SINGLE-NUCLEOTIDE POLYMORPHISMS OF CALCIUM-SENSING RECEPTOR ENCODING GENE ASSOCIATED WITH CALCIUM KIDNEY STONE DISEASE IN BABYLON PROVINCE

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INTRODUCTION

Kidney stone could be formed a hard crystal accumulation from nutritional minerals within the kidneys when minerals in urine are in high concentration. The stones are typically aching related to hematuria and harm to kidney tissue and may be developed a renal failure [1]. The formation of most kidney stones is a multifactorial illness resulting from the mutual effect of epidemiologic, organic chemistry, environmental, and hereditary risk factors [2]. Threat factors include high urine calcium levels, calcium supplements, certain foods, obesity, hyperparathyroidism, gout, some medications, and not drinking enough fluids. The calcium-sensing receptor (CASR) is a member of Class C or III that associated with G-protein-coupled receptor. It is chiefly expressed in the parathyroid gland and the renal tubules of the kidney. It has a potential role to sense the change in the extracellular levels of calcium ion in these two organs. It regulates the parathyroid hormone (PTH) secretion and renal tubular calcium reabsorption. Inactivating and activating CASR gene due to mutations severally caused hypercalcemia or hypocalcemia disorders. The aim of the study was to investigate the risk factor of CASR rs1801725 (Ala896Ser) patients with renal disease.

METHOD

The blood samples were collected from 100 patients and divided into two groups, each one containing 50 samples; chronic kidney disease and end-stage renal disease, who admitted Mjeran Teaching Hospital in Babylon Province, Iraq, from February to July 2016. In addition, healthy persons as a control group (50 samples). Genotyping of CASR single-nucleotide polymorphisms (SNP) was performed using a polymerase chain reaction technique, followed by single-strand conformation polymorphism. Accordingly, these DNA polymorphisms were confirmed using DNA sequencing.

RESULTS

The conformational haplotypes of CASR, exon7 NCBI Primer3plus reference were obtained in three patterns, including two, three, and four bands, due to the presence SNPs within the studied region. These SNPs leads to change three amino acid residues of CASR, including amino acid substitutions were Ala 128→ Ser 128, Leu 155→ Tye 155, and Leu 156→ Ser 156 that may affect or modified the tertiary structure of the receptor, subsequently the function like the affinity to calcium ion may be effected.

Conclusion: These results suggest that the variants of CASR SNPs namely, rs1801725 might be involved in susceptibility to kidney stone disease.

Keywords: Chronic and end-stage renal disease, Polymerase chain reaction, rs1801725, Calcium-sensing receptor, Single-nucleotide polymorphisms.

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who admitted to Merjan Teaching Hospital (Dialysis Center), Babylon Province. All the participants were evaluated by a certified physician, who was also responsible for getting the most analytical and clinical values. The patients with hepatitis were excluded.

Genotypic identification using single-strand conformation polymorphism (SSCP)-polymerase chain reaction (PCR) amplification

The genotyping of the study groups was performed using the SSCP-PCR technique after DNA extraction from blood samples. DNA of blood was extracted and purified using extraction and purification kit from Geneaid company (UK).

The targeted sites of DNA were amplified using design specific primers which used to identify CASR (rs1801725), obtained from Bioneer, IDT DNA (USA). Forward primer: 5’-AGCCCAGATGCAAGCAGAAG-3’, and reverse primer: 5’-CAGACCTGTTTCCGAGG-3’.

PCR was carried out in 20 µl reaction volumes containing 1 µl from each forward and reverse primer, 12.5 µl of Green Master Mix, 3 µl of Genomic DNA, and the reaction volume was completed up to 20 µl by adding 2.5 µl of the nuclease-free water. Amplification was carried out in a thermocycler (Biometra, Germany) programmed as pre-denaturation for 2 min at 94°C; 30 cycles, each cycle the denaturation for 5 min at 94°C, annealing 1 min at 64°C, extending for 1 min at 72°C; and a final extension of 5 min. PCR products were electrophoresed using gel electrophoresis (Cleaver scientific – UK) in 1% agarose at 75 V for one hand visualized by ethidium bromide. Photos were taken using gel documentation system) Cleaver Scientific –UK).

The sharp and obvious bands were found after performing electrophoresis regarding exon7 of the CASR PCR fragment. These amplified fragments are subsequently suitable for downstream SSCP tests.

All the obtained SSCP gels were aligned with each other to show how many haplotypes were, two types of SSCP band patterns were observed in SSCP gels. The single-stranded (ssDNA) DNA bands, which occupy the upper portion of the gel and the double-stranded (dsDNA), which occupies the lower portion of the gel was observed. The variation of ssDNA in SSCP gels is relied on to identify the genetic pattern of each amplicon, as shown in Fig. 3.

