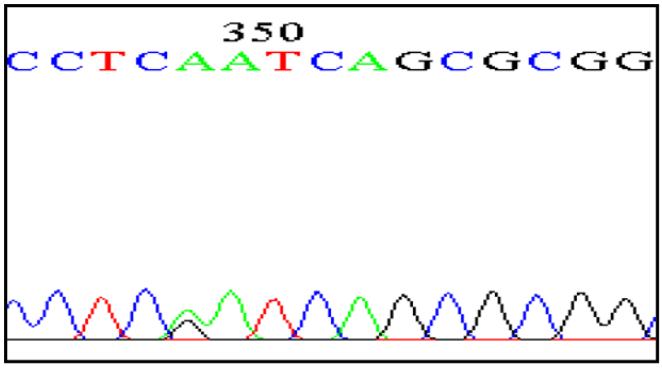

Figure 5: Analysis of VDR (rs731236) SNP

Results

Demographic and Clinical Data with Relation to Vitamin D in Studying Groups

The data in Table 4 represent the relation between demographic data and clinical parameters for the participants and serum levels of vitamin D. Patients' group and control group were divided into two subgroups according to vitamin D level (below 20nmole/L and above 20 nmole/L). Group-1 and Group-2 related to patients. Group 3 and Group 4 are related to the control group. There is a non-significant difference between patients' groups (osteoporotic postmenopausal women) with vitamin D levels below 20nmple/L and above 20nmole/L for the estimated demographic parameters (BMI, HT, and duration menopause) with p-value (0.38, 0.08, 0.37); respectively.

In the control group, there is a non-significant difference between Group-3 and Group-4 in measured demographic parameters (BMI, HT, DM, duration of menopause) with p-value (0.46, 0.96, 0.54, and 0.39); respectively.

Table 4: Demographic and clinical data of the participants regarding vitamin D level

Parameter	Patients (n=40)			Control (n=30)		
	Vitamin D <20ng/dl Group-1	Vitamin D >20ng/dl Group-2	P-value	Vitamin D <20ng/dl Group-3	Vitamin D >20ng/dl Group-4	P-value
BMI	26.37±4.5	29.11±7.1	0.38	30.57±5.3	32.5±5.7	0.46
HT	12 (75%)	4 (25%)	0.08	6 (40%)	9 (60%)	0.96
DM	2 (100%)	0	----	4 (33.33%)	8 (66.67%)	0.54
Duration of Menopause (years)	10.37±5.2	8.33±3.8	0.37	7.3±4.4	8.5±4.1	0.39
PTH pg/ml	36.8±14.6	36.4±13.6	0.43	38.08±16.5	33.19±9.7	0.48
OC ng/ml	9±6.1	9.4±6.6	0.49	6.4± 5.1	4.8±5.1	0.45
Ca mg/dl	9.5±0.4	9.5±0.6	0.34	9.7±0.5	9.6±0.5	0.41
PO4= mg /dl	4.09±0.4	3.95±0.4	0.43	4.06±0.4	3.8±0.4	0.24

Data are presented as mean± SD, BMI: body mass index, HT: hypertension, DM: diabetes mellitus, PTH: parathyroid hormone, OC: osteocalcin, Ca: calcium, PO4: phosphate.

Osteocalcin Level in Participated Groups Regarding Genotypes of Studied SNPs

Table 5 shows the serum level of OC in each genotype for studied SNPs of CYP24A1 and VDR genes with related to vitamin D level. As related to VDR SNP, serum OC in Group-1 is higher than that in Group-2 (11.7±4.1, 2.05; respectively) with homozygous GG genotype, while for heterozygous AG genotype, the percent of patients in Group 1 is higher than that of Group 2 with serum OC (9.5±7.1, 7.56±3.8; respectively). With the mentioned genotypes in CYP24A1, rs2762934 showed a higher percentage of homozygous GG genotype in which serum OC in Group-1 and Group-2 was equal to 8.2±5.8, 8.7±7.9, respectively. Group 3 and Group 4 for this SNP showed lower OC levels; their values were 1.2±0.7 and 2.7±2.1, respectively.

The other SNP for CYP24A1, rs4809957, Group-1 showed OC serum level equal to 9.1±5.3 in homozygous AA genotype-related patients, while Group-2 showed serum OC equal to 11.6±7.3 for the same genotype. Groups-3 and Group-4 showed lower OC, 6.6±6.9 and 5.2±6.1, respectively.

