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Calcium-Sensing Receptor Gene Polymorphisms among Saudi Adults: The Possible Relation with Calcium and Bone Mineral Density

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ABSTRACT

The calcium-sensing receptor (CaSR) gene A986S polymorphism is believed to play an important role in maintaining serum calcium concentration within a narrow physiological range. The CaSR gene A986S polymorphism was associated with bone mineral density (BMD). In this study, we investigated whether the CaSR gene A986S (rs1801725) polymorphism was related to BMD in healthy Saudi adults of both sexes. Fifty males and sixty-three females were recruited from the Center of Excellence for Osteoporosis Research in a quantitative cross-sectional study design. Anthropometric measurements were taken for all participants. BMD was determined for the anteroposterior lumbar spine (L1-L4) and the neck femurs by dual-energy X-ray absorptiometry. The genotypes were divided into the presence (61%) or absence of the S allele (39%). Female subjects with the S allele had a higher BMD at the lumber spine (LS) and femur neck (FN) (g/cm^2), while males had higher serum calcium (mmol/L) and parathyroid hormone (pmol/L) and lower body mass index (kg/m^2) and 25-$(OH)_2$D (nmol/L) compared with subjects lacking the S allele. Using bivariate correlations, we found that weight and height were significantly associated with higher LS BMD in males. In females, there was a significant correlation between FN BMD and weight and a negative correlation between LS BMD and serum calcium. There were no significant differences in BMD observed between the AS/SS and AA genotypes. We concluded that, no clinical significance of the CaSR genotype on BMD in both sexes. Further studies demonstrating a functional effect of this CaSR polymorphism are necessary before additional association studies are performed.

Key words: Calcium-sensing receptor gene; Gene polymorphism; Bone mineral density; Saudi adults.

Introduction

The calcium-sensing receptor (CaSR) was first cloned and described by Dr. Edward M. Brown and coworkers (Brown and MacLeod, 2001). CaSR is a surface G protein-coupled receptor that is composed of three major domains, the Venus Flytrap module, a cysteine-rich domain, and a seven-helix transmembrane region. CaSR sustains extracellular Ca^{2+} homeostasis by regulating the secretion of parathyroid hormone (Geng *et al.,* 2016). The structure of CaSR shows several binding sites for Ca^{2+} and PO_4^{3-} ions. Ca^{2+} ions stabilise the active state, and the PO_4^{3-} ions support the inactive state. This protein plays important functions in body fluid and cellular processes, such as Ca^{2+} and fluid reabsorption, acid secretion, and osteoblast and keratinocyte differentiation (Alfadda *et al.,* 2014).

CaSR has very important functions in calcium homeostasis. Many tissues that are not linked to calcium metabolism, such as the skin, brain, and breast, contain CaSR. In cells, CaSR controls cell differentiation, proliferation, death, and gene expression (Campos-Verdes *et al.,* 2018).

The major cells and tissues that play a role in extracellular ionised calcium concentration homeostasis include the parathyroid cells, the thyroidal calcitonin-secreting C-cells, the kidney, bone, and the intestine (Magno *et al.,* 2011). The CaSR gene is a marker that may detect osteoporosis (Cetani *et al.,* 2003). The CaSR A986S genotype was reported to influence serum calcium and was associated with bone mineral density (BMD) in healthy Caucasian girls (Brown *et al.,*1993). Common allelic CaSR variants have been associated with determinants of bone mineral homeostasis (Toka and

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Pollak, 2014). The relationship between CaSR and BMD showed no or minor effects in most association studies in healthy volunteers (Yamauchi *et al.,* 2001; Corbetta *et al.,* 2006; Jung *et al.,* 2009). However, data concerning the association between CaSR gene polymorphisms and BMD in the Saudi population are lacking.

Osteoporosis is a major health condition that results in fractures. Osteoporosis is defined as a bone density T-score of -2.5 or below. This condition can be prevented by exercise and diet (Eastell, 2017). Since the mid-1990s, the World Health Organization (WHO) has defined osteoporosis based on the measurement of BMD by dual-energy X-ray absorptiometry (DXA) (Curtis *et al.,* 2017). Many web-based tools have been developed to allow the enclosure of clinical risk factors, with or without BMD, in fracture estimation algorithms to increase the detection of people at high fracture risk (Curtis *et al.,* 2017). It was estimated that over 200 million people worldwide have osteoporosis (Kanis, 2007; Aaseth *et al.,* 2012). In Saudi Arabia, epidemiological analysis indicated that 34% of all women and 30.7% of all men 50-79 years of age have osteoporosis (Sadat-Ali *et al.,* 2012). A study conducted in Riyadh, Saudi Arabia showed higher rates of osteoporosis in females (40.93%) compared to (24.56%) males (Awwad *et al.,* 2017).

