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Role of rs2762934 and rs4809957 polymorphisms of CYP24A1 on outcomes of vitamin D treatment in postmenopausal osteoporotic women in Iraq

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ABSTRACT

Osteoporosis is widely distributed condition characterized by bone weakness making it more fragile and easier to be fractured. Various elements enter in bone structure making it rigid to tolerate body weight and any accident. Many factors had a role in progression of osteoporosis such as age, gender, medications, and genetic variations in enzymes responsible for vitamin D metabolism. The aim of the current study is to evaluate role of polymorphism of metabolic enzyme CYP24A1 on responding to vitamin D in postmenopausal osteoporotic women. Forty postmenopausal women were followed up for 2 months and took 50000IU/week of vitamin D during study period, and 30 postmenopausal women without osteoporosis as a control group. Blood samples were taken pre- and post-treatment to determine responder to vitamin D therapy. Regarding vitamin D level, there was a significant difference between responder and non-responder at pre-treatment levels. GG genotype of rs2762934 SNP and AA genotype of rs4809957 SNP are the predominant in non-responder group, and both genotypes showed negative correlation to being responsiveness to vitamin D therapy. The homozygote wild GG genotype of rs2762934 predict vitamin D non-responsiveness in sample of osteoporotic postmenopausal women. The AA genotype of the rs2762934 substantially increases the responsiveness while AA of rs4809957 is associated with non-responsiveness. In conclusion, this study will implicate in correct choosing the desired dose of vitamin D for desired osteoporotic women to get better response and avoid any harmful effect of vitamin D overdose.

INTRODUCTION

Osteoporosis, a widely distributed disease is known by reduction of bone density and changing of bone fine-structure resulting in more bone fragility and fracture risk and it also can result from reaction of genetic and environmental factors that affect bone mineral density and make bony tissue easy to be fractured [1, 2]. The prevalence of osteoporosis is suspected to increase significantly in the future because of the aging population and it affects mostly postmenopausal women leading to a higher rate of mortality due to fracture risk [3-5]. Osteoporotic patients get fracture either in hip or vertebrae, that increase morbidity, and mortality and decrease life quality [6]. Furthermore, it is considered as one of the major considerations of the health care systems due to the growing economic burden. Osteoporosis fractures can cost 13.8$ billion in US [7]. However, the knowledge on osteoporosis among general people and women is very poor, as reported in a study among osteoporotic female [8].

Osteoporosis can be classified into 2 types. Primary osteoporosis, which involves both postmenopausal osteoporosis (1st type) and senile osteoporosis. Secondary osteoporosis

(2nd type), which has a specific etiological sequence, such as poor absorption, medications such as corticosteroid, and some conditions that effect on bone architecture such as hyperparathyroidism [9, 10].

Vitamin D is produced naturally through skin when exposed to sunlight. However, the exposure to sunlight is not enough to synthesis vitamin D due to a sedentary lifestyle, and its deficiency carried out several disease like renal problems, liver disease, causing decrease in seasonal concentrations of 25-Hydroxy-vitamin D_3 (25(OH)D_3), especially in winter and spring [11, 12]. Bone health is mainly depend on the optimum level of vitamin D and calcium, and osteoporosis may develop if there is an impairment or deficiency in their level in bone [13]. Vitamin D plays an important role in strengthen the bone and skeletal system, and helps in enhancing calcium absorption from intestine which act together in improvement of bone health [14]. Also, vitamin D and calcium both act on bone healing post fracture [15].

Vitamin D level is regulated by several enzymes that act to control its level, like CYP27A1 responsible for activation step in vitamin D metabolic pathway, whereas CYP24A1 is responsible for inactivation of vitamin D to prevent its toxicity [16]. CYP24A1 is a mitochondrial enzyme responsible for breakdown of vitamin D, it presents in many tissues like kidney, lung, bone, and colon. Any mutation or polymorphism in the gene responsible for this enzyme can cause different diseases to the affected organs [17]. The endogenous synthesis and metabolism of vitamin D through metabolic enzymes that present in liver (CYP2R1) or in kidney (CYP27B1), and then CYP24A1 to inactivate vitamin D and help in excretion [18]. Controlling of vitamin D levels in the body and reaching the desired concentration might be affected by various factors, such as resistance in response or alteration in enzyme function by polymorphism, which is the main subject in the current study.

