Study of rs17251221 single nucleotide polymorphism of calcium sensing receptor gene and its association with calcium nephrolithiasis in a cohort of Egyptian calcium nephrolithiasis patients.

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Introduction:
Calcium nephrolithiasis is the most common type of renal stones representing about 70-80% of all cases. (Reynolds, 2005) Since calcium stones are a multifactorial disorder, both genetic and environmental factors predispose to disease occurrence. Environmental factors including lifestyle, obesity, and dietary habits are suggested as significant elements that seem to participate in stone development. (Leaf 2010; Coe et al., 2005)

Urine supersaturation is essential for stone formation, therefore, the physiological processes that influence calcium delivery to the kidney may influence stone formation. Alteration in calcium homeostasis is considered the most important risk factor for calcium nephrolithiasis. (Cole et al., 2009).

Genetic polymorphisms of the CASR, vitamin D receptor, and osteopontin genes are reported to be highly associated with stone formation in many studies. (Vezzoli et al., 2011; Ferreira et al., 2010; Gao et al., 2007)

The human calcium sensing receptor (CASR) gene located on chromosome 3q13.3-q21.1 encoding a protein of 1078 amino acids, is approximately 103 kb in length and contains 8 exons and 2 functional promoters. (Cole et al., 2009; Rodriguez et al., 2005).
rs17251221 polymorphism of the CASR gene is located in an intron of the CASR gene and is considered a gain of function mutation. (Bai et al., 1998).

The CASR is a member of subfamily C of G protein-coupled receptors (Brauner-Osborne et al., 2001) which is expressed in the parathyroid hormone-producing chief cells of the parathyroid gland and the cells lining the kidney tubule. It can sense any minor changes in circulating calcium concentration and through intracellular signaling pathways modifies parathyroid hormone secretion or renal cation handling to maintain physiological calcium homeostasis. (Hendy et al., 2000).

In the kidney, CASR prevents kidney stone formation through banning the reabsorption of divalent cations in the thick ascending limb and triggering the inhibitory actions of hypercalcemia on the urinary-concentrating mechanism, thus leading to the prevention of kidney stone formation. (Brown et al., 1998; Renkema et al., 2009)

The present study aimed at investigating whether CASR gene polymorphism rs17251221 is associated with the development of calcium nephrolithiasis in a cohort of Egyptian calcium nephrolithiasis patients.

Materials and Methods:-
This study was approved by the Ethics Committee of the Faculty of Medicine, Alexandria University. Consent was obtained from all the participants in the study.
The current study was conducted on 80 subjects divided into 2 groups. The first group included 40 Egyptian patients with calcium nephrolithiasis who were consecutively recruited from Urosurgery Department of Alexandria Main University Hospital-Egypt.

All the studied patients were subjected to full history taking and clinical examination. All patients with renal stones were also subjected to routine imaging, plain x ray abdomen and pelvis, ultrasound abdomen and pelvis. Non-contrast CT abdomen and pelvis were performed for all included patients to diagnose accurately the stone location, type and multiplicity.

All stones were retrieved by either percutaneous nephrolithotripsy (PCNL), or by open surgery in the form of pyelolithotomy or pyelonphrolithotomy, then referred to chemical analysis to be proven as calcium stones. Radiolucent stones were excluded by imaging and other types of stones other than calcium stones were excluded after chemical stone analysis.

Patients were excluded if they had a history of chronic urinary tract infection, renal failure, chronic diarrhea, gout, renal tubular acidosis, regular intake of diuretics, vitamin D, or calcium supplements more than or equal to one time per week within the previous six months. All participants had a normal parathyroid hormone level. The second group included 40 healthy individuals with matched age and sex who served as a control group. They had no past history or family history of calcium nephrolithiasis.

Serum creatinine, calcium, phosphorus and uric acid were assessed for all those participating in the study. Random urine samples were collected from every participant in a sterile container and used for complete urine analysis and also for assessment of creatinine, calcium, phosphorus and uric acid. All urinary parameters were corrected using urinary creatinine levels. All participants in the study were Egyptians living in the northern region of Egypt.

Detection of CASR gene SNP rs17251221:–
Genomic DNA was extracted from whole blood EDTA samples using PureLink® Genomic DNA Kit (Life Technologies, CA, USA) as instructed by the manufacturer. Concentration and purity of DNA were assessed using NanoDrop ND-1000 spectrophotometer (Thermoscientific, USA).

The single nucleotide polymorphism rs17251221 within calcium sensing receptor gene was detected in the present study by allelic discrimination using 5’ nuclease assay, on the Mx3000P™ Real-Time PCR System (Stratagene, CA, USA).

Calcium Sensing Receptor Gene assay mix (rs17251221) were purchased from Applied Biosystems, CA, USA; Assay ID C_32771445_10. Amplification was carried out in 20 uL reaction volumes containing TaqMan® Master Mix (Applied Biosystems, Foster City, CA, USA) and 1 uL of primers/probes mixes. DNA was added in a concentration of 1–20 ng per reaction.

