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Understanding of Vitamin D receptors Polymorphisms (FokI and ApaI) Role in Kidney Stones Formation

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Abstract: Kidney stones are a multifactorial disease results from complex interaction of the environmental, dietary and genetics factors. Vitamin D receptor (VDR) polymorphisms have indirect impact on kidney stone formation via regulation of Ca2+ metabolism. This study investigated the association of the Vit.D3 receptor gene polymorphisms in ApaI (T>G) rs7975232 and FokI (T>C) rs2228570 in the Iraqi kidney stones formers. The study included (96) samples, 64 patients with kidney stones, and the control group consisted of 32 apparently healthy individuals. The samples of this study were collected from The AL-Yarmok hospital in Baghdad/Iraq; the period of this study was from October 2024 to July 2024. Calculi were chemically analyzed. Biochemical investigations of kidney function tests: Urea, Creatinine, Uric acid, also Vit. D3 and Ca2+serum levels were measured. The target sequences of *VDR* gene were amplified by PCR technique and directly sequenced by Sangers method to identify the polymorphic genotypes. The odds ratio (OR) for TG genotype was (OR1.53, 95% CI 0.06 - 38.75) which may be increasing the risk factor for kidney stone formation. The odds ratio (OR) for TT genotype was (OR0.26, 95% CI 0.03-2.23), which indicates that TT genotype does not constitute a risk factor for kidney stones. Understanding In this study, it was found that weak of statistically significant association between genotype distribution FokI (C>T) in exon 2, and ApaI (T>G) in intron 8 of *VDR* gene and the risk of calcium urolithiasis development in the Iraqi population. The relationship between genetic variations in the vitamin D receptor helps support and develops sustainable health care through several axes, including reducing health costs and promoting sustainable nutrition. Keywords: Stone formation, single-nucleotide polymorphisms (SNPs), VDR gene.

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Introduction

Kidney stones, nephrolithiasis, are a high prevalence medical condition in the world. The stones may be seen as solid masses in different sizes and shapes within the kidney pelvis, ureter or bladder, as a result of the deposition of minerals and salts (1). It is considered as a multi-causal disease, where the environment, diet, and genetic factors all play a role in the formation of stones (2). The stones are formed through slow complex steps including super saturation of urine with minerals and salts, nucleation then growth of the aggregation ending with sold stone formation (3,4).The estimated prevalence of kidney stones is approximately (1-15%) of populations (5). According to statistics issued by the Iraqi Ministry of Health, the infection statistics in the last six years have been continuously increasing, reaching (85835) in 2018, (93635) in 2019, (63602) in 2020, (81160) in 2021,

(110818) in 2022 and (123880) in 2023 (Iraqi ministry of health statistics).

According to their chemical composition kidney stones can be classified into five different types: calcium stones, which are the most common among stones, account for 80 % of all urinary calculi in most of the world's countries, While uric acid or urate stones (~ 3–10) %, cysteine stones accounts less than 2% of all stone types , struvite stones (~10–15) %, and drug-Induced Stones accounts for about 1% of all kidney stones types. Calcium metabolisms a key player in stone formation which is affected by the several genes have an impact on the metabolism of calcium, oxalate and uric acid. The Vitamin D receptor gene, among the genetic loci that play a role in the formation and development of kidney stone (6-9).Vitamin D receptor (VDR), which is also referred to as calcitriol receptor, is a member of nuclear receptor family. VDR is expressed in most tissues in cell specific manner of the body , It has crucial role in calcium / phosphate balance and controls bone metabolism(7,10,11).The activation of genomic mechanism of action is started by binding of VDR to the active form of vitamin D3 (1, 25(OH) 2D3, result in Initiate heterodimeric interactions with the nuclear receptor RXR (retinoid X receptor) and formation the complex (VDR-RXR heterodimer), then the (VDR-RXR) complex enters the nucleus and binding with and activate vitamin D responsive elements (VDREs) , to enhance gene expression or transcription. There more than 20,000 genomic VDR binding sites known in the human genome , the fact that just a few hundred of these sites are permanently bound by VDR and the majority of loci are discovered temporarily after the ligand is activated (10-13). Activated VDR alters the transcription of genes that encode proteins that propagate the usual genomic effects of vitamin D, which include affecting bone and calcium homeostasis by signaling intestinal calcium and phosphate absorption (14, 15).