Single nucleotide polymorphism (SNP) is considered one of the factors that are responsible for affecting diseases or resistance to therapy. It has been shown that even autoimmune disorders or not being respond to biological agents can be related to specific SNP(s) in the enzyme or receptors that enter in drug metabolism or disease activation [19-22]. Polymorphism of CYP24A1 gene plays an essential role in countering vitamin D level reflected by its resorption effects on bone tissue, which eventually leads to osteoporosis. SNP in CYP24A1 may act on its main function and the resulting high level of vitamin D in blood due to the metabolism of vitamin D and controlling its level by CYP24A1. This pathway has direct or indirect effect on extent of several conditions, like renal diseases, cancer, non-alcoholic fatty liver disease, depending on the affected SNP [23, 24].

According to our knowledge, there is no previous study in Iraq about CYP24A1 gene polymorphism in relation to osteoporosis. Thus, the present clinical study tried to find the relative effect of polymorphism of CYP24A1 gene rs2762934 and rs4809957 on vitamin D level in osteoporotic postmenopausal females.

MATERIALS AND METHODS

Study subjects

According to the World Health Organization (WHO) criteria, osteoporosis is diagnosed by measuring bone mineral density (BMD), in which T-score is the tool which aids in helping to reach diagnosis. Here, T-score≥-1 is considered as normal and within the reference standard deviation (SD) of young population. If T-score is between -1 and -2.5, it is diagnosed as osteopenia. Further, T-score ≤-2.5 is diagnosed as osteoporosis [25].

This study is a prospective patient-control study, in which the participant patients were diagnosed with osteoporosis based on their BMD values (T-score ≤-2.5 are considered osteoporotic patients, and T-score ≥-2.5 are considered non-osteoporotic patients). The other inclusion criteria were lack of menstrual cycle for at least two years, did not receive any therapy for osteoporosis, and diagnosed with primary osteoporosis. The selected patients started high dose of vitamin D (50000 IU/week) for two months duration, without missing or prior vitamin D regimen. However, patients with following criteria were excluded: those with co-existing autoimmune disease, like rheumatoid arthritis, systemic lupus erythematosus, any renal or hepatic impairment, any secondary cause of osteoporosis, those using anti-resorptive agents or incomplete vitamin D course therapy.

A total number of 70 postmenopausal females (who had no menstrual cycle at least for two years and aged over 60 years) were involved in this study. Forty of them were osteoporotic females who met the inclusion criteria and participated in this study, in addition to 30 postmenopausal non-osteoporotic females. Females with osteoporosis were diagnosed by DXA-scan according to WHO-osteoporotic criteria. These forty women considered as patient group were classified into two groups, responder and non-responder to vitamin D therapy who received it for 2 months. Patients who had 100% or more increments in vitamin D concentration at post treatment with vitamin D were considered as "responders" and who had less than 100% increments considered as "non-responders".

Blood specimens were taken from the participants at study beginning and after 2 months of vitamin D treatment, to obtain serum to be used for estimating serum vitamin D level, upon which the response to vitamin D therapy was determined. Blood samples of 2 ml from each participant were taken and kept at freezing temperature in EDTA tube for further analysis of DNA.

Ethical approval

The Ethical and Scientific Committee in the College of Pharmacy at University of Baghdad, Iraq accepted to order the acceptance for this observational case-control study (approval number: RECAUBCP24112021B). This study was applied at Rheumatology Unit/Basra Teaching Hospital, Basra, Iraq from March 2022 to January 2023. Acceptance from each participant was taken before study started.

Clinical evaluation

Analyze of disease signs and symptoms, medical history, and investigational laboratory data for all participants in the study were recorded by direct interview. The disease state of patients was measured depending on BMD which was measured by dual x-ray absorptiometry (DEXA-scan, Lunar Prodigy Advance, Belgium).