Serum levels of Vitamin D in Different Genotypes of VDR & CYP24A1 genes Polymorphism

In Table 6, serum levels of vitamin D in different genotypes of studied alleles, rs2762934, rs4809957, and rs731236, showed non-significant differences,

Correlation of Vitamin D level with Osteocalcin

Pearson correlation coefficient (r) was calculated between vitamin D level and osteocalcin post-treatment with vitamin D regarding the studied genotypes of VDR & CYP24A1 genes.

There is a negative correlation between serum vitamin D level and serum osteocalcin in the GG genotype of rs2762934, AG genotype of rs4809957, and AA genotype of rs731236 (r=-0.18, -0.66, and -0.31, respectively). The other genotypes showed a positive correlation with different degrees of strength.

Frequency of Genotypes and Alleles in Participating Groups

Genotyping and Alleles distribution of CYP24A1 (rs2762943 G/A, rs4809957 G/A) and VDR (rs731236 A/G) in OP patients and Control group are explained in Table 8. The GG homozygote genotype is prevalent in the patients' group (62.5%), and this genotype is also present in 46.6% of the control group. The homozygote AA genotype presented a lower percentage in the patients' group (2.5%) and the control group (6.66%). In contrast, the mutant allele A was 20%, whereas the G allele, the wild one, had a higher percentage in both patients and control groups, 80% and 70%, respectively.

About another SNP of CYP24A1, rs4809957, the homozygote AA genotype is the prevalence in the patients' group (65%) and 50% for the control group, while the lower percent was for the homozygote GG with 2% prevalence for patients. Noticeably, the A allele is prevalent, with 64% in the patients and 75% in the control groups.

The heterozygote GA is prevalent in rs731236 for the VDR gene, with 57.5% and 46.5% in the patients and control groups, respectively. An allele is the predominant one, with 58.75% in patients and 60% in the control groups.

Table 5: Serum Level of OC in Different Genotypes of VDR and CYP24A1 Gene Polymorphism

VDR									
			Patients (n=40)				Control (n=30)		
SNP	Genotype		OC				OC		
		No. (%)	Vitamin D <20ng /dl Group-1	No. (%)	Vitamin D >20ng /dl Group-2	No. (%)	Vitamin D <20ng /dl Group-3	No. (%)	Vitamin D >20ng /dl Group-4
rs731236	GG	3 (10.7%)	11.7±4.1	1(9%)	2.05	2(15%)	1.2±0.7	3(18%)	2.7±2.1
	AG	17(60.7%)	9.5±7.1	6(54%)	7.56±3.8	7(53%)	6.9±5	7(41%)	7.2±5.8
	AA	8(28.5%)	7.9±4.3	4(36%)	14.78±8.2	4(31%)	8±5.9 I	7(41%)	3.4±4.6
CYP24A1									
rs2762934	GG	19 (67.8%)	8.2±5.8	6(50%)	8.7±7.9	7(53.8%)	6.4±5.2	7(41%)	4.3±6.4
	AG	9 (32%)	11.4±6.7	5(41.6%)	10.3±5.5	6(46%)	6.4±5.3	8(47%)	4.5±3.2
	AA	0	--------	1(8.3%)	8.7	0	------	2(12%)	8.06±8.2
rs4809957	GG	2(6.8%)	5.5±1.1	0	-----	0	--------	0	--------
	AG	8(27.5%)	10.6±8.4	4(36.3%)	5.6±3	9(69%)	6.3±4.5	6(35%)	4.2±2.8
	AA	19(65.5%)	9.1±5.3	7(63.6%)	11.6±7.3	4(31%)	6.6±6.9	11(65%)	5.2±6.1

Data are expressed as mean ± SD, significance when p-value<0.05. VDR=Vitamin D Receptor, OC=Osteocalcin, homozygous pattern GG, heterozygous pattern AG, homozygous pattern AA

Table 6: Serum levels of Vitamin D in Different Genotypes of VDR & CYP24A1 genes Polymorphism in OP patients

SNP	Genotype	No.	Serum Vitamin D Post Treatment	P-Value
rs2762934	AG+AA	15	48.35±26.4	0.12
	GG	25	37.88±15	
rs4809957	AA	26	39.9±21.6	0.35
	AG+GG	14	46.58±17	
rs731236	AA	12	49.6±29.5	0.23
	AG	23	38.5±14.5	
	GG	5	35.8±6	

Data expressed as mean± SD, significance when p-value<0.05. wild genotype with a bold line

Table 7: Correlation of Vitamin D level with Osteocalcin in regarding to Genotypes of Genes

SNP	Genotypes	r	R^2
rs2762934	AG	0.33	0.11
	GG	-0.18	0.033
rs4809957	AA	0.21	0.043
	AG	-0.66	0.44
rs731236	AA	-0.31	0.098
	AG	0.17	0.029
	GG	0.24	0.058