The vitamin D receptor gene, is located on the large arm of chromosome 12 (12q12–q14) , contains of 14 exons, its spanning approximately 75 kilobases of genomic DNA. It encodes for a VDR protein that composed of (427) amino acids (16, 17). The function of the VDR protein can be affected by single-nucleotide polymorphisms (SNPs), which are genetic variations found in either exons or introns of genes, and affect at least 1% of the population. There are many (SNPs) in the VDR gene were discovered to be relevant with kidney stones disease (18). Four single nucleotide polymorphisms (SNPs) are among the functional polymorphisms of the VDR gene such as: (TaqI) rs731236 (T/C) in the 9th exon of the 3′ terminal, (FokI) rs2228570 (C/T) in exon 2, (ApaI) rs7975232 (T>G) and (BsmI) rs1544410 (G/A) Polymorphisms, are in intron 8 between exon 8 and9 (19-21). These Polymorphisms associated with kidney stone formation and conceder a risk factors for renal diseases, and may contribute to the formation of kidney calcium-containing stone disease (22-24, 6).

The aim of this study is to clarify the relationship between genetic variations of the vitamin D receptor and kidney stone formation in Iraqi people with kidney stones.

Material and method

1- Subjects and samples

This study was conducted from November to July 2024 at Yarmouk Hospital in Baghdad, Iraq; the studied

population of 96 individuals (60 males and 36 female) was divided into two groups: the patients were 64 (42 males and 22 female), and the healthy groups were 32 (18 males and 14 female). For diagnosis, both patients and control participants were diagnosed by kidney ultrasound, X- Ray to confirm the presence or absence of the kidney stones, general urine examination and chemical analysis of kidney stones were performed. For kidney function investigation, five ml of venous blood was collected from each patient and control; each blood sample was divided into two sections: 2 ml of whole blood was kept in EDTA tubes for DNA extraction and used for the genetic study and the other 3 ml in GEL tubes and centrifuged at 5000 rpm for 5 minutes and the serum was obtained to use for measure biochemical Investigation for each patient and control which included the measuring of kidney function test (Urea, Creatinine, Uric acid) , Also measuring the level of calcium in the blood Ca2+, and Vitamin D level , then exclusion of samples with normal vitamin D levels. On the other hand, Urine samples were collected from each individual in the control group to examine urinary tract infection and make sure there was no sand microscope in the urine, as well as conducting CBC blood tests and measure biochemical tests (Urea, Uric acid, Ca2+, and Vitamin D level) for each patient and controls. Family history of KSD was collected from all patients, and make sure no family history of kidney stone disease (KSD) was found for any people in the control group and collected all the details related to the disease by conducting a questionnaire that included details about the nature of the food, the amount of fluids taken, and weight, and ensured that there were no chronic, genetic, or immune diseases.

2- Genetic study

The genetic study included the extraction of genomic DNA from Peripheral blood samples According of the kit (ReliaPrep(TM)) Blood DNA Miniprep system/Promega/USA), then DNA concentration and the purity was measured by Nano Drop spectrophotometers Cleaver /UK stained with Ethidium bromide ,and then electrophoresis for genomic DNA .The targeted DNA sites were amplified using design-specific primers Provided by from Macrogen Company in South Korea, which designed using (Geneious primer) software) (http ://WWW.macrogen.com), As shown in Table(1).

The master mix composed of 12.5 µl of One Taq (NEB®) master mix, 3 µl of DNA sample, 1.5 µl from each F and R primers and 6.5 µl of free-nuclease water. Amplification was conducted in a Gradient thermocyclers (Eppen-drop, Germany).

The targeted DNA sites rs7975232 in intron 8 between exon 8 and9 of *VDR* gene, and exon 2 of *VDR* gene were amplified using PCR technique, PCR thermal program for *VDR* rs7975232 was (35) cycles with the following settings: Initial denaturation Stage at 94°C for 3 minutes., denaturation Stage at 94°C for 30 seconds, annealing Stage began with 57 and gradually decreases to 47°C for 45 seconds and extension stage at 68°C at 30 seconds, at last, final extension Stage at 45°C for 5 minutes. After completing the reaction, PCR thermal program for exon 2 of *VDR* gene was (35) cycles with the following settings: Initial denaturation Stage at 94°C for 3 minutes., denaturation Stage at 94°C for 30 seconds, annealing Stage began with 58 and gradually decreases to 48°C for 45 seconds and extension

stage at 68°C at 30 seconds, at last, final extension Stage at 45°C for 5 minutes.

