Original Research Article

Genetic association of CaSR (Calcium Sensing Receptor) gene polymorphism with kidney stone formation in Vindhyan region of Madhya Pradesh

Vandana Tiwari[1], Ajay Kumar Pathak[2], Daya Shankar Parauha[3], Akanksha Verma[4], Rashmi Arnold[5], Arvind Kumar Tripathi[6*]

[1]Centre for Biotechnology Studies, APS University, Rewa, M.P, India
[2]Shyam Shah Medical College, Rewa, M.P, India
[3]Department of Medicine, Vindhya Superspeciality Hospital, Rewa, M.P, India
[4]Centre for Biotechnology Studies, APS University, Rewa, M.P, India
[5]Department of Botany, GDC College, Rewa, M.P, India
[6]Centre for Biotechnology Studies, APS University, Rewa, M.P, India

Received: 30-11-2021 / Revised: 21-12-2021 / Accepted: 01-01-2022

Abstract

Calcium (Ca2+) is an important mediator of multicellular homeostasis and is involved in several diseases. The interplay among the kidney, bone, intestine, and parathyroid gland in Ca2+ homeostasis is strictly modulated by numerous hormones and signaling pathways. The calcium sensing receptor (CaSR) is a G protein–coupled receptor, that is expressed in calcitropic tissues such as the parathyroid gland and the kidney, plays a pivotal role in Ca2+ regulation. CaSR is importantfor renal Ca2+, as a mutation in this receptor leads to hypercalciuria and calcium nephrolithiasis. Aberrant Ca2+ sensing by the kidney and VSMCs, owing to altered CaSR expression or function, is associated with the formation of nephrolithiasis and vascular calcification. Basedon emerging epidemiological evidence, patients with nephrolithiasis have a higher risk of vascular calcification, but the exact mechanism linking the two conditions is unclear. However, a dysregulationin Ca2+ homeostasis and dysfunction in CaSR might be the connection between the two. This reviews summarizes renal calcium handling and calcium signaling in the vascular system, with a special focus on the link between nephrolithiasis and vascular calcification. The age, sex, BMI, WHR were the parameters. As expected the nephrolithiatic patients had markedly higher levels of weight of men (P=0.3916) then women (P=0.0723) and BMI of Women (P=0.2247) and Men (P=0.3499) but both was not significantly different between patient and healthy population. Concentration of IL-18 level in malarial patient (case) and healthy population (control) is showing elevated level during infection and it was statistically significant associated. Overall distribution of *CaSR*Ala 986 Ser genotypes was significantly different in healthy control group as compared to disease group (χ^2=7.253, P=0.0266*). HC group showed an decrease of mutant 'TT' genotype as compared to Patients of Nephrolithiatic (*1.11% vs.* 4.37%). Similarly, wild type '*GG*' genotype was present in significantly high frequency in HC as compared to Nephrolithiatic patients group (72.22% *vs.* 54.37%). An odds ratio of 0.4584 in Nephrolithiatic group.

Keywords: BMI, CaSR gene, Ca^{2+}, VDR , Nephrolithiatic.

Introduction

Kidney stone is a solid crystal aggregation formed in the kidneys from dietary minerals in the urine. Kidney stones are often a painful experience associated with hematuria and if severe or chronic, damage to kidney tissue and renal failure occur. In India, kidney disease is a rising incidence and major problem for healthcare and the economy. Kidney stone formation is a multi-factorial urologic disorder resulting from the combined influence of epidemiological, biochemical and genetic risk factors[1,2]. The tendency of stone formation is largely attributed to primary renal calcium leak, excessive calcium absorption or an imbalance between bone resorption and formation. Vitamin D receptor (VDR) plays an important role in regulating calcium homeostasis by affecting bone resorption and increasing calcium absorption. It has been reported that the intestine, bones and kidneys of genetic hypercalciuric stone-forming rats exhibited increased numbers of vitamin D receptor. A growing amount of epidemiological evidence has suggested that allelic variation of VDR gene may be involved in the etiology of kidney stone disease[1-4].

*Correspondence
Dr. Arvind Kumar Tripathi
Centre for Biotechnology Studies, APS University, Rewa, M.P, India

Several polymorphisms have been identified in the VDR gene. Among them four (Apa I, Bsm I, Taq I and Fok I) are particularly studied throughout the globe but with controversial results. Parathyroid hormone is the main regulator of minute to minute calcium balance and a key regulator of its release iscalcium sensing receptor (CaSR), a G-protein coupled receptor expressed in the parathyroid gland and renal tubular cells. Its activation induced increased calcium excretion in the kidney. In accordance with this, mutation in the CaSR gene has been shown to cause abnormalities in blood calcium ion (Ca2+) levels. The presence of an activating and inactivating mutation of CaSR gene cause autosomal dominant hypocalcemia or familial hypocalciuric hypercalcemia respectively. Three single-nucleotide polymorphisms causing nonconservative amino acid changes have been described on exon 7, encoding the intracellular domain of CaSR[2,6,7]. Urolithiasis is a multifactorial disease which is considered to be associated with the effects of multiple genes incombination with lifestyles and environmental influences. Although no specific gene has been declared to be the underlying cause of urolithiasis, many functional genes suchas urokinase, vitamin D receptor gene (VDR), and calcium sensing receptor gene (CaSR) have been verified to be relatedto urolithiasis. The CaSR gene, located on chromosome3q13.3-21, spans 103 kb and encodes for a protein of 1078amino acids present in the plasma membrane. CaSR is amember of the G-protein coupled receptors and its structurehas 3 different domains. It is widely accepted that

CaSRmay be connected with urolithiasis, since it decreases calcium reabsorption in thick ascending limbs and distal convoluted tubules, increases phosphate reabsorption in proximal tubules, and decreases water and proton reabsorption incollecting ducts. As a consequence, the CaSR gene is acandidate to explain the susceptibility to Urolithiasis[8-13].The CaSR gene is composed of seven exons, the first six coding for the extracellular domain of the CaSR protein and exon 7 coding for the transmembrane and the intracellular domains. Three single-nucleotide polymorphisms (SNPs), A986S (rs1801725, G > T), R990G (rs1042636, A > G), and Q1011E (rs1801726, C > G), located on exon 7, are extensively studied. Shakhssalim and his colleagues observed a significantly higher frequency of the 986S, 990G, and 1011Q alleles in stone formers[6,14,15]. Another study puts forward similar conclusions. These findings confirm that CaSR gene polymorphisms may be involved inurolithiasis, but the impact of these amino acid changes on the function of CASR is not well defined. Unfortunately, we have no sufficient knowledge to resolve these puzzles. At present, several studies have attempted to investigate associations between CaSR gene variants with Urolithiasis and urinary calcium concentration. However, the results were inconsistent or even contradictory. To date, no one has conducted a meta-analysis to further probe the associations. To fill this gap, we performed a meta-analysis of all eligible studies to derive more reliable estimation of associations between calcium-sensing receptor gene polymorphisms with urolithiasis and urinary calcium concentration[16-19].

