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Association of Calcium Sensing Receptor Gene Polymorphism(rs3804594) With Parathyroid hormone levels in Patients With Urinary Calcium Oxalate Stones:A Cross-sectional Study

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Abstract
Background: Calcium oxalate nephrolithiasis may be considered as a complex disease having multiple pathogenetic mechanisms. Calcium homeostasis is crucially regulated by the calcium sensing receptor(CaSR) expressed mainly in PTH glands , which reduces passive and active calcium reabsorption in distal tubules, and hence a candidate gene for calcium nephrolithiasis. Objective of the study was to determine the association of CaSR gene polymorphism(rs3804594) with calcium oxalate stone formation and also to assess the association between CaSR gene polymorphism and serum levels of PTH in patients with urinary oxalate stones.
Methodology: Two Hundred subjects were recruited in the cross-sectional study,out of which 100 were patients with calcium oxalate stones & 100 were healthy controls. Analysis of the Single Nucleotide Polymorphism of CaSR was performed with Polymerase Chain Reaction-Restriction Fragment Length Polymorphism(PCR–RFLP).Chisquare test was applied for the analysis of associations using Graph pad prism.
Results: The genotype frequencies of the cases and controls were in accordance with the Hardy–Weinberg equilibrium with no significant difference in the expected and observed allele frequencies (chi square 4.73, p=0.73). There was no significant association between the CaSR gene polymorphisms and renal stone disease (chi square 0.32,p=0.57) with Odd's Ratio=1.174. Frequencies of CC (wild) and CT+TT (mutant) genotypes were 47% and 42% in normal serum PTH group, 4% and 7% in patients with elevated serum PTH group. No significant association was observed between the gene polymorphisms and serum PTH levels. Haplotype analysis shows that C allele is the risk allele that seems to have a dominant genetic effect over the T allele.
Conclusion: It could be concluded from the study there was no significant association between CaSR gene polymorphism with oxalate stone disease. CaSR gene polymorphism was insignificantly associated with the serum levels of PTH in patients with urinary oxalate stones. However the polymorphism of the calcium-sensing receptor (CaSR) can impact kidney stone formation by altering the receptor's sensitivity to calcium, potentially leading to increased urinary calcium levels and an increased risk of stone development.

Keywords- Calcium sensing receptor, gene polymorphism, renal stone, haplotypes

I. INTRODUCTION

The incidence of urinary stone disease varies by continent and climate (5-20% in Asia, 5-9% in Europe, and 7-13% in North America).It is suggested that stone development is influenced by dietary practices, employment, metabolic diseases, abnormalities of the urinary tract, climate, and genetic factors[1].

The human calcium sensing receptor gene (CASR) is located on chromosome 3q 13.3-21.A Cellular calcium homeostasis is crucially regulated by the CASR, which is found in the plasma membrane of renal tubule cells and it is involved in the pathogenesis of diabetes mellitus (DM) due to its expression in pancreatic β-cells[2].CaSR has been found to play an important role in the formation and growth of kidney stones[3]. Kidney stones are formed when substances such as calcium, oxalate, and uric acid accumulate in the urine and crystallize to form a stone[4]. The CaSR helps to regulate the amount of calcium that is excreted by the kidney, and if it is not functioning properly, it can lead to an imbalance in calcium levels and an increased risk of kidney stone formation[5].

An loss of function Heterozygous inactivating mutation of CASR causes familial hypocalciuric hypercalcemia (FHH), that is accompanied by absolute calciuria or hypocalciuria, according to a prior study, while neonatal severe hyperparathyroidism is the most common manifestation of

homozygosity[3,4]. A study by Liu et al. discovered that CaSR single nucleotide polymorphisms (SNPs) were associated to hypercalciuria[5]. A study by Guha et al indicate a change in the CaSR-CLDN14 signalling pathway, which most likely plays a substantial role in the onset of hypercalciuria and the development of KSD[6].

