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Association Study of Genetic Polymorphism of MGP (T138C) Gene with Calcium Nephrolithiasis in Vidhyan Population Madhya Pradesh, India.

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ABSTRACT

*The role of matrix Gla protein (MGP) is not completely known but, its expression within sub-endothelial macrophages and vascular smooth muscle cells is suggested to be involved in vascular calcification. MGP is a mineral-binding extracellular matrix protein secreted by chondrocytes and vascular smooth muscle cells. It is thought to be a key regulator of vascular calcification. MGP-deficient mice rapidly developed extensive vascular calcification and died due to blood vessel rupture. In humans, nonsense mutations in MGP cause Keutel syndrome, a rare autosomal recessive disorder characterized by abnormal cartilage calcification. The MGP gene is located on the short arm of chromosome 12 (12p12.3). There is increasing evidence that genetic variation at the MGP locus could modulate the development of vascular calcification and atherosclerotic disease. MGP, a vitamin K-dependent extracellular matrix protein, was originally isolated from the bone and also expressed in several soft tissues including the lung, heart, vascular smooth muscle cells of the blood vessel wall and kidney. MGP is an 84-amino-acid protein that contains five γ-carboxyglutamic acid (Gla) residues which has a high affinity for calcium and phosphate ions, and hydroxyapatite crystals. The work done using PCR-RFLP based method. Significant level of change has been seen in overall distribution of MPG genotypes in Healthy control group as compared to disease group although Healthy control group showed little increase in 'TT' genotype as compared to Patients of Nephrolithiatic (69.44% vs 56.25%). Similarly, mutant type 'CC' genotype was present in low frequency in Nephrolithiatic patients group 3.75% and also in control group 4.44% (**P**= 0.0243*), P= 7.436). An odds ratio of TT genotype is 0.5657 which indicates little protective effect whereas an odds ratio of TC genotype is 1.887 of Nephrolithiatic patients group respectively indicate little or no effect and association of this mutant genotype with the Nephrolithiatic susceptibility. Overall allele 'C' was found little lower frequency in disease group as compared to HC group whereas allele 'T' was present in little high frequency in the disease group but the difference is nominal and was not significant (χ^2 = 0.0437*, P=4.069). the data shows association of genetic polymorphism of MGP gene with susceptibility to Calcium nephrolithiasis.*

Introduction

Calcium-containing kidney stones are the most common type of kidney stone. A collection of systemic causes may predispose toward calcium stone formation and include inflammatory bowel disease, primary hyperparathyroidism, and renal tubular acidosis; however, in most people these are absent. Instead, metabolic factors within the urine, the commonest being hypercalciuria, contribute toward a stone-forming environment [1,2]. The evidence points toward calcium oxalate stones forming on anchored sites in the renal papillae made of interstitial apatite deposits and known as Randall plaques. However, in conditions where there is urinary supersaturation of calcium salts, formation of stones may develop from crystal deposits at the tip of the Bellini duct and within inner medullary collecting ducts. The most obvious environmental risk factor for stone formation is dehydration from low fluid intake and other risk factors include high dietary animal protein and sodium.

Monogenic forms of kidney stones are rare but their study offers a chance to Renal stones are common; the overall lifetime risk of stone disease in the United States is 8.8%. Renal stones may be associated with both metabolic syndrome2 and ESRD3 and provide a huge burden on health services [3]. Most stones are calcium-containing, accounting for approximately 75% of all stones. Calcium-containing kidney stones are the most common type of kidney stone. A collection of systemic causes may predispose toward calcium stone formation and include inflammatory bowel disease, primary hyperparathyroidism, and renal tubular acidosis; however, in most people these are absent. Instead, metabolic factors within the urine, the commonest being hypercalciuria, contribute toward a stone-forming environment. The evidence points toward calcium oxalate stones forming on anchored sites in the renal papillae made of interstitial apatite deposits and known as Randall plaques. However, in conditions where there is urinary supersaturation of calcium salts, formation of stones may develop from crystal deposits at the tip of the Bellini duct and within inner medullary collecting ducts [3-6].