Materials and Methods

Study population

The study population consisted of 400 unrelated subjects comprising of 190 T2D patients and 210 ethnically matched controls of central Indian population were included in this study. In this region Hindu, Muslim and some Sikh peoples are mainly living but most peoples belong to Hindu religion in this region.

Inclusion and Exclusion criteria for Cases

Cases included consecutive patients who attended the Department of Medicine, Shyam Shah Medical College and Sanjay Gandhi Memorial Hospital, Rewa, Ayurveda Medical College, Rewa, Ranbaxy pathology Regional collection centre Rewa, District hospital Satna, Shahdol, Sidhi. Type 2 diabetes was diagnosed in accordance with World Health Organization (WHO Expert committee 2003) criteria. Pregnant women, children under age of 18 years and any patients with type 1 diabetes were excluded from the study.

Inclusion and Exclusion criteria for Controls-Control group composed of non-diabetic healthy individuals that were collected during "Diabetes Awareness Camps" organized in urban regions in and around SSMC Rewa and many volunteers were also included to collect control sample. The control subjects were recruited from the regions that from homogenous cluster in Vindhyan region India in accordance with a recent report of genetic landscape of the people of India.. All the participants were asked to fill a detailed questionnaire at the time of recruitment, seeking information regarding individual's age, sex, ethnicity, dietary habits, physical activity, and life style, personal and family medical history.

Anthropometric and Biochemical Measurements-Height and Weight were measured in light clothes and without shoes in standing position as per standard guidelines. Body Mass Index (BMI) was calculated as weight in kilograms divided by height in meters squared. Waist circumference was measured in standing position midway between iliac crest and lower costal margin and hip circumference was measured at its maximum waist to hip ratio (WHR) was calculated using waist and hip circumferences. Systolic and diastolic blood pressures were measured twice in the right arm in sitting position after resting for at least 5 minute using a standard sphygmomanometer and the average of the two reading was used.Biochemical parameters related to type 2 diabetes were estimated for both cases and controls subjects. Measurement of Serum levels of Total cholesterol (TC), Triglycerides (TG), HbA1c, High density lipoprotein-cholesterol (HDL-C), Low density lipoprotein-cholesterol (LDL-C), Urea, Uric acid, C-reactive protein (CRP) and Creatinine were measured based on spectrophotometric method using automated clinical chemistry analyzer Cobas Integra 400 plus (Roche Diagnostics, Mannheim, Germany).

Blood collection and plasma/serum separation-Venous blood samples were obtained from the subjects after 12 hours of overnight fasting in vacutainers with and without appropriate anti-coagulants. Immediately, plasma and serum from the respective vacutainers were separated by centrifuging the tubes at 1000 rpm for 10 min. at 4˚C

Molecular Laboratory Analysis: Genomic DNA was extracted from whole blood by the modification of salting out procedure described by Miller and coworkers (Miller *et al*. 1988). Frozen blood sample was thawed at room temperature. 0.5 ml. of whole blood sample was suspended in 1.0 ml. of lysis buffer (0.32 M Sucrose, 1 mM Mgcl2, 12 mM Tris and 1% Triton-X-100) in a 1.5 ml. microcentrifuge tubes. This mixture was mixed gently by inverting the tube upside down for 1 min. The mixture was than allowed to stand for 10 min. at room temperature to ensure proper lysis of cells. The mixture was centrifuged at 11,000 rpm for 5 min. at 4˚C to pellet the nuclei. The supernatant was discarded carefully in a jar containing disinfectant, as pellet formed is loosely adhered to the bottom of centrifuge tube. The pellet was resuspended in 0.2 ml. of lysis buffer and recentrifuge at 11,000 rpm for 5 min. The pellet was than dissolved in 0.2 ml. of deionized autoclaved water and mixed thoroughly on vortexer. The mixture was centrifuged at 14,000 rpm for 1 min. at 4˚C. Supernatant was discarded to gain an intact pellet. To the above pellet, 80 µl. of proteinase K buffer (0.375 M Nacl, 0.12 M EDTA, pH 8.0) and 10 µl. of 10% SDS (10% w/v SDS, pH 7.2) was added. Mixture was well frothed with the help of micro tip to allow proper lysis of pelleted nuclei. After digestion was complete, 100 µl. of saturated cold 5M Nacl was added and shaken vigorously for 15 sec. To the above mixture 0.2 ml. of deionized, autoclaved water and 0.4 ml. of phenol-chloroform (4:1 v/v) was added to remove most of the non nucleic acid organic molecules. Microcentrifuge tube was inverted upside down until the solution turned milky. Phases were separated by centrifuging the above mixture at 12,000 rpm for 10 min. at 4˚C. Aqueous (top) layer was saved and transferred in another microcentrifuge tube. Transferring of any interface layer was avoided. To the aqueous layer, 1 ml. chilled absolute ethanol was added and the tube was inverted several times until the DNA precipitated. DNA precipitates like thread. This was centrifuged at 14,000 rpm for 4 min. at 4˚C to pellet the DNA thread. Supernatant was discarded. The pellet was washed twice with 1 ml. of 70% alcohol. The mixture was again centrifuged at 14,000 rpm for 1 min. 4˚C. Supernatant was discarded and pellet was air dried for 10-20 min. The pelleted DNA was rehydrated in 100-200 µl. of TE buffer pH 7.4 (10 mM Tris-HCL pH 7.4, 1mM EDTA, pH 8.0). DNA was allowed to dissolve overnight at 37˚C before quantization.

Determination of quality and quantity of isolated DNA-The isolated DNA is to be used for PCR based study. Therefore its suitability for PCR along with its size heterogeneity is among the most important criterion for purity. As a matter of general practice all DNA preparations were tested for quality and quantity measures, as described in the following paragraphs.

Quantitation by UV spectrophotometry-The isolated genomic DNAs were then tested for purity by measuring their absorbance values at 230 nm, 260 nm, 280 nm and 300 nm using a UV visible spectrophotometer (Systronic, India). A DNA preparation was considered to be good if it had A 260 nm / A 280 nm ratio as approximately 1.8 and A 300 nm was 0.1 or lesser. The absorbance at 260 nm was used to calculate the amount of DNA, using the relationship that double stranded DNA at 50µg/ml concentration has an absorbance= 1.0 at 260 nm.