After completing the reaction PCR products were electrophoresed in 1.2 gm agarose at 80 V for 80 minute using gel electrophoresis (cleaver science – UK) and visualized with µl Red safe (dyeIntron/South Korea). A gel documentation system with a high-resolution camera (Cleaver Scientific – UK) was used to capture the images.

Table (1): Primers used in the study

VDR	Sequence (5'……….3')	Product Size (bp)
rs2228570 (C>T)	F:CTTCTGTTTGCAGTCACTGAT	637bp
	R:GCTTGAGGTTATTTGCTGCTA	
rs7975232 (T>G)	F: GATCCTAAATGCACGGAGAAG	682bp
	R: ACAAACAGCAACTCCTCATG	

2.3 Sequencing

For each sample, the PCR products were subjected to Sanger sequencing by (Macrogen company /South Korea) for both the forewords and reverse primers to identify the single nucleotide polymorphism. Analysis of sequence FASTA files has been done by Geneious Prime software (Macrogen – South Korea) http: //WWW.macrogen.com and aligned to Ref Seq of (CASR) gene.

2.4 Biochemical tests

The kidney stones collected from patients and subjected in to chemical quantitative method to determine the main mineral components of urinary stones. Biochemical analysis of kidney functions: including Urea, Uric acid, and Creatinine levels in the sera of both patients and control groups were performed as rotten work in hospital laboratories. Serum vitamin D3 (Elecsys® Vitamin D3, Roche Diagnostic, Ref.Code: 09038078, Germany), and Calcium levels (Roche Diagnostic, Ref.Code: 05061504190, Germany),levels were measured using a biochemical automated analyzer (Cobas e411, Roch Diagnostics, Germany) according to manufacturer's instructions.

2.5 Statistical

Statistical analyses have been done by SPSS statistical software (version 26; SPSS IBM), genetic statistical analyses have been done by the medical online, Odd ratio calculator software(https://www.medcalc org/calc/odds_ratio.php) to compare the association between the Odd ratio for each genotype with kidney stones disease.

At the statistically significant table value **p**$\leq$ 0.05,$\chi 2<3.84$.

3. Results and discussion

As a first step, the extracted genomic DNA samples of patients and controls were separated in agarose gel using an electrophoresis device the electrophoresis of genomic DNA Agarose in TBE (Tris-Borate EDTA) for 45 minutes at 80 Volt as shown in(Figure.1)

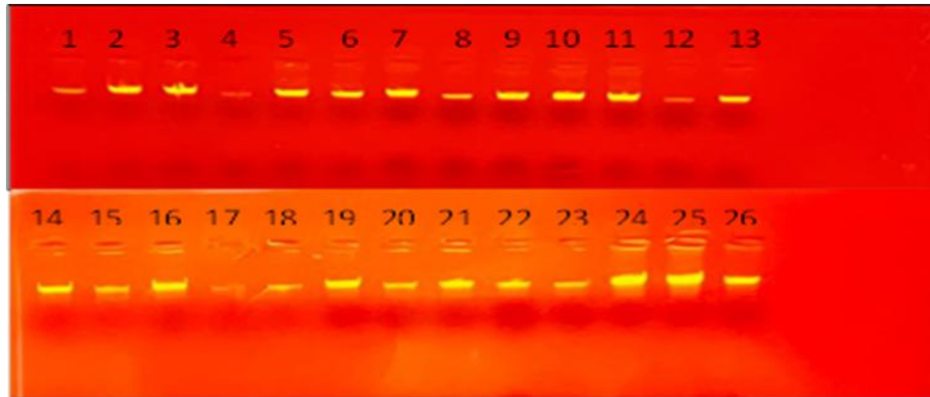

Figure (1): Genomic DNA extracted from blood samples of kidney stone disease from patients and controls groups on an agarose gel electrophoresis pattern. (1%) agarose, for 45 minutes at 80 Volt

PCR product electrophoresed on a gel. (1 %) agarose, 75Volt, for 1hour) shown in (Figure.2)