Many factors, such as family history of fracture, slender habitus, early menopause, treatment with medications that affect bone, diseases known to affect bones, age, sex, BMD, spine fracture in the past, a recent non-spine fracture, a non-recent fracture above the age of 50, and a low body weight, are risk factors for osteoporosis and osteoporosis-related fractures ((Eastell, 2017; Hernlund *et al.,* 2013).

Analysis of osteoporosis by BMD is a standard procedure for the assessment of fracture risk. Fractures mostly occur when the BMD is within the osteopenic range (Unnanuntana *et al.,* 2010).

The genotype frequencies of the CaSR polymorphism rs1801725 were 75.21% for the normal genotype AA, 92.51% for the heterozygous genotype AS and 32.29% for the homozygous genotype SS. The frequencies of the A and S alleles were 121.46 and 78.54%, respectively. We recently studied the genotype frequencies of the CaSR polymorphism rs1801725 in Saudi adults. The A986S polymorphism of the CaSR gene occurs frequently in Saudi adult males and females. The heterozygous genotype was more frequently found in males, whereas the normal genotype was more frequently found in females (Sonbol and Otaibi, 2016).

In the present study, we investigated whether the CaSR gene A986S (rs1801725) polymorphism was related to BMD in healthy Saudi adults.

Subjects and Methods

Subjects:

The study consisted of 113 randomly selected Saudi males (50) and females (63) between 20 and 60 years of age who participated in quantitative cross-sectional study, as described previously [20]. All study subjects were of Saudi origin without any known ancestors of other ethnic origins. The subjects were recruited from the Center of Excellence for Osteoporosis Research (CEOR). Participants were included if they were not taking medication known to affect bone metabolism and did not have an existing medical condition or known renal disease, liver disease, thyroid disorders, diabetes mellitus and pregnancy. Informed consent was obtained from each individual, and the study protocol was in compliance with CEOR ethical standards. The Human Ethics Research Committee at the CEOR approved the study. Informed written consent was obtained from all subjects who participated in this study. At baseline, each subject was medically examined and interviewed using a standardised questionnaire that included questions on socioeconomic status, lifestyle, smoking habits, level of physical activity in leisure time, sun exposure, and the use of vitamins and medications. Dietary intakes of calcium and vitamin D supplementation were also recorded**.**

Anthropometric measurements:

Anthropometric measurements were performed in the morning, before breakfast. The measurements were taken with the subjects wearing light clothes and bare footed. Weight and height measurements were performed using a digital scale and stadiometer. The body mass index (BMI) was

calculated by dividing the body weight (in kilograms) by the square of the height (in metres). The waist circumference (between rib cage and iliac crest) and the hip circumference (the maximum standing horizontal circumference of the buttocks) were also recorded. Waist to hip ratio was also calculated by dividing waist circumference by hip circumference.

Bone densitometry:

BMD (g/cm^2) was determined for the anteroposterior lumbar spine (LS; L1-L4) and the neck femurs by DXA (LUNAR Prodigy Model, Lunar Corp., Madison, WI). BMD values were classified according to WHO criteria: a T-score between -1 and -2.5 is indicative of osteopenia, while a T-score of -2.5 and below reflects osteoporosis. A T-score of -1 and above is considered normal.

Biochemical analysis:

Calcium, glucose, magnesium, sodium, phosphate, uric acid and bone marker serum osteocalcin (s-OC) were measured using an ECLIA Elecsys autoanalyser (Roche Diagnostics GmbH, D-68298 Mannheim, Germany). Serum bone-specific alkaline phosphatase (s-bone ALP) was measured with a Metra Biosystem immunoassay kit in a microtiter strip (Alkphase-B, Metra Biosystems, Inc., Mountain View, CA, USA). Serum procollagen type 1 N-terminal propeptide (s-PINP) was measured using ECLIA Elecsys autoanalyser (Roche Diagnostics GmbH, D-68298 Mannheim, Germany). Serum crosslinked C-terminal telopeptide of type 1 collagen (s-CTX) was measured by Elecsys β-CrossLaps assays using an ECLIA Elecsys autoanalyser. All analyses were conducted at the CEOR, King Abdulaziz University, Jeddah, Saudi Arabia.