Table 8: Frequency of Genotypes and Alleles Related to CYP24A1 (rs2762943 G/A, rs4809957 G/A), VDR (rs731236 A/G)

rs2762934				
Genotype	Patients (n=40)		Control (n=30)	
	No.	Percentage	No.	Percentage
AA	1	2.5	2	6.66
GA	14	35	14	46.66
GG	25	62.5	14	46.66
A	16	20	18	30
G	64	80	42	70

rs4809957				
Genotype	Patients (n=40)		Control (n=30)	
	No.	Percentage	No.	Percentage
AA	26	65	15	50
GA	12	30	15	50
GG	2	5	0	0
A	64	80	45	75
G	16	20	15	25

rs731236				
Genotype	Patients (n=40)		Control (n=30)	
	No.	Percentage	No.	Percentage
AA	12	30	11	36.6
GA	23	57.5	14	46.6
GG	5	12.5	5	16.6
A	47	58.75	36	60
G	33	41.25	24	40

Discussion

Vitamin D deficiency can be a promoter for number of diseases other than skeletal system, and correction of its level can ameliorate bad prognosis (5).

Polymorphism in VDR and/ or CYP24A1 supposed to responsible for vitamin D deficiency even the required dose is consumed. (8).

In the present study, the participants were divided according to the serum level of vitamin D. Choosing a vitamin D level below or above 20nmole/L due to this level can separate groups into sufficient or deficient and can be used as a marker for dividing patients' groups (40, 41).

Body mass index shows a non-significant difference in the patients' group between Group-1 and Group-2 (p=0.38) and a non-significant difference to that of the control group between Group-3 and Group-4 (p=0.46). Such results were presented in Bindayel 2021, which studied the effect of BMI on adult vitamin D levels (42). A local study found that the BMI of osteoporotic patients is lower than that of non-osteoporotic patients (43), as the study revealed, this making bone easy to fracture.

When comparing BMI between Group-1 and Group-3, we assumed there is a difference between patients and control's BMI with vitamin D levels lower than 20nmole/L, suggesting the inverse relation between osteoporotic state and BMI.

Hypertension was prevalent in 75% of OP patients in Group-1 and 25% of patients in Group-2, while in the control group, 40% had HT in Group-3, and 60% of Group-4 had HT. The prevalence of HT in osteoporotic women can be related to vitamin D levels. Vitamin D affects blood pressure by regulating the renin-angiotensin-aldosterone system at pathological and physiological levels(44, 45), which helps as aiding factor for lowering HT. So, as a result, the incidence of HT can be linked to a vitamin D deficiency.

None of the OP women who participated in the study had DM in Group-1, while only two patients had DM in Group-2. Meanwhile, the control group showed 33% in Group-3 and 66% in Group-4. Vitamin D is essential in diabetes patients in controlling their glycemic state(46), and type II diabetes carries a high risk of osteoporosis, increased risk of fractures, and more deficient vitamin D(47). In present study, women with diabetes over 50 years old with vitamin D levels over 20nmole/L are still inadequate., so correction of vitamin D level is essential for their glycemic state. Vitamin D can be helping in adjustment of blood glucose level, and through its anti-inflammatory effect can help in wound healing if diabetic foot occurred(48).

In this study, there was a non-significant difference in menopausal duration in both patients' groups and control groups (Group-1 and 2, Group-3 and 4) with p-value= (0.37 and 0.39); respectively, but the duration of menopause in OP patients is longer when compared to that of control, as the more deficient of vitamin D can play a role in reduction the reproduction age(49).

Regarding bone-related biomarkers (Ca, PTH, OC, and PO_4) levels in both patients and the control group, there is a non-significant difference in among groups. Serum levels of bone-related markers (Ca, PO4, and PTH) were evaluated to exclude any secondary osteoporosis causes, which can be resulting from endocrine disorder, like hyperparathyroidism, that characterized by hypocalcemia, hyperparathyroidism, and hyperphosphatemia(50). As the level of these markers was within the average value in present study, osteoporosis is related to post-menopause and is primary cause.

OC level in the patients' group is higher than in the control group, related to higher remodeling process and increased bone resorption in osteoporotic women(51). Such results were found in case-control studies on OP postmenopausal females (52, 53), which revealed that measuring OC with DXA-scan results helps to complete diagnosis,

and its level helps to reach the desired diagnosis of osteoporosis even without BMD measurement.

About genotypes of the studied genes, three SNPs will be involved in our study, and the relation between each genotype and osteocalcin level in both the control and patients' groups was explained. These groups are divided into two subgroups according to vitamin D level.