This increase relates to a whole range of environmental effects, including dietary changes such as high animal protein and high sodium intake. In addition, the traditional pattern of more stone disease in men compared with women is being narrowed, due to a rise in stones among women. Here we will discuss the basic aspects of calcium stone disease and review the current understanding of monogenic and complex disease causes of calcium stone disease within the context of known metabolic risk factors [2,4]. Kidney stone is a complex disease resulting from an interaction between environmental and genetic factors. About 2–5% of the population in Asia and 8–15% in Europe and North America develop renal stones in their lifetime. Wide geographical variations and racial differences exist in stone incidence and composition. Racial genetic polymorphisms may affect intricate interactions between promoting and inhibiting stone formation factors in renal tubules and are associated with the risk of kidney stone disease [4,7]. We previously identified a variant of the human MGP (matrix Gla protein) gene associated with the individual sensitivity to kidney stone disease within the Japanese population. This association needs to be confirmed by further replication studies, particularly in other ethnic populations. The differences in risk allele frequencies and linkage disequilibrium (LD) structure across ethnicities can provide further insights to exact the association information and identify the true risk variant. MGP is a molecular determinant regulating calcification of the extracellular matrix, and is expressed at high levels in the kidney, bone, lung and heart. A previous study showed that MGP expression was higher in calcified human atherosclerotic plaques and inhibited calcification [7-10].

Nephrolithiasis, the formation of stones in the kidneys, is the third most common affliction of the urinary tract and is associated with a high rate of recurrence with an average rate of 6.78 years. Stone formation is controlled by many factors, including urinary supersaturation, with respect to the crystallization of the stone-forming salts. Calcium oxalate (CaOx) is the most common type of kidney stone [11,9]. CaOx supersaturation is controlled by the urinary calcium, oxalate, pH as well as the concentration of various mineralization modulators present in the urine. Pathogenesis involves not only. crystallization but also crystal retention within the kidneys which is a highly regulated process requiring cellular changes and expression of a variety of macromolecules including the modulators of crystallization and inflammation such as osteopontin (OPN), Tamm– Horsfall protein (THP), bikunin (BK), hyaluronic acid, CD 44 and matrix GLA protein (MGP) MGP is a vitamin K-dependent protein functioning primarily as an inhibitor of vascular calcification. Although first isolated from bone, MGP mRNA expression is tenfold higher in lungs and heart and five times higher in kidneys. MGP gene polymorphism is associated not only with vascular calcification but also with kidney stone formation. Results of studies show increased expression of MGP in renal tubules of rats fed ethylene glycol for 28 days as well as in rat renal proximal tubular cell line, NRK-52E, after the exposure to Ox or CaOx monohydrate crystals [12-16].

Materials & 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.

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

Biochemical Measurements

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).

Molecular Laboratory Analysis

Method for DNA isolation:

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 then 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 MGP (Matrix Gla Protien) Single Nucleotide Polymorphism
The MGP (Matrix Gla Protien) Gene has been amplified by PCR. This polymorphism is a functional polymorphism causing change in T138C (rs1800802). The oligonucleotide sequence (primers) was designed to amplify the gene wild type gene is 473bp having restriction site for *BsrI* enzyme cleaves in to 428 and 45 bp fragment.

Primer sequence: The oligonucleotides sequences (primers) used were those described by F Yukcu ***(Yukcu F et. al. 2015).***
Forward primer: 5'- AAGCATACGATGGCCAAAACTTCTGCA-3'
Reverse Primer: 5' -GAACTAGCATTGGAACTTTTCCCAACC-3'

PCR Mix
The PCR was carried out in a final volume of 25 µl, containing 50-100 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 (Bangalore genei).

PCR Thermal Program
After an initial denaturation of 5 min at 95˚C, the samples were subjected to 35 cycles at 95˚C for 1 min, at 58˚C for 45 s, and 72˚C for 45 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 2.5 % agarose gel electrophoresis.

Restriction digestion
The amplified product size of 473 base pairs (bp) was digested by the specific restriction enzyme, *Bsr1.* for 16 h at 37˚C. The mutated genotype was digested, in to 428 bp and 45 bp. The wild-type genotype (CC) was not digested, whereas the mutated homozygous genotype (TT) was cut as a doublet of 428 and 45 bp. The heterozygous genotype (TC) was represented as 2 fragments of 473 and 428 bp whereas 45 bp of DNA fragments are run out from the gel because of its too short fragments. The digestion products were then separated by electrophoresis on a 2.5% agarose gel. The results were documented by digital camera and further saved by gel documentation system.

