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Association of FokI vitamin D receptor gene polymorphisms with clinical parameters in Iraqi CKD patients.

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Abstract

(VDR) "a member of the steroid/nuclear receptor superfamily of ligand activated transcription factors" these biological effects of 1-25,dihydroxyvitamin D3 include regulation of ca homeostasis, cellular development, and immune-function. The VDR gene's coding region contains a FokI restriction fragment length polymorphism that leads to the synthesis of a 3 amino acid longer VDR protein. The FokI restriction enzyme was used to find the T>C SNP in exon2 start codon, one of the VDR single nucleotide polymorphisms (SNPs), that was being studied. This study was examined to evaluate association between VDR polymorphisms and clinical parameters in Iraqi CKD patients. 30 Iraqi healthy controls and 60 patients with stage 5 clinically confirmed CKD. Genomic DNA was isolated from blood and genotyped for the FokI (T/C) single nucleotide polymorphism SNPs (rs2228570) by using (PCR) polymerase chain reaction and restriction fragment length polymorphism (PCR_RFLP) analysis. Statistical Analysis Used; between patients and controls, the genotype distribution and allelic frequencies were examined. Using SPSS for windows (version 28) the mean values and odds ratios (ORs) with 95% confidence intervals (CI) were determined. Patients chronic kidney disease have(rs,10735810) polymorphisms in the VDR. Homozygous CC was found to be as a preventative genotype of CKD (OR < 1) whereas, CT and TT were found to be associated with CKD risk (OR ≥1). The allele T functions as an etiological factor (risk factor), while The allele C acts as a preventative allele (preventive factor).

1. Introduction

The discovery of the active vitamin D hormone's affinity for a protein came at the same time as the hormone's chemical identification and kidney origin(1). The biggest transcription factor family in humans

and a member of the nuclear receptor superfamily is VDR(2). Vitamin-D's active form (1-25 D) interacts with the VDR to effect the tissue. The retinoid-X receptor (RXR) and the 1,25D-VDR-RXR heterodimer create a dimer in this complex, which then translocates to the nucleus where it binds to Vitamin-D responsive elements (VDREs) in the promoter region of Vitamin D responsive genes and activates them(3). The sole known mechanism by which the 1-25(OH)2D3 hormone is regulated in higher vertebrates in the vitamin D receptor. It controls the in the expression of genes whose products control various, cell-type specific biological processes, such as mineral homeostasis in the nucleus of vitamin D target cells(4). The VDR gene has been found to include a number of single nucleotide polymorphisms (SNPs), including FokI in exon 2 (C/ T) (rs 10735810), TaqI (T to C) rs731236 in exon 9, BsmI in intron 8 (G/ A)(rs 1544410) and ApaI in intron 8 (C/ A) (rs 7975232), among others(5). The start codon polymorphism FokI (T/C) in exon II is one of the most prevalent allelic variants examined(6). RNA polymerase skips the start codon (ATG to ACG) in exon 2 as a result of the VDR FokI polymorphism. As a result, a different start site is employed, resulting in a protein with a shorter length (424 aa) and higher transcriptional activity than the longer length (427 aa)(7). The VDR gene's FokI polymorphism is recognized as one of the strongest indicators of disturbed vitamin D signaling pathway(8). The FokI polymorphism may modify how well the VDR binds to vitamin D3, which may change how downstream molecules are produced. Additionally, it is hypothesized that VDR polymorphism may alter PTH levels, which would disrupt the control of calcium and phosphate levels(9).

In recent years, chronic-kidney disease (CKD) has become a significant public health concern. For people around the world, End-stage renal disease (ESRD) is a significant health and financial concern (10). Patients with CKD commonly lack adequate amounts of Vitamin D(11). The vitamin-D receptor, also known as the VDR , is responsible for the actions of vitamin D. In the proximal renal tubule, vitamin D3 is controlled by its receptor. It has been shown that the VDR serves to "sense" the amount of circulating (VD) and regulates the activity of 1,α-hydroxylase and 24-hydroxylase in the proximal convoluted tubule cells(12).