Many genes and their associated genetic variants have been found in many ethnic populations around the world, but no chromosomal mapping has yet been developed specifically for nephrolithiasis[7].Using gene panels or exome sequencing, a detrimental CaSR variation was not found in a global cohort of 697 NL families. It was suggested that the risk calcium-sensing receptor gene (CASR) genotype in rs380 could serve as markers to identify people at risk for Ca nephrolithiasis [8].As a result, finding new NL-associated alleles can help us comprehend the function of CaSR in calcium management and kidney stone disease.

There are multiple causative factors contributing to calcium oxalate stone formation including environmental and genetic factors. Role of calcium sensing receptor (CaSR) gene polymorphism has been least explored. Single nucleotide polymorphism of CaSR gene may alter PTH levels, in turn contributing to the pathogenesis of renal stones. There are only a few studies available in this area to the best of our knowledge.

II. OBJECTIVES

1. To determine the association of CaSR gene polymorphismwith urinary calcium oxalate stones formation.
2. To compare serum PTH levels in patients with and without urinary calcium oxalate stones.
3. To assess the association between CaSR gene polymorphism and serum levels of PTH in patients with urinary oxalate stones.

III. METHODOLOGY

The study cross-sectional study included hundred patients with calcium renal stones who met the diagnostic criteria for nephrolithiasis. Patients were recruited after obtaining the central ethics board, Nitte DU approval. Informed consent was also taken. Study included patients with calcium nephrolithiasis and patients with other stones were excluded.
Patients' demographic profile, other relevant data were recorded in the proforma.

DNA Extraction
Two ml of EDTA blood sample was collected from the subjects. Blood cells were treated with 7000 µl RBC Lysis buffer. Centrifuged at 3500rpm for 5 mins, decanted the supernatant. 2000µl of cell lysis buffer was added for 2-4hrs and 700µl of protein precipitation solution was added. Centrifuged for 10 mins at 3000rpm then 2 ml isopropanol was added to the clean new tube, decanted the supernatant to a new isopropanol tube , 50-60 times mixed to see thread like structure, leave DNA to precipitate. Decanted the supernatant and washed the pallets with 4ml of 70% eathnol and decanted the supernatant. Tubes were let to get dry for overnight. 50µl of TRIS EDTA Buffer was transferred to the tubes and stored in -20℃.

Genotyping of CASR Polymorphism
Genotyping was performed by PCR-RFLP method.
Forward and reverse oligonucleotide primers were designed to amplify the CASR polymorphism- 5'-CAAGGACCTCTGGACCTCCCTTTGC-3' and 5'-GACCAAGCCCTGCACAGTGCCCAAG-3'respectively using Primer 3Plus.

Amplification was performed in MiniAmp plus Thermal cycler (Thermofischer Scientific).The PCR Process was performed in 35 cycles under initial denaturation at 94°C for 5 minutes denaturation at 94°C for 30 secs,annealing at 68°C for 30 secs,extension at 72°C for 5 minutes. The PCR Product was checked by gel electrophoresis system (Gel DocTM EZ imager Biorad).

Using Gel Blotting setup(Mini-PROTEAN Tetra Cell,Bio-Rad,USA) ,containing 0.5 µg/ml ethidium bromide and DM012-R500 50 bp DNA Ladder Ready to use(Gene Direx,Inc.) in TAE Buffer(1X).

PCR products was digested with 0.5 µl BseRI(NEB) restriction endonucleases overnight at 37°C,and the digested products were separated by 3% agarose gel electrophoresis and visualized using ethidium bromide. Individuals with a genotype CC [dominant homozygous]showed bands of 320 bp, CT[heterozygote] had three bands of 320bp,260bp & 60bp, subjects with a genotype of TT [recessive homozygous] indicate two bands of 260bp & 60bp.

Biochemical Evaluation
Serum calcium, phosphorus, uric acid, creatinine, albumin, were evaluated by using semi automated chemistry analyzer, serum parathyroid hormone levels was assayed by ELISA.
Statistical Analysis

- Statistical analysis was done by using SPSS version23 software.
- $\chi 2$ test was used to find the association of genetic polymorphism and kidney stone disease.

