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GRHPR gene variations in Iraqi patients infected with calcium oxalate kidney stones

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Abstract

The alterations in glyoxylate reductase and hydroxy-pyruvate reductase concentrations in the sera and the genetic alterations associated with calcium oxalate kidney stones in Iraqi patients were not studied previously so this study aimed to focus on these points. This study included 80 subjects; they were 50 patients with calcium oxalate stones compared to 30 apparently healthy controls. Biochemical investigations for kidney functions (creatinine, urea, and uric acid), were performed on the sera of both groups. Also, complete blood count, random blood sugar, and blood group tests. Furthermore, urine had been collected for General Urine Examination to visualize oxalate crystals in the urine of the patient. Also, the GRHPR enzyme concentration was measured by ELISA for both groups. The DNA was isolated from whole blood and the target DNA was amplified by PCR then the pathogenic mutations at c.295C>T (rs119490108), c.165G>A (rs180177314) and c.904C>T (p. Arg302Cys) rs180177322 were investigated by direct sequencing of the product, and then the results were analyzed. This study found that the concentration of the enzyme in the controls (4.78 ± 1.06 mg/dl) was significantly higher than its concentration in the patients (0.411 ± 0.02mg/dl). The pathogenic mutations were not found in both studied groups, but other positions were found polymorphic; at exon 4 the rs2768659 (A>G), rs1294628807 (G>A) and rs2736664 (C>T), at exon 6: c.579A>G (p. Ala193=) rs309458 and c.494-68A>G rs309459 and at exon 9 c.*146A>G rs1057507. In conclusion, this study found that calcium oxalate stones were associated with decrease GRHPR enzyme concentration in the patients compared to the control group which may be caused by mutations or epigenetics masking of the gene expression.

Keywords: ELISA, GRHPR enzyme concentration, *GRHPR* gene variation, kidney stone, PCR.

Introduction

Urolithiasis or nephrolithiasis widely known as kidney stone disease (KSD)[1] has increased prevalence and recurrence rates worldwide, particularly over the past 30 years[2,3] and these rates have varied greatly in different countries, reaching nearly 5–9% in Europe, 12% in Canada, and 13–15% in the USA and 20. % in Saudia Arabia[4,5]. It is related to high financial costs, as well as high recurrence rates[6]. The recurrence rates are very high[7], leading to an increase in the number of patients who need adequate therapy and a proper prevention method for the recurrence[8].

Kidney stone is formed by a slow complex process including the formation of crystals by

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nucleation then they increase in size and aggregation to sold stone[9]. The crystals can be single or multiple and come in a variety of shapes, sizes, structures, and locations[10]. Kidney stones are classified into the following types, calcium oxalate (CaOx) is the most predominant type of all kidney stones around the world[11, 12], in Iraq; previous studies have reached the same results indicating that CaOx stones are the most common type[13]. It is followed by calcium phosphate, struvite, uric acid, and cysteine which are the least common[14,15]. Kidney stones diseases are characterized by the accumulation of oxalate crystals in the kidneys and eventually forming calcium oxalate stones, there are also several other genetic diseases that lead to the formation of other types of kidney stones, such as distal renal tubular acidosis forming cysteine stones and calcium phosphate stones respectively[16].

The disease is quite complex and has several possible etiologies [17]including sex, age, race[18], pregnancy[19], diabetes and hypertension[20,21], high temperature degrees[22], nutrition regime[23] like depending on animal protein-rich diet and consuming foods that contain high amounts of oxalates, Vitamin C and D deficiency and salt intake with low potassium and low citrate content in the nutrition are main factors that lead to stones formation[23-28] and insufficient drinking water intake[29,30]Also, biofilm forming microorganisms have a role in stone forming; including *Pseudomonas spp., Klebsiella spp., Proteus spp., Oxalobacter formigenes* play a role in calcium oxalate formation and other certain bacterial species[30-32]. Some drugs have a potential role in promoting kidney stone formation like nephrocalcin, osteopontin, urinary prothrombin fragment-1, bikunin and glycosaminoglycans[33]. Moreover, genetic factors have an important role in stone formation[29]. Hereditary kidney stone disease could be associated with monogenic rare recessive, and X-linked transmission genes that inherited in mendelian manner with distinct phenotype with full penetrance and lead to severe congenital disorders in newborns and adolescents, such as *CASR*, *SLC34A1*, *AGXT*, *GRHPR*, and *HOGA1* genes[34,35], while dominant polygenic risk variants and single nucleotide polymorphism are more frequent in adults with less penetrance[36,37].

One of KSD associated genes is the *GRHPR*, it's a protein coding gene, located in the pericentromeric region of chromosome 9, composed of 21280 base pairs with 9 exons and 8 introns spanning 9 Kbp, encodes for 328 amino acid, 36 kDa, this gene encodes for GRHPR enzyme and mutations in *GRHPR* gene cause its deficiency[38]. The enzyme catalyzes glyoxylate and hydroxyl-pyruvate reduction using NADPH coenzyme, glyoxylate is normally removed through the alteration to glycolate in the liver cytosol and mitochondria also hydroxyl-pyruvate is typically reduced to D-glycerate by this enzyme, the absence of this enzyme results in the oxidation of glyoxylate to oxalate and hydroxyl-pyruvate is reduced to L-glycerate by L-lactate dehydrogenase effect[39]. Numerous reported polymorphisms and mutations in the *GRHPR* gene have been linked to primary hyperoxaluria type2[40-42]. The *GRHPR* gene has a potential protective effect as a tumor suppressor gene in association with a number of cancers, including breast cancers and kidney cancers[43]. Additionally, low *GRHPR* expression has been linked to a high degree of risk of hepatocellular carcinoma (HCC)[44]. A recent study found that its expression is regulated by miR-138-5p[45], as well as, that it is expressed at low levels in the malignant tissues compared to neighboring normal tissues[46] Moreover, *GRHPR* expression is downregulated by methylation of CpG regions and chromatin remodeling in the human steatohepatitis leading to metabolic pathway disorders[46].

Materials and Methods

Patients and controls:

The study extended from February 2022 until August 2022 and included 80 individuals; the patients' group included 50 subjects distributed as 25 males (50%) and 25 females (50%), and their ages ranged from 19 to 60 years. They were diagnosed as having calcium oxalate stones by specialists, in Al Karama Teaching Hospital, according to an abdomen ultrasound, stone analysis,

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biochemical tests, and the presence of oxalate in the urine. The healthy control group consisted of 30 individuals. They were 17 males (57%) and 13 females (43%), their ages ranged from 15 to 53 years. All participants in the two groups were not smokers, did not have hypertension, diabetes, or obesity. They filled out a prepared questionnaire, and provided oral consent. The ethical approval was registered in the Iraqi Ministry of Health.

Clinical samples and Laboratory investigations

The clinical samples were collected from both patients and controls at Al Karama Teaching Hospital they included urine samples for general urine examination (GUE). The urine was collected in sterile screw caped container and examined within two hours by centrifuging the samples in a bench centrifuge for 10 minutes at 1500 rpm then the supernatant was thrown and the sediment was put on the glass sild and covered by a coverslip then examined by the compound microscopic under the power of 40X, to conform the oxalate crystals presence in the patients' urine. Also, five milliners of peripheral whole blood samples were collected from both patients and controls by vein puncture in sterile plan tube (3ml) then left for 20 minutes to coagulate then centrifuged by bench centrifuge for 5 minutes to collect the sera. The renal functions were investigated by testing the sera for Urea, Creatinine and Uric acid, by using uric acid measuring kit (JTC Diagnostics/Germany), urea measuring kit (JTC Diagnostics/Germany) and creatinine measuring kit (Linear/ Spain) in the sera of both of the patients and controls.

Enzyme Linked Immunosorbent Assay (ELISA) for GRHPR

The concentration of human Glyoxylate Reductase/Hydroxy pyruvate Reductase (GRHPR) enzyme kit (Shanghai YL Biotec / China) was measured in the sera of both patients and controls by sandwich enzyme -linked immune-sorbent assay (Biotech, Greece) according to the manufacture instructions.