Agarose Gel Electrophoresis

Gel electrophoresis of the genomic DNAs was carried out for qualitative estimation of samples prepared. A good DNA preparation appears as single band. A horizontal agarose slab gel electrophoresis apparatus (Bangalore Genei, Bangaore, India) was used. In brief, 4-5 µl of each genomic DNA was loaded on 0.8 agarose (0.8 % w/v, Sigma) containing ethidium bromide solution (0.5 µg/ml) and

electrophoresis was done at 80 V in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). Lambda DNA *Eco*RI / *Hind* III double digest (Bangalore Genei, Bangalore, India) was used as molecular weight marker after completion of electrophoresis, the DNA bands were visualized and photographed using an UV transilluminator (312 nm) and gel documentation system (Vilber Lourmate, Cedex 1, France) respectively.

Polymorphism screening

In general, the genomic DNA extracted from peripheral blood of healthy individuals and diseased individuals was subjected to PCR followed by restriction digestion and electrophoresis to genotype both the groups for relevant gene of interest. All the PCRs were carried out in a PTC 200 thermal cycler (MJ Research Inc. USA). PCR is a rapid, inexpensive and simple mean of producing relatively large copy number of DNA molecules from the small amounts of source DNA material, even when the source DNA is of relatively poor quality. Due to the extreme sensitivity, precautions were taken against contamination of the reaction mixture with the trace amounts of DNA, which could serve as an unwanted template. Appropriate negative control was included in each PCR run carried out for each gene, to monitor this contamination of PCR mix to avoid any false positive results. The negative control used for PCR contained whole PCR reaction mix except target DNA which was replaced by HPLC purified water free of RNase, DNase, and any contamination from any other source resembling the gene sequence.Subsequently restriction enzyme digestion was performed by incubating the double stranded DNA with appropriate amount of restriction enzyme, in its respective buffer as recommended by the supplier and at optimal temperature for that specific enzyme. A typical digestion includes one unit of enzyme per microgram of starting DNA. One enzyme unit is usually defined as the amount of enzyme needed to completely digest one microgram of double stranded DNA in one hour at the appropriate temperature. Their biochemical activity of the restriction enzyme is the hydrolysis of phosphodiester backbone at specific sites in a DNA sequence. Precaution was taken to avoid star activity of restriction enzymes. When DNA is digested with certain restriction enzymes under non-standard conditions, cleavage can occur at sites different from the normal recognition sequence. Such aberrant cutting is called "star activity" which can be due to high pH (>8.0) or low ionic strength, extremely high concentration of enzyme (>100 U/µg of DNA) and presence of organic solvents in the reaction (e.g. ethanol, DMSO).The PCR and restriction digestion conditions were optimized for specific locus of relevant segment of the gene to be studied. The PCR products as well as the digested products were separated on either agarose gel or polyacrylamide gel depending on their size. Gels were stained with ethidium bromide solution (0.5 µg/ml) and subsequently visualized and photographed under UV transilluminator.

Detection of CaSR (Calcium Sensing Receptor) Polymorphism

The Ala 986 Ser polymorphism of CaSR gene has been amplified by PCR. This polymorphism is a functional polymorphism causing change of Amino acid from Ser to Ala. Primer sequences oligonucleotide sequence (primers) was designed to amplify the gene wild type gene is lack of restriction site for *HinII* enzyme but mutant allele contains a restriction site.

PCR Primer: The oligonucleotides sequences (primers) used were those described by Z Isam***(Isam Z, et. al. 2018).***

Forward primer- 5`- CAAGGACCTCTGGACCTCCCTTTGC-3`
Reverse primer- 5`- GACCAAGCCCTGCACAGTGCCCAAG-3`

PCR Mix

The PCR was carried out in a final volume of 25 µl, containing 100 ng of genomic DNA(4-5 µl), 2.5 µl of 10X *Taq* polymerase buffer (10 mM Tris HCl pH 8.8, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 0.005% Tween-20, 0.005% NP-40; final concentration 1X; Genetix Biotech Asia Pvt. Ltd.,India), 1 µl of 10 mM dNTPs (Banglore Genei, Bangalore, India), 1 µl of 25 pmol/µl of forward and reverse primers specific for and 1 µl of unit of 1U/ µl Red Taq DNA polymerase (Bangalre genei).

PCR Thermal Program

After an initial denaturation of 5 min at 94˚C, the samples were subjected to 35 cycles at 94˚C for 1 min, at 55˚C for 40 s, and 72˚C for 40 s, with a final extension of 10 min at 72˚C in a thermal cycler. A 100bp ladder with amplified product has been run under 1 % agarose gel electrophoresis. 238bp product will be generated after PCR.

Restriction Digestion

Restriction Digestion The 238-bp product was digested with AvaII enzyme (New England Biolabs, overly, MA) for 16 h at 37˚C. The PCR products when digested by restriction enzyme and wild type allele 269bp segment which were generated by PCR but the mutant allele shows 241 and 28 bp segments. The product sizes are Wild type homozygote, 269 bp; mutant Ala 986 Ser homozygote, 241 and 28 bp; and heterozygote, 269, 241, and 28 bp respectively. Samples will analyzed by electrophoresis using 2.5% agarose gels to analyze the genotype pattern of the gene.

Statistical Analysis-Statistical analysis was done by comparing the distribution of genotype frequencies, allele frequencies and carriage rates of all the four polymorphism in diseased and control group. Disease group included Diabetic patients whereas control group included all healthy controls (HC) enrolled in the study. The proportions of different genotypes for a gene in a population are known as genotype frequencies. The proportion of genotype in a sample will be the ratio of the number of individuals having that genotype to the total number of individuals in the sample. The proportions of different alleles for a gene present in a population are known as allele frequencies. The proportion of an allele in a sample will be the ratio of number of occurrences of the investigated allele in the population to the total number of alleles. The carriage rate was calculated as the number of individuals carrying at least one copy of the test allele divided by the total number of individuals. Data was analyzed using Microsoft Excel 2002, Microsoft Corporation.

Results

Anthropometric results: The descriptive data and comparison of anthropometric and biochemical parameters of nephrolithiatic patients versus controls are presented in Table no. 1. The age, sex, BMI, WHR were the parameters. As expected the nephrolithiatic patients had markedly higher levels of weight of men (P=0.3916) then women (P=0.0723) and BMI of Women (P=0.2247) and Men (P=0.3499) but both was not significantly different between patient and healthy population. Thus WHR in Women (P=0.1741) and Men (P=0.0973) were not found significantly different between case and control group **(See Table No. 1).**

Table No-1: Comparison of anthropometric parameters of nephrolithiatic patients and healthy controls

Characteristics	Cases (160)	Controls(180)	P-value
n(Men/Women)	160(104/56)	180(118/62)	
Age(years)	52.5±12.5	52.6±12.4	0.9411,ns
Height(m)	162.50±11.3	161.2±12.4	0.3152,ns
Weight (Kg)			
Women	62.5 ±4.7	61.6 ± 4.5	0.0723,ns
Men	68.4±5.6	67.8±7.1	0.3916,ns
BMI (kg/m^2)			
Women	25.6±3.1	26.1 ± 4.3	0.2247,ns
Men	24.6±4.7	25.1± 5.1	0.3499,ns