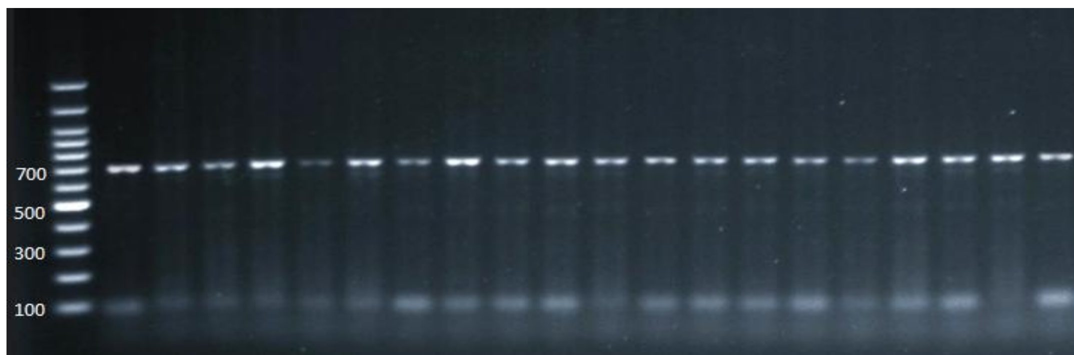

Figure2. PCR product gel electrophoreses for *VDR* gene at 2% Agarose in TBP for 80 minutes at 80 Volt for patient and controls groups , band size 651bp.

3.2 Sequence alignment of (VDR) gene rs2228570 (T>C)

After analyzing the sequence results for the vitamin D receptor (VDR) gene, it was found that the single nucleotide polymorphism (T>C), displays the genotype and allele frequency of both control and patient groups. The observed value is the same as the expected value in both control and patient groups, and the Chi-Square $\chi 2$ Values is significantly less than the table value of 3.84, therefore the alleles are consistent with Hardy Weinberg law in this population, as shown in (Table2).

Table (2): Chi square analysis of the studied groups at the position rs2228570 of VDR gen

Groups		TT	TC	CC	Chi square $\chi 2$
Control	Observed genotype	26	5	1	1.25
	Expected genotype	25.38	6.23	0.38	
Patient	Observed genotype	53	10	1	0.41
	Expected genotype	52.56	10.87	0.56	

By analysis of genotype distribution between the control and Patient groups, the study found three genotypes (TT, TC, and CC.). In the exon 2 at the position rs2228570 of VDR gene in both groups. The results of TT genotype were close in both studied population, appearing as (83%)in Patient and(81%)

in control groups; the P value is (0.85) significantly greater than 0.05. Therefore, showed no significant differences between control and cases in the TT genotype; the odds ratio (OR) for the TT genotype was (1.11) with a Confidence interval (0.37 - 3.33), therefore TT genotype may be a risk factor with kidney stone formation. TC genotype was equal in both Patient and control grope as (16%) with a P value (1.00), significantly greater than 0.05,Therefore showing no significant differences between control and cases in the TC genotype; the odds ratio (OR) for the TC genotype was (1.00) with a Confidence interval (0.31-3.21). Therefore, this genotype was no association with stones formation.CC genotype was (2%) in patients and (3%) in control with a P value (0.62) significantly greater than 0.05,also showed no significant differences between control and cases in CC genotype, the odds ratio (OR) for the CC genotype was (0.49) with a Confidence interval (0.02 - 8.13), this indicates that CC genotype does not constitute a risk factor for kidney stones. As shown in (Table 3).

Table (3): Genotype and allele frequency of *VDR* rs2228570 (T>C) SNP in control and patients groups

Genotype	Patients N=64(%)	Control N=32(%)	OR	95%CI	P-value
TT	53(83%)	26 (81%)	1.11	0.37 - 3.33	0.85
TC	10 (16%)	5 (16%)	1.00	0.31 - 3.21	1.00
CC	1 (2%)	1 (3%)	0.49	0.02 - 8.13	0.62

The allele frequency of the T allele in patient was (91%) close to control which was (89%); the odds ratio (OR) was (1.18) with a Confidence interval (0.44- 3.17) Therefore, the T allele may be constitute a risk factor for kidney stones. The C allele frequency was (9%) in the patient group compared to the control group (11%).

The odds ratio (OR) was (0.84) with a Confidence interval (0.44- 3.17). Therefore, the C allele does not constitute a risk factor for kidney stones. The p value of T and C allele were (0.73), showing no significant differences in the T and C allele between Control and cases (Table 4).