DNA extraction and Real time PCR analysis

In DNA extraction, 2 ml of blood samples were used. DNA extraction was performed by using Kit purchased from Promega, USA. After this step, purification of DNA was done by using ReliaPrepTM (Promega Corporation, USA) which offers a simple and precise technique. The position of CYP24A1 (rs2762934 and rs4809957) genes were amplified by Real time PCR using Thermal Cycler (Kyratec, Australia). Conventional PCR analysis was applied to amplify the isolated genomic DNA. The PCR process

included three fundamental steps: denaturation (95°C for 30 sec), annealing (55°C for 30 sec), as well as extension (72°C for 30 sec). In the first step, denaturation of the DNA occurs at high temperatures (from 90 - 97°C for 5 min). In the next step, primers are annealed with the strands of the DNA template in order to prime extension. Finally, an extension is carried out at the terminal of the annealed primers in order 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.

To design PCR primers, specific software (Primer 3) was used. The studied primers for CYP24A1 SNPs (rs2762934 and rs4809957): Forward (f): 5‘-ttcatgggaggcctgataac-3‘, and Reverse (r): 5‘-agcatcccaaccaacagaac-3‘. PCR results were sent for Sanger sequencing by using DNA sequencer (ABI3730XL, Macrogen Corporation South Korea). The results of this work were then analyzed by using software Geneious prime (Scientific laboratory, Basra City, Iraq).

Analysis of vitamin D

Vitamin D is measured by using a specific ELISA test kit (Elecsys® Vitamin D total II by Cobas, Roche Diagnostics, Belgium). First, the calibrators and patient samples were diluted with biotin-labelled 25-OH vitamin D and added to microplate wells coated with monoclonal anti-25-OH vitamin D antibodies. During the incubation an unknown amount of 25-OH vitamin D in the patient sample and a known amount of biotin-labelled 25-OH vitamin D competed for the antibody binding sites in the microplate wells. Unbound 25-OH vitamin D was removed by washing. For the detection of bound biotin-labelled 25-OH vitamin D, a second incubation was performed using peroxidase-labelled streptavidin. In a third incubation using the peroxidase substrate tetramethylbenzidine, the bound peroxidase promoted a color reaction. The color intensity was inversely proportional to the 25-OH vitamin D concentration in the samples. Results for the samples was calculated directly using a standard curve.

Statistical analysis

Statistical analysis was made by using SPSS version 29 software. Data with continuous value was presented as mean ± SD. Distinct variables were presented as numbers and frequency. Direct counting was used for detecting allele and genotype percentages and frequencies. $P \leq 0.05$ was considered as significant. The tests used here were Chi-square test and Fischer exact test to analyze data. Phi coefficient correlation was applied to find the relation between each genotype and having a role on responsiveness.

RESULTS

Demographic and clinical data

Demographic and clinical data of the participants in this study were explained in Table 1. Mean age of the groups was 58.4±5.9, 61.34±6.46, and 57.27±5.7 years for control, responder, and non-responders, respectively with non-significant difference (p=0.09). The body mass index (BMI) of these groups showed significant differences (p=0.001).

Table 1. Demographic and clinical data.

Variable	Category	Control (n=30)	Responder (n=29)	Non-Responder (n=11)	P value
Age		58.4±5.9	61.34±6.46	57.27±5.7	0.09
BMI		31.67±5.6	26.11±5.3	29.79±5	0.001*
Duration of menopause		8±4.2	10.3±5	9.09±4.7	0.181
Previous fracture	Yes	(4)13.4%	(5)17%	(1)9%	0.33
	no	(26)86.6%	(24)83%	(10)91%	
Area of living	City center	80%	76%	81.8%	0.133
	Rural	20%	24%	18.2%	
Smoking state	Yes	(2)5%	(1)4%	0%	0.001*
	no	(28)95%	(28)96%	100%	
Presence of	HT	(15)37.5%	(11)37.9%	(5)45.4%	
	DM	(12)30%	(1)3.4%	(1)9%	
	Asthma	(3)7.5%	(2)6%	0%	
T-score		-1.11±0.85	-2.7±0.71	-2.4±0.31	0.001*

Data are presented as Mean ±SD, otherwise as numbers and (%) for each group, * is significant when p value <0.5. ANOVA test and Chi-square were used.