Genotyping of A986S SNP:

Total genomic DNA was extracted from whole blood (freshly collected or stored at -80ºC) using a DNA extraction kit (Gentra Puregene Blood Kit, Qiagen, Valencia, CA, USA) with the manufacturer's protocol. Identification of the polymorphism was determined by a restriction fragment length polymorphism method. The DNA concentration was determined as described earlier (Cetani *et al.,* 2003). The region containing the polymorphism was amplified by polymerase chain reaction (PCR) by using a previously described primer pair ((Brown *et al.,*1993). Genotyping of A986S SNP was conducted as described previously (Cetani *et al.,* 2003).

Statistical analysis:

Statistical analysis of the data was performed using Statistical Package for Social Sciences (SPSS for Windows, version 18) (SPSS Inc., Chicago, IL, USA). Descriptive data are given as the mean ± standard deviation (SD). Correlations between different variables were assessed using Pearson's correlation. *P* values <0.05 were considered significant. Hardy-Weinberg equilibrium was used to determine the allele frequency and genotype frequency in the population.

Results

All participants underwent a complete physical examination and routine blood biochemical analysis. Allele and genotype frequencies for the CaSR gene A986S polymorphism were determined (Cetani *et al.,* 2003). Homogeneity of the population (P value was >0.05) was confirmed by performing Levene's test for homogeneity of variance. The mean±SD values for age, anthropometric characteristics, bone density, serum calcium concentration, 25-$(OH)_2$D and PTH of 113 males and females are presented in Table 1.

Forty-four of the 113 subjects (39%) had the AA genotype, and 69 (61%) had the AS or SS genotype. The genotypes were found to be out of Hardy-Weinberg equilibrium (Cetani *et al.,* 2003). Analysis of the polymorphism and its relation to BMD was performed by using an independent samples t-test.

Subjects with the S allele had a lower age (years), height (cm) and weight (kg) for females, BMD at the LS and femur neck (FN) for males (g/cm^2), BMI (kg/m^2), and 25-(OH)$_2$D (nmol/L) (3%, 0.1%, 8%, 7, 2, 6% and 24%, respectively) than subjects without the S allele. Subjects with the S allele had a higher weight (kg) and height (cm) in males, BMD at the LS and FN (g/cm^2) in females, and serum calcium (mmol/L) and PTH (pmol/L) in males compared with subjects lacking the S allele (0.1%, 2%, 2%, 5.6%, 2% and 5.7%, respectively) (Table 1).

Table 1: Calcium-sensing receptor polymorphism, age, anthropometric characteristics, biochemical analysis and bone density in 113 males and females. Mean values, standard deviations and P values are presented.

Subjects	**Male**			**Female**		
Genotypes	**AA (n=13)**	**AS SS (n=37)**	***P* value**	**AA (n=31)**	**AS SS (n=32)**	***P* value**
Physical characteristics						
Age (years)	43.4±14.4	43.3±13.9	0.978	45.8 ±12.1	43.0±14.3	0.402
Weight (kg)	83.1±18.4	83.2±18.9	0.987	76.3± 15.1	70.4±15.3	0.128
Height (cm)	164.7±4.6	168.3±7.8	0.120	154.6±7.4	154.5±6.6	0.963
BMI (kg/m^2)	30.6±6.5	29.3±6.2	0.526	32.1±7.0	29.7±7.1	0.182
BMD (g/cm^2)						
BMD LS (L2-L4)	1.149±0.158	1.07±0.15	0.137	1.039±0.195	1.06±0.171	0.659
BMD FN	0.989±0.187	0.97±0.12	0.727	0.918±0.149	0.969±0.132	0.193
Biochemical analysis						
Serum calcium (mmol/L)	2.30±0.12	2.4±0.11	0.103	2.33±0.11	2.32±0.14	0.892
PTH (pmol/L)	5.1±2.8	5.12±2.3	0.997	5.8±2.2	6.4±4.6	0.478
25-(OH)2D (nmol/L)	32.8±10.6	31.6±15.2	0.796	42.1±33	28.6±27	0.081

Data are given as the mean±SD. Values in brackets denote number of subjects assessed. BMI: body mass index, BMD: bone mineral density, PTH: parathyroid hormone, LS: lumbar spine, FN: femoral neck

There were no significant differences in age, body weight, height, BMD, serum calcium, PTH and 25-(OH)$_2$D between subjects with the two allelic variants (Table 1). Pearson correlation analysis of BMD at the LS and FN and other physical and biochemical factors was performed, and data were divided into two groups according to gender. Using bivariate correlations, we found that weight and height were significantly associated with higher BMD at the LS in the male group (*P*=0.008, 0.017, respectively). In the female group, there was a significant correlation between FN BMD and weight (0.007) and a negative correlation between LS BMD and serum calcium (Table 2).