- $\chi 2$ test was to find the association of gene polymorphism and metabolic parameters.
- Metabolic parameters was compared between cases & controls using Mann Whitney U test

IV. RESULTS:

Among the patients, 62 % were males and 38 % were females, indicating males having high frequency of occurrence of kidney stones than females.

Table 1: Association of CaSR gene polymorphism between cases and controls

	Wild Type(CC)	Mutant (CT+TT)	Chi square	P value	Odds ratio
CASE	49	51	0.3212	0.57	1.174
CONTROL	45	55			

There is no significant association between cases and cases (chi square 0.32,p=0.57) with Odd's Ratio=1.174(table 1). Frequencies of CC and CT+TT genotypes were 47% and 42% in normal serum PTH group,4% and 7% in high serum PTH group(table 2).

Table 2: Association of serum PTH level and CaSR gene polymorphism

Serum PTH level(pg/ml)	Wild Type (CC)	Mutant (CT+TT)	Chi Square,df	P value	OR(95% CI)
<63 pg/ml	47(47%)	42(42%)	$X^{2=}$1.06,df=1	0.30	1.958(0.5548 to 6.278)
>63 pg/ml	04(4%)	07(7%)			

The genotype frequencies of the cases and controls were in accordance with the Hardy–Weinberg equilibrium.Genotype of Hardy weinberg equilibrium in cases and controls didn't show significance difference between observed and expected value (chi square 4.73,p=0.73)(table 3).

Table 3:Hardy weinberg equilibrium in cases and controls

	Case		**Chi square**	**P value**	**Control**		**Chi square**	**P value**
	Observed	**Expected**			**Observed**	**Expected**		
CC	**49**	**53.29**			**45**	**49.13**		
CT	**48**	**39.42**	**4.7374**	**0.73**	**53**	**34.72**	**5.1066**	**0.73**
TT	**03**	**7.29**			**02**	**6.13**		

Table 4 shows the genotype frequencies of CASR polymorphism between cases and controls The genotypes or allele frequencies of the SNPs did not show significant difference between the cases and controls.But divided subjects into CC and CT+TT genotypes shows higher risk compare to the allele frequencies C & T.

Table 4. Genotype distribution and alleles frequencies of CasR

CasR (C>T) Genotype	Case (n=100)%	Control(n=100) %	OR(95% CI, p value)
CC CT TT	**49(49%)** **48(45%)** **3(3%)**	**45(45%)** **53(53%)** **2(2%)**	
CC CT+TT	**49(49%)** **51(51%)**	**45(45%)** **55(55%)**	**1.17(**0.6694 to 2.072) P=0.570
Alleles C T	**122(61%)** **30(15%)**	**116(58%)** **30(15%)**	**1.05(**0.6073 to 1.821) P=0.86

We compared clinical risk factors including serum uric acid,creatinine,calcium,PTH between cases and controls. Patients with kidney stones had lower uric acid and phosphorous compared with control p<0.0001**(Table 5)

Table 5:comparison of Biochemical parameter between cases and controls group

	Case (n=100)	Controls (n=100)	P value
Demographics			
Age(years)	46(37-55)	37(26-48)	0.0011**
Sex(%)			
Male	62(62%)	78(78%)	
Female	38(38%)	22(22%)	
Blood Biochemistry			
Uric acid(mg/dL)	4.23(2.15-5.77)	5.13(4.3-6.14)	<0.0001***
Creatinine(mg/dL)	1(0.83-1.27)	0.8(0.56-0.96)	<0.0001***
Phosphorous	5.35(4.7-6.2)	6.11(4.8-7.3)	0.0071**
Calcium	10(8.95-11.35)	9.97(8.83-12.39)	0.739

Albumin(g/dL)	4.31(2.76-4.9)	4.02(3.13-4.64)	0.226
PTH(pg/ml)	47.8(32-71.96)	40.6(27.8-69.4)	0.0575

Table 6 shows the comparison of biochemical parameters and Casr gene polymorphism in cases and .None of the clinical variables were significant at the nominal level of 0.05.