The thermal profile included an initial denaturation at 95°C for 10 minutes, followed by 45 cycles of denaturing at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute.
Statistical analysis:
Data were analyzed using IBM SPSS software package version 20.0. Comparison between different groups regarding categorical variables was tested using Chi-square test. For normally distributed data, comparison was done using independent t-test. For abnormally distributed data, comparison was done using Mann Whitney test, statistical significance was considered at $p<0.05$.

Results:
The mean age of calcium nephrolithiasis patients was 47.03 ± 10.64 years. Sixty-five percent (n=26) of calcium nephrolithiasis patients were males with a male to female ratio of 1.9:1. No statistically significant difference was detected between both groups as regards age ($p=0.333$) or gender ($p=0.115$).

No statistically significant difference in serum creatinine ($p=0.103$), uric acid ($p=0.07$), calcium (0.706) or phosphorous ($p=0.998$) was noticed on comparing both studied groups.

A statistically significant increase in urinary calcium ($p<0.001$) and phosphorus ($p<0.001$) was detected among patients with calcium nephrolithiasis, while no significant difference was observed as regards urinary uric acid ($p=0.243$).

Chemical analysis of calcium stones showed diverse stone composition form one patient to another. Table 1.

Most of the studied patients (60%) had single stone, with 85% of studied nephrolithiasis patients having stones for the first time. Family history of renal stones was detected in only 25% of cases.

As the GG genotype was detected in only two participants, those carrying the G allele were gathered in one group (GG+GA).

There was no statistically significant difference between the two studied groups as regards the genotypes or allele frequency. Table 2.

On studying the association of rs17251221 CASR gene polymorphism and different patients' characteristics including family history of stones, number of stones and history of recurrent stones. Asignificant association was observed between the gene polymorphism rs17251221 and stone multiplicity ($p<0.001$). No statistically significant difference was detected as regards the relation between genotype and stone frequency ($p=0.067$), family history of stone disease ($p=0.066$) or stone type ($p=0.290$).

No significant association was detected between the CASR SNP rs17251221 and the studied biochemical data (serum and urine parameters) among cases or control group.

Discussion:
The estimated lifetime risk of urolithiasis is elevated up to 25% in the middle east representing a considerable health problem (Pak, 1998) particularly owing to the high recurrence rate in 20-75% of cases within ten years of the first disease occurrence (Basiri et al., 2010), thus imposing a social impact in addition to the influence on patients' quality of life.

As it was reported that calcium stones are the most prevailing cause of urolithiasis in up to 80% of cases (Moe 2006), serum calcium modifiers are considered important calcium urolithiasis risk factors. (Vezzoli et al., 2010)

Gain or loss of function genetic polymorphisms of CASR gene are reported to play a substantial role in calcium homeostasis, thus the current study aimed to investigate the potential association of one of CASR gene polymorphisms; rs 17251221, with the development of calcium nephrolithiasis in a cohort of Egyptian calcium nephrolithiasis patients.

In the present study, a statistically significant increase in urinary calcium and phosphorus was detected in cases with calcium nephrolithiasis in association with normal serum calcium and phosphorus.
The pathophysiological factors behind development of calcium urolithiasis are diverse, still, most common metabolic risk factor encountered in cases of calcium stone formation is urine supersaturation by crystals including cases of hypercalciuria resulting from alteration in calcium homeostasis that was reported by many studies to be either idiopathic, thus associated with normal calcium serum level, or secondary to other medical conditions as bowel diseases or genetic disorders of oxalate metabolism. (Coe et al., 2005) It was even noticed that lowering urinary calcium excretion reduces the rate of stone recurrence. (Coe et al., 2000)

In the current study, phosphaturia might be explained by the diversity in chemical stone composition with many combined calcium/phosphate stones or by the potential change of renal phosphate threshold in stone formers. (Prié et al., 2001)

A study by Prié et al. (Prié et al., 2001) measured renal phosphate threshold in 207 stone formers with normal parathyroid hormone (PTH) serum concentration and in 105 healthy control subjects. The study reported that a low renal phosphate threshold was more frequently detected in stone formers than in control subjects and was associated with a high urinary Ca excretion.

In the present study, most of nephrolithiasis patients (85%) were presented for the first time by a calcium stone. Stone recurrence rates have been shown to be diminished by 50% or more via proper treatment and dietary interventions, thus suggesting that recurrent stone disease is preventable. (Borghi et al., 2002)

Being a multifactorial disease, 25% of the studied cases had a positive family history of stones emphasizing on the role of genetic background behind the disease pathogenesis.

rs17251221 SNP of CASR gene had been associated to higher serum calcium levels in a previous study through regulation of parathyroid hormone secretion and calcium reabsorption. (O’Seaghdha et al., 2010) In addition, it has been associated to various cancers as cancer prostate (Jorde et al., 2013) and cancer breast. (Li et al., 2014)

In the current study, the frequency of the mutant G allele detected in Egyptian Calcium nephrolithiasis patients exceeded that revealed in controls (21.3% vs 16.3%).