Table 2: Correlation between BMD and other variables in the study groups.

	Males				**Females**			
Variables	BMD LS (L1-L4)		BMD FN		BMD LS (L1-L4)		BMD FN	
	Pearson correlation	*P* value	Pearson correlation	*P* value	Pearson correlation	*P* value	Pearson correlation	*P* value
Age (years)	-0.019	0.861	-0.214	0.148	-0.157	0.216	-0.124	0.369
Weight (kg)	0.345	0.008*	0.166	0.265	0.053	0.215	0.360	0.007*
Height (cm)	0.290	0.017*	-0.035	0.816	0.143	0.125	0.204	0.136
BMI (kg/m2)	0.254	0.064	0.206	0.164	-0.027	0.695	0.237	0.082
ALP (U/L)	-0. 211	0.098	-0.219	0.139	-0.186	0.163	-0.048	0.729
Serum Calcium(mmol/L)	-0. 134	0.525	0.173	0.244	-0.244	0.024*	-0.234	0.086
PTH (pmol/L)	-0.147	0.373	-0.273	0.063	0.064	0.770	-0.048	0.727
25-(OH)2D (nmol/L)	0.162	0.385	0.054	0.719	-0.029	0.746	-0.085	0.536

Asterisk indicates statistical significance at: P<0.05, ALP: serum bone-specific alkaline phosphatase, PTH: parathyroid hormone, LS: lumbar spine, FN: femoral neck*

The comparison between rs1801725 SNPs and BMD is summarised in Table 3. There were no significant differences in BMD observed between subjects with the AS/SS genotypes and the AA genotype. Independent samples t-tests were used to compare the physical and biochemical parameters

between the normal and osteopenia groups. The results showed significant differences in LS BMD, FN BMD (P=0.0001), weight (P=0.035) and height (P=0.003) between the two groups (Table 4).

Table 3: Comparison of the mean LS BMD and FN BMD values (SD) in those with the AS/SS vs. AA alleles of the calcium-sensing receptor gene polymorphism rs1801725.

BMD	A986S genotype			*P**
	AA	AS	SS	
BMD LS (g/cm^2)	1.071 (0.18)	1.076 (0.16)	1.03 (0.15)	0.98^{AS} 0.78^{SS}
BMD FN (g/cm^2)	0.93 (0.16)	0.96 (0.12)	0.98 (0.13)	0.72^{AS} 0.50^{SS}

*LS: Lumbar spine, FN: Femoral neck. Data are the mean (SD), *P: probability that the AS and SS group mean differs from AA group, by ANOVA and Tukey multiple comparison test.*

Table 4: Comparison between normal and osteopenic groups.

Groups	Normal (n=74) Mean± SD	Osteopenia (n=38) Mean± SD	*P* value
Age (years)	41.36±12.53	48.38±13.94	0.008
Weight (kg)	80.01±18.08	72.67±15.46	0.035*
Height (cm)	162.12±8.67	156.52±10.12	0.003*
BMI (kg/m2)	30.46±6.55	30.01±7.22	0.740
BMD LS (g/cm2)	1.15±0.14	0.90±0.04	0.0001*
BMD FN(g/cm2)	1.00±0.13	0.85±0.09	0.0001*
Serum Calcium (mmol/L)	2.32±0.10	2.35±0.13	0.167
ALP (U/L)	88.25±30.79	95.02±27.18	0.255
s-OC (ng/ml)	49.52±201.42	25.05±7.57	0.207
βCTx (ng/ml)	292.54±167.19	325.35±137.73	0.420
sP1NP (ng/ml)	43.30±19.40	47.32±14.90	0.362
sBALP(ng/ml)	19.72±8.77	19.83±8.55	0.976
PTH (pmol/L)	5.35±2.34	6.31±4.29	0.207
25-(OH)2D (nmol/L)	33.13±25.09	34.89±24.71	0.724

Data are given as the mean±SD. Asterisk indicates statistical significance at: P<0.05. SD: std. deviation, BMI: body mass index, BMD: bone mineral density, PTH: parathyroid hormone, LS: lumbar spine, FN: femoral neck, ALP: serum bone-specific alkaline phosphatase, s-OC: marker serum osteocalcin, βCTX: crosslinked C-terminal telopeptide of type1, s-PINP: collagen serum procollagen type 1 N-terminal propeptide.*