Table 6: comparison of clinical risk factors within cases and controls.

	Case		p value	Control		p value
	CC	CT+TT		CC	CT+TT	
Age(years)						
Uric acid(mg/dL)	2.7(1.76-5.16)	4.41(2.4-5.89)	0.13	5.17(4.65-6.14	5.04(3.96-6.04)	0.11
Creatinine(mg/dL)	1.05(0.84-1.41)	1(0.79-1.21)	0.23	0.71(0.51-0.95)	0.81(0.65-0.98)	0.10
Phosphorous	5.15(4.51-5.9)	5.6(4.8-6.3)	0.23	0.60(4.85-7.05)	6.27(4.9-7.89)	0.40
Calcium	10.1(8.6-12.39)	9.9(9-12.64)	0.55	9.66(8.94-11.3)	10.14(8.96-11.37)	0.43
Albumin(g/dL)	4.35(2.7-4.89)	4.26(2.62-4.91)	0.89	3.54(2.52-4.53)	4.2(3.32-4.68)	0.08
PTH(pg/ml)	45.6(29.2-69.2)	42.4(28.35-77.6)	0.62	40.6(26.2-76)	34(25.65-49.85)	0.37

Global result:
Haplotype analysis shows that C allele of the polymorphism rs3894594 is a risk allele that seems to have a dominant genetic effect over the T allele (table 7).

Table 7: Haplotype analysis of casr gene polymorphism in cases and controls.

Haplotype	Case(freq)	Control(freq)	Chi2	Fisher's p	Pearson's p	OR [95% CI]
C	143(0.737)	137(0.706)	0.461	0.571	0.496	1.166 [0.747~1.819]
T	51(0.262)	57(0.293)	0.461	0.571	0.496	0.857 [0.549~1.337]

Global Chi2 is 0.461, Fisher's p is 0.571, Pearson's p is 0.496.

V. DISCUSSION

PTH, calcitonin and vitamin D are the three most important Calcium regulating hormones [9]. CaSR and PTH have an important interplay in the regulation of calcium homeostasis. When serum calcium levels are low, PTH is secreted to increase calcium levels. PTH acts on the CaSR in the parathyroid gland to decrease its sensitivity to calcium, which leads to further secretion of PTH[10]. PTH also acts on the CaSR in the kidney to increase calcium reabsorption. In addition, PTH can activate the CaSR in bone, which can stimulate the release of calcium from the bone matrix[11].

Gene polymorphisms are variations in the DNA sequence of a particular gene that can lead to differences in the function or expression of the gene. In the case of the Calcium Sensing Receptor (CaSR), several studies have investigated the association between CaSR gene polymorphisms and kidney stone disease[12].

Previous study on CaSR gene polymorphism has reported the association of kidney stone disease with the A986S polymorphism.This single nucleotide variation leads to a change in the amino acid sequence of the CaSR protein[13]. Some studies have suggested that individuals who carry the A986S polymorphism may have a higher risk of developing kidney stones compared to those who do not carry the polymorphism[14].

However, other studies have not found a significant association between the A986S polymorphism and kidney stone disease.

Another CaSR gene polymorphism that has been studied in relation to kidney stone disease is the rs17251221 polymorphism[6]. This polymorphism is located in a regulatory region of the CaSR gene and has been shown to affect CaSR expression levels in vitro. Some studies have suggested that individuals who carry the rs17251221 polymorphism may have a higher risk of developing kidney stones, although the evidence is not consistent across all populations.