Genetic investigation

The genetic investigation included the extraction of genomic DNA from peripheral whole blood samples. The remaining blood (2 ml) was poured into EDTA tubes and kept at -20C˚ till it is used for DNA extraction for the genetic study by using a Reliaprep™ kit (Promega, USA) in accordance with the manufacturer's instructions. The efficacy of the extraction method was assessed directly by measuring the purity of the extracted DNA samples using a Nano-spectrophotometer (Thermo Science, USA). The purity of DNA samples ranged from 1.6 to 2, and the findings were computed using the Nanodrop method. The target DNA fragments were amplified by the Polymerase chain reaction (PCR) technique using a thermal cycler (Applied Biosystems, USA). For a single reaction, the PCR's components were master mix (12.5 µl) and forward primer (1µl), reverse primer (1 µl), DNA (2 l) the final volume was adjacent to 25 µl by adding nuclease-free water. The oligonucleotide primers were designed using primer 3 plus software. the oligonucleotide primers were designed using primer 3 plus software for the studied fragments for exon 4, 6 and 9 as shown in Table 1.

Table 1. Primers sequences and fragments size.

The exon	Primers sequences	Fragment size
Exon 4	F1=5'CAGAGATGTCAGCCTCTTATTC3' R1=5'ACCTGGTCTGCGTTCACTTAC3'	876 bp
Exon 6	F2=5'TCTCCAGTTCTCAGAGGTTAG3' R2=5'TAGTGCTTGCCAGTTTAT3'	765 bp
Exon 9	F3=5'TCTCTCTCTCTCTCCTTTC3' R3=5'CCATTTACAGGCCAAGACGG3'	376 bp

The amplification reaction included, the following: the first step was initial denaturation at 95°C for 1 minute, the second step was repeated 35 cycles composed of: denaturation for 15 seconds then

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annealing of the primers at 58°C for 30 seconds then the extension at 72°C for 60 seconds and the final extension was performed at 72°C for 7 minutes. Agarose gel electrophoresis was used to separate PCR products, and then they were visualized using ethidium bromide staining under the UV-trans-illuminator apparatus (OPTEMA /Japan) to ensure the accurate size of the DNA bands. The DNA sequencing of the PCR products was performed by using the Sanger method (Macrogen/Korea), the PCR products sequences were analyzed by Geneious Prime software, and then the variations of the sequence were calculated and statistically analyzed by T-test and least significant difference - LSD (analysis of variance-ANOVA) were used to compare the means. Chi-square test was used to compare the significant percentage in this study according to the statistical analysis system -SAS (2018) program.

Results and Discussion

General Urine Examination:

The microscope examination is a basic examination used to identify crystals' type and shape in the urine[41]. Calcium oxalate monohydrate (thermostable form) and dihydrate (soluble form) are the most common crystals found in the urine of urolithiasis infected patients, while anhydrous and trihydrates forms are rarely seen forms in the patients' urine[47]. The initial step for calcium oxalate crystallization is the supersaturation of urine, the attachment of the saturated urine to a sold surface such as red or white blood cells, bacterium or tubular epithelial cells induce the nucleation of the crystals spontaneously, then the crystals increase in size and number facilitate their aggregation, leading to hyperoxaluria and calcium oxalate kidney stone[48]. Basically, crystallization is started due to urine supersaturation with minerals, mainly calcium, and proteins which act as a glue to gather the crystals[49]. Supersaturated urine occurred by several factors include: the high consumption of animal protein which has high purines that are metabolized to oxalate and uric acid. Animal protein consumption and their metabolism lower urine pH, which is between 5.0 and 6.5 thus promoting the growth of calcium oxalate stones[17]. Also, less fluid intake decreases urine flow, hypercalciuria, high salt consumption, Vitamin D deficiency, hyperthyroidism, low calcium intake, hypocitraturia and hyperoxaluria[50,51]. Alternatively, it may be caused by the absence of the GRHPR enzyme, which results in calcium oxalate crystal formation and consequently kidney stone formation Fig. 1.

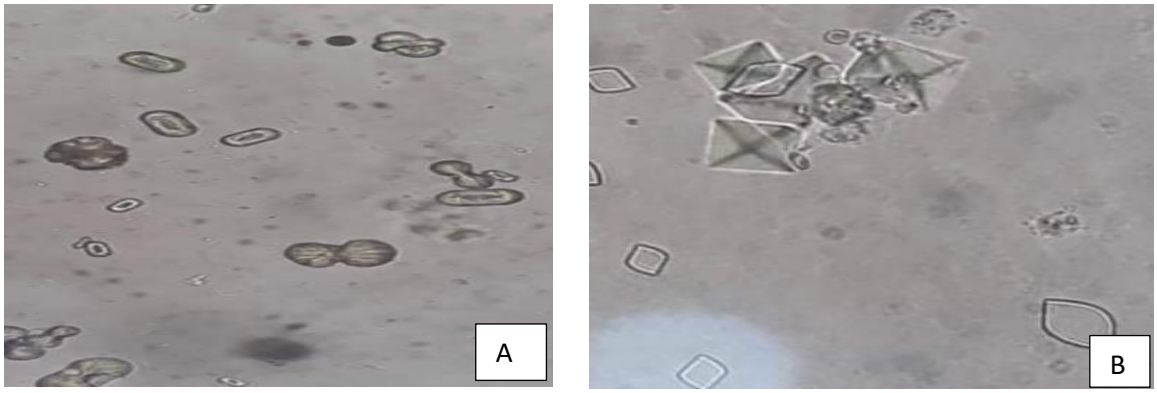

Figure 1. A: Calcium Oxalate monohydrate crystals and B: calcium oxalate dihydrate crystals under 40x power microscopic examination

Kidney functions tests

The results of urea showed highly significant differences between the control (15.95 ±0.19) and the patients' group (31.44 ±2.17), the normal ranges are (7-37 mg\dl). The results of creatinine have also showed highly significant differences between the control (0.721 ±0.04) and the patients' group (1.29

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±0.11) as they were above the normal range, the standard normal ranges are (0.5-1.2 mg\dl). The results of uric acid between the control group and the patients' group were non-significant (4.45 ±0.19) and (5.16 ±0.48) respectively, the standard normal range is (2.4-6 mg\dl) as shown in Table 2.

Laboratory testing of serum urea and creatinine is rottenly used to diagnose kidney stone[46]as it may indicate that the patients have kidney dysfunctions. Shen and his colleagues, found that serum urea, creatinine and uric acid (5.43 ± 2.50, 83.40 ± 38.56 and 333.68 ± 90.44) were significantly high in stone formers, at p≥0.001, than non-stone formers (4.90 ± 2.09, 78.57 ± 33.47 and 322.62 ± 85.97) reaspectivelly[51]. Waikar and his colleagues found comparable outcomes. The high levels of urea and creatinine products may be the result of a metabolic disorder in the kidneys caused by direct renal epithelia injury and reduced glomerular filtration rate (GFR), which is attributed to the precipitation of calcium oxalate crystals in the kidney tubules lumen that obstructed the nephrons. Renal dysfunction reduces waste excretion and leads to an accumulation of toxic compounds and waste products in the blood and urine [52,53]. In this study, the results of uric acid in the control were non-significant compared to the patients may be related to genetic factors. In Park et al study including the serum metabolites, they found that serum uric acid in was not significantly differing between families with stone history than those without stone family history [54]. The risk of urolithiasis had been found to increase in gout patients suffering from serum uric acid and calcium high levels as a consequence of thyroid hormone dysregulation[55]. In this study, the uric acid concentration was not significantly differed between the two groups may be related to the normal function of the thyroid hormones that maintain normal calcium levels in the blood.

Table 2. concentration of Urea, Creatinine, and Uric acid by mg/dl in patients with renal stones compared to controls.