1. MATERIALS AND METHODS

Population of the study

The study was carried out on patients with chronic kidney disease at Al-Ramadi Teaching Hospital's dialysis center (synthetic kidney center). Between the first of December 2021 to the end of June 2022, in the genomic medical laboratory. The study protocol was accepted by the Anbar university's scientific research ethics committee. All participants gave their consent after being fully informed. The study groups included: 30 healthy controls (15 men and 15 women) and 60 hemodialysis patients (30 pre-dialysis and 30 post-dialysis) equally divided between men and women. Hemodialysis patients were excluded from the study if they had a C virus (Hepatitis). Taken from patients (2ml) blood were put into (EDTA) for DNA extraction. Each patients was stable and receiving hemodialysis on a regular basis for 3-4 hours, from 2-3 times per week, for at least 2 years.

Methods (vitamin D Receptor):

Real time PCR and (HRM) High resolution melting Analysis For Genotyping

To study the connection between Iraqi CKD patients and the genetic variation of the vitamin D3 gene, the SNP, FokI SNP (rs2228570) C to T was identified using HRM (real-time PCR) .

Whole blood samples were used to extract genomic DNA using the MagPurix® Blood DNA Extraction Kit 1200. The FokI polymorphism was genotyped using PCR-RFLP analysis (polymerase chain reaction, restriction fragment length polymorphism). The obtained DNA product was amplified using the proper primers (Forward: 5' CTGCTTGCTGTTCTTACA 3' and Reverse: 5' CAAAGTCTCCAGGGTCAG 3' with 60 $\mathring{C}$ annealing temperature). Gene Rotor qPCR was carried out using a Q-Real time PCR System (QIAGEN), and then an HRM analysis with 0.2 °C scaling from 55 to 95 °C was conducted. qPCR Master (EVA green) WizPure™ duplicates were used to test Synthetic SNP sequences. The HRM Tool, a component of the integrated software, was used to create normalized melting curves (NMC) and differential curves (DC) using duplicate synthetic controls in order to detect allelic variations. Following the program in(Table 1).

Table 1: The HRM (SNPs) experiment uses quantitative real-time PCR components.

Component	**(20) µl rxn**
(SYBR)qPCR Master	10 (µl)
ROX-Dye (50X)* optional	0.4(µl)
10µM Forward-Primer	0.2-2.0(µl)
10µM Reverse-Primer	0.2-2.0(µl)
DNA	3
Nuclease free water	5

The cycling protocol was programmed for the following optimized cycles based on the thermal profile, as given in (Table 2).

Table 2 : The HRM genotyping for FokI thermal profile.

Step	Temp(^{0}C)	time(sec.)	cycles
enzyme activation	94	30	1
denaturation	94	5	40
annealing	60	15	
extension	72	20	
(HRM)	65-95	2-5 sec./step	

Statistical Analyses

To conduct statistical analyses, SPSS (version 28) was employed. Using the Students-t test for differences between two independent means or the Paired-t test for differences between (pairs of observations or two dependent means), the significance of differences between various means (quantitative data) was investigated. Using Pearson Chi-square-test (x^2-test), the significance of variations between distinct percentages (qualitative data) were examined. P values less than 0.05 were regarded as significant. The correlation was calculated using pearson correlation. The ANOVA test's determination of the correlation between biochemical parameters.

2. RESULTS AND Discussion

In our study, we discovered a relationship between Age, Gender and BMI in chronic kidney disease patients. The statistical analysis in age showed no significant-difference (P >0.05), in the age between the CKD group (p= 0.209). The statistical analysis showed no significant difference between gender and CKD patients was (p= 0.796). BMI was not correlated with FokI polymorphisms; in contrast, there was no significant correlation (P=959) in CKD patients. Shown in(Table 3). This result agreement with Gokhan F et al, (13).