Furthermore, studies have suggested that changes in the expression and activity of CaSR in the kidney can lead to abnormal calcium deposition and crystal formation, which are key steps in the development of kidney stones[15]. In particular, downregulation of CaSR in the distal tubules of the kidney may impair the ability of the kidney to regulate calcium and magnesium reabsorption, leading to increased urinary calcium excretion and stone formation[16].

There were few evidence to show that one in regulatory region of the CASR polymorphisms may be associated with an increased risk for renal disease.So the study aimed to investigate the CaSR gene polymorphism and its association with kidney stone patients.

The present study showed no association of CaSR gene polymorphism with the kidney stones, but it has a slightly higher risk (odd's ratio 1.174). Study by Elezníková et al investigated the CaSR gene with respect to etiopathogenesis of non diabetic renal disease ,further showed that intron 4 gene polymorphism of CaSR gene is a risk factor for the occurrence of DM and CKD[2].

Calcium sensing receptor (CaSR) gene polymorphisms have been shown to be associated with alterations in parathyroid hormone (PTH) levels, in turn contributing to the pathogenesis of renal stones[17].

In Japanese hemodialysis patients, the progression of secondary hyperparathyroidism was due to the correlation of CaSR polymorphisms with parathyroid hormone (PTH) secretion[2].

One such polymorphism is the A986S variant, which has been associated with increased PTH secretion in response to decrease in extracellular calcium levels. Another variant, R990G, has been associated with decreased PTH secretion in response to increases in extracellular calcium levels[13].

Another study by Walker MD et al found that the same polymorphism was associated with lower levels of PTH[18].Another study by Carpenter TO,et al in 2012 found that individuals with the CC genotype (the homozygous variant) had significantly higher PTH levels compared to those with the TT genotype (the homozygous wild-type) or the CT genotype (heterozygous)[19]. Another study by Arenas MD et al. found a similar association, with individuals with the CC genotype having higher PTH levels and a greater risk of secondary hyperparathyroidism.our study is consistent with all the results showing that CC genotype have higher Serum PTH level compare to CT+TT[20].

However, not all studies have found a significant association between the rS3804594 polymorphism and PTH levels.

The CaSR in the kidneys is involved in sensing changes in blood calcium levels and adjusting the body's response accordingly. It influences the reabsorption of calcium and other minerals in the renal tubules, the tiny structures in the kidneys where urine is formed.

Genetic variations in the CaSR gene can lead to altered receptor function. Some polymorphisms may result in CaSR that is more sensitive to changes in blood calcium levels, while others may lead to reduced sensitivity. These variations can impact how the kidneys handle calcium reabsorption and excretion, and consequently, influence the risk of kidney stone formation.

Influence of Polymorphism on Kidney Stone Formation: Certain CaSR polymorphisms have been associated with an increased risk of kidney stone formation. For example, if a person carries a CaSR variant that leads to decreased receptor sensitivity to calcium, the kidneys might reabsorb more calcium from the urine back into the bloodstream. This could potentially result in higher levels of calcium in the urine, increasing the likelihood of calcium-based kidney stone formation.

Conversely, other CaSR variants with increased sensitivity to calcium may lead to increased excretion of calcium in the urine, which could also contribute to kidney stone formation, particularly when accompanied by other factors such as elevated levels of oxalate or phosphate.

It's important to note that kidney stone formation is a complex process influenced by multiple genetic, dietary, and environmental factors. CaSR polymorphisms are just one piece of the puzzle and may interact with other factors to determine an individual's risk of developing kidney stones.

In conclusion, the polymorphism of the calcium-sensing receptor can influence kidney stone formation by altering the receptor's sensitivity to calcium and affecting how the kidneys regulate calcium levels in the urine. However, the development of kidney stones is multifactorial, and additional research is needed to fully understand the intricate interactions between genetics, receptor function, and other contributing factors.

VI. CONCLUSION

We could conclude from the study that there is no significant association of CaSR gene polymorphism with calcium oxalate stones formation. It was also found that PTH levels may be one of the marker to identify the CaSR gene polymorphism in kidney stone patients.

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Conflicts of interest:None

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