Group	Mean ± SE		
	Urea (mg\dl)	**Creatinine (mg\dl)**	**Uric acid (mg\dl)**
Control	15.95 ±0.91	0.721 ±0.04	4.45 ±0.19
Patients	31.44 ±2.17	1.29 ±0.11	5.16 ±0.48
T-test	5.782 **	0.294 **	1.289 NS
P-value	0.0001	0.0001	0.278

**** significant at (P≤0.01), NS: Non-Significant at (P≤0.05).**

Enzyme Linked Immunosorbent Assay (ELISA) for GRHPR:

Both patient and control groups had their GRHPR enzyme concentrations evaluated, by comparing the two groups the results revealed highly significant differences between the two groups Fig. 2. The results revealed that the concentration Mean ± SE of the controls was (4.78 ± 1.06) while the patients group lacked this enzyme's activity (0.411 ± 0.02) Fig. 2, for that reason the comparison between the two groups was highly significant which is consistent with the findings of Takayama and his collegues[35]. This significant difference between the two groups suggests that a deficiency in GRHPR may play a role in the formation of calcium oxalate kidney stones. Previous studies have reported similar associations between GRHPR deficiency and the formation of kidney stones[56]. This may be because the enzyme's primary function is to keep levels of glyoxylate and hydroxypyruvate relatively low in order to prevent the production of oxalate stones, and it also converts glyoxylate to glycolate, in the case of enzyme absence, glyoxylate is oxidized to oxalate through lactate dehydrogenase (LDH) effect[56]. Since the human body cannot hydrolyze oxalate; calcium oxalate crystals will precipitate and aggregate as calcium oxalate stones[57].

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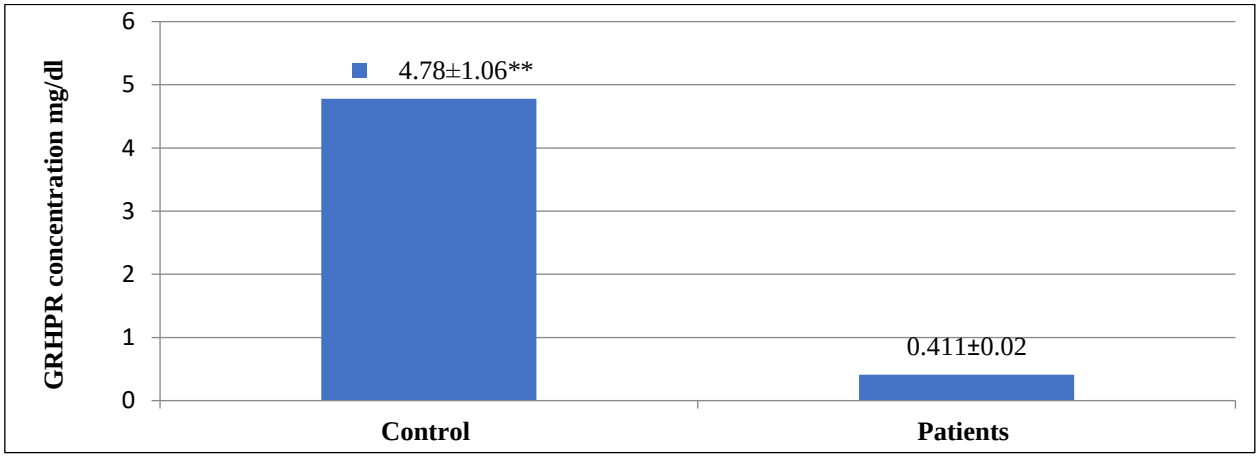

Figure 2. GRHPR enzyme concentration (mg/dl) in patients with renal stones compared to controls. ** significant at (P≤0.01).

The genetics investigation

The amplified PCR products of exon 4, exon 6 and exon 9 are shown in Fig. 3, 4 and 5 respectively. The amplified fragments were subjected to the Sanger sequencing technique to identify possible mutations and /or SNPs for both of patients and controls. Table 3 shows the interpretation of SNPs detected in the amplified products of exon 4, 6 and 9 by sequencing technique.

Table 3. The Variants found in the patients and controls GRHPR exon 4,6 and 9.

The exon	The variants	Interpretation
Exon 4	rs2768659 (A>G)	Benign
	rs1294628807 (G>A)	Intronic variant
	rs2736664 288-11 (C>T)	Benign
	rs119490108 c.295 C>T (Arg99ter)	pathogenic
Exon 6	rs309458c.579A>G (p. Ala193=)	Benign
	rs309459 c.494-68A>G	Intronic Variant
	rs180177314(G>A) c.494G>A (G165D Gly165Asp	Pathogenic
Exon 9	rs180177322 c.904C>T (p. Arg302Cys)	Pathogenic

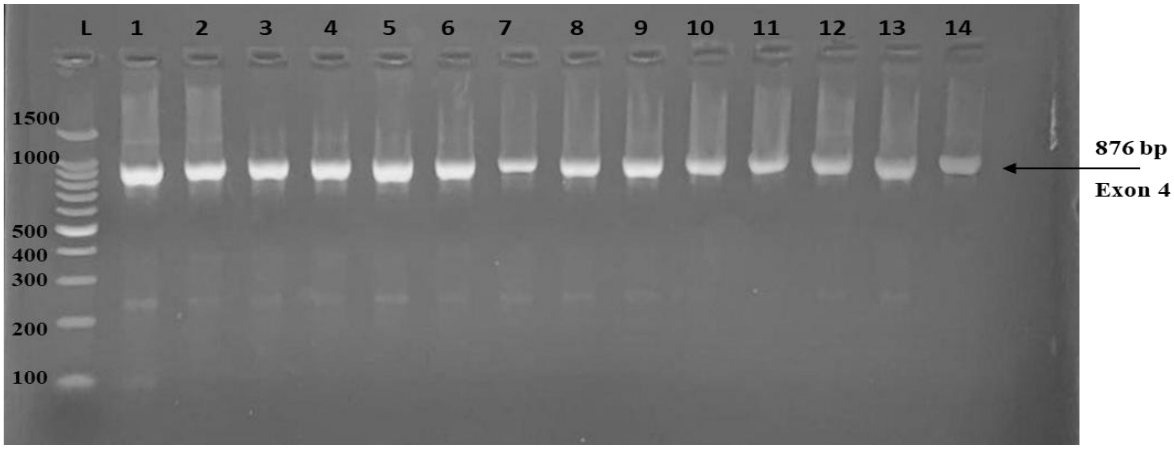

Figure 3. PCR product gel electrophoreses at 2% Agarose using 100 bp DNA ladder in TBP for 40 minutes at 80 Volt for patient's exon 4, band size 879bp.

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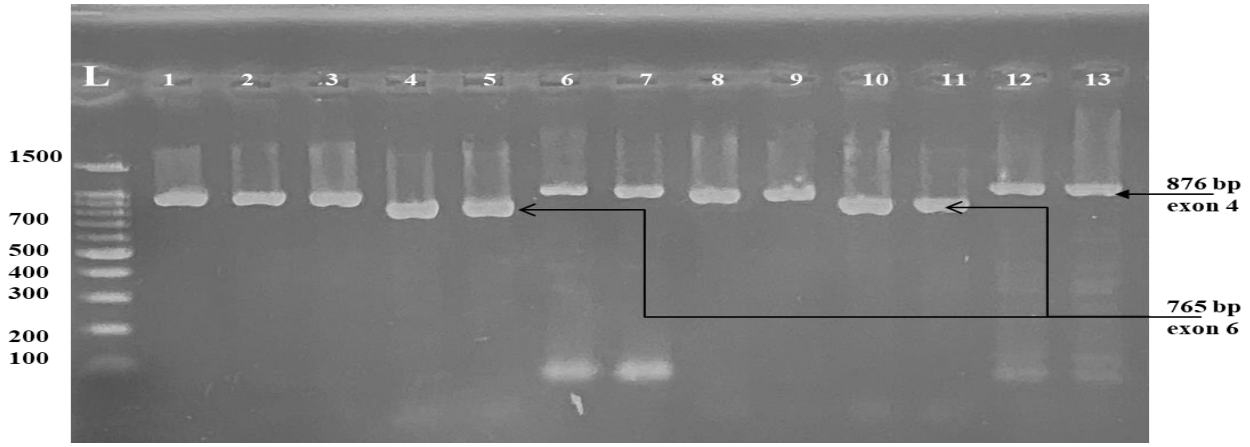

Figure 4. PCR product gel electrophoreses at 2% Agarose using 100 bp DNA ladder in TBP for 40 minutes at 80 Volt for patient's exon 4 and 6, bands sizes 876bp and 765 bp respectively.