Table 3 : Age, Gender and BMI in chronic kidney disease patients

		CKD patients		Control		P value
		No	%	No	%	
Age (years)	40---49	13	43.3	14	46.7	0.209
	50---59	9	30.0	11	36.7	
	60---69	8	26.7	5	16.7	
Gender	Male	15	50.0	14	46.7	0.796
	Female	15	50.0	16	53.3	
BMI (Kg/m2)	Underweight (<18.5)	1	3.3	1	3.3	0.959
	Normal (18.5-24.9)	9	30.0	8	26.7	
	Overweight (25-29.9)	11	36.7	10	33.3	
	Obese (=>30)	9	30.0	11	36.7	

*Using the Pearson Chi-square test (x2-test) at the (0.05) level, there is a significant difference in both percentages. # Using Students' t-test and the 0.05 level, there is a Significant difference between two independent means .

DNA concentration

The nucleic acid was identified using a UV spectrophotometer , as shown in (Figure 1).

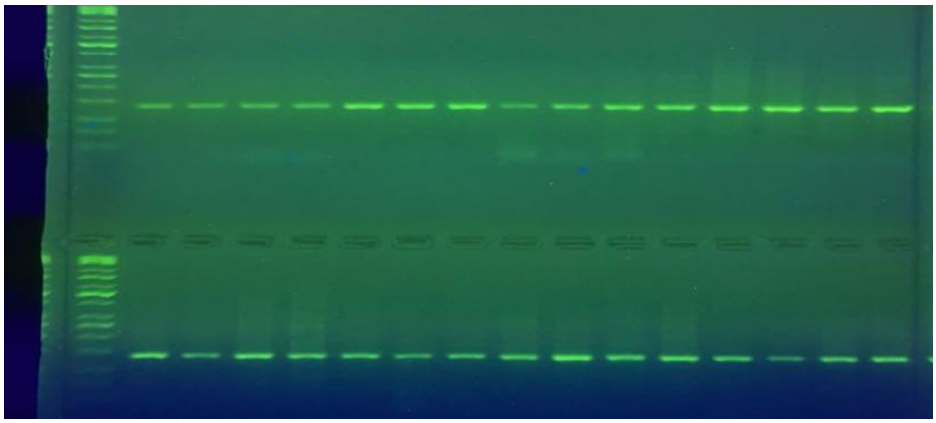

Figure 1 : Genomic(DNA) extraction from blood was Gel electrophoresis on a 1.5% agarose gel, at(5) Vol /cm for 1:15 hours.

FokI polymorphism rs (10735810)

Using the PCR method, The FokI rs10735810 (exon 2) of Vit.D Receptor was amplified. The frequency of the VDR FokI rs(10735810) gene-polymorphism (Genotype and allele) in CKD patients and controls shown a highly significant differences ($p>0.05$) was $p\leq0.0001$. Using (HRM)-PCR technique to genotyping samples and analyze the results , as shown in (Table 4).

Table 4 : Genotype counts, frequency, and Hardy-Weinberg equilibrium (HWE) in the Control and Patient groups.

Genotype	control-group (n=26)		P value	(Patient) group (n=46)		P value
	observed n %	expected n %		observed n %	expected n %	
CC	22 (84.61)	20.3 (78.25)	≤0.0015	18 (39.1)	11.5 (25)	≤0.0001
CT	2 (7.69)	5.3 (20.4)		10 (21.7)	23 (50)	
TT	2 (7.69)	0.35 (1.33)		18 (39.1)	11.5 (25)	

For the Chi-squared distribution, there is one degree of freedom (d.f.).

The results revealed a rang in genotyping frequency, with controls having a lower CC genotype ratio than CKD patients, differences between study groups were shown to be highly significant ($p=0.0001$) ($P\leq0.05$), $OR>1$ ($OR=0.12$), and CI 95%(0.04-0.32). When comparing CT genotypes of CKD patients and controls, the results are non-significant ($P=0.190$), $OR>1$ ($OR=3.3$), and CI 95% (0.69-16.10). The TT genotype ratio in CKD patients was highly significant compared to controls, and a highly significant difference between study groups was discovered ($P=0.005$), $OR>1$ ($OR=7.71$), and CI 95% (1.67-35.1), as shown in (

Table 5).

Table 5 : In the Control and Patient group, The frequency of the gene's genotype and Alleles.

