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CLINICAL STUDY

Calcium sensing receptor gene polymorphism, circulating calcium concentrations and bone mineral density in healthy adolescent girls

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Abstract

Objective: Bone mineral density (BMD) in adolescence is under strong genetic control. The calcium sensing receptor (CASR) is involved in the regulation of calcium homeostasis and bone resorption. The A986S polymorphism of the CASR has recently been associated with serum calcium levels, in one hitherto unconfirmed report. We investigated whether this polymorphism was related to BMD, circulating calcium and parathyroid hormone (PTH) concentrations in girls.

Design: BMD, plasma calcium and serum PTH were measured in adolescent girls and compared with regard to CASR genotype.

Methods: In 97 healthy Caucasian girls (mean age 16.9 ± 1.2 years (mean ± S.D.)), the A and S alleles were determined using PCR with a mismatched primer and the restriction enzyme BsaHI. BMD (g/cm^2) of the total body, humerus, femoral neck and lumbar spine was measured using dual energy X-ray absorptiometry.

Results: The genotype frequencies were 71% AA, 26% AS and 3% SS. The genotypes were divided into presence (29%) or absence of S allele (71%). Subjects with the S allele had higher levels of plasma calcium, corrected for albumin ($2.17 \pm 0.06 > 2.14 \pm 0.06$; $P < 0.05$, using independent samples *t*-test), lower BMD at the lumbar spine ($P = 0.02$) and total body ($P = 0.04$), and were significantly less physically active (2.9 ± 2.6 vs 4.3 ± 2.6 h/week; $P = 0.01$) than the subjects lacking the S allele. PTH levels were not significantly different between the two allelic groups. A multiple regression analysis, including age, height, weight and physical activity, revealed that the CASR allelic variants were not independent predictors of BMD at any site measured ($\beta = -0.03$–0.09; $P > 0.05$). Physical activity was an independent predictor of BMD, was significantly different between the CASR genotypes, and could therefore have a role in explaining the difference in BMD between the CASR genotypes.

Conclusions: The CASR alleles are related to BMD, but it cannot be definitely concluded whether the CASR polymorphism has a direct influence on BMD, or whether the differences in BMD were mediated via an influence of the amount of physical activity.

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Introduction

The calcium sensing receptor (CASR) senses extracellular calcium concentrations and mediates alterations in parathyroid hormone (PTH) secretion and renal calcium re-absorption in order to keep serum calcium levels within the narrow physiological range (1). It is predominantly expressed in the parathyroid chief cells (2) and in the tubular epithelial (3) cells but also in bone cells and new data indicate that this receptor is involved in regulation of osteoclastic bone resorption (4). Inherited abnormalities in the CASR gene can result in either hyper- or hypocalcemia (5), and recently the A986S polymorphism of the CASR was found to be associated with serum levels of total and ionized calcium (6). The latter of these results has not yet been confirmed. The CASR gene consists of seven exons and the corresponding protein is a 1078 amino acid G-protein-coupled receptor. The A986S polymorphism is located in exon 7 and results in an amino acid shift (alanine or serine at codon 986) in the intracellular C-terminal tail of the receptor (1).

Genetic factors are believed to regulate a large part of the age-specific variation in bone mineral density (BMD) (7–9) and genetic polymorphisms, and their association to BMD, have been investigated in many

candidate genes (10). The achieved peak bone mass in adolescence and young adulthood is probably a major determinant of the risk of contracting osteoporosis and resulting fracture later in life (11). Finding markers for low bone mass in adolescence could provide means for early preventive measures, such as dietary optimization and increased physical activity (12). A recent report revealed an association of radial BMD with a CA-repeat polymorphism in the CASR locus, in postmenopausal Japanese women (13).

Since the A986S polymorphism of the CASR appears to be involved in regulation of the calcium homeostasis, we hypothesized that this polymorphism could be associated with bone mass. In the present study we tested this hypothesis and investigated whether or not the A986S polymorphism is related to BMD or to circulating calcium or PTH levels in healthy adolescent girls.