Prevalence of *CYP24A1* gene polymorphism (Rs2762934 and Rs4809957) in all studied groups

In Table 2, the frequency of genotypes and alleles was related to Rs2762934 and Rs4809957 SNPs in participated patients. The GA heterozygote genotype of Rs2762934 in control group was higher than that of patient group (46.66%), while GG genotype is higher in the patient group (62.5%) than in control group. In the 2nd SNP, Rs4809957, the homozygote AA genotype in patient group (65%) is higher than that of control group, while GA heterozygote in control group (50%) is higher than that of patients group.

Table 2. Frequency of genotypes and alleles to rs2762934 and rs4809957 in osteoporotic patients.

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
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

Data are presented as numbers and (%) for each group.

Vitamin D levels at pre- and post-treatment in participated groups

In Table 3, pre- and post-treatment of vitamin D in participated groups showed a significant difference between pre- and post-level both in responder and non-responder groups, while non-significant difference between patients' groups (responder and non-responder) at post-treatment level.

Table 3. Vitamin D level in osteoporotic patients.

Vitamin D level	Responder (n=29)	Non-responder (n=11)	Control (n=30)	P value
Pre-treatment	11.3±5.4	33.51±21.12	28.8±24.2	0.001*
Post-treatment	40.8±20.3	43.2±19.9	--------	0.931
p-value	0.001*	0.001*		

Data are presented as Mean ±SD, * is significant when p-value <0.05.

Distribution of genotypes and alleles in rs2762934 and rs4809957 among participants

In Table 4, the distribution of each genotype and allele in s2762934 and rs4809957 was explained. For rs2762934 SNP, GG genotype was the highly prevalent in the participated groups, with non-significant difference (p=0.33), and G allele exerted its higher percent in the responder, non-responder, and control groups at 77.55%, 86.3%, and 70%, respectively. While the 2nd SNP (rs4809957) exerts AA genotype predominant in the participated groups with non-significant difference (p=0.37), with the prevalence of A allele in the groups at 45%, 86.3%, and 75% in responder, non-responder, and control groups, respectively.

Table 5 explained the phi-correlation which measure the strength of correlation and probability of responsiveness in patients who received therapy. Here, a positive correlation in homozygote AA in rs2762934 with responsiveness to vitamin D while GG genotype showed a negative correlation with the responsiveness without significance (p=0.53, and p-0.41, respectively) when applied phi-coefficient. Related to the 2nd SNP, rs4809957, AA genotype showed a negative correlation to the responsiveness to vitamin D treatment.

Table 4. Distribution of rs2762934 and rs4809957 SNP and each allele among the participants.

		Responder (n=29)		**Non-responder (n=11)**		**Control (n=30)**		
SNPs	Genotype	No.	%	No.	%	No.	%	P value
rs2762934	AA	1	3.4	0	0	2	6.66	0.53
	GA	11	37.9	3	27.27	14	46.66	0.51
	GG	17	58.6	8	72.72	14	46.66	0.30
Allele	A	13	22.45	3	13.6	18	30	0.28
	G	45	77.55	19	86.3	42	70	
rs4809957	AA	18	62	8	72.72	15	50	0.37
	GA	9	31	3	27.27	15	50	0.22
	GG	2	6.8	0	0	0	0	-----
Allele	A	45	77.58	19	86.3	45	75	1.21
	G	13	22.4	3	13.6	15	25	0.54

Data are presented as numbers and % under each group.

Table 5. Phi-coefficient correlation finding the responder probability.