Table (4): Allele frequency of *VDR* gene rs2228570 (T > C) SNP in control and patients groups

Allele Frequency	Patients N=64(%)	Control N=32(%)	OR	95%CI	P-value
Causative allele T	116 (91%)	57 (89%)	1.18	0.44- 3.17	0.73
Protective allele C	12 (9%)	7 (11%)	0.84	0.31-2.25	

3.3 Sequence alignment of *VDR* gene rs7975232 (T>G) SNP

The results of analyzing the sequence of the vitamin D receptor *VDR* gene at the position rs7975232, which exhipt a substitutional SNP from T toG in intron 8 between 8 and 9 exons. The genotype and allele frequency of both control and patient groups showing in table (5). There is a clear difference between the observed and expected distribution in both groups, and the chi-square values are higher than the table value of 3.84, indicating that the distribution does not consistent with Hardy Weinberg law in the studied population.

Table (5): Chi square analysis of the studied groups at the position rs7975232 of VDR gen.

Groups		TT	TG	GG	Chi square χ2
Control	Observed genotype	31	0	1	32
	Expected genotype	30.03	1.93	0.03	
Patient	Observed genotype	57	1	6	53.51
	Expected genotype	51.66	11.67	0.66	

By analysis of genotype distribution between the controls and Patients groups, the study exhibit three genotypes (TT, TG, and GG) at the position rs7975232 in the intron 8 of VDR gene. TT genotype was carried by (89%) in Patient and (97%) in control groups; the P value is (0.22) significantly greater than 0.05. Therefore, showed no significant differences between control and Patients in the TT genotype; the odds ratio (OR) for the TT genotype was (0.26) with a Confidence interval (0.03-2.23), which indicates that TT genotype does not constitute a risk factor for kidney stones. TG genotype were (2%) in Patient and (0%) in control groups; the P value is (0.79) significantly greater than 0.05. Therefore, showed no significant difference TG genotype may be a risk factor with kidney stone formation, and there was a high genotype was (1.53) with confidence interval (0.06 -38.75), which indicates that genotype and stones formation. GG genotype were (9%) in Patient and (3%) in between control and cases in the TG genotype; the odds ratio (OR) for the TG probability of association between the TG control groups; the P value is (0.29) significantly greater than 0.05. Therefore, showed no significant differences between control and cases in the GG genotype; the odds ratio (OR) for the GG genotype was (3.20) with confidence interval (0.36 - 27.84), which indicates that GG genotype may be a risk factor with kidney stone formation, and there was a high probability of association between the GG genotype and stones formation, as shown in (Table 6).

Table (6): Genotype and allele frequency of *VDR* gene rs7975232 (T>G) SNP in control and patients groups

Genotype	Patients N=64(%)	Control N=32(%)	OR	95%CI	P-value
TT	57(89%)	31(97%)	0.26	0.03 - 2.23	**0.22**
TG	1 (2%)	0 (0%)	1.53	0.06 -38.75	**0.79**
GG	6 (9%)	1 (3%)	3.20	0.36 - 27.84	**0.29**

The allele frequency of the T allele in patient was (90%) ,while in control was (97%); the odds ratio (OR) was (0.2854) with a Confidence interval (0.06 -1.30). Therefore, the T allele was not constitute a risk factor for kidney stones. The C allele frequency was (10%) in the patient group compared to the control group (3%), the odds ratio (OR) was (3.50) with a Confidence interval (0.76-16.02). Therefore, the C allele was constitute a high risk factor for kidney stones. The PValue of T and C allele were (0.73), showing no-significant Significant differences in the T and C allele between control and cases.As shown in (Table 7).

Table (7): Allele frequency of VDR gene rs7975232 (T >G) in control and patients groups

Allele Frequency	Cases N=64(%)	Control N=32(%)	OR	95%CI	P-value
Causative allele T	115 (90%)	62 (97%)	0.2854	0.06 -1.30	0.10
Protective allele G	13 (10%)	2 (3%)	3.5043	0.76 - 16.02	

3.4 Screening for genetic variations

In this study, the sequencing results showed No SNPs in the exone2 of *VDR* gene at FokI (rs2228570),but show several SNPs in intron 8 at ApaI (rs7975232) of *VDR* gene (Fig. 3) and (Fig. 4).