Waist circumference (cm)			
Women	92.5±6.2	93.6±6.7	0.1186,ns
Men	90.0±7.0	89.0±6.0	0.1571,ns
Hip (cm)			
Women	95.9±2.4	96.1±2.2	0.4233,ns
Men	90.8±4.3	91.2±1.5	0.2426,ns
WHR			
Women	0.97±0.05	0.98±0.08	0.1741,ns
Men	0.98±0.08	0.99±0.01	0.0973,ns

(*denotes level of significant change between case and control)

Biochemical and clinical findings

Biochemical test performed in the blood sample for following clinical parameters and the findings were tabulated. Statistical analysis was done by using student's t-test and p value suggests the level of significant were changed here. The descriptive data and comparison of biochemical parameters of nephrolithiatic patients versus healthy controls are presented in Table no. 4.2. As expected the nephrolithiatic patients had markedly higher levels of Blood Urea (P<0.0001) and Serum creatinine (P<0.0001) and Urinary calcium excretion (P<0.0001) compared to that of control subject. Whenever rest of all parameters were not significantly different between patient and healthy population (**See Table No. 2).**

Table No-2: **Comparison of Biochemical and clinical findings of nephrolithiatic patients and healthy controls.**

Characteristics	Cases (160)	Controls(180)	P-value
Post-Prandial Glucose (mg/Dl)	118.7±12.4	119.4±11.6	0.5912,ns
HbA1C(%)	5.9±0.7	5.8±0.8	0.2235,ns
HDL-C(mmol/L)	108.8±12.2	109.3±11.6	0.6989,ns
LDL-C (mg/dL)	42.1±2.6	41.8±3.7	0.3932,ns
TG(mg/dL)	125.9±13.2	126.2±12.2	0.8278,ns
Systolic BP (mmHg)	125.4±8.1	124.8±5.7	0.4263,ns
Diastolic BP (mmHg)	87.1±5.8	86.5±6.2	0.3593,ns
Blood Urea(mg/dL)	28.5±1.6	16.8±1.8	P<0.0001***
Urinary Citrate (mmol/24 h)	2.58±0.96	2.62±0.57	0.6365,ns
Spot urine pH	5.72±0.41	5.75±0.35	0.4673,ns
Serum creatinine (mg/dl)	1.45±0.47	0.71±0.26	P<0.0001***
Serum calcium (mg/dl)	9.42±0.32	9.46±0.38	0.2978,ns
Urinary calcium excretion (mmol/24 h)	7.87±0.59	4.04±0.68	P<0.0001***
Urinary potassium (mmol/24 h)	64.21±4.7	64.39±4.3	0.7125,ns
Urinary Phosphate (mmol/24 h)	27.45±4.2	26.81±3.3	0.1172,ns
Urinary Oxalate (mg/24 h)	28.11±3.7	27.51±3.4	0.1202,ns
Urinary Urate (mmol/24 h)	2.89±0.47	2.88±0.86	0.8961,ns

(* denotes the level of significant change between case and control)

Biochemical Analysis

IL-18 is a pro-inflammatory cytokine associated with increased inflammatory response and elevated level during plasmodium infection. IL-18 level in blood serum reveals significant association with nephrolithiatic infection. Concentration of IL-18 level in malarial patient (case) and healthy population (control) is depicted in table no.-4.3, is showing elevated level during infection and it was statistically significant associated as P<0.0001*** **(See Table No. 4.3)**..

Figure no. 3 (a) and 3 (b) are showing ELISA of IL-18 result. This is kit based ELISA result reveals elevated IL-18 level in malarial infection. In figure no. 3 (a), two columns ELISA analysis having four standards A, B, C, D containing 25, 50, 100, 200 Pg/ml concentration respectively read absorbance at 450 nm. G for Patient (Case) and H for Healthy (control) showing differences in IL-18 level as 23.97 Pg/ml and 11.82 Pg/ml respectively.

Table No-3: Comparison of Biochemical Factor between Nephrolithiatic Cases and Healthy Controls:

Biochemical Factor	Cases(160)	Controls(180)	P-value
IL-18 **Cytokine Level in serum**(pg/mL)	23.97 ± 9.52	11.82 ± 6.41	(P<0.0001)***

(*Denotes level of significant change between malarial cases and healthy controls.)

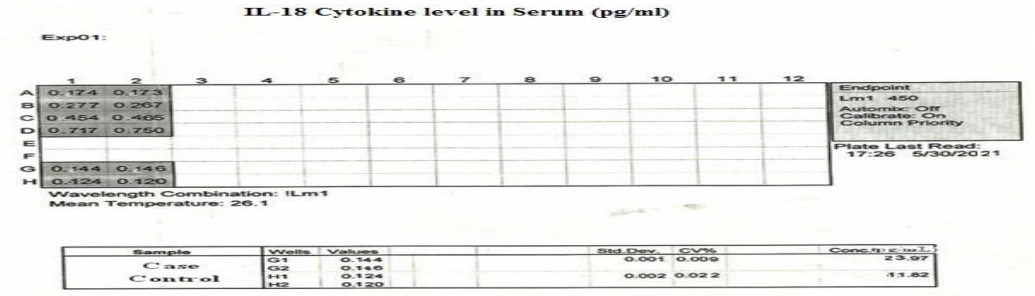

Figure No.-1 (a): Result of ELISA, showing IL-18 Cytokine Level in both case and control.

Standards (pg/mL)

Sample	Conc	Wells	Value	MeanValue	SD	CV
St01	25.000	A1	0.174	0.173	0.0007	0.004
		A2	0.173			
St02	50.000	B1	0.277	0.272	0.0070	0.257
		B2	0.267			
St03	100.000	C1	0.454	0.459	0.0077	0.016
		C2	0465			
St04	200.000	D1	0.717	0.733	0.0233	0.031
		D2	0.750			

Smallest standard value: 0.173
Largest standard value: 0.733

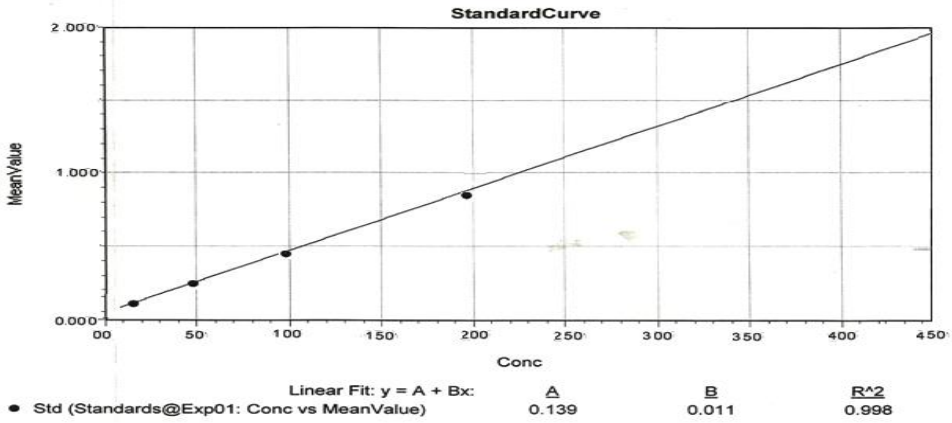

Figure No.-1 (b); Straight line graph is showing concentration change accordance to absorbance.