About VDR SNP (rs731236**),** in Group-1, the heterozygous AG genotype was prevalent in 18 patients (9.3±7.1), while in control groups (Group-3 and Group- 4), the AG genotype was seen in 7 patients each. These genotypes and allelic frequency are similar to the study applied to osteoarthritis patients and the role of VDR polymorphism in this condition's susceptibility (54).

When related to another gene, CYP24A1. The 1st SNP (rs2762934), homozygous GG genotype, is prevalent in 19 patients in Group-1 and Group-2; there is a non-significance difference between the two groups (p=0.28). There are numerous studies about the polymorphism of CYP24A1 in different SNPs on OP, but rs2762934 has never been studied for its role in osteoporosis risk or treatment resistance. This SNP has been analyzed in rheumatoid arthritis(23) and the risk of ischemic stroke in Chinese(55). The another SNP (rs4809957) showed a higher prevalence of homozygous AA genotype in Group-1, and Group-2 showed a prevalence of AA genotype with a non-significant difference between them (p=0.91). The prevalence of these wild genotypes in different SNPs is considered the primary factor in non-responding to vitamin D treatment, which needs to be investigated before any treatment with vitamin D for OP women. This SNP did not apply to osteoporosis studies before. Still, it was involved in other conditions in which CYP24A1 SNPs play a role in disease progression, like a study by Mallah N. et al., 2022, which studied the role of rs4809957 in upper GIT hemorrhage and AA genotype was prevalent(56).

Serum levels of vitamin D in postmenopausal women have a positive relation with bone strength and patients whose vitamin D level increments after a period of therapy can be caused by the presence of mentioned SNPs. SNPs of VDR or CYP24A1 will lead to poor response or over-serum vitamin D levels, respectively(57, 58).

When explaining the serum level of vitamin D in the studied SNPs in the current study, there was a non-significant difference in vitamin D level post-treatment regarding rs2762934, rs4809957, and rs731236. The wild genotypes of SNPs showed lower vitamin D levels than the others, which can explain their lower responsiveness to therapy.

The correlation coefficient is a parameter to measure the linear relation between two variants and explains the effect of each other(59). This study illustrated the r coefficient between vitamin D and osteocalcin with regarding to the studied genotypes. The negative correlation can show the impact of polymorphism on vitamin D levels and osteocalcin, which increases the risk of osteoporosis and poor response to vitamin D.

The prevalence of genotypes and alleles in OP patients were explained in present study, and the prevalent genotypes were shown to be responsible for non-responding to vitamin D or poorly responding for the high dose of vitamin D. In genotype studies, there is no previous study involving the role of these genotypes in osteoporosis and detecting their response to vitamin D in Iraq, but either finding the prevalence of related genotypes. There is a study made by Zhang TP et al, 2021 on rheumatoid arthritis (RA) showed the higher prevalence of homozygous GG genotype in CYP24A1 SNP rs2762934 with alteration in vitamin D metabolic pathway and its effect on RA progression, even though the result was non-significant-((23), the 2nd SNP of CYP24A1 (rs4809957) was seen to carry a risk factor for getting cancer(60). Still, the prevalence was similar in this study. While VDR SNP, Taq1 showed the prevalence of heterozygous AG genotype and carried the risk of being obese in Diabetes patients(61). These findings can put in mind the importance of genetic variation that places people at risk of OP, being non-respond to treatment, and the benefit of using vitamin D supplements for OP to control disease progression and reverse its bad prognosis on patients.

Conclusion

This study is the first that applied in Iraq on postmenopausal osteoporotic women to investigate genetic polymorphism related to VDR and CYP24A1 and their impact on the prognosis of osteoporosis in postmenopausal women through studying some genotypes of SNPs. Through our investigations, the GG genotype of rs2762934 and AA genotype of rs4809957 related to CYP24A1gene, and the AG genotype of rs731236 of VDR gene were the prevalent and they associated with lower response to vitamin D supplementation. Osteocalcin levels were inversely related to serum vitamin D level in patients with osteoporosis and showed non-significant difference after supplementation of vitamin D, making it unsuitable in osteoporosis follow-up after supplementation with vitamin D.

Acknowledgments

The authors thank Basra Teaching Hospital healthcare providers for their help completing this work**.**

Funding

This research did not receive any specific fund.

Conflict of Interest

Authors declare no conflict of interest.

Data availability

Data are available upon reasonable request.

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To cite this article: Abdulrahman NM, Ali SH. Correlation of Osteocalcin with Vitamin D Level in Postmenopausal Women Concerning CYP24A1 and VDR Gene Polymorphisms. Al-Kindy College Medical Journal (. 2024;20(2):135-144. https://doi.org/10.47723/sdfrzk14