Li et al. (Li et al., 2014) reported a prevalence of the minor G allele to be higher in Chinese breast cancer patients (25.81%) than in the controls (15.15%) using 5’ Nuclease assay in SNP genotyping analysis. The GG genotype was carried by only 0.43% of control participants and 4.15% of the cancer breast patients, while the heterozygous form was carried by 14.72% of healthy volunteers and 21.66% of cases.

Chou et al. (Chou et al., 2011) genotyped CASR polymorphism rs17251221 in 480 Taiwanese participants including 189 calcium nephrolithiasis patients and 291 healthy controls. The study used TaqMan allelic discrimination assay for CASR polymorphism genotyping and reported a prevalence of the mutant G allele of 4.0% in patients and of 3.1% in the healthy volunteers with only one patient carrying the homozygous GG genotype and the rest carrying the heterozygous one.

Kapur et al. (Kapur et al., 2010) in their genome-wide association study of serum calcium, reported a frequency of the minor G allele of 19.24% in participants of European and Indian-Asian descent.

The difference in frequency of CASR polymorphism rs17251221 from one population to another reflects ethnic variation due to diverse genetic background.

Although no association was detected in the present study between the rs17251221 CASR SNP and occurrence of calcium nephrolithiasis or between the gene polymorphism and various patients’ characteristics including family history of stones or history of recurrent stones, a significant association was observed between rs17251221 and stone multiplicity.

In accordance with the current work, the study of Chou et al. (Chou et al., 2011) reported a significant association between rs17251221 and stone multiplicity with higher risk of stone multiplicity in those carrying the mutant G allele, while no association was detected between rs17251221 and the susceptibility to calcium nephrolithiasis.
No significant association was detected between the CASR SNP rs17251221 and all studied biochemical data (serum and urine parameters) among cases or control group participating in the current work.

A study by O’Seaghdha et al (O’Seaghdha et al., 2010) investigated in their genome-wide association studies the common genetic variants associated with serum calcium levels in 20,611 participants. The study concluded that the G allele of rs17251221 was associated with higher calcium level, higher serum magnesium levels and lower serum phosphate levels.

The different results detected in the present study may be attributed to smaller sample size compared to the study of O’Seaghdha et al. (O’Seaghdha et al., 2010)

The novelty of the current study is mainly due to the investigation of rs17251221 polymorphism in Egyptian calcium nephrolithiasis patients.

### Table 1 Distribution of the studied cases according to chemical composition of calcium stones.

<table>
<thead>
<tr>
<th>Stone composition</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca oxalate</td>
<td>13</td>
<td>32.5</td>
</tr>
<tr>
<td>Ca phosphate</td>
<td>3</td>
<td>7.5</td>
</tr>
<tr>
<td>Ca oxalate, phosphate</td>
<td>8</td>
<td>20.0</td>
</tr>
<tr>
<td>Ca oxalate, carbonate</td>
<td>3</td>
<td>7.5</td>
</tr>
<tr>
<td>Ca oxalate, phosphate, carbonate</td>
<td>6</td>
<td>15.0</td>
</tr>
<tr>
<td>Ca oxalate, uric acid</td>
<td>5</td>
<td>12.5</td>
</tr>
<tr>
<td>Ca oxalate, phosphate, uric acid</td>
<td>2</td>
<td>5.0</td>
</tr>
</tbody>
</table>

### Table 2 Comparison between the two studied groups according to genotype and allele frequency

<table>
<thead>
<tr>
<th></th>
<th>Cases (n = 40)</th>
<th>Control (n = 40)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td><strong>Genotype</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA+GG</td>
<td>17</td>
<td>42.5</td>
<td>11</td>
</tr>
<tr>
<td>AA</td>
<td>23</td>
<td>57.5</td>
<td>29</td>
</tr>
<tr>
<td><strong>Allele frequency</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>17</td>
<td>21.3</td>
<td>13</td>
</tr>
<tr>
<td>A</td>
<td>63</td>
<td>78.8</td>
<td>67</td>
</tr>
</tbody>
</table>

χ²: Value for Chi square  
MC: Monte Carlo test

**Conclusion:**
Although no association was detected between calcium sensing receptor genes rs17251221 polymorphism and development of calcium nephrolithiasis, the mutant G allele of the polymorphism might be considered a marker for stone multiplicity in Egyptian calcium nephrolithiasis patients.

**Author Disclosure Statement:**
No conflicts of interest to be reported.
References: