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Original Article

Molecular assessment of calcium-sensing receptor gene polymorphism rs1801725 in Iraqi women with osteoporosis

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Abstract

Calcium-sensing receptor (CaSR) gene polymorphism A986S (rs1801725) is a genetic factor of the calcium homeostasis and susceptibility of osteoporosis. Although, its role in premenopausal and postmenopausal women with osteoporosis is yet to be investigated. Therefore, this study was conducted to assess the CaSR gene polymorphism A986S and evaluate its correlation with biochemical parameters in premenopausal and postmenopausal Iraqi women with osteoporosis. Blood samples were obtained from 100 women (53 premenopausal and 47 postmenopausal) diagnosed with osteoporosis by specialist physicians and 70 healthy women of the same age as the control group. Serum calcium and phosphorus concentrations were estimated. Genomic DNA was extracted from the whole blood and used for polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis to detect CaSR A986S polymorphism. The results obtained showed that calcium and phosphorus levels were significantly lower (p value < 0.01) in the osteoporosis women compared with healthy groups. Frequencies of T allele and TT genotype were significantly higher (p value < 0.01) in the osteoporosis patients compared with controls, while there were no significant differences in the frequencies of the patient group. Conversely, there were no differences in the calcium and phosphorus levels and there was the presence of T allele of the CaSR A986S genotypes. Thus, our finding revealed that the CaSR polymorphism A986S was one of the genetic susceptibility factors for the premenopausal and postmenopausal in Iraqi women with osteoporosis and had little effects on mineral levels.

Keywords: CaSR gene polymorphism A986S, Osteoporosis, Iraqi Women

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Introduction

Osteoporosis is a disease accompanied by decreasing bone mass, defects in the microarchitecture of the bone tissue and a raised risk of fragility fractures (Mondockova et al., 2018). It causes 8.9 million fractures each year worldwide (Pouresmaeili et al., 2018). The disease is relatively common in women, especially in post-menopause. About one-third of women over fifty years of age suffer from fragility fractures (Zhu et al., 2017). The relationship between genetic and environmental aspects is central to the

etiology of osteoporosis (Kanwar et al., 2014). The environmental risk factors such as weak calcium intake, exercise, body mass index, menopause and smoking play a central role in osteoporosis development (Moura et al., 2014; Sassi et al., 2015). Also, the genetic aspect has a strong effect on bone formation and several important genes correlated with bone mineral density (Young et al., 2003). The single nucleotide polymorphism (SNP) is the most significant genetic factors examined in association researches (Hosseinpanah et al., 2014). SNP plays an active role in the development of osteoporosis (Al-Azzawie et al., 2020) and some SNPs in candidate osteoporosis genes involved in bone physiology (Boroňová et al., 2014).

The human chromosomal region 3q13.3–21 contains CaSR gene (Tang et al., 2014). This gene contains about 24000 genetic variants including germline and somatic mutations as well as SNPs (Masvidal et al., 2017). Clinically, mutations of the CaSR gene result in either hypercalcemia (if the receptor is inactivated) leading to hypocalciuric hypercalcemia in the family. Alternatively, it can also lead to hypocalcemia when the receptor is stimulated to yield a hypersensitive receptor that results in autosomal-dominant hypocalcemia (Yan et al., 2015; Rasmussen et al., 2018). Recent studies have revealed a correlation between CaSR gene SNPs and serum calcium levels (Majid et al., 2015). The exon 7 of CaSR gene comprises three SNPs which include Ala986Ser (rs1801725, A986S, base change 2956G >T), Gln1011Glu (Q1011E, base change 3031C > G, rs1801726) and Arg990Gly (rs1042636, R990G, base change 2968A > G) (Di Nisio et al., 2018). From these SNPs, the A986S (2956G > T) induces a shift in amino acid from alanine (A) to serine (S), and S (T) allele causes higher calcium levels (Jeong et al., 2016) and correlated to a decrease in calcium excretion in urine (Sonbol and Al Otaibi, 2016).

The CaSR is a coupled G-protein comprising three major components: a domain spanning seven-transmembrane, an extracellular domain, and the intracellular domain (Alkukhun et al., 2017). It plays very important roles in calcium homeostasis in particular (Fahad et al., 2018) when there is a high concentration of extracellular calcium (Assimos, 2019). Also, it can induce phospholipases, ion transport, bicarbonate, acid transport, protein kinases activated by mitogen, and fluid secretion (Alkukhun et al., 2017). Calcium is an important cation that is contributed in several natural processes, for example, in bone formation, neurotransmission and muscle contraction (Toka et al., 2012). Approximately 99 % of the calcium content of the body is found in the bones and teeth, the remaining 1 % is in the bloodstream where it participates in the intracellular signals (Vinayagamoorthy et al., 2015). The body needs a large amount of calcium during growth, pregnancy and lactation. Thus, calcium deficiency has various effects on females from the fetus to the post-menopausal period, such as stunted growth and decreased bone density causing osteoporosis (Almaghamsi et al., 2018).