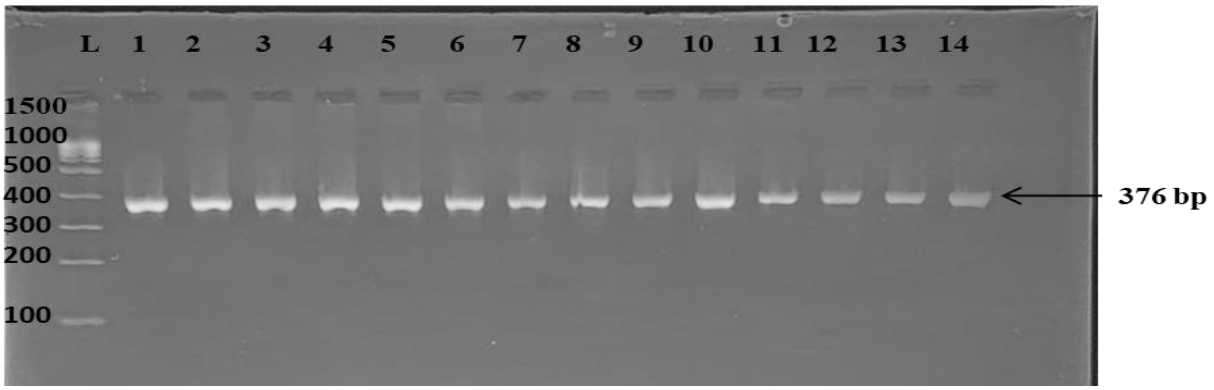

Figure 5. PCR product gel electrophoreses at 2% Agarose using 100 bp DNA ladder in TBP for 40 minutes at 80 Volt for patient's exon 9, band size 376 bp.

The rs2768659 (A>G) intronic single nucleotide variant with benign effect, is located upstream of the exon4 displays the genotype and allele frequency of both control and patient groups. The study found three genotypes (AA, AG, and GG) in both groups, with varying frequencies. The distribution of genotypes in both the control and patient groups was consistent with Hardy-Weinberg's law at a significance level of 0.05, indicating that the population was in genetic equilibrium. The Chi-Square test showed no significant differences in the number of genotype distribution and allele frequency between the control and patient groups for the AA and AG genotypes. However, the dominant AA genotype accounted for almost half of the genotype in the controls (40%) while in the patients' group it was (28%) therefore, the Chi-Square value 0.154 was non-significant. The GG genotype was significantly higher in the patient group compared to the control group, with a Chi-Square value of 7.758 and a p-value of 0.0053. The odds ratio (OR) for the GG genotype was 1.47 (0.88-2.08), indicating a moderate association with kidney stone formation. The allele frequency of the A allele was higher in the control group (0.58) compared to the patient group (0.42), while the G allele frequency was higher in the patient group (0.58) compared to the control group (0.42). This suggests that the G allele may be associated with an increased risk of kidney stone formation in this population as shown in Table 4. This study may provide evidence for an association between this variant and kidney stone formation.

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Table 4. Genotype and allele frequency of *GRHPR* gene/SNP (rs2768659) in control and patients' groups

Genotype	Control No. (%)	Patients No. (%)	Chi-Square ($\chi 2$)	P-value	O.R. (C.I.)
AA	12 (40%)	14 (28%)	0.154 NS	0.694	Ref. =1
AG	11 (37%)	14 (28%)	0.360 NS	0.548	0.507 (0.22-1.36)
GG	7 (23%)	22 (44%)	7.758 **	0.0053	1.47 (0.88-2.08)
Total	30	50	--		
P-value	0.492 NS	0.273	--		
Allele	**Frequency**				
A	35 (0.58)	42 (0.42)	--		
G	25 (0.42)	58 (0.58)	--		

*** Significant at (P≤0.05), ** significant at (P≤0.01), NS: Non-Significant at (P≤0.05).**

The rs1294628807 is a single nucleotide variant, its located upstream exon 4 was identified in the patients and in the controls. The wild type AA was not found in any group the results were non-significant between the two groups, the results of the hetero type AG was highly significant (10.89), it is considered as the causative type, finally the mutant type GG was non- significant (0.580), as shown in Table 5. The Chi-Square test showed a significant difference in the number of genotype distribution and allele frequency between the control and patient groups for the AG and GG genotypes. The AG genotype was significantly higher in the patient group compared to the control group, with a Chi-Square value of 10.89 and a p-value of 0.001. The odds ratio (OR) for the AG genotype was 1.74 (0.86-3.16), indicating a moderate association with kidney stone formation. In contrast, the GG genotype was significantly higher in the control group compared to the patient group, with a Chi-Square value of 0.580 and a non-significant p-value of 0.446. The OR for the GG genotype was 0.492 (0.21-0.89), indicating a protective effect against kidney stone formation in this population. The allele frequency of the A allele was higher in the patient group (0.16) compared to the control group (0.03), while the G allele frequency was higher in the control group (0.97) compared to the patient group (0.84). This suggests that the A allele may be associated with an increased risk of kidney stone formation, while the G allele may be protective against kidney stone formation in this population.

Table 5. Genotype and allele frequency of *GRHPR* gene/SNP (rs1294628807) in control and patients' groups

Genotype	Control No. (%)	Patients No. (%)	Chi-Square ($\chi 2$)	P-value	O.R. (C.I.)
AA	0 (0%)	0 (0%)	0.00 NS	1.00	Ref. =1
AG	2 (7%)	16 (32%)	10.89 **	0.001	1.74 (086-3.16)
GG	28 (93%)	34 (68%)	0.580 NS	0.446	0.492 (0.21-0.89)
Total	30	50	--		
P-value	0.0001 **	0.0001 **	--		
Allele	**Frequency**				
A	2 (0.03)	16 (0.16)	--		
G	58 (0.97)	84 (0.84)	--		

*** Significant at (P≤0.05), ** significant at (P≤0.01), NS: Non-Significant at (P≤0.05).**

The c.288-11 C>T (rs2736664) variant was found at exon 4 of the patients and controls. The study found three genotypes (TT, TC, and CC) in both groups, with varying frequencies. None of the individuals in

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the control or patient groups had the TT genotype. The Chi-Square test showed no significant difference in the number of genotype distribution and allele frequency between the control and patient groups for the TC genotype. The OR for the TC genotype was 0.071 (0.03-26), indicating no association with kidney stone formation. However, the CC genotype was significantly higher in the patient group compared to the control group, with a Chi-Square value of 5.261 and a p-value of 0.0218. The OR for the CC genotype was 1.29 (0.78-1.84), indicating a moderate association with kidney stone formation. The allele frequency of the T allele was similar between the control group (0.03) and the patient group (0.02), while the C allele frequency was higher in the patient group (0.98) compared to the control group (0.97). This suggests that the C allele may be associated with an increased risk of kidney stone formation in this population as shown in Table 6.