Hardy Weinberg Equilibrium Test

The genotype frequencies of each gene in each study group were tested to be in accordance with Hardy Weinberg equilibrium using chi squre ($\chi 2$) test for independence. When the calculated value of $\chi 2$ was less than tabulated value of $\chi 2$ at degree of freedom 1 (d.f. = 1) and level of significance (P = 0.05), the population is at equilibrium for the gene and vice versa. The standard tabulated value of $\chi 2$ at degree of freedom 1 and level of significance 0.05 is 3.84. All the tabulated $\chi 2$ values for the genes were compared to this value. The genotype frequencies of all the study groups included in the study were in accordance with Hardy Weinberg equilibrium **(See Table No. 4.4).**

Table No-4: Hardy Weinberg Equilibrium Test for both nephrolithiatic patient and Healthy control population.

Gene	X^2 value for Case	X^2 value for control
CaSR	1.60	1.1211

The X^2 value indicates the difference between expected and observed values for genotype counts.
Tabulated Value = 3.84

Detection of Genetic Polymorphism in CaSR (Calcium Sensing Receptor) gene

The nucleotide position Ala 986 Ser polymorphism in CaSR(Calcium Sensing Receptor) gene create restriction site for *HinII*. The PCR products when digested by restriction enzyme and wild type allele 269bp segment which were generated by PCR but the mutant allele shows 241 and 28 bp segments. The product sizes are Wild type homozygote, 269 bp; mutant Ala 986 Ser homozygote, 241 and 28 bp; and heterozygote, 269, 241, and 28 bp respectively. In gele picture too short fragmant was The 28-bp fragments are not visualized because of run out from the gel **(Depicted in figure no.2.)**

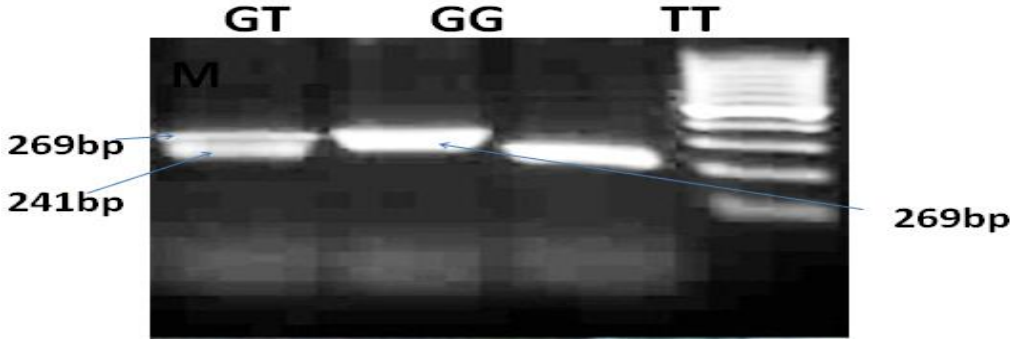

Figure No.-1: Representative gel picture of CaSR polymorphism. Lane M represents 50 bp molecular marker, Lane GG Wild type genotype, Lane GT heterozygous genotype and Lane TT variant genotype.

The distribution of polymorphic genotype was strongly under HWE. The observed genotype frequencies, allele frequencies and carriage rates for *CaSR* Ala 986 Ser polymorphism are depicted in table 6 and table 7 and graph no. 4, 5, 6. Overall distribution of *CaSR*Ala 986 Ser genotypes was significantly different in healthy control group as compared to disease group (χ^2=7.253, P=0.0266*). HC group showed an decrease of mutant 'TT' genotype as compared to Patients of Nephrolithiatic (*1.11*% *vs.* 4.37%). Similarly, wild type '*GG*'

genotype was present in significantly high frequency in HC as compared to Nephrolithiatic patients group (72.22% *vs.* 54.37%). An odds ratio of 0.4584 in Nephrolithiatic group respectively for 'GG' genotype indicated a protective effect of this type genotype in our population whereas an odds ratio of 4.072 for Mutant TT Nephrolithiatic patients group respectively indicated a positive association of this wild type genotype with the disease, heterozygous is also significantly different but may be not protective because of odds ratio of 0.7094. Overall allele 'T' was found to be in significantly low frequency in disease group as compared to HC group whereas allele '*G'* was present in significantly high frequency in the healthy control group (χ^2 =12.07 P= 0.0005***). Overall G allele shows an odds ratio of 0.5065 which indicates its protective association.Carriage rate of allele 'G' was high in HC group whereas carriage rate of allele 'T' was high in disease group (χ^2 =6.180 P=0.0129*) but the values were not significant. .The pattern of genotype and allele distribution in disease and control group suggested a significant association of *CaSR* Ala 986 Ser wild type allele 'G' carriage (carriage of 'GG') in Susceptibility to Nephrolithiatic and not show the protective effect **(See Table No. 7 and 8).**

Table No-5: Frequency distribution and association ofGenotype, allele frequency and carriage rate of CaSR gene polymorphism in population of Vindhyan regionusing Chi Square Test

CaSR GENE	CASE N= 160		CONTROL N=180		CHI SQUARE VALUE χ^2 (P Value)
	N	%	N	%	
Genotype					
GG	87	54.37	130	72.22	
GT	66	41.25	48	26.66	
TT	7	4.37	2	1.11	13.01 (0.0015**)
Allele					
G	240	75.00	308	85.55	
T	80	25.00	52	14.44	12.07 (0.0005***)
Carriage Rate					
G	153	67.69	178	78.07	
T	73	32.30	50	21.92	6.180 (0.0129*)

(- denotes the level of significant association between case and control.)*
(N – Number of individuals in study group.)
(% - Genotype allele frequency and carriage rate expressed in percentage.)