Statistical Analysis
To check the difference in the anthropometric and biochemical parameters of diabetic patients and the non-diabetic controls we used student's t test.

Statistical Analysis of Genotype
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.

Hardy-Weinberg equilibrium

According to Hardy Weinberg law, gene and genotype frequencies in Mendelian population remain constant generation after generation, if there is no migration, mutation, selection or random drift. Since at any autosomal locus, an individual carries two alleles, for example 'A' and 'a' , and if the relative frequency of the 'A' allele in the population is 'P' and 'q' is denoted as the relative frequency of the allele, than the following equation holds true:

$$p2 + 2pq + q2 = 1$$

Results

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. 1. 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. 1).

Table 1. 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. 2, is showing elevated level during infection and it was statistically significant associated as P<0.0001*** 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 2. 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.)*

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 3. Hardy Weinberg Equilibrium Test for both nephrolithiatic patient and Healthy control population.

Gene	X^2 value for Case	X^2 value for control
MGP	**1.7439**	**1.6492**

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

Detection of Genetic Polymorphism in MGP (Matrix Gla Protien) Gene

PCR amplification with specific primers gave 473-bp product which was digested with *BsrI* enzyme (New England Biolabs, Boverly, MA) for 16 h at 37˚C. The wild-type genotype (CC) was not digested, whereas the mutated homozygous genotype (TT) was cut as a doublet of 428 and 45 bp. The heterozygous genotype (TC) was represented as 2 fragments of 473 and 428. bp whereas 45 bp of DNA fragments are run out from the gel because of its too short fragments **(Depicted in figure no. 1.)**

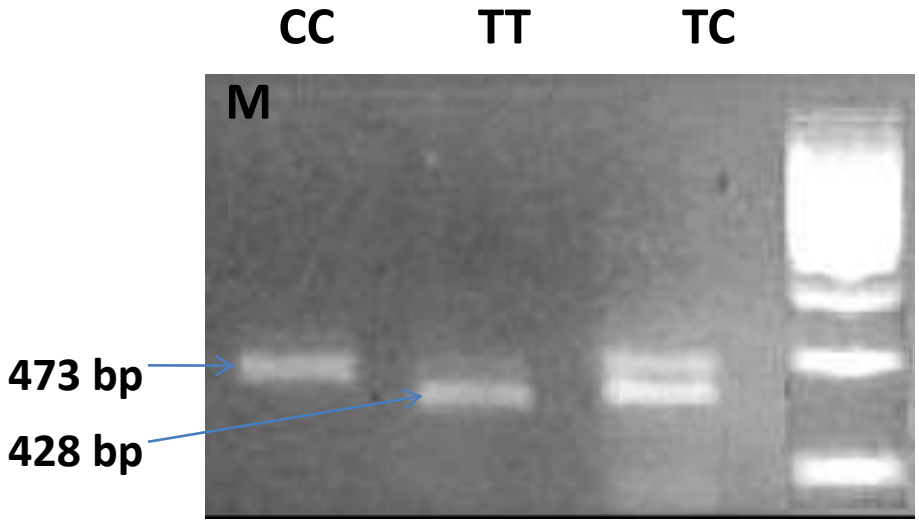


Fig 1: Representative gel picture of MGP T138C polymorphism. Lane M represents 50 bp molecular marker, Lane CC Wild type genotype and Lane TC heterozygous genotype and Lane TT variant genotype.

The distribution of the polymorphisms of MGP was consistent with Hardy- Weinberg equilibrium (HWE) in healthy controls. The observed genotype frequencies, allele frequencies and carriage rates for MGP polymorphism are depicted in table 4.5 and table 4.6 and Graph 1, 2, 3. significant level of change has been seen in overall distribution of MPG genotypes in Healthy control group as compared to disease group although Healthy control group showed little increase in 'TT' genotype as compared to Patients of Nephrolithiatic (69.44% *vs* 56.25%). Similarly, mutant type 'CC' genotype was present in low frequency in Nephrolithiatic patients group 3.75% and also in control group 4.44% (χ^2 = 0.0243*), P= 7.436). '*TT'* genotype is higher in control group and may be protective in our population and statistically significantly different between both groups.