To the best of our knowledge, numerous studies have been carried out to estimate the relationship between CaSR gene polymorphism in the Caucasian healthy girls (Lorentzon et al., 2001), healthy Saudi adults of both sexes (Fahad et al., 2018), healthy postmenopausal (Young et al., 2003) and postmenopausal women from the Italian population with and without bone fragility (Cetani et al., 2003). However, there are no any study that included determination of CaSR gene polymorphism in premenopausal and postmenopausal women with osteoporosis. Therefore, the aim of this study was to determine the CaSR gene polymorphism A986S and evaluate its correlation with biochemical parameters in premenopausal and postmenopausal Iraqi women with osteoporosis in Salah Al-Din Province.

Material and Methods

Study population

In this study, 170 women aged 40-70 years volunteered from Salah al-Din Province. One hundred woman with osteoporosis (53 premenopausal and 47 postmenopausal) were diagnosed by specialist doctors in some private clinics and 70 healthy women as control group.

Sample collection

An aliquot of 5 ml of blood samples were collected, of which 3 ml was used to recover serum for calcium and phosphorus tests measured by spectrophotometric method using kits (Biomeurx Company, France). The remaining 2 ml of blood samples were collected into EDTA tubes, stored at -20 ^{o}C and used for DNA extraction as described by Ali et al. (2008).

PCR-RFLP analysis to determine polymorphism

CaSR gene polymorphism was determined using PCR-RFLP (polymerase chain reaction-restriction

fragment length polymorphism) analysis. A 269 bp fragment of the CaSR gene was PCR-amplified according to the method reported by Dabiri et al. (2016). The forward primer sequence was 5'-CTGAGCTTTGATGAGCCTCAGAAGGAC-3' and the reverse primer sequence was 5'-CACTGATGACAAGCTCTGTGAACTGGA-3'.
The PCR was set up using the 2X Go Taq green master mix (Promega Company, USA) as follows: 4 µL (100 ng) of genomic DNA, 1 µL (10 picomole) of each primer, 10 µL of master mix and 4 µL of DNase/RNase free water in a total reaction volume of 20 µL. PCR cycling consisted of primary denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 45 sec, 63 °C for 30 sec and 72 °C for 45 sec. The reaction was concluded with one cycle of final extension at 72 °C for 5 min. An aliquot of 5 µL of the PCR product was digested with 10 U of *Hin1I* enzyme (New England, BioLabs, Inc.) at 37 °C for 3 hours and visualized on 3.5 % agarose gel electrophoresis containing red stain with the inclusion of 100 bp DNA ladder (Biolabs-England).

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS version 20 PC program. One-way ANOVA and students' t-test were used for comparing means of biochemical parameters of the patient and control groups in relation to CaSR A986S genotypes. Allelic and genotypic frequencies, odds ratios (OR) and their 95 % confidence intervals (CI) of the patient and control groups were determined by utilizing Pearson's chi-square test. P value (<0.05) was viewed as significant and (<0.01) as highly significant.

Results

Levels of calcium and phosphorus in the patient and healthy control groups

The results of calcium and phosphorus levels in blood serum for 100 Iraqi women with osteoporosis and 70 healthy women control group are presented in Table 1. The osteoporosis patients were further subdivided into two classes: premenopausal and postmenopausal women with osteoporosis. Serum calcium level in the control group was significantly higher (10.5±1.38 mg/dl) compared with the osteoporosis women group (8.02±1.18 mg/dl). Also, phosphorus level in the control group was significantly higher (3.11 ± 0.547 mg/dl) than the patient group (2.03 ± 0.598 mg/dl). There were no significant differences between calcium levels of premenopausal women (8.00+1.16 mg/dl) and postmenopausal women (8.04+1.21 mg/dl) with osteoporosis. More so, there was no significant difference between the levels of phosphorus of the premenopausal (2.02+0.600 mg/dl) and postmenopausal (2.05+0.601 mg/dl) women with osteoporosis. Conversely, the results of the calcium and phosphorus parameters indicated a higher significance at (p-value <0.001) compared with the control and premenopausal women with osteoporosis or osteoporosis and control postmenopausal women.

Table-1. Comparison between calcium and phosphorus levels of the study groups

Comparison between control and women with osteoporosis			
Parameter	**Mean ± SD**		***p* value**
	Patients (100)	**Control (70)**	
Calcium mg/dl	8.02 ± 1.18	10.5 ± 1.38	0.001**
Phosphorus mg/dl	2.03 ± 0.598	3.11 ± 0.547	0.001**
Comparison between premenopausal and postmenopausal women with osteoporosis			
Parameter	**Mean ± SD**		***p* value**
	Premenopausal women (53)	**Postmenopausal women (47)**	
Calcium mg/dl	8.00 + 1.16	8.04 + 1.21	0.88
Phosphorus mg/dl	2.02 + 0.600	2.05 + 0.601	0.82
Comparison between control and premenopausal women with osteoporosis			
Parameter	**Mean ± SD**		***p* value**
	Premenopausal women (53)	**Control (70**	
Calcium mg/dl	8.00 + 1.16	10.5 + 1.38	0.001**
Phosphorus mg/dl	2.05 + 0.601	3.11+ 0.547	0.001**
Comparison between control and postmenopausal women with osteoporosis			
Parameter	**Mean ± SD**		***p* value**
	Postmenopausal women (47)	**Control (70)**	
Calcium mg/dl	8.04 + 1.21	10.5 + 1.38	0.001**
Phosphorus mg/dl	2.02 + 0.600	3.11+ 0.547	0.001**

*p<0.05 significant and **p<0.01 highly significant.

Polymorphic analysis revealed three genotypes of CaSR A986S gene

Analysis of PCR-RFLP products of the CaSR A986S gene revealed three genotypes: GG, GT and TT as revealed in Fig. 1. The frequency of alleles and genotypes is presented in Table 2 as a percentage (%). There were highly important variances between the number and % of genotypes and alleles of CaSR A986S polymorphism (p-value <0.01) compared with osteoporosis patients and control or premenopausal and postmenopausal osteoporosis groups with control. The percentage of osteoporosis women with TT genotype was 60 % compared with 21.42 % observed in the control group. On the contrary, GG genotype was 10 % in the patient compared with 36 % in the healthy groups. Our results also showed an increase in the odd ratio (OR) of the TT genotype (14.40) and T allele (5.571). On the other hands, there were no significant differences (p-value 0.519) between premenopausal and postmenopausal women with osteoporosis.

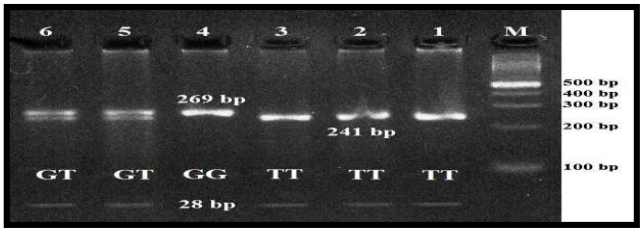

Figure-1. PCR-RFLP products of CaSR A986S genotypes fractionated on 3.5 % agarose gel electrophoresis.

Lane M: 100 bp DNA ladder; Lanes 1-3: TT homozygote (241 and 28 bp); Lane 4: GG homozygote (269 bp); Lanes 4- 5: GT heterozygote (269, 241 and 28 bp).

Table-2. Number and percentage of genotypes and alleles of the CASR A986S polymorphism for the study groups

Comparison between women with osteoporosis and control group							
Genotypes	**Patients (100)**		**Control (70)**		***p* value**	**OR**	**95% IC**
	No.	**%**	**No.**	**%**			
GG	10	10	36	51.43		1 Ref.	-
GT	30	30	19	27.15	<0.01**	5.684	2.296-14.067
TT	60	60	15	21.42		14.40	5.851 - 35.438
Allele Frequency	**No.**	**%**	**No.**	**%**	***p* value**	**OR**	**95% IC**
G	50	25	91	65	<0.01**	1 Ref.	-
T	150	75	49	35		5.571	3.474 - 8.934
Comparison between premenopausal and postmenopausal women with osteoporosis							
Genotypes	**Premenopausal women (53)**		**Postmenopausal women (47)**		***p* value**	**OR**	**95% IC**
	No.	**%**	**No.**	**%**			
GG	4	24.5	6	23.4		1 Ref.	-
GT	18	26.4	12	34.0	0.519	2.250	0.522 - 9.697
TT	31	49.1	29	42.6		1.603	0.410 - 6.264
Allele Frequency	**No.**	**%**	**No.**	**%**	***p* value**	**OR**	**95% IC**
G	26	24.5	24	25.5	0.870	1 Ref.	-
T	80	75.5	70	74.5		1.054	0.555 - 2.002
Comparison between premenopausal women with osteoporosis and control group							
Genotypes	**Premenopausal women (53)**		**Control (70)**		***p* value**	**OR**	**95% IC**
	No.	**%**	**No.**	**%**			
GG	4	24.5	36	51.43		1 Ref.	-
GT	18	26.4	19	27.15	<0.01**	8.526	2.523 - 28.813
TT	31	49.1	15	21.42		18.600	5.585 - 61.937
Allele Frequency	**No.**	**%**	**No.**	**%**	***p* value**	**OR**	**95% IC**
G	26	24.5	91	65	<0.01**	1 Ref.	-
T	80	75.5	49	35		5.714	3.256 - 10.028
Comparison postmenopausal women with osteoporosis and control group							
Genotypes	**Postmenopausal women (47)**		**Control (70)**		***p* value**	**OR**	**95% IC**
	No.	**%**	**No.**	**%**			
GG	6	24.5	36	51.43		1 Ref.	-
GT	12	26.4	19	27.15	<0.01**	3.789	1.228 - 11.691
TT	29	49.1	15	21.42		11.600	3.996 - 33.670
Allele Frequency	**No.**	**%**	**No.**	**%**	***p* value**	**OR**	**95% IC**
G	24	25.5	91	65	<0.01**	1 Ref.	-
T	70	74.5	49	35		5.416	3.035 - 9.667