Subjects and methods

Subjects

Ninety-seven Caucasian girls, mean age 16.9 ± 1.2 years (mean±S.D.), who were at least 2 years post menarche were recruited from advertisement and information in schools and local sports clubs and included in the present study. None of the girls were related or had any disease or medication known to affect bone metabolism. Using a standardized questionnaire, the amount (h/week) of weight-bearing physical activity per week during the last year was assessed (14). Informed written consent was given by all the participants and the study protocol was approved by the Ethical Committee of the Medical Faculty, Umeå University.

Techniques for estimating bone density

Height and weight were measured using standardized equipment. BMD (g/cm^2) of the total body, humerus, femoral neck and lumbar spine, and total bone mineral (g) were measured using a Lunar DPX-L (Lunar Co, WI, USA) dual energy X-ray absorptiometer, software version 1.3y. Total bone calcium was calculated from the measurement of total bone mineral, using the same software. The precision of this method has previously been discussed in detail by others (15, 16). The CV value (S.D./mean) for repeated measurements is 0.7–2.0% in our laboratory, depending on application (17).

Genomic DNA analysis

Genomic DNA from the 97 girls was isolated from EDTA stabilized blood, using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Determination of the A986S gene polymorphism of the calcium-sensing receptor was performed using 30 ng of genomic DNA, 0.4 μmol/l forward primer 5′-CTGAGCTTTGATGAGCCTCAGAAGGAC-3′, 0.4 μmol/l reverse primer 5′-CACTGATGACCAAGCTCTGTGAACT GGA-3′, 0.2 mmol/l each of dATP, dCTP, dGTP, dTTP, 1× PCR buffer and 2.5 U of Taq polymerase in a 50 μl reaction mixture (Roche Biochemicals, Stockholm, Sweden), in 30 cycles of 30 s denaturation at 95 °C, 30 s annealing at 60 °C, and 30 s elongation at 72 °C (Peltier Thermocycler, MJ Research, Watertown, MA, USA). The forward primer contained a mismatched nucleotide (underlined), creating a cut site for the restriction enzyme BsaHI. The PCR products were cleaved overnight in BsaHI (New England Biolabs, Stockholm, Sweden), electrophoresed and analyzed on ethidium bromide stained agarose gel.

Presence of the BsaHI restriction fragment site represents alanine (A), while absence of restriction fragment site represents serine (S), rendering the allelic variants SS, AS, AA. To validate the accuracy of the genotyping, ten random subjects (of the total 97 subjects) were re-determined for the polymorphic site and no discrepancies were found.

Biochemical analysis

Blood samples were collected after overnight fast. Intact PTH was measured in all 97 girls, using Imulite intact PTH, solid-phase sandwich chemiluminescent immunological assay (DPC, CA, USA). Calcium was measured in plasma samples from 94 of the subjects using atomic absorption spectroscopy (18) with 1.2% intra-assay precision. Albumin was measured with complexocolometric dry chemistry by Bromcresol Green on Vitros 950 (Ortho-Clinical diagnostics, NY, USA). Osteocalcin was analyzed in plasma samples from 92 girls by a commercially available radioimmunoassay kit (Dia-Sorin, Stillwater, MI, USA). All samples were analyzed in duplicate. The sensitivity of this assay was 0.8 ng/ml.

Statistical analysis

The CASR genotypes were divided into two groups, on the basis of presence or absence of S allele. Differences in physical characteristics, circulating calcium, osteocalcin, PTH and bone density between the two groups defined by the A986S genotypes were investigated using an independent samples *t*-test. Bivariate correlations were tested using Pearson's coefficient of correlation. The independent predictors of bone density were tested using multiple regression. The SPSS package for PC was used for the statistical analysis. A P value less than 0.05 was considered significant.