SNPs	**Genotypes**	**Phi-coefficient**	**P value**
rs2762934	AA	0.99	0.53
	GA	0.11	0.52
	GG	-0.13	0.41
rs4809957	AA	-0.1	0.53
	GA	0.037	0.81
	GG	0.14	0.37

DISCUSSION

Pharmacogenetic helps the researchers and scientists to get benefit from genetic data of each patient for helping in prevent disease progression, improve diagnostic tests, choose the desired medication and reduce any harmful adverse events of the used drugs [26].

In the current study, mean age of three groups (responder, non-responder, and control) did not show a significant difference (p=0.09), while the BMI showed a significant difference (p<0.001) among participated groups. There is a negative relation between

BMI and the incidence of osteoporosis. People with BMI> 30 got protection from osteoporosis. It is reported that people with higher BMI will be higher BMD and then lower risk of fracture or osteoporosis [1].

Regarding the frequency and prevalence of genotypes in patients group related to rs2762934, the homozygote GG is more frequent (62.5%) than the heterozygote GA (35%) and homozygote AA (2.5%) genotypes. While in control group, GA and GG genotypes present in the same percent (46.6%). The prevalence of GG genotype associated with disease is also reported previously [27]. In Iraq, there is no previous study on prevalence of rs2762934 or their genotypes in osteoporotic patients.

Now, in case of 2nd SNP, rs4809957, the homozygote AA showed higher percent (65%) than GA (30%) and GG (5%), while in control group the AA and GA genotypes showed equal percent (50%) and no case reported GG genotype in control group. Also, role of rs4809957 in disease progress was reported in a study [28].

Vitamin D level had been increased both in responder and non-responders' groups after two months duration of therapy in the current study, but the difference is in percent of this increments that make total patients classified into two groups depending on responsiveness. There is no specific role to adjust or evaluate responsiveness, never less; more than theory or experimental method to consider response to therapy, as explained previously [29-31]. As seen in responders' group, that their vitamin D level increased more than 100%, while the non-responder did not meet this increment.

Regarding rs2762934, the current data showed a higher percentage of homozygote GG genotype, that occurred in 58.6% of the responder patients. Additionally, G allele was found in more than 77% while A allele was found in 22% of the responders, this gave the effectiveness and active role of G allele. Prevalence of GG genotype is also seen in a study for risky of hypertension in Chinese people with regarding to *CYP24A1* gene [32].

Regarding rs4809957, the wild homozygote AA showed a higher percent with non-significant difference (p=0.37) among participated groups, with higher percent of A allele (more than 77% and 86% in responders and non-responders, respectively).

Table 5 reveals the probability of responding with the genotyping of selected SNPs and phi correlation coefficient analysis. These findings help in assessing responsibility to vitamin D and identify the responsible SNP for this. Phi-correlation is one of the correlation relations used in statistics to help to find the positive or negative correlation of the affected agent with the applied factors. Regarding rs2762934, genotype GG showed negative correlation for responsiveness to vitamin D, while for rs4809957, the AA genotype showed negative correlation of responsiveness and GA genotype exert positive correlation of responsiveness. The other data was either positive or negative relations with no significant level.

There are some limitations in the current study findings as following: a) small patient size, b) one center was involved in this study and need more centers and areas in future work, and c) the duration of study was 2 months which may be one of the reasons not to get highly significance prevalence.

CONCLUSION

The current study found the relationship between SNPs of CYP24A1 gene and vitamin D supplements in postmenopausal osteoporotic women. Wild GG homozygote genotype of rs2762934 predicted vitamin D non-responsiveness in sample of osteoporotic postmenopausal women. While AA of rs4809957 is associated with non-

responsiveness. These results may help to identify the presence of these SNPs before administration of vitamin D, especially with high dose and long period, as therapeutic option for osteoporotic patients.

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AUTHOR CONTRIBUTION

NM designed outlines and drafted the manuscript. NM performed the experiments and analyzed the data. NM and SH wrote the initial draft of the manuscript. SH reviewed the scientific contents described in the manuscript. All authors read and approved the final submitted version of the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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