Table No-6: Fisher Exact Test values of CaSR gene polymorphism

CaSR GENE	CASE N= 160		CONTROL N=180		P Value	Odds Ratio (95% confidence interval)
	N	%	N	%		
Genotype						
GG	87	54.37	130	72.22	0.0007***	0.4584 (0.2920 to 0.7195)
GT	66	41.25	48	26.66	0.0057**	1.931 (1.223 to 3.048)
TT	7	4.37	2	1.11	0.0894ns	4.072 (0.8332 to 19.90)
Allele						
G	240	75.00	308	85.55		0.5065 (0.3437 to 0.7465)
T	80	25.00	52	14.44	0.0006***	1.974 (1.340 to 2.910)
Carriage Rate						
G	153	67.69	178	78.07		0.5887 (0.3869 to 0.8958)
T	73	32.30	50	21.92	0.0151*	1.699 (1.116 to 2.585)

(- denotes the level of significant association between case and control.)*
(N – Number of individuals in study group.)
(% - Genotype allele frequency and carriage rate expressed in percentage.)

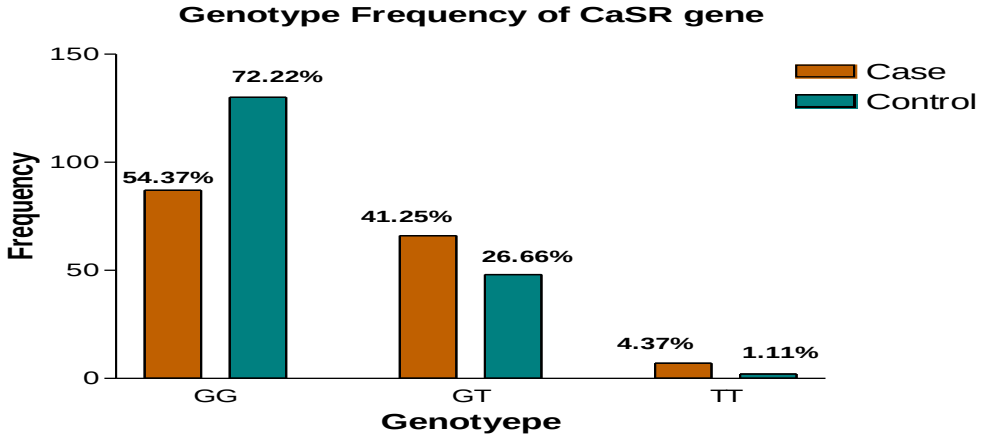

Figure No.-2: Genotype Frequency of CaSR gene

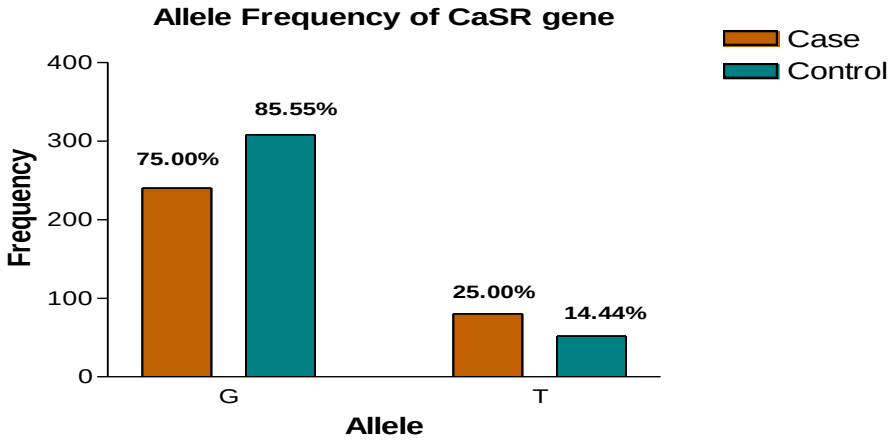

Figure No.-3: Allele Frequency of CaSR gene

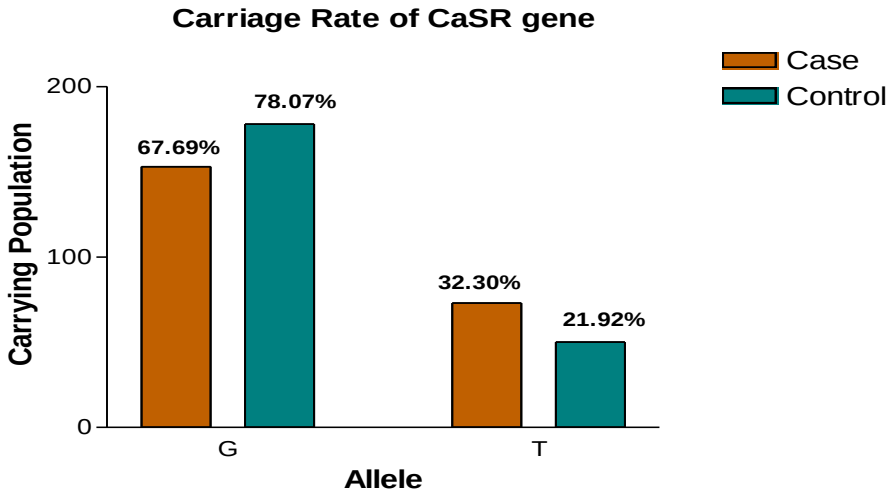

Figure No.-4: Carriage rate of CaSR gene

Discussion

Kidney stones are a prevalent clinical condition imposing a large economic burden on the healthcare system. Hypercalciuria remains the major risk factor for development of a Ca^{2+}containing stone. The kidney's ability to alter Ca^{2+} excretion in response to changes in serum $Ca^{2+}$is in part mediated by the Ca^{2+}sensing receptor (CaSR). Recent studies revealed renal claudin-14 (Cldn14) expression localized to the thick ascending limb (TAL) and its expression to be regulated via the CaSR. We find that Cldn14 expression is increased by high dietary Ca^{2+} intake and by elevated serum Ca^{2+} levels induced by prolonged 1,25-dihydroxyvitamin D3 administration[20,18]. Consistent with this, activation of the CaSR in vivo via administration of the calcimimetic cinacalcet hydrochloride led to a 40-fold increase in Cldn14 mRNA. Moreover, overexpression of Cldn14 in two separate cell culture models decreased paracellular Ca^{2+} flux by preferentially decreasing cation permeability, thereby increasing transepithelial resistance. These data support the existence of a mechanism whereby activation of the CaSR in the TAL increases Cldn14 expression, which in turn blocks the paracellular reabsorption of Ca^{2+}. This molecular mechanism likely facilitates renal Ca^{2+} losses in response to elevated serum Ca^{2+}. Moreover, dysregulation of the newly described CaSR-Cldn14 axis likely contributes to the development of hypercalciuria and kidney stones[21-24].