Table 6. Genotype and allele frequency of *GRHPR* gene/SNP (rs2736664) in control and patients' groups

Genotype	Control No. (%)	Patients No. (%)	Chi-Square ($\chi 2$)	P-value	O.R. (C.I.)
TT	0 (%)	0 (%)	0.00 NS	1.00	Ref. =1
TC	2 (7%)	2 (4%)	0.00 NS	1.00	0.071 (0.03-26)
CC	28 (93%)	48 (96%)	5.261 *	0.0218	1.29 (0.78-1.84)
Total	30	50	--		
P-value	0.0001 **	0.0001 **	--		
Allele	**Frequency**				
T	2 (0.03)	2 (0.02)	--		
C	58 (0.97)	98 (0.98)	--		

*** Significant at (P≤0.05), ** significant at (P≤0.01), NS: Non-Significant at (P≤0.05).**

The results of genotype and allele frequency of the c.295C>T(Arg99ter) rs119490108 nonsense transition in exon 4 in both groups indicated that there was no significant difference in the genotype distribution of this variant between patients with kidney stones and control individuals. All individuals in both groups had the TT genotype, and no individuals had the CC or CT genotypes. The allele frequency of the T allele was 1 in both groups, indicating that the T allele is the dominant allele for the studied population, and the C allele was not detected in either group as shown in Table 7, and this study may provide some evidence that this variant may not play a significant role in kidney stone formation in the Iraqi population. This variant was found in a PH2 patient by Garrelfs et al[42]. Moreover, the result did not agree with the findings of Konkoľová, et al [35]and Takayama, et al that found it was common in Caucasian individuals of American and European origen[18], because the patients in this study initially did not have PH2, so, it most likely they did not have the pathogenic mutations. The single nucleotide variants in exon 4 are shown in the fig. 6.

Table 7. Genotype and allele frequency of *GRHPR* c.295C>T(Arg99ter) rs119490108 in control and patients' groups

Genotype	Control No. (%)	Patients No. (%)	Chi-Square ($\chi 2$)	P-value	O.R. (C.I.)
TT	30 (100%)	50 (100%)	5.00 *	0.0253	Ref. =1
CT	0(%)	0 (%)	0.00 NS	1.00	---
CC	0 (%)	0(%)	0.00 NS	1.00	---
Total	30	50	--		
P-value	0.0001 **	0.0001 **	--		
Allele	**Frequency**				
T	**60 (1)**	**100 (1)**	--		
C	**0 (0.00)**	**0 (0.00)**	--		

*** Significant at (P≤0.05), ** significant at (P≤0.01), NS: Non-Significant at (P≤0.05).**

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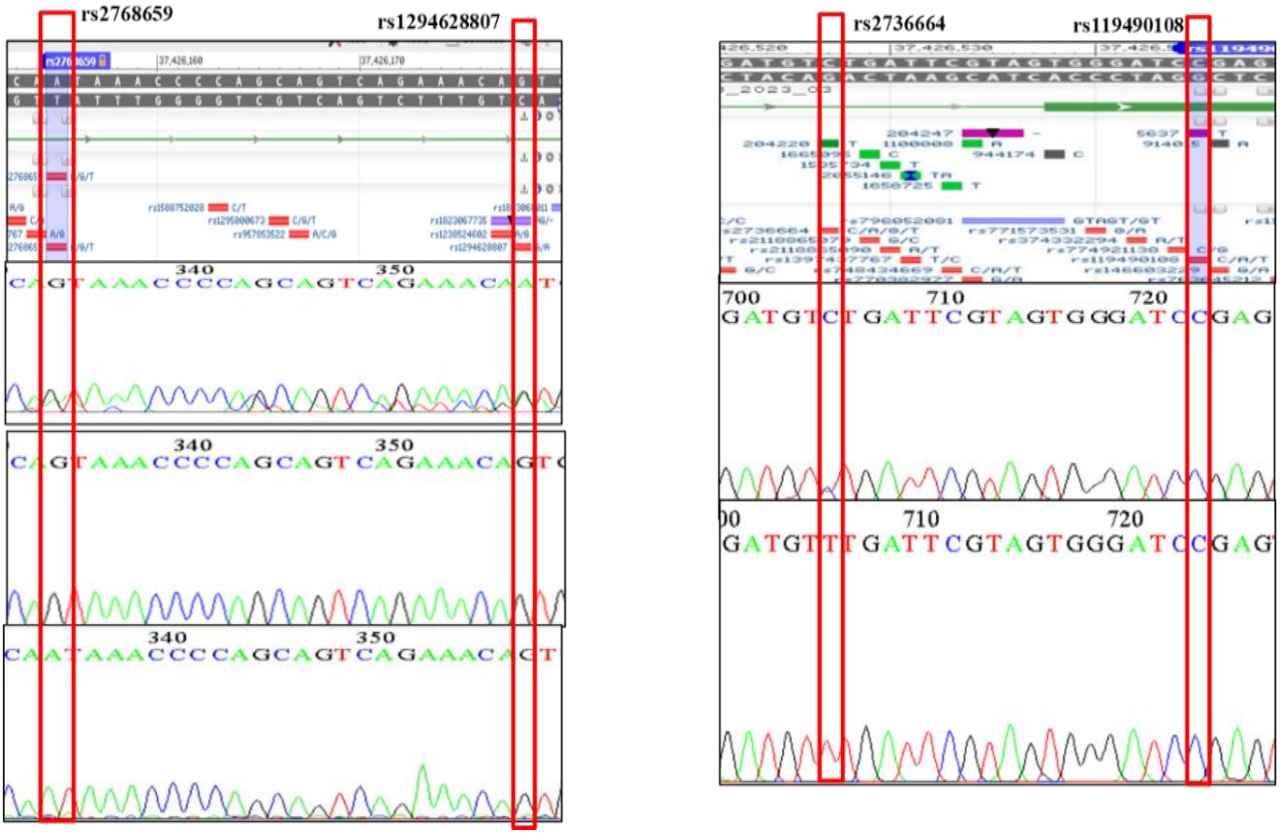

Figure 6. The chromatograms for the nucleotide sequences of the GRHPR exon 4 showed the reference sequences of the rs2768659, rs1294628807, rs2736664, and rs119490108 respectively. The chromatograms also demonstrated the transitions from A to G in rs2768659, G to A in rs1294628807, C to T in rs2736664, and the dominant allele C in rs119490108 respectively.

The c.579A>G (p. Ala193=) rs309458 is located within exon 6[53], it's a benign single nucleotide variant. This variant was identified in both groups. The genotype frequencies in the control group were AA=0%, AG=7%, and GG=93%, while in the patient group, the genotype frequencies were AA=0%, AG=22%, and GG=78%. The allele frequencies of A and G were 0.03 and 0.97 in the control group, respectively, and 0.11 and 0.89 in the patient group, respectively. The chi-square test was used to evaluate the significance of the differences in genotype frequencies between the two groups. The results showed a statistically significant difference between the control and patient groups in the distribution of genotypes for this SNP ($\chi 2$=6.231, p=0.0126). The odds ratio (OR) for the AG genotype compared to the AA genotype was 0.98 (95% CI: 0.43-2.18), indicating that there is no significant association with the disease. On the other hand, the OR for the GG genotype compared to the AA genotype was 0.335 (95% CI: 0.19-0.77), which suggests a protective effect against the disease. The results indicate that the GG genotype of this SNP in the gene is associated with a lower risk of the disease, while the AG genotype does not appear to be associated with the disease as shown in Table 8. Cregeen, et al. found that, the c.579A>G (A193A) rs309458 had no effect on the GRHPR enzyme activity as it does not form a splicing site in 68% of total alleles in non PH2 patients[58].

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Table 8. Genotype and allele frequency of *GRHPR* c.579A>G (p. Ala193=) rs309458 in control and patients' groups.