*p<0.05 significant and **p<0.01 high significant.

OR = odd ratio

Effects of CaSR A986S polymorphism on calcium and phosphorus levels in study groups

The results of the effects of CaSR A986S polymorphism on calcium and phosphorus concentrations in osteoporosis women (Table 3) showed that there were no significant differences between all the CaSR A986S genotypes, although there were differences in the calcium levels. The phosphorus levels were highly significant (p-value 0.022) between the GG compared with GT genotypes. On the other hands, there was no significant difference between the calcium and phosphorus concentrations of the premenopausal and postmenopausal women with osteoporosis in relation to CaSR A986S polymorphism as depicted in Table 4.

Table-3. Effect of CaSR A986S polymorphism on biochemical parameters

Parameter	**GG (No. 10)**	**GT (No. 30)**	**P. value**
Calcium	8.24 ± 0.377	7.68 ± 0.696	0.332
Phosphorus	1.77 ± 0.158	2.25 ± 0.147	0.022*
Parameter	**GG (No. 10)**	**TT (No. 60)**	**P. value**
Calcium	8.24 ± 0.377	8.146 ± 0.824	0.727
Phosphorus	1.77 ± 0.158	2.049 ± 0.172	0.15
Parameter	**GT (No. 30)**	**TT (No. 60)**	**P. value**
Calcium	7.68 ± 0.696	8.146 ± 0.824	0.091
Phosphorus	2.25 ± 0.147	2.049 ± 0.172	0.765

Table-4. Comparison between calcium and phosphorus levels of premenopausal and postmenopausal osteoporosis women in relation to CaSR A986S polymorphism

Parameter	**Premenopausal women (53)**	**Postmenopausal women (47)**	**p value**
	GG No. 4	**GG No. 6**	
Calcium	8.1±0.483	8.33±0.301	0.368
Phosphorus	1.87±0.282	1.71±0.264	0.762
Parameter	**GT No. 18**	**GT No. 12**	**p value**
Calcium	7.483±0.702	7.975±0.592	0.467
Phosphorus	2±0.297	2.183±0.175	0.869
Parameter	**TT No. 31**	**TT No. 29**	**p value**
Calcium	8.280±0.105	8.003±0.126	0.195
Phosphorus	2.063±0.128	2.035±0.218	0.836

Discussion

Homeostasis of calcium plays a vital role in controlling bone makeup and alters the regulatory mechanisms that causes development of metabolic bone diseases (Cetani et al., 2003). This study showed a significant decrease in the concentration of serum calcium (8.02±1.18) and phosphorus (2.03 ±0.598) of the women with osteoporosis compared with the control group. These observations are consistent with the studies of Shakoor et al. (2014) and Li et al. (2020) and inconsistent with other studies (Hamdi, 2013; Ali, 2018). The concentrations of serum calcium and phosphate can vary according to physiological, biochemical and pathological variations (Ikechukwu et al., 2005). Although, phosphorus levels showed a significant variance between the osteoporosis (2.03 ± 0.598) and control (3.11 ± 0.547) groups, while serum phosphorus concentration was still within a normal range. This result agrees with previously published work which revealed that there was no noticeable differences in the analysis of osteoporosis (Omrani et al., 2006; Mutlu et al., 2007). Meanwhile, there was no significant difference in the calcium and phosphorus levels between premenopausal and postmenopausal women with osteoporosis. These results support the finding of Pandey et al. (2013). This observation may be due to the calcium hemostasis which is regulated by multifactor, like parathyroid hormone, vitamin D, and dietary intake (Martins et al., 2017; Wasilewski et al., 2019).