An odds ratio of TT genotype is 0.5657 which indicates little protective effect whereas an odds ratio of TC genotype is 1.887 of Nephrolithiatic patients group respectively indicate little or no effect and association of this mutant genotype with the Nephrolithiatic susceptibility. Overall allele 'C' was found little lower frequency in disease group as compared to HC group whereas allele '*T'* was present in little high frequency in the disease group but the difference is nominal and was not significant (χ^2 = 0.0437*, P=4.069). Carriage rate of allele 'T' was slightly high in nephrolithiatic group as compared to healthy control (75.77% Vs 68.75%) whereas carriage rate of allele '*C*' was approximately similar in both control and disease group and no significant level of change has been seen. The pattern of genotype distribution, allele frequency and carriage rate in disease and control group suggests MPG polymorphism is significantly associated with Nephrolithiatic in our population (See Table No. 4 and 5).

Table 4. Frequency distribution and association of Genotype, allele frequency and carriage rate of MGP gene polymorphism in population of Vindhyan region using Chi Square Test

MGP GENE	CASE N= 160		CONTROL N=180		CHI SQUARE VALUE χ^2 (P Value)
	N	%	N	%	
Genotype					
TT	90	56.25	125	69.44	
TC	64	40.00	47	26.11	
CC	6	3.75	8	4.44	7.436 (0.0243*)
Allele					
T	244	76.25	297	82.50	
C	76	23.75	63	17.50	4.069 (0.0437*)
Carriage Rate					
T	154	68.75	172	75.77	
C	70	31.25	55	24.22	2.774 (0.0958ns)

(- 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 5. Fisher Exact Test values of MGP gene polymorphism

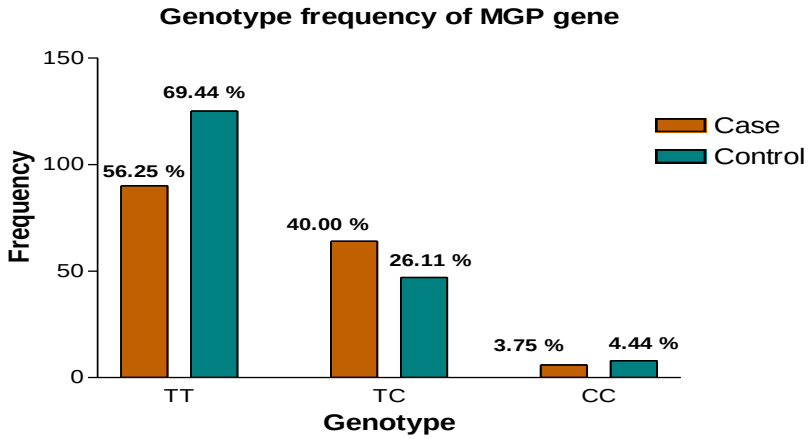
MGP GENE	CASE N= 160		CONTROL N=180		P Value	Odds Ratio (95% confidence interval)
	N	%	N	%		
Genotype						
TT	90	56.25	125	69.44	0.0133*	0.5657 (0.3624 to 0.8830)
TC	64	40.00	47	26.11	0.0077**	1.887 (1.192 to 2.986)
CC	6	3.75	8	4.44	0.7914ns	0.8377 (0.2842 to 2.469)
Allele	244	76.25	297	82.50		0.6810 (0.4683 to 0.9904)
T	76	23.75	63	17.50	0.0459*	1.468 (1.010 to 2.135)
C						
Carriage Rate						
T	154	68.75	172	75.77		0.7035 (0.4646 to 1.065)
C	70	31.25	55	24.22	0.1144ns	1.421 (0.9388 to 2.152)