Results

Sixty-nine out of the 97 subjects (71.1%) had the AA genotype, 25 (25.8%) the AS, and only 3 (3.1%) had

Table 1 Calcium-sensing receptor polymorphism, age, anthropometric characteristics, biochemical analysis and bone density in 97 adolescent Caucasian girls. Mean values, standard deviations and *P* values are presented.

	Subjects		
A986S allelic variants	Without S	With S	***P* values**
Number of subjects (97 total)	69	28	
Physical characteristics			
Age (years)	17.0±1.2	16.9±1.2	0.64
Body weight (kg)	60.4±6.1	58.2±5.5	0.10
Height (cm)	167±5	166±5	0.25
Years post menarche	4.1±1.3	4.1±1.7	0.95
Physical activity (h/week)	4.3±2.6	2.9±2.6	0.01
Bone mineral density (g/cm^2)			
Femoral neck	1.10±0.13	1.04±0.13	0.06
Humerus	1.01±0.09	0.97±0.10	0.07
Lumbar spine	1.24±0.11	1.18±0.11	0.02
Total body	1.17±0.06	1.14±0.08	0.04
Total bone calcium (g)	1009±110	956±120	0.04
Biochemical analysis			
Osteocalcin (ng/ml)	11.3±10.8	10.5±8.5	0.73
PTH (pmol/l)	3.5±1.5	3.9±2.4	0.32
Plasma calcium (mmol/l)	2.14±0.08	2.16±0.08	0.28
Plasma calcium corrected for albumin (94 subjects, 68 without S, 26 with S)	2.14±0.06	2.17±0.06	<0.05

the SS genotype. The genotypes were found to be in Hardy–Weinberg equilibrium, calculated using chi-square test ($P = 0.91$). In order to analyze the polymorphism and relation to BMD, circulating calcium, osteocalcin, PTH and anthropometric characteristics using independent samples *t*-test, the groups were divided into presence (29%) or absence of S allele (71%). There was no significant difference in age, body weight, height, or years post menarche between the two allelic variants (Table 1). Subjects with the S allele were significantly less physically active (2.9 ± 2.6 vs 4.3 ± 2.6 h/week; $P = 0.01$).

Subjects with the S allele had 5.0% lower BMD (g/cm^2) at the femoral neck ($P = 0.06$), 4.7% lower BMD of the lumbar spine ($P = 0.02$), 2.7% lower BMD of the total body ($P = 0.04$) and 5.3% lower total bone calcium ($P = 0.04$) compared with subjects lacking the S allele (Table 1).

Subjects with the S allele had higher plasma calcium (mmol/l), corrected for albumin, than subjects lacking the S allele ($2.17 \pm 0.06 > 2.14 \pm 0.06$; $P < 0.05$), while no significant difference was observed in total plasma calcium ($2.16 \pm 0.08 > 2.14 \pm 0.08$; $P = 0.28$), not corrected for albumin. Serum levels of osteocalcin and PTH were not significantly different in the two allelic groups (Table 1).

Using bivariate correlations, higher levels of PTH were associated with lower levels of plasma calcium, corrected for albumin, in the whole group ($n = 94$; $P = 0.004$), in the group with the S allele ($n = 26$; $P = 0.03$), and in the group without the S allele ($n = 68$; $P = 0.01$).

The independent contributors to the variation in bone density were investigated using multiple regression. Based on bivariate correlations, age, body height, body weight, physical activity, and the CASR allelic variants were used as explanatory variables (Table 2).

Physical activity was found to predict BMD at all sites but the humerus ($\beta = 0.31$–0.46; $P < 0.01$) and body weight predicted BMD of the humerus, lumbar spine

Table 2 The independent predictors of BMD in 97 17-year-old girls. Body weight, the CASR allelic variants, physical activity and years after menarche were used as explanatory variables. β values, *P* values and R^2 values are presented.

	Bone density							
Explanatory variables	Femoral neck	*P*	Humerus	*P*	Lumbar spine	*P*	Total body	*P*
Age	0.11	0.22	0.08	0.41	0.12	0.19	0.18	0.04
Body height	0.19	0.06	−0.07	0.55	0.08	0.47	0.01	0.94
Body weight	0.12	0.28	0.36	<0.01	0.24	0.03	0.33	<0.01
CASR allelic variants	−0.03	0.77	−0.08	0.41	−0.09	0.31	−0.06	0.49
Physical activity	0.46	<0.01	0.17	0.09	0.31	<0.01	0.32	<0.01
R^2	0.35	<0.01	0.21	<0.01	0.28	<0.01	0.34	<0.01

and total body ($\beta = 0.24$–0.36; $P < 0.05$). Age was only an independent predictor of total body BMD ($\beta = 0.18$; $P = 0.04$). The CASR allelic variants were not independent predictors of BMD at any site measured ($\beta = -0.09$–0.03; $P > 0.05$).

Discussion

BMD is under strong genetic control (7–9) and a major determinant of fracture risk (19). The risk of contracting osteoporosis and resulting fracture is probably partly determined by achieved bone mass in adolescence and young adulthood (11). Despite the knowledge that up to 60–80% of the age-specific variation in BMD is due to genetic factors (11), the specific genes regulating BMD have not been revealed. Polymorphisms, in relation to BMD, have been investigated in many candidate genes for osteoporosis, including the genes for the estrogen receptor α (10, 20), transforming growth factor beta (21), type I collagen and vitamin D receptor (VDR) (22). The VDR polymorphism has been extensively examined, indicating an association in girls and young women, but the many studies are not conclusive (23–26).

In the present study, we show that the CASR polymorphism is related to BMD (unadjusted for influencing factors), where the subjects lacking the S allele have higher BMD than the S allele subjects. However, our multivariate analysis revealed that the CASR alleles did not independently predict BMD at any site. Age, body weight and physical activity were found to be independent predictors of BMD, in agreement with previous findings (12).

Experimental studies in rat, and in humans, have revealed that CASR mRNA is expressed in many tissues and cells, including thyroid C-cells, lung, ileum, large intestine, adrenal gland, and in the central nervous system (1). In the rat brain, the highest regional expression of CASR mRNA has been found in the corpus striatum and in the hypothalamus, a region believed to be involved in regulating motivation, such as sodium hunger, thirst, sexuality and hostility (27, 28). In the present study, physical activity was clearly a confounding factor when analyzing the CASR association with BMD. Whether the differences in physical activity seen between the genotypes were only coincidental or due to a possible biological role in motivational behavior, the need or urge to exercise, remains to be determined.

In the present study, the genotype frequencies were very similar to those reported by Cole and colleagues (6), who found that the frequencies in their population of women was 70.6% AA, 26.3% AS and 3.1% SS, compared with 71.1% AA, 25.8% AS and 3.1% SS in our population of girls.

In our population, the rare S allele was associated with higher concentrations of circulating calcium than absence of S allele. These results are the first to confirm the very recent findings by Cole *et al.* (6). Since the CASR is involved in maintaining calcium homeostasis via the regulation of PTH secretion (2, 29) we tested whether the A986S genotypes were related to circulating levels of PTH, but found no significant difference between the two allelic variants. However, subjects with the S allele had higher levels of serum PTH, as well as circulating calcium, compared with subjects lacking the S allele. Constantly high levels of PTH have been shown to increase bone resorption and demineralization, while pulsative, high levels of PTH have been reported to stimulate trabecular bone formation (30). The rather low number of subjects ($n = 97$) in the present study may have contributed to this difference not being significant.

In conclusion, we could demonstrate that the A986S polymorphism of the CASR is related to circulating calcium levels and to BMD in healthy adolescent girls. Despite the difference in BMD between the CASR allelic variants, a multivariate analysis showed that when taking factors such as physical activity and weight into account, the effect of the CASR allelic variants on BMD could not be detected, implicating that further studies in other and larger populations are required to determine the role of the CASR in predicting BMD.

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