Genotype and allele	Control group (n=26) no. (%)	Patient group (n=46) no. %	(OR)	C I (95%)	P value
CC	22 (84.61)	18 (39.1)	0.12	(0.04-0.32)	0.0001
CT	2 (7.69)	10 (21.7)	3.3	(0.69-16.10)	0.190
TT	2 (7.69)	18 (39.1)	7.71	(1.67-35.1)	0.005
C	46 (88.46%)	46(50%)	0.13	0.05-0.33	0.0001
T	6(11.54%)	46(50%)	7.67	3.01-19.45	0.0001

(OR), odd ratio; (CI), confidence interval.

Additionally, the CKD group's prevalence for the FokI polymorphism in this study was CC (39.1%) , TT (39.1%) and CT (21.7%) . According to the allele frequency data for the newly identified T there were extremely substantial differences between CKD patients and control group (P=0.0001). Allele T is represented as a risk allele (risk factor), when the odds ratio OR>1 (OR=7.67) to allele (T>1) is greater than one. According the detected C allele frequency, there were extremely substantial differences between CKD patients and control group (P=0.0001). When the odds-ratio OR>1 (OR=0.13) to allele (C<1) is less than one, this ratio indicates that allele C is not a protective factor. According to the odds ratio calculation, patients with either the CT or TT genotype had a three-fold or about seven-fold increased risk of developing chronic kidney disease, respectively. Instead, having the CC wild-type genotype was linked to a less than one-fold lower chance of developing chronic kidney disease, indicating that the C allele may have a protective effect, whereas the T allele has a (risk factor). This result agreement with Zhou T et al, (14). But among Asians, the Fok1 f(T) allele, ff(TT) genotype and FF(CC) genotype were associated with the risk of CKD. This agree with Mason D et al,(15). Al-shaer O et al, (16).

In the study's participants, the relationships between vitamin D and calcium, phosphorus and PTH were as follows: vitamin D , calcium, Phosphorus and PTH levels in CKD patients didn't significant correlate. Therefore, a strong negative association between serum vitamin D and calcium and a positive correlation between vitamin D and phosphorus and PTH levels were anticipated. shown in (Table 6). This result agree with Valizadeh S et al, (17).

Table 6 : The relationships between vitamin D and the minerals phosphorus, calcium and PTH in CKD patients.

parameters	Vitamin D3	
	r	p
Vitamin D3	-	-
Calcium	-0.113	0.553
Phosphorus	0.175	0.356
PTH	0.181	0.340

The 0.05 level of significance for correlation.

Table 7 : Serum levels of phosphorus, vitamin D, calcium and intact parathyroid hormone in CKD patients who had FokI (gene) polymorphisms.

parameters		FokI			P(value)
		CC	TT	CT	
Vitamin D (ng/ml)	(mean ± SD)	20.5 ± 5.4	25.0 ± 5.6	24.1 ± 8.6	0.50
Calcium (mg/dl)	(mean ± SD)	8.6 ± 0.5	8.5 ± 0.4	8.5 ± 0.5	0.60
Phosphorus (mg/dl)	(mean ± SD)	6.4 ± 1.0	5.5 ± 0.7	5.7 ± 0.10	0.02
PTH (pg/dl)	(mean ± SD)	446.5 ± 125.0	342.5 ± 116.3	318.5 ± 125.9	0.02

The ANOVA test was used to evaluate group differences; SD stands for Standard deviation.

Individuals with either the CC homozygous or TT genotype had considerably higher PTH readings than those with the CT heterozygous genotype, according to the analysis of the biochemical parameters between-groups variability, the p value ($p<0.05$) indicating that this difference was statistically significant. Patients in the CC group exhibited significantly higher serum PTH levels than those in both the CT and TT groups, although no significant variations in serum levels of calcium or vitamin D were detected among genotypes, indicating that Serum PTH levels in CKD patients were also impacted by FokI polymorphism. This result agree with Santoro D et al, (18).

3. Conclusion

Our research showed that CKD patients had vit. D receptor (FokI) gene polymorphisms. The relationships between vitamin D3 and the minerals calcium, phosphorus and PTH in individuals with CKD. And, studies were conducted in Iraqi CKD patients to assess the relationship between VDR polymorphisms and clinical parameters.

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