Recent study on genetic SNPs in osteoporosis will continue to be interesting and useful because of the potential impact of race and ethnic factors (Haryono et al., 2019). Some mutations and SNPs of CaSR gene are related to many benign diseases, so, detection of the genotyping of CaSR gene will help in assessing susceptibility to osteoporosis in adults and patients (Sonbol and Al Otaibi, 2016). Some researchers investigated the association between osteoporosis and SNP of some genes, but there has been no report on the comparison between CaSR A986S polymorphism of premenopausal and postmenopausal women with osteoporosis. We investigated the CaSR A986S polymorphism among Iraqi women with osteoporosis in premenopausal and postmenopausal status. The frequency of T mutant allele (75 %) of the CaSR gene was higher than the G wildtype allele (25 %) of the osteoporosis patient compared with the frequency of T mutant allele (35 %) and the wildtype allele A (65 %) in the control. No significant difference was observed in the CaSR A986S genotypes, although there were differences in the number and percentage between premenopausal and postmenopausal women with osteoporosis. The number of patients was relatively small and in addition to the differences in the genetic family history that altered the results of the association. Researches have shown that not only multiple genetic factors are responsible, but also environmental factors affect osteoporosis disease

(Fahad et al., 2018). As a result of the role of CaSR in bone and mineral metabolism, many genetic studies have been achieved to explore its effect on bone mass variability. A few of these studies are in agreement with our results and indicated that CaSR gene can affect bone mineral density variations. An example of such study is the work of Wang et al. (2006) that found a significant correlation between the CaSR and bone mineral density in the Chinese population. Another study was on the Japanese postmenopausal women which revealed a significant relationship between the CaSR polymorphism and bone mineral density (Tsukamoto et al., 2000). In a study on the Jewish Israelites, CaSR heterozygote polymorphism, A986S was associated with lower bone mineral density in premenopausal women (Eckstein et al., 2002). Meanwhile, the findings obtained from some studies were different as in Mo et al. (2004) who found no important relationship between CaSR A986S polymorphism and bone mineral density in premenopausal women. Also, there was no significant difference in the bone mineral density in a study on 164 Italian postmenopausal women CaSR A986S polymorphism (Cetani et al., 2003). More so, from the study of 113 Saudi adults from both sexes, it was concluded that there was no clinical importance in the CaSR genotype on bone mineral density (Fahad et al., 2018).

We tested the possibility of CaSR A986S polymorphism being linked to biochemical concentrations, but found that the mutant T allele in osteoporosis women was not linked with increased levels of circulating calcium. Although, the TT genotype of the osteoporosis women had higher levels of serum calcium (8.146 ± 0.824) than osteoporosis women with GT genotype (7.68 ± 0.696). There was a difference in the calcium levels between the GG and GT genotypes but the difference was non-significant. This was because of the small number of women with GG genotype (ten persons) which represented 5 % of all the patients, that could be related to participant's lifestyle and environment (Al-Azzawie et al., 2019). Thus, there was an indication of a biological effect of the S allele depending on calcium results between GG, GT, and TT genotypes as shown in Table 1 and 3. These results are in agreement with a study by Lorentzon et al. (2001) which was carried out on 97 Caucasian healthy girls and they found that the subjects with S allele had high levels of calcium. Another study indicated that the CaSR A986S polymorphism might be correlated with calcium levels in the healthy subjects of the Chinese population (He et al., 2012). The small number of patients (100) in our study may be the reason for the insignificant differences between women with osteoporosis. Reports have shown that differences in calcium levels were detected by the CaSR which conserved calcium levels, but led to decreasing mineral content of the bone (Di Nisio et al., 2018). This observation could also be attributed to the homeostasis of serum calcium and phosphorus which were affected by many factors. These factors include specific organ interactions, mainly the skeleton, kidneys and intestines. Also, hormones such as calcitonin and parathyroid hormone, vitamin D have also been reported to help preserve homeostasis of the mineral calcium and phosphorus (Berndt et al., 2005; Allen and Burr, 2014).

Conclusion

The results from this study revealed that A986S polymorphism of CaSR is one of the genetic susceptibility factors for the premenopausal and postmenopausal Iraqi women with osteoporosis and it had non-significant effects on mineral levels. Therefore, further studies involving a large number of people in different provinces of Iraq are required in order to confirm our findings.

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