After the amplification of the target site, conformational polymorphism of the exon7 region of the CASR gene using the PCR-SSCP method. All the obtained SSCP gels were aligned with each other to show how many haplotypes obtained. The results revealed the presence of the three haplotypes of SSCP band patterns were observed in SSCP gels. The single-stranded (ssDNA) DNA bands, which occupy the upper portion of the gel and the dsDNA, which occupies the lower portion of the gel was observed. The variation of ssDNA in SSCP gels is relied on to identify the genetic pattern of each amplicon, as shown in Fig. 3.

All the statistical analyses were done with the SPSS statistical software (version 17.0; SPSS Inc., Chicago, IL), p<0.05 was considered statistically significant.

RESULTS

The DNA was extracted from the blood sample as a first step to isolate the genomic DNA of patients and healthy control. The Fig. 1 revealed the gel electrophoresis of genomic DNA (1% agarose, 75 V; 20 mA for 1 h).

For CASR genotyping using the RCR-SSCP method, the genomic DNA of renal patients and healthy control groups was amplified using specific primers, which were designed according to the exon7 region of Homo sapiens reference ID: DQ327728.1 (https://www.ncbi.nlm.nih.gov/nuccore/DQ327728.1).

The genomic DNA amplification was accomplished by the thermocycler apparatus under the optimal conditions. The results revealed that the presence a single band (221 bp) of the target sequence of an exon7 region of CASR in agarose gel (Fig. 2), which contains a risk region (rs rs1801725).
Electrophoresis conditions: 8% polyacrylamide gel concentration; 200V (7.5V/cm) – 100 mA, time to run: 90–120 min. Staining method; ethidium bromide.

The results of SSCP gel showed that the presence of different conformational DNA polymorphisms according to the number of the bands constitutes two (homozygous), three bands (homozygous), and four bands (heterozygous) (Fig. 3).

The results showed that conformational polymorphism distributions among the haplotypes were homozygous (2-bands) 38%, homozygous (3-bands) 50%, and heterozygous (4-bands) 12%, respectively, in CKD group. Whereas, conformational haplotypes in ESRD group were homozygous (2-bands) 28%, homozygous 60%, and heterozygous (4-bands) 12%, respectively, in comparison with the healthy control group was 18% homozygous, 62% homozygous (3-bands), and 20% heterozygous (4-bands), respectively. The results demonstrate that there is an association between DNA polymorphisms according to the number of bands with patients as compared with the control groups, as shown in Tables 1 and 2.

### Table 1: Conformational haplotype polymorphism distribution of CASR gene by the number of bands and their association with CKD and control groups

| Conformational haplotype polymorphism | n (%)     | p value | OR     | 95% CI      |
|--------------------------------------|-----------|---------|--------|-------------|
|                                       | CKD group | Control group |       |             |
| Homozygous 2-bands                  | 19 (38)   | 9 (18)  | 0.037  | 2.61  1.01-6.78 |
| Homozygous 3-bands                  | 25 (50)   | 31 (62) | 0.05   | 3.51  0.97-12.72 |
| Heterozygous 4-bands                | 6 (12)    | 10 (20) | 0.41   | 1.34  0.42-4.20 |
| Total number                         | 50        | 50      |        |             |

*aReference; *p≤0.05, OR=95% CI

### Table 2: Conformational haplotype polymorphism distribution of CASR gene by the number of bands and their association with end-stage renal disease and control groups

| Conformational haplotype polymorphism | n (%)     | p value | OR     | 95% CI      |
|--------------------------------------|-----------|---------|--------|-------------|
|                                       | ESRD group | Control group |       |             |
| Homozygous 2-bands                  | 14 (28)   | 9 (18)  | 0.23   | 1.60  0.60-4.26 |
| Homozygous 3-bands                  | 30 (60)   | 31 (62) | 0.13   | 3.88  0.69-9.64 |
| Heterozygous 4-bands                | 6 (12)    | 10 (20) | 0.56   | 1.62  0.52-4.99 |
| Total number                         | 50        | 50      |        |             |

*aReference; *p≤0.05; OR=95% CI

### Table 3: The association of PCR-SSCP polymorphisms of CASR gene in renal disease groups with physiological parameter

| PCR-SSCP haplotypes band (n) | Mean±SE            | p value | OR     | 95% CI      |
|------------------------------|---------------------|---------|--------|-------------|
| ESRD                         | Urea (mg/dl)        | Creatinine (µmol/l) | Calcium (mmol/l) |
| Homozygous 2-bands (n=14)    | 26.9±1.36           | 427.5±7.07 | 0.90±0.082 |
| Homozygous 3-bands (n=30)    | 24.6±1.20           | 448.4±12.44 | 1.85±0.091 |
| Heterozygous 4-bands (n=6)   | 17.4±2.69           | 428.0±12.76 | 1.85±0.07 |
| p value                      | 0.018*              |         | 0.44   |             |
| CKD                          | Urea (mg/dl)        | Creatinine (µmol/l) | Calcium (mmol/l) |
| Homozygous 2-bands (n=19)    | 25.9±1.66           | 438.1±9.86 | 2.24±0.17 |
| Homozygous                   | 26.5±1.58           | 428.2±5.22 | 2.17±0.08 |
| Heterozygous 4-bands (n=6)   | 26.1±0.46           | 456.1±13.01 | 2.16±0.20 |
| p value                      | 0.96                |         | 0.18   |             |

n: Patient number, CKD: Chronic kidney disease, ESRD: End-stage renal disease, P≤0.05, the results represent as mean±SE, SE: Standard error

Table 3 summarizes to the present significant differences (p≤0.05) in the serum levels of urea, creatinine, and calcium among 2, 3, and 4 bands for the CASR gene in end-stage renal disease groups, while there are no significant differences in the concentrations of calcium, creatinine, and urea between 2, 3, and 4 bands of the CASR gene in CKD groups.

However, it might be difficult to determine the pattern of all resolved SSCP bands using only the gel visualization. Accordingly, these DNA polymorphisms must be confirmed using sequencing. The sequencing results observed that many SNPs between the one resolved haplotypes and between the CASR exon7 for Primer3plus reference sequences. The results appeared in the presence of two SNPs (Fig. 4) which revealed that which located at position 81 (C/T) as a substitution mutation and the second was a deletion mutation (T) at the location 163 according to the reference sequence alignment of the human CASR gene ID: DQ327728.1 (https://www.ncbi.nlm.nih.gov/nuccore/DQ327728.1).

When translating the DNA sequence using by BioEdit program version 7.2.5, according to the reference sequence alignment of the
human CASR gene ID: DQ372728.1. The results appeared change three amino acid residues of CASR protein as shown in Figs. 5 and 6. These amino acid substitutions were Ala 128→ Ser 128, Leu 155→Tyr 155, and Leu 156→ Ser 156 that may affect or modified the tertiary structure of the receptor; subsequently, the function like the affinity to calcium may be affected or inhibit reabsorption on tubular calcium and PTH excretion [Fig. 6].

DISCUSSION

KSD could be a communal and a complicated impressive clinical circumstance an outsized load on economic the healthcare systems. Hypercalcuria is the main danger issue for the progress of a stone containing calcium. A change of kidney systems of calcium eliminations is the foremost cause for hypercalcuria. The G-protein-coupled CASR as a receptor is expressed within the cell basolateral shows essential roles within the directive secretion of PTH and excretory organ canicular Ca biological process in reaction to Ca blood levels [14]. The CASR reduction calcium reabsorption renal tubules and hypercalcuria caused by defeating the action of calcium potassium sublimate channels. The gene encoding CASR is additionally controlled by VDR, and raise levels of VDR are determined in hypercalcuric genetic variation forming stone rats are involved within the multiplied urinary organ. The CASR informational RNA induced by one, 25 dihydroxy aliment D3 and Leu 156→ Ser 156 that may affect or modified the tertiary structure of the receptor and thereby influencing its function or affects on protein stability [15]. The mutation of CASR factor an activating and inactivating source of the complex and could affect the regulation of the cytosolic part of ions in tissues or blood. These results resemble the structural modulation of other proteins, particularly the receptors which leads to modulation or dysregulation of receptors through an increase in inorganic precipitation of phosphate within the kidney and created stones.

Finally, the change of some amino residues of receptor protein due to SNPs formation in exons of the encoding gene may change or modulate the function of receptor or dysregulation of the homeostasis of calcium concentrations were in individuals carrying 986Ser (T) allele subjects. To modulation or dysregulation of receptors through an increase in polar residues rises the Van der Waals force, which could be the donating factor to increase in free energy and thereby to reduce the stability. The SNP incidence interferences with the overall conformation of the complex and could affect the regulation of the cytosolic part of the receptor and thereby influencing its function or affects on protein stability [19,20].

CONCLUSION

These results suggest that the variants of CASR SNP, namely, rs1801725 might be involved in susceptibility to KSD.

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