(* - 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.)*

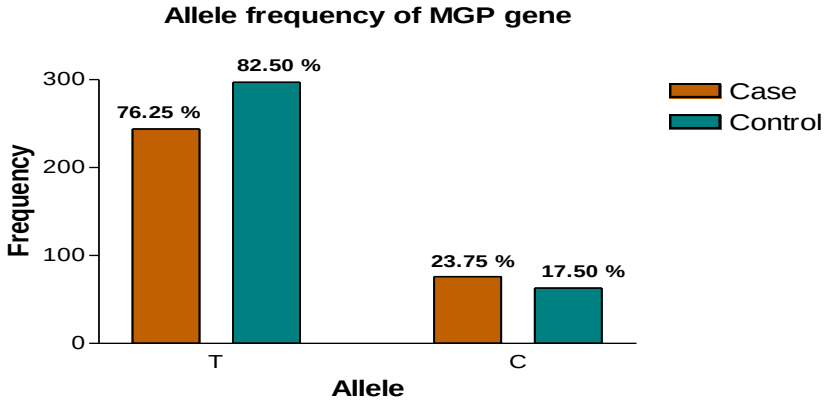
Discussion

Interleukin (IL)-18, a member of the IL-1 superfamily, is a pro-inflammatory cytokine that is structurally similar to IL-1b. IL-18 promotes the production of interferon gamma (IFN-g) and strongly induces a Th1 response. IL-18 drives the same myeloid differentiation factor 88 (MyD88)/nuclear factor kappa B (NF-kB) signaling pathway as IL-1b. In physiological conditions, IL-18 is regulated by the endogenous inhibitor IL-18 binding protein (IL- 18BP), and the activity of IL-18 is balanced. It is reported that in several inflammatory diseases, the IL-18 activity is unbalanced, and IL-18 neutralization by IL-18BP is insufficient. IL-18 acts synergistically with IL-12 to induce the production of IFN-g as a Th1 cytokine, and IL-18 acts alone to induce the production of Th2 cytokines such as IL-4 and IL-13. In addition, IL-18 alone enhances natural killer (NK) cell activity and FAS ligand expression. The biological and pathological roles of IL-18 have been studied in many diseases [17-19]. Our data from cytokine estimation revealed that 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***. 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. Interleukin (IL)-18 was originally discovered as a factor that enhanced IFN- production from anti-CD3-stimulated Th1 cells, especially in the presence of IL-12. Upon stimulation with Ag plus IL-12, naïve T cells develop into IL-18 receptor (IL-18R) expressing Th1 cells, which increase IFN- production in response to IL-18 stimulation. Therefore, IL-12 is a commitment factor that induces

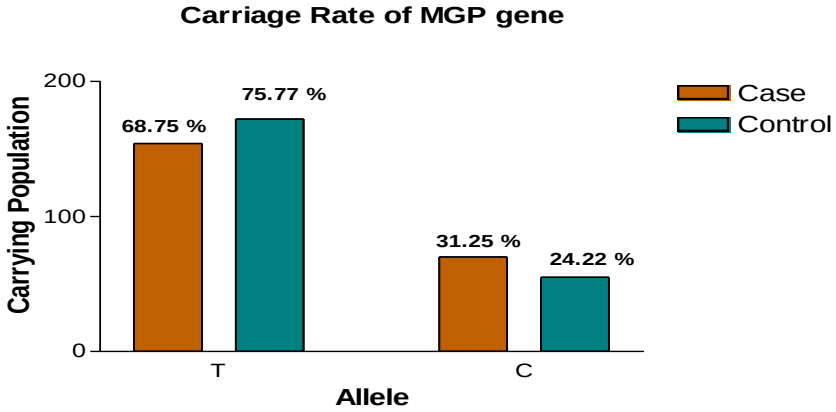
the development of Th1 cells. In contrast, IL-18 is a proinflammatory cytokine that facilitates type 1 responses [19,20]. However, IL-18 without IL-12 but with IL-2, stimulates NK cells, CD4+ NKT cells, and established Th1 cells, to produce IL-3, IL-9, and IL 13. Furthermore, together with IL-3, IL-18 stimulates mast cells and basophils to produce IL-4, IL-13, and chemical mediators such as histamine. Therefore, IL-18 is a cytokine that stimulates various cell types and has pleiotropic functions. IL-18 is a member of the IL-1 family of cytokines. IL-18 demonstrates a unique function by binding to a specific receptor expressed on various types of cells. In this review article, we will focus on the unique features of IL-18 in health and disease in experimental animals and humans [21-23].



Graph 1: Genotype Frequency of MGP gene.



Graph 2: Allele Frequency of MGP gene.



Graph 3: Carriage rate of MGP gene.