Genotype	Control No. (%)	Patients No. (%)	Chi-Square (χ2)	P-value	O.R. (C.I.)
AA	0 (%)	0 (%)	0.00 NS	1.00	Ref. =1
AG	2 (7%)	11 (22%)	6.231 *	0.0126	0.98 (0.43-2.18)
GG	28(93%)	39 (78%)	1.806 NS	0.179	0.335 (0.19-0.77)
Total	30	50	--		
P-value	0.0001 **	0.0001 **	--		
Allele	**Frequency**				
A	2 (0.03)	11 (0.11)	--		
G	58 (0.97)	89 (0.89)	--		

***significant at (P≤0.05), **significant at (P≤0.01), NS: Non-Significant at(P≤0.05).**

he c.494-68A>G rs309459 intronic variant, is located upstream exon 6 (ncbi.nlm.nih.gov/SNP/). This benign mutation was identified in the patients and in controls, the genotype and allele frequencies showed three genotypes (AA, AG, and GG) in both groups, with varying frequencies. None of the individuals in the control or patient groups had the AA genotype. The Chi-Square test showed no significant difference in the number of genotype distribution and allele frequency between the control and patient groups for the GG genotype. The OR for the GG genotype was 0.521 (0.23-1.09), indicating no association with kidney stone formation. However, the AG genotype was significantly higher in the patient group compared to the control group, with a Chi-Square value of 4.571 and a p-value of 0.0325. The OR for the AG genotype was 1.06 (0.72-1.61), indicating a slight association with kidney stone formation, although the effect size is small. The allele frequency of the A allele was higher in the patient group (0.11) compared to the control group (0.05), while the G allele frequency was higher in the control group (0.95) compared to the patient group (0.89). This suggests that the A allele may be associated with an increased risk of kidney stone formation in this population as shown in Table 9. Cregeen, et al. found that, the c.494-68A>G rs309459 had an effect on the GR activity but less on the HPR enzyme activity [58].

Table 9. Genotype and allele frequency of *GRHPR* c.494-68A>G in control and patients' groups.

Genotype	Control No. (%)	Patients No. (%)	Chi-Square (χ2)	P-value	O.R. (C.I.)
AA	0 (%)	0 (%)	0.00 NS	1.00	Ref. =1
AG	3 (10%)	11(22%)	4.571 *	0.0325	1.06 (0.72-1.61)
GG	27 (90%)	39 (78%)	2.182 NS	0.139	0.521 (0.23-1.09)
Total	30	50	--		
P-value	0.0001 **	0.0001 **	--		
Allele	**Frequency**				
A	3 (0.05)	11 (0.11)	--		
G	57 (0.95)	89 (0.89)	--		

***significant at(P≤0.05), **significant at (P≤0.01), NS: Non-Significant.**

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The results of the genotype and allele frequency of the c.494G>A (p. Gly165Asp) rs180177314 (G>A) variant in exon 6 of the *GRHPR* gene in both control and patient groups indicated that there was no significant difference in the genotype distribution of this variant between patients with kidney stones and control individuals. All individuals in both groups had the AA genotype, and no individuals had the AG or GG genotypes. The allele frequency of the A allele was 1 in both groups, indicating that A allele is the major allele for this variant in the population, and the G allele was not detected in either group as shown in Table 10.

Table 10. Genotype and allele frequency of *GRHPR* c.494G>A (p. Gly165Asp) rs180177314 in control and patients' groups

Genotype	Control	Patients	Chi-Square ($\chi 2$)	P-value	O.R. (C.I.)
	No. (%)	No. (%)			
AA	30 (100%)	50 (100%)	5.00 *	0.0253	Ref. =1
AG	0 (%)	0 (%)	0.00 NS	1.00	---
GG	0(%)	0 (%)	0.00 NS	1.00	---
Total	30	50	--		
P-value	0.0001 **	0.0001 **	--		
Allele	**Frequency**				
A	60 (1)	100 (1)	--		
G	0 (0.00)	0 (0.00)	--		

*** Significant at (P≤0.05), ** significant at (P≤0.01), NS: Non-Significant at (P≤0.05).**

In this study it had been found that three single nucleotide intronic variants at rs 309458 and rs309459 as well as the pathogenic variant at c.494G>A (p. Gly165Asp) rs180177314 which is restricted in the GG genotype in both of the patients and the controls, Fig. 7 shows the sequences of the nucleotides of all GRHPR SNPs that were found at exon 6.

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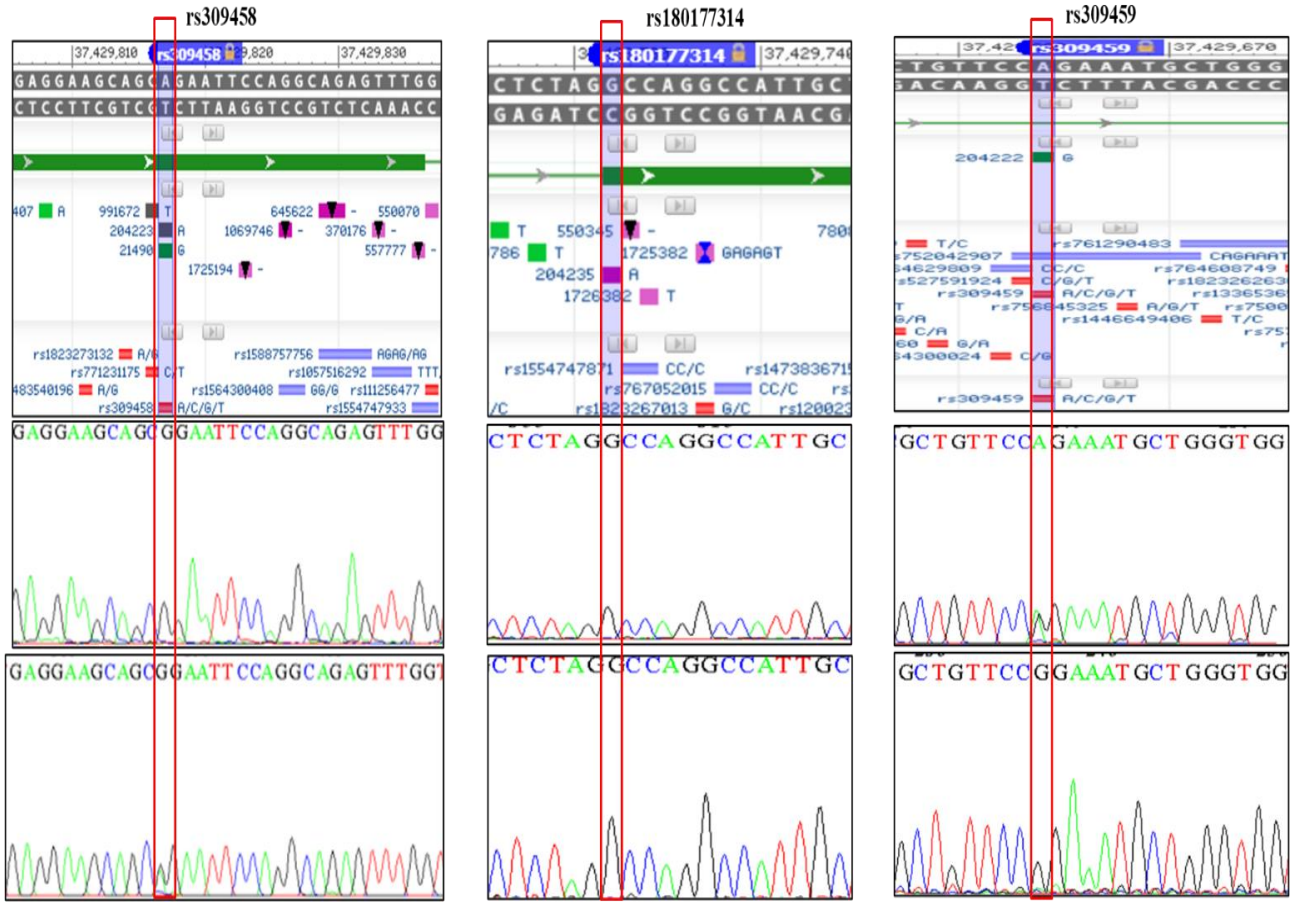

Figure 7. The chromatograms of nucleotide sequences of *GRHP* exon 6, showing the reference sequences of c.579A>G (p. Ala193=) rs309458, rs180177314 and c.494-68A>G rs309459 intronic variant respectively. Also, chromatograms show dominant allele A transition to G at the c.579A>G (p. Ala193=) rs309458, the dominant allele G of rs180177314 and the transition from A to G the transition from A to G in the c.494-68A>G rs309459.