Discussion

Heredity controls BMD, and it is a major predictor of osteoporosis (Zheng *et al.,* 2015). Heritability of BMD differs with different locations in the skeleton (Kemp *et al.,* 2014). CaSR expression in cartilage and bone controls skeletal homeostasis (Goltzman and Hendy, 2015). This receptor is important for bone cell activity in skeletal development and bone remodelling (Vezzoli *et al.,* 2018). The A986S variant is associated with increased serum levels of Ca^{2+} and PTH and with decreased serum levels of phosphorus and BMD (O'Seaghdha *et al.,* 2010 and 2013). Age, female gender, BMI, a history of fracture, heredity, cortisone use, smoking, and excessive alcohol use are independent clinical risk factors for fractures (Kanis *et al.,* 2008; Bonnick *et al.,* 2010).

In the present study we found no association of the calcium-sensing receptor gene polymorphism rs1801725 with BMD. However, male subjects lacking the S allele had a higher BMD than the S allele subjects. In addition, female subjects lacking the S allele had a lower BMD than the S allele subjects. These results could be due to perimenopause and menopause in some female subjects. However, our multivariate analysis revealed that the CaSR alleles did not independently predict BMD at any site.

Lorentzon et al. performed a study of healthy young adolescent girls and found similar results to those of the males and contradictory results to those of the females. Their findings indicated that the subjects lacking the S allele had a greater BMD than the subjects with the S allele. However, their multivariate analysis indicated that the CaSR alleles were not an independent predictor of BMD. The

independent predictors of BMD were age, body weight and physical activity. There were variations in BMD between the CaSR allelic variants, and a multivariate analysis showed that when physical activity and weight were considered, the effect of the CaSR allelic variants on BMD was not observed (Lorentzon *et al.,* 2001).

Several studies have found an association of A986S polymorphisms with BMD and are consistent with our data. Takacs et al. was unable to find an association between the CaSR gene A986S polymorphism and BMD in Hungarian postmenopausal women (Takács *et al.,* 2002). Young et al. assessed bone mass at baseline and after 2 years of calcium therapy in a group of 135 postmenopausal women, observing no relationship in either case (Young *et al.,* 2003). Furthermore, in Italian women, no differences were observed (Cetani *et al.,* 2003). In a study of 1252 postmenopausal Australian women, researchers found no relations among the polymorphism, bone mass and the presence of fractures due to fragility. In healthy Chinese premenopausal women (Bollerslev *et al.,* 2004), there were no significant differences in the BMD or bone size of either the spine or hip between CaSR polymorphisms (Mo *et al.,* 2004). Moreover, there was a study on CaSR polymorphisms of codon 986 in 110 adult, Caucasian female dizygotic twin pairs, and the results showed that the CaSR polymorphisms of codon 986 did not significantly influence serum calcium corrected for albumin; serum PTH; serum 25OHD3; serum 1, 25$(OH)_2$D3; urinary calcium: creatinine ratios; and BMD at the total lumbar spine, forearm and total hip (Harding *et al.,* 2006).

Differences in the genetic background of mixed populations may alter the outcome of association studies. However, our study population was homogeneously of Saudi origin. Not only multiple genetic factors but also environmental factors have an influence on BMD. If the genetic effect was weak, the environmental factors may have masked the actual genetic influence of the CaSR gene in our association study.

In our population, the rare S allele in males was associated with increased concentrations of circulating calcium. Additionally, subjects with the S allele had higher levels of serum PTH than subjects lacking the S allele. The interaction of skeletal CaSR activation with PTH may cause net bone formation in trabecular bone or net bone resorption in cortical bone (Goltzman and Hendy, 2015). If PTH is administered intermittently, it has anabolic functions on bone (Augustine and Horwitz, 2013). The low number of subjects (n=113) in the present study may have contributed to the insignificant difference in BMD. In conclusion, we found that the CaSR A986S polymorphism was not related to BMD in Saudi subjects.

In summary, we found no evidence to support a relationship between the CaSR gene A986S polymorphism and BMD in Saudi males and females. This finding indicates that further studies in other and larger populations are required to determine the role of CaSR in predicting BMD.

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Disclosure Summary:

The authors have nothing to disclose. This manuscript describes original work and is not under consideration by any other journal.

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