Association between the MGP gene rs1800801, rs1800802, rs4236 polymorphisms and vascular calcification and atherosclerotic disease was inconsistent. To clarify precise association, we performed this meta-analysis. A total of 23 case-control studies, consisting of 5280 cases and 5773 controls, were included [15,24]. The overall results suggested that the -7A polymorphism was associated with an increased risk for vascular calcification and atherosclerotic disease in the recessive model. Subgroup analyses of Caucasians showed significant associations in the allelic model, recessive model, and homozygote model: allelic model (OR = 1.19, 95% CI 1.06–1.34, P = 0.004), recessive model (OR = 1.60, 95% CI 1.26–

2.03, P < 0.001), homozygote model (OR = 1.83, 95% CI 1.18–2.81, P = 0.006). Subgroup analysis of the Asian population did not demonstrate any significant associations in any of the genetic models. No significant association was found in any genetic model amongst the rs1800802 and rs4236 polymorphisms. The findings of this meta-analysis indicate that the MGP gene rs1800801 polymorphism is significantly associated with vascular calcification and atherosclerotic disease, especially in the Caucasian population [25-29].

Our statistical data revealed PCR amplification with specific primers gave 473-bp product which was digested with *BsrI* enzyme (New England Biolabs, Boverly, MA) for 16 h at 37˚C. The wild-type genotype (CC) was not digested, whereas the mutated homozygous genotype (TT) was cut as a doublet of 428 and 41 bp. The heterozygous genotype (TC) was represented as 2 fragments of 473 and 428. bp whereas 41 bp of DNA fragments are run out from the gel because of its too short fragments. The distribution of the polymorphisms of MGP was consistent with Hardy- Weinberg equilibrium (HWE) in healthy controls. The observed genotype frequencies, allele frequencies and carriage rates for MGP polymorphism are depicted in table 4.5 and table 4.6 and Graph 1, 2, 3. significant level of change has been seen in overall distribution of MPG genotypes in Healthy control group as compared to disease group although Healthy control group showed little increase in 'TT' genotype as compared to Patients of Nephrolithiatic (69.44% *vs* 56.25%). Similarly, mutant type 'CC' genotype was present in low frequency in Nephrolithiatic patients group 3.75% and also in control group 4.44% (χ^2 = 0.0243*), P= 7.436). '*TT'* genotype is higher in control group and may be protective in our population and statistically significantly different between both groups. An odds ratio of TT genotype is 0.5657 which indicates little protective effect whereas an odds ratio of TC genotype is 1.887 of Nephrolithiatic patients group respectively indicate little or no effect and association of this mutant genotype with the Nephrolithiatic susceptibility. Overall allele 'C' was found little lower frequency in disease group as compared to HC group whereas allele '*T'* was present in little high frequency in the disease group but the difference is nominal and was not significant (χ^2 = 0.0437*, P=4.069). Carriage rate of allele 'T' was slightly high in nephrolithiatic group as compared to healthy control (75.77% Vs 68.75%) whereas carriage rate of allele '*C*' was approximately similar in both control and disease group and no significant level of change has been seen. The pattern of genotype distribution, allele frequency and carriage rate in disease and control group suggests MPG polymorphism is significantly associated with Nephrolithiatic in our population. Matrix Gla protein, a potent calcification inhibitor in arterial vessels, is also expressed in the kidney and is up-regulated following the administration of ethylene glycol, a precursor of oxalate. Considering the analogous characteristics between arterial calcification and kidney stones, we identified variants of the human matrix Gla protein gene and investigated whether there is an association between [21,30]. MGP genetic polymorphisms and kidney stones. The single nucleotide polymorphisms in matrix Gla protein in 122 kidney stone cases and 125 controls. A single nucleotide polymorphism was associated with kidney stones (OR 0.51, 95% CI 0.30–0.87; p =0.012). The G allele carrier had a 2-fold decreased kidney stone risk compared with A allele carriers in single nucleotide polymorphism 11 (OR 0.55, 95% CI 0.31–1.00, p =0.047). We found no association between the polymorphism and kidney stone clinical characteristics. An *MGP* gene polymorphism is associated with kidney stones and influences genetic susceptibility to kidney stones. In the future functional assays of the polymorphism should permit better understanding of the role of matrix Gla protein genetic variants and kidney stones [31-33].

Conclusion

MGP gene polymorphism is associated with kidney stones and influences genetic susceptibility to kidney stones. In the future functional assays of the polymorphism should permit better understanding of the role of matrix Gla protein genetic variants and kidney stones [31-33].

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