The nonpathogenic c.*146A>G rs1057507, intron variant was identified in both groups, the results of genotype frequencies in the control group were AA=87%, AG=13%, and GG=0%, while in the patient group, the genotype frequencies were AA=90%, AG=10%, and GG=0%. The allele frequencies of A and G were 0.975 and 0.025 in the control group, respectively, and 0.95 and 0.05 in the patient group, respectively. The chi-square test was used to evaluate the significance of the differences in genotype frequencies between the two groups. The results showed a significant difference between the control and patient groups in the distribution of genotypes for this SNP ($\chi 2=5.084$, $p=0.0241$). The odds ratio (OR) for the AG genotype compared to the AA genotype was 0.317 (95% CI: 0.15-0.80), indicating a significant protective effect against the disease. The results suggest that the AG genotype of this SNP is associated with a lower risk of the disease. The absence of individuals with the GG genotype in both control and patient groups suggests that this genotype may be rare in the population studied or may be associated with severe phenotypes that are not included in the study as shown in Table 11. This variant was found to be dominant in several other populations like European, African American, African and others (ncbi.nlm.nih.gov/SNP/). The nucleotide sequence for the SNPs that had been found at exon 9 is shown in Fig. 8.

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Table 11. Genotype and allele frequency of *GRHPR* c.*146A>G (rs1057507) in control and patients' groups

Genotype	Control No. (%)	Patients No. (%)	Chi-Square ($\chi 2$)	P-value	O.R. (C.I.)
AA	26(87%)	45 (90%)	5.084 *	0.0241	---
AG	4 (13%)	5 (10%)	0.111 NS	0.739	0.317 (0.15-0.80)
GG	0 (%)	0 (%)	0.00 NS	1.00	---
Total	30	50	--		
P-value	0.0001 **	0.0001 **	--		
Allele	**Frequency**				
A	56 (0.05)	95 (0.95)	--		
G	4 (0.95)	5 (0.05)	--		

*** Significant at (P≤0.05), ** significant at (P≤0.01), NS: Non-Significant at (P≤0.05).**

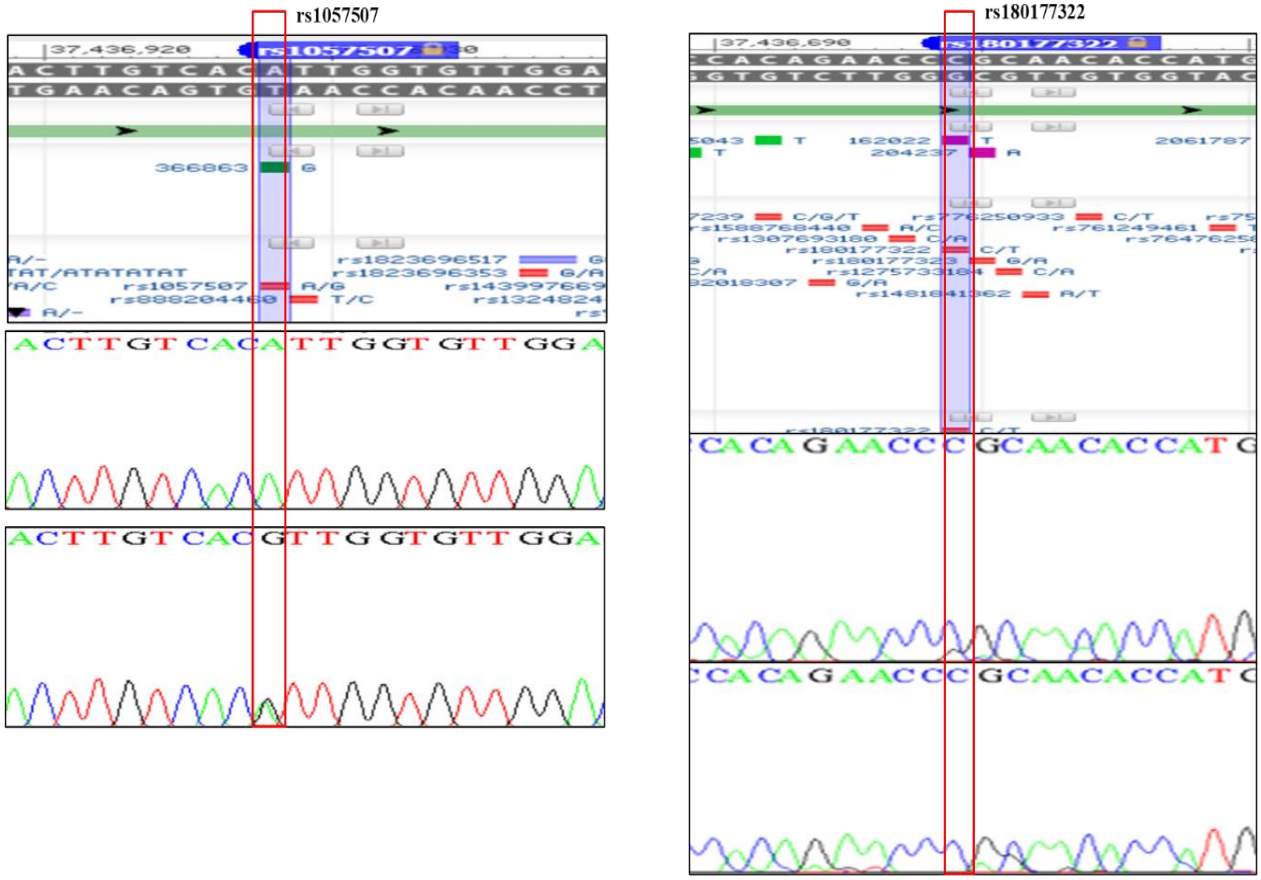

Figure 8. The chromatogram of nucleotide sequence of *GRHPR* exon 9. The chromatogram shows the intronic variant c.*146A>G rs1057507 nucleotide sequence and the transition from A to G. and the pathogenic variant at c.904C>T (p. Arg302Cys rs180177322, only the C dominant allele is appeared in both patients and controls.

The results of genotype and allele frequency of *GRHPR* c.904C>T (p. Arg302Cys) rs180177322 in exon 9 in the control and patient groups indicated that there were no significant differences in the genotype or allele frequency of this pathogenic variant between the control and patient groups. Both groups had a 100% frequency of the dominant CC genotype and a 0% frequency of the CT and TT

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genotypes as shown in Table 12. This suggests that this particular SNP may not be associated with an increased risk of kidney stone formation in the studied population.

Table 12. Genotype and allele frequency of *GRHPR* c.904C>T (p. Arg302Cys) rs180177322 in control and patients' groups.

Genotype	Control No. (%)	Patients No. (%)	Chi-Square ($\chi 2$)	P-value	O.R. (C.I.)
CC	30 (100%)	50 (100%)	5.00 *	0.0253	Ref. =1
CT	0 (%)	0 (%)	0.00 NS	1.00	---
TT	0 (%)	0 (%)	0.00 NS	1.00	---
Total	30	50	--		
P-value	0.0001 **	0.0001 **	--		
Allele	**Frequency**				
C	60 (1)	100 (1)	--		
T	0 (0.00)	0 (0.00)	--		

*** Significant at (P≤0.05), ** significant at (P≤0.01), NS: Non-Significant at (P≤0.05)**

The pathogenic missense mutation at c.165 G>A (Gly165Asp) rs180177314, the c.295C>T (Arg99ter) rs119490108, and the c.904C>T p. (Arg302Cys) rs180177322 had been associated with reduced GRHPR enzyme activity and an increased risk of PH2, which lead to the formation of kidney stones[37,58]. These pathogenic mutations are rare in the studied population and they were not detected due to the presence of the wild genotype and the absence of the heterozygous genotypes, the patients did not have PH2 because of the protective alleles are the dominant alleles while the causative alleles were not shown in the studied population.