Our findings on CaSR polymorphism suggested that nucleotide position Ala 986 Ser polymorphism in CaSR(Calcium Sensing Receptor) gene create restriction site for *HinII*. The PCR products when digested by restriction enzyme and wild type allele 269bp segment which were generated by PCR but the mutant allele shows 241 and 28 bp segments. The product sizes are Wild type homozygote, 269 bp; mutant Ala 986 Ser homozygote, 241 and 28 bp; and heterozygote, 269, 241, and 28 bp respectively. In gele picture too short fragmant was The 28-bp fragments are not visualized because of run out from the gel. The distribution of polymorphic genotype was strongly under HWE. The observed genotype frequencies, allele frequencies and carriage rates for *CaSR* Ala 986 Ser polymorphism are depicted in table 6 and table 7 and graph no. 4, 5, 6. Overall distribution of *CaSR*Ala 986 Ser genotypes was significantly different in healthy control group as compared to disease group (χ^2=7.253, P=0.0266*). HC group showed an decrease of mutant 'TT' genotype as compared to Patients of Nephrolithiatic (*1.11% vs.* 4.37%). Similarly, wild type '*GG*' genotype was present in significantly high frequency in HC as compared to Nephrolithiatic patients group (72.22% *vs.* 54.37%). An odds ratio of 0.4584 in Nephrolithiatic group respectively for 'GG*'* genotype indicated a protective effect of this type genotype in our population whereas an odds ratio of 4.072 for Mutant TT Nephrolithiatic patients group respectively indicated a positive association of this wild type genotype with the disease, heterozygous is also significantly different but may be not protective because of odds ratio of 0.7094. Overall allele 'T' was found to be in significantly low frequency in disease group as compared to HC group whereas allele '*G'* was present in significantly high frequency in the healthy control group (χ^2 =12.07

P= 0.0005***). Overall G allele shows an odds ratio of 0.5065 which indicates its protective association.Carriage rate of allele 'G' was high in HC group whereas carriage rate of allele 'T' was high in disease group (χ^2 =6.180 P=0.0129*) but the values were not significant. .The pattern of genotype and allele distribution in disease and control group suggested a significant association of *CaSR* Ala 986 Ser wild type allele 'G' carriage (carriage of 'GG') in Susceptibility to Nephrolithiatic and not show the protective effect.

The calcium-sensing receptor gene (CaSR) is a candidate to explain urolithiasis. A number of case-control studies were conducted to investigate associations between CaSR polymorphisms with risks of hypercalciuria and urolithiasis in humans. But the results were still inconsistent. A meta-analysis was performed to address this issue. Crude odds ratios (ORs) with 95% confidence intervals (CIs) were calculated to estimate the strength of associations between CaSR polymorphisms and the risk of Urolithiasis[25]. The pooled standardized mean difference (SMD) with 95% CI was used for the meta-analysis of CaSR polymorphisms and urine calcium concentration. For urolithiasis association, the SS genotype of A986S polymorphism was a risk factor for urolithiasis in Asians and PHPT patients, but a protective factor in Caucasians. The GG genotype of R990 polymorphism was associated with an increased risk of urolithiasis, especially in Caucasians and healthy population. Regarding urine calciumconcentration association, individuals with the G allele had a higher level of urine calcium than the noncarriers. This meta-analysis revealed that the G allele of CaSR R990G polymorphism increases susceptibility to urolithiasis and hypercalciuria. The A986S and Q1011E polymorphisms were associated with urolithiasis and hypercalciuria in specific populations[26-28].

The calcium-sensing receptor (*CaSR*) gene plays an important role in regulating the Ca2+ balance and reducing the risk for calcium stones. In this study, we evaluated the association of *CaSR* polymorphisms with calcium nephrolithiasis in the population of Yi nationality in Southwestern China. Biochemical variables were evaluated in 624 calcium nephrolithiasis patients and 470 age-matched healthy controls without a history of nephrolithiasis. *CaSR* polymorphisms rs7652589, rs1501899, rs1801725 (Ala986Ser), rs1042636 (Arg990Gly) and rs1801726 (Gln1011Glu) were investigated between the calcium nephrolithiasis patients and healthy controls, using direct sequencing. Compared with the healthy controls, serum creatinine and 24-hour urine calcium levels were significantly higher in calcium nephrolithiasis patients. Among these five polymorphisms, the genotypic and allelic frequency distributions of rs7652589 SNP was significantly associated with the risk of calcium nephrolithiasis[4,13,18]. However, there were no genotypic or allelic distribution differences for rs1501899, rs1801725, rs1042636, and rs1801726 polymorphisms between calcium nephrolithiasis patients and healthy controls. The Calcium nephrolithiasis is one of the most common causes of renal stones. While the prevalence of this disease has increased steadily over the last 3 decades, its pathogenesis is still unclear. Previous studies have indicated that a genetic polymorphism (rs17251221) in the calcium-sensing receptor gene (CASR) is associated with the total serum calcium levels. In this study, we collected DNA samples from 480 Taiwanese subjects (189 calcium nephrolithiasis patients and 291 controls) for genotyping the CASR gene. His results indicated no significant association between the CASR polymorphism (rs17251221) and the susceptibility of calcium nephrolithiasis. The risk of stone multiplicity was higher in patients with the GG+GA genotype than in those with the AA genotype[29,9]. The calcium-sensing receptor gene is a candidate to explain nephrolithiasis. The potential role of CaSR in lithogenesis according to findings of functional and genetic studies. CaSR is a cation receptor located in the tubular cell plasma membrane. Its activation decreases calcium reabsorption in the ascending limb and distal convoluted tubule, but increases phosphate reabsorption in proximal tubules and decreases water and proton reabsorption in collecting ducts. Its effects in proximal tubules and collecting ducts can limit the calcium phosphate precipitation risk induced by the increase in calcium excretion. The nonconservative CaSR gene Arg990Gly polymorphism was associated with nephrolithiasis and hypercalciuria in different populations. Arg990Gly is located on exon 7 and produces a gain of the CaSR function. rs7652589 and rs1501899 were also associated with nephrolithiasis in patients with normal citrate excretion. These polymorphisms are located in the CaSR gene regulatory region and may modify CaSR gene promoter activity[26,27]. The activating Arg990Gly polymorphism may predispose to nephrolithiasis by increasing calcium excretion. Polymorphisms at the regulatory region may predispose to nephrolithiasis by changing tubular expression of the CaSR. CaSR genotype may be a marker to identify patients prone to develop calcium nephrolithiasis. Claudin-16 and -19 are proteins forming pores for the paracellular reabsorption of divalent cations in the ascending limb of Henle loop; conversely, claudin-14 decreases ion permeability of these pores. Single-nucleotide polymorphisms in gene coding for claudin-14 were associated with kidney stones and calcium excretion. This study aimed to explore the association of claudin-14, claudin-16, and claudin-19 singlenucleotide polymorphisms with calcium excretion[30].

References

1. Coe FL, Keck J, Norton ER. The natural history of calcium urolithiasis. JAMA 1977;238:1519-23.
2. Stamatelou KK, Francis ME, Jones CA, Nyberg LM, Curhan GC. Time trends in reported prevalence of kidney stones in the United States: 1976-1994. Kidney Int 2003;63:1817-23.
3. Curhan GC, Willett WC, Rimm EB, Stampfer MJ. Family history and risk of kidney stones. J Am Soc Nephrol 1997; 8:1568-73.
4. Jawalekar S, Surve VT, Bhutey AK. The composition and quantitative analysis of urinary calculi in patients with renal calculi. Nepal Med Coll J 2010;12:145-8.
5. Pak CY, Poindexter JR, Adams-Huet B, Pearle MS. Predictive value of kidney stone composition in the detection of metabolic abnormalities. Am J Med 2003;115:26-32.
6. Moe OW, Bonny O. Genetic hypercalciuria. J Am Soc Nephrol 2005;16:729-45.
7. Renkema KY, Lee K, Topala CN, Goossens M, Houillier P, Bindels RJ, et al. TRPV5 gene polymorphisms in renal hypercalciuria. Nephrology Dialysis Transplantation 2009; 24:1919-24.
8. Scott P, Ouimet D, Proulx Y, Trouvé ML, Guay G, Gagnon B, et al. The 1 alpha-hydroxylase locus is not linked to calcium stone formation or calciuric phenotypes in French-Canadian families. J Am Soc Nephrol 1998;9:425-32.
9. Schurgers LJ, Cranenburg EC, Vermeer C. Matrix Gla-protein: the calcification inhibitor in need of vitamin K. Thromb Haemost 2008;100:593-603.
10. Cancela L, Hsieh CL, Francke U, Price PA. Molecular structure, chromosome assignment, and promoter organization of the human matrix Gla protein gene. J Biol Chem 1990;265:15040-8.
11. Fraser JD, Price PA. Lung, heart, and kidney express high levels of mRNA for the vitamin K-dependent matrix Gla protein. Implications for the possible functions of matrix Gla protein and for the tissue distribution of the gamma-carboxylase. J Biol Chem 1988;263:11033-6.
12. Yasui T, Fujita K, Sasaki S, Sato M, Sugimoto M, Hirota S, et al. Expression of bone matrix proteins in urolithiasis model rats. Urol Res 1999; 27:255-61.
13. Lu X, Gao B, Liu Z, Tian X, Mao X, Emmanuel N, et al. A polymorphism of matrix Gla protein gene is associated with kidney stone in the Chinese Han population. Gene. 2012;511:127-30.
14. Gao B, Yasui T, Itoh Y, Tozawa K, Hayashi Y, Kohri K. A polymorphism of matrix Gla protein gene is associated with kidney stones. J Urol. 2007;177:2361-5.

15. Farzaneh-Far A, Davies JD, Braam LA, Spronk HM, Proudfoot D, Chan SW, et al. A Polymorphism of the Human Matrix γ-Carboxyglutamic Acid Protein Promoter Alters Binding of an Activating Protein-1 Complex and Is Associated with Altered Transcription and Serum Levels. J Biol Chem. 2001;276:32466-73.
16. Herrmann SM, Whatling C, Brand E, Nicaud V, Gariepy J, Simon A, et al. Polymorphisms of the Human Matrix Gla Protein (MGP) Gene, Vascular Calcification, and Myocardial Infarction. Arteriosclerosis, Thrombosis, and Vascular Biology. 2000;20:2386-93.
17. Proudfoot, D. Calcium Signaling and Tissue Calcification. Cold Spring Harb. Perspect. Biol. 2019, 11.
18. Sirajudeen, S.; Shah, I.; Al Menhali, A. A Narrative Role of Vitamin D and Its Receptor: With Current Evidence on the Gastric Tissues. Int. J. Mol. Sci. 2019, 20, 3832.
19. Riccardi, D.; Valenti, G. Localization and function of the renal calcium-sensing receptor. Nat. Rev. Nephrol. 2016, 12, 414–425.
20. Bertero, E.; Maack, C. Calcium Signaling and Reactive Oxygen Species in Mitochondria. Circ. Res. 2018, 122, 1460–1478.
21. Jadiya, P.; Kolmetzky, D.W.; Tomar, D.; Di Meco, A.; Lombardi, A.A.; Lambert, J.P.; Luongo, T.S.; Ludtmann, M.H.; Pratico, D.; Elrod, J.W. Impaired mitochondrial calcium efflux contributes to disease progression in models of Alzheimer's disease. Nat. Commun. 2019, 10, 3885.
22. Demaurex, N.; Distelhorst, C. Cell biology. Apoptosis—The calcium connection. Science 2003, 300, 65–67.
23. Kim, M.K.; Kim, G.; Jang, E.H.; Kwon, H.S.; Baek, K.H.; Oh, K.W.; Lee, J.H.; Yoon, K.H.; Lee, W.C.; Lee, K.W.; et al. Altered calcium homeostasis is correlated with the presence of metabolic syndrome and diabetes in middle-aged and elderly Korean subjects: The Chungju Metabolic Disease Cohort study (CMC study). Atherosclerosis 2010, 212, 674–681.
24. Varghese, E.; Samuel, S.M.; Sadiq, Z.; Kubatka, P.; Liskova, A.; Benacka, J.; Pazinka, P.; Kruzliak, P.; Busselberg, D. Anti-Cancer Agents in Proliferation and Cell Death: The Calcium Connection. Int. J. Mol. Sci. 2019, 20, 3017.
25. Zemel, M.B. Calcium modulation of hypertension and obesity: Mechanisms and implications. J. Am. Coll. Nutr. 2001, 20, 428S–435S; discussion 440S–442S.
26. Shavit, L.; Girfoglio, D.; Vijay, V.; Goldsmith, D.; Ferraro, P.M.; Moochhala, S.H.; Unwin, R. Vascular calcification and bone mineral density in recurrent kidney stone formers. Clin. J. Am. Soc. Nephrol. 2015, 10, 278–285.
27. Pasch A, Frey FJ, Eisenberger U, et al. PTH and 1.25 vitamin D response to a low-calcium diet is associated with bone mineral density in renal stone formers. Nephrol Dial Transplant 2008;23:2563–70.
28. Liu K, Wang X, Ye J, et al. The G allele of CaSR R990G polymorphism increases susceptibility to urolithiasis and hypercalciuria: evidences from a comprehensive meta-analysis. Biomed Res Int 2015;1–2.
29. Brown EM. Physiology and pathophysiology of the extracellular calcium-sensing receptor. Am J Med 1999;106:238–53.
30. Riccardi D, Park J, LeeWS, et al. Cloning and functional expression of a rat kidney extracellular calcium/polyvalent cation-sensing receptor. Proc Natl Acad Sci USA 1995;92:131–5.