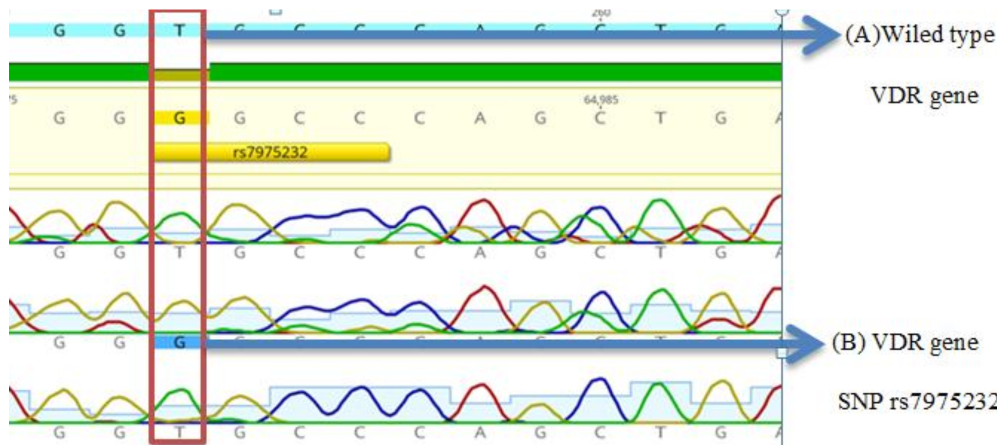

Figure (3): Chromatograms of VDR gene (A) Wiled type VDR gene (B) VDR gene SNP at (rs7975232) from T to G.

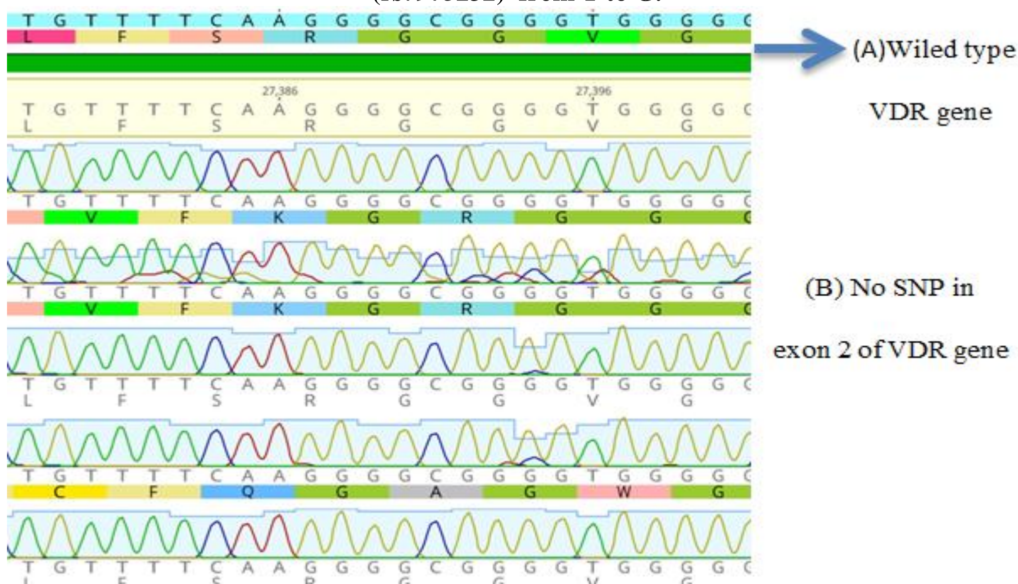

Figure (4):Chromatograms of VDR gene (A) Wiled type VDR gene (B) No SNP in exon 2 of VDR gene

3.5 Biochemical tests

The chemical Analysis of kidney stones showed 87% calcium kidney stone (calcium oxalate and 13% uric acid stones.The results of the biochemical tests were found no differences between in patient with kidney stones compared to controls in terms of most variables (Calcium, vitamin D, uric acid, and creatinine).

However, they showed differences in Urea between the patient and control group. The results of Calcium were (9.63 ± 0.83) in the patients com-pared (to 9.51 ± 0.31) in control with a P value (0.31); the standard normal ranges are (8.5-10 mg\dl). The results of Vitamin D were (15.71 ±5.80) in the patients compared (to 16.16 ± 6.491) in control with a P value (of 0.74); the normal ranges are (30-70 mg\dl). The results of Uric Acid were (4.92 ± 1.52) in the patients compared to (6.26 ± 0.99) in control with a P value (0.07); the standard normal ranges are (2.4-6 mg\dl)). The results of Creatinine were (0.94 ± 0.42) in the patients compared to (0.89 ± 0.24) in control with P value (0.471); the standard normal ranges are (0.5-1.2 mg\dl). The results of Urea were shown high significance (30.51 ± 9.45) in the patients compared to (26.75 ±5.52) in control with P value (0.01); the standard normal ranges are (7-37 mg\dl) show in (Table 8).

Table (8): Mean ± S.D. of Serum calcium, vitamin D3, Urea, Uric Acid and Creatinine of the study groups

Group				
Variables	**Patients (N=64) (mean ± SD)**	**Control (N=32) (mean ± SD)**	***P*-value**	**Significance**
Calcium	9.63 ±0.83	9.51±0.31	0.31	N.S
Vitamin D	15.71 ±5.80	16.16±6.49	0.74	N.S
Urea	30.51 ± 9.45	26.75±5.52	0.01	S
Uric Acid	4.92 ±1.52	6.26± 0.99	0.07	N.S
Creatinine	0.94± 0.42	0.89± 0.24	0.47	N.S

Vitamin D receptor (VDR) has biological and physiological importance for the body including the control of calcium and phosphate hemostasis, innate and adaptive immunity, cellular differentiation (25). It also plays an important role in the kidneys by regulating the renal electrolyte balance through renal reabsorption and excretion of both phosphorus and calcium after their association with vitamin D (10).

Genetic variants within the population may play a significant role in the risk of manifesting multifactorial disorders, such as kidney stone formation and recurrent kidney stones. polymorphisms in *VDR* genes were associated with urinary stone formation, including FokI (rs2228570), ApaI(rs7975232) (26,27).The SNP FokI (rs2228570) located at the translational start site of exon 2 of the *VDR* gene therefore changes the codon and alter the VDR protein sequence and associated with a reduced response to vitamin D in target cells. The SNP ApaI(rs7975232) occur in the intron sections, have also been proven to enhance messenger RNA (mRNA) stability or transcriptional activity, and therefore they may lead to an increase in vitamin D activity(22). In this study, no deviation from HWE was found in the genotype distribution of the SNPs under study; we did not find an increase in serum calcium in cases of idiopathic stones. Through the results of the P value , it becomes clear to us that there is no association between rs7975232 SNP and stones formation, and they were not associated with serum calcium; this indicates that these polymorphisms did not alter the

function of the vitamin D receptor, and this is consistent with the results of both studies, while the results of the Odd ratio of rs7975232 SNP refer there was a probability of association between the GG and GT genotype and the risk of stones formation and the G allele was the risk allele. At the same time, there was no SNPs found at (rs2228570) of vitamin D receptor in this study and no significant differences in genotype of both study groups. This results of genotype and allele frequency were agree with some researchers in many countries around the world (28). And disagree with other research (17,19.29). Despite the absence of statistical differences in Biochemistry tests between the patients and control group, the results are still clinically significant because the disease remains present and still poses a risk to the kidneys. When linking the results of biochemical tests with the results of the study of genetic variations in the vitamin D receptor gene at positions rs7975232 and rs2228570, these findings suggest that the genetic polymorphisms are ineffective and do not have any influence on regulating the function of Vitamin D receptors in the body.. Patient care requires specialized resources when kidney disease is not detected and treated early, which drives up costs, puts many people at risk of catastrophic health expenditures, and poses high opportunity costs for health systems (30). Understanding the genetic variations of the vitamin D receptor helps develop treatments and improves the quality of healthcare. It also allows for the implementation of preventive measures to reduce the recurrence of stone formation, which reduces medical waste and ensures better use of resources. All of this reduces the burden on the healthcare system.

Conclusion

In summary, there was no statistically significant association detected between polymorphism FokI (rs2228570), and ApaI(rs7975232) of the VDR gene and the risk of calcium urolithiasis development in the Iraqi population in the pooled analysis results. Although analysis showed that the of association between the GG and GT genotype of rs7975232 SNP and the risk of stones formation and the G allele of rs7975232 SNP was the risk allele. we suggest conducting more studies to determine the relationship between VDR gene polymorphism and kidney stone disease.

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Ethical statement

Ethical approval for this study was obtained from the local ethics council at the University of Baghdad, under the official letter No 73283 in 30/11/2023

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