Table 13 elucidates the association of the GRHPR single nucleotide variants with enzyme concentration in control and patient. The results showed that all SNPs were significantly associated with enzyme concentration in both control and patient at ($p<0.0001$). In the control group, the mean enzyme concentrations for each genotype were significantly different for all SNPs. The highest mean enzyme concentrations were observed for the homozygous wild-type genotypes (AA, GG, TT and CC) while the lowest mean concentration was found in the heterozygous genotypes (AG, CT).

In the patient group, the mean enzyme concentrations for each genotype were also significantly different for all SNPs. However, the pattern of association was different compared to the control group, and the lowest mean enzyme concentrations were consistently observed in the homozygous mutant genotypes, while the highest mean concentrations were found in the heterozygous genotypes. These results suggest that the SNPs are associated with changes in enzyme concentration in both control and patient populations, and this association is likely to be mediated by the effect of these genetic variants on the activity or expression of the enzyme[59].

The reasons that may have led to these results are the existence of mutations in another place that was not studied in this study [56]. On the other hand, the presence of microRNA (miR-138-5p) could attack *GRHPR* mRNA and inhibits its expression[45]. It may be led to DNA methylation of the CpG sequences in the promoter region or other CpG islands, which may affect the *GRHPR* gene expression[40]. However, it is important to note that the study has some limitations, such as the small sample size and the lack of information on other potential confounding factors that may influence enzyme concentration. Therefore, further studies with larger sample sizes and more comprehensive analyses are necessary to confirm these findings and elucidate the underlying mechanisms of these associations. However, it is important to note that the study has some limitations, such as the relatively small sample size, and further studies with larger sample sizes are needed to confirm these findings.

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Table 13. The association analysis of SNPs with GRHPR enzyme concentration.

SNP	Variable	Genotype	N	Mean± SE	P-value
rs2768659	Control	AA	12	7.96±2.32[a]	0.0001
		AG	7	2.25±0.42[b]	
		GG	11	3.31±1.12[b]	
	Patients	AA	14	0.38±0.01[b]	
		AG	14	0.42±0.04[b]	
		GG	22	0.43±0.02[b]	
rs1294628807	Control	GG	28	4.83±1.14[a]	0.0001
		GA	2	3.33±2.39[ab]	
	Patients	GG	34	0.42±0.02[b]	
		GA	16	0.39±0.03[b]	
rs2736664	Control	TT	28	5.02±1.13[a]	0.0001
		CT	2	1.56±0.56[ab]	
	Patients	TT	48	0.41±0.02[b]	
		CT	2	0.41±0.03[b]	
rs119490108	Control	CC	30	4.78±1.07[a]	0.0001
	Patients	CC	50	0.41±0.02[b]	
c.494G>A	Control	GG	30	4.78±1.07[a]	0.0001
(p. Gly165Asp)	Patients	GG	50	0.41±0.03[b]	
rs180177314					
c.494-68A>G	Control	GG	27	4.99±1.17[a]	0.0001
rs309459		AG	3	2.93±1.44[ab]	
intronic variant	Patients	GG	39	0.41±0.02[b]	
		AG	11	0.43±0.05[b]	
c.579A>G	Control	GG	28	5.02±1.13[a]	0.0001
(p. Ala193=)		AG	2	1.54±0.59[ab]	
rs309458	Patients	GG	39	0.41±0.02[b]	
		AG	11	0.43±0.05[b]	
c.904C>T	Control	CC	30	4.78±1.07[a]	0.0001
(p. Arg302Cys)	Patients	CC	50	0.41±0.02[b]	
rs180177322					
rs1057507	Control	AA	26	5.17±1.21[a]	0.0001
		AG	4	2.27±0.83[ab]	
	Patients	AA	45	0.41±0.02[b]	
		AG	5	0.39±0.02[b]	

***significant at (P≤0.05), ** significant (P≤0.01), NS: Non-Significant**

Conclusion

This study found that GRHPR enzyme concentration was lower in the sera of patients than in the sera of the control group. The pathogenic mutations at c.295C>T, c.165G>A and c.904C>T (p. Arg302Cys) rs180177322 were not found in the kidney stone patients in this study and the alleles were in the dominant not mutant type, therefore they did not have PH2. The wild genotypes were dominant in the studied groups and the SNP variants and the benign intron variants collectively may lead to reduce enzyme gene expression and eventually lead to stone formation.

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Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Authors sign on ethical consideration's approval.
- Ethical Clearance: The project was approved by the local ethical committee at University of Baghdad.

Authors' Contribution Statement

This study was performed by the cooperation between the authors. The first author D. A. M. Al. contribution: collect the samples, perform the biochemical tests, genetic investigation, the statistics and writ the original draft. The second author L. H. A. Al. contribution: the idea of the research, study design, molecular genetic analysis of the results, writing, reviewing and editing the manuscript.

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تغايرات مورث *GRHPR* في المرضى العراقيين المصابين بحصى أكسالات الكالسيوم في الكلى

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الخلاصة

حصى الكلى هو مرض شديد الخطورة له تهديد خطير لكل من الصحة والاقتصاد العالميين. توجد عدة أنواع مختلفة من الحصى، ولكن حصوات أكسالات الكالسيوم هي الأكثر انتشارًا في العراق. لم يتم دراسة التغيرات في تركيزات اختزال الجليوكسيلات واختزال هيدروكسي البيروفات في المصل والتغيرات الجينية المرتبطة بحصوات أكسالات الكالسيوم في المرضى العراقيين سابقًا لذا تهدف هذه الدراسة إلى التركيز على هذه النقاط. اشتملت هذه الدراسة على 80 شخص, كانو 50 مريضاً مصابا بحصى أكسالات الكالسيوم مقارنة بـ 30 لمجموعة السيطرة. تم اجراء بعض الاختبارات البيوكيميائية لوظائف الكلى (الكرياتينين واليوريا وحمض البوليك) في مصل كلا المجموعتين، فضلا عن تحاليل تعداد الدم الكامل، وسكر الدم العشوائي، واختبارات فصائل الدم. كما تم جمع البول من أجل الفحص العام للبول، لتأكيد رؤية بلورات الأكسالات في بول المريض. بالإضافة إلى قياس تركيز إنزيم اختزال الجليوكسيلات واختزال هيدروكسي البيروفات في المصل كلا المجموعتين بطريقة الايلايزا. و تم استخلاص الدنا من الدم الكامل وتم التحري عن الطفرات الممرضة c.295C>T (rs119490108) وc.165G>A (rs180177314) و rs180177322في مورث GRHPRعن طريق تضخيم الحمض النووي المستهدف عن طريق تفاعل البلمرة المتسلسل وتحري التعاقبات المباشر للمنتجات ثم تم تحليل النتائج. وجدت هذه الدراسة أن تركيز الإنزيم في مجموعة السيطرة (4.78 ± 1.06 مجم / ديسيلتر) كان أعلى بكثير من تركيزه في مجموعة المرضى (0.411 ± 0.02 مجم / ديسيلتر). لم تظهر الطفرات الممرضة في كلا المجموعتين، ولكن وجد بان عدة مواقع ضمن القطع المدروسة كانت ذات تعدد بالأشكال: في اكسون 4: rs2768659 (A>G) و rs1294628807 (G>A)و rs2736664 (C>A) اكسون 6: rs309459 (A>G) وc.494-68A>G rs309458 (A>G) واكسون 9 : . rs1057507 (A>G) و كأستنتاج وجد بان تكوين حصى اوكزالات الكالسيوم ارتبط بانخفاض انزيم اختزال الجليوكسيلات واختزال هيدروكسي البيروفات في المرضى مقارنة بمجموعة السيطرة والتي ربما حدثت بسبب الطفرات او الوراثة الفوقية الكابحة للتعبير الجيني.

الكلمات المفتاحية: الايلايزا ، تركيز انزيم GRHPR ، تغايرات مورث GRHPR ، حصى الكلى، تفاعل البلمرة التسلسلي.