

ORIGINAL ARTICLE

Functional characterization of calcium sensing receptor polymorphisms and absence of association with indices of calcium homeostasis and bone mineral density

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Summary

Objectives Associations between calcium-sensing receptor (CaSR) polymorphisms and serum calcium, PTH and bone mineral density (BMD) have been reported by six studies. However, three other studies have failed to detect such associations. We therefore further investigated three CaSR coding region polymorphisms (Ala986Ser, Arg990Gly and Gln1011Glu) for associations with indices of calcium homeostasis and BMD and for alterations in receptor function.

Patients and design One hundred and ten adult, Caucasian, female, dizygotic twin pairs were investigated for associations between the three CaSR polymorphisms and serum calcium, albumin, PTH, 25-hydroxyvitamin D₃ (25OHD₃), 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], urinary calcium excretion and BMD. Each polymorphic CaSR was also transfected into HEK293 cells and functionally evaluated.

Results There was a lack of association between each of these three CaSR polymorphisms and serum calcium corrected for albumin, PTH, 25OHD₃, 1,25(OH)₂D₃, urinary calcium excretion or BMD at the hip, forearm and lumbar spine. These findings were supported by a lack of functional differences in the dose–response curves of the CaSR variants, with the EC₅₀ values (mean ± SEM) of the wild-type (Ala986/Arg990/Gln1011), Ser986, Gly990 and Glu1011 CaSR variants being 2.74 ± 0.29 mM, 3.09 ± 0.34 mM (*P* > 0.4), 2.99 ± 0.23 mM (*P* > 0.4) and 2.96 ± 0.30 mM (*P* > 0.5), respectively.

Conclusions Our study, which was sufficiently powered to detect effects that would explain up to 5%, but not less than 1%, of the variance has revealed that the three CaSR polymorphisms of the coding region have no major influence on indices of calcium homeostasis in this female population, and that they do not alter receptor function.

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Introduction

The extracellular calcium-sensing receptor (CaSR) is a 1078-amino-acid G-protein-coupled receptor (GPCR) that is predominantly expressed in the parathyroids and kidneys, where it allows regulation of PTH secretion and renal tubular calcium reabsorption appropriate to the prevailing extracellular calcium [Ca²⁺]_o concentration.^{1,2} The CaSR consists of: a large extracellular domain that binds ionized calcium, magnesium, amino acids and other compounds; seven transmembrane domains that are characteristic of GPCRs; and an intracellular domain that has a role in cell surface expression of the CaSR.^{1–3} Ligand binding by the CaSR results in G-protein-dependent stimulation, through G_{q/11}, of phospholipase C (PLC) activity, causing an accumulation of inositol 1,4,5-trisphosphate (IP₃) and rapid release of calcium ions from intracellular stores [Ca²⁺]_i, which is followed by an influx of [Ca²⁺]_o ions.² The key role of the CaSR in the regulation of [Ca²⁺]_o homeostasis has been further demonstrated by the identification of CaSR mutations in human disorders and by studies of CaSR mouse models.^{4–7} In humans, inactivating CaSR mutations result in familial (benign) hypocalciuric hypercalcaemia (FBHH) and neonatal severe primary hyperparathyroidism (NSHPT), whereas activating CaSR mutations result in autosomal dominant hypocalcaemia with hypercalciuria (ADHH) and Bartter's syndrome type V^{4,5,8,9}. Functional expression studies of mutant CaSRs, in HEK293 cells, assessing the dose–response curves and the [Ca²⁺]_o needed to produce a half-maximal (EC₅₀) response in total [Ca²⁺]_i, have revealed that the inactivating CaSR mutations associated with FBHH and NSHPT result in a rightwards shift of the dose–response curve and a significantly higher EC₅₀, whereas the activating CaSR mutations associated with ADHH result in a leftwards shift of the dose–response curve and a significantly lower EC₅₀ when compared to the wild-type CaSR.¹⁰ Furthermore, mice heterozygous for a

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Table 1. Summary of findings from nine reports investigating for associations with CaSR polymorphisms

Reference and year	1999 ¹¹	2000 ¹²	2000 ¹³	2001 ¹⁴	2001 ¹⁵	2002 ¹⁶	2003 ¹⁷	2003 ¹⁸	2004 ¹⁹
Population	Canadian normal white	Japanese haemo-dialysis	Japanese post-m	Swedish teenagers	Canadian women	Hungarian post-m	New Zealand post-m	Australian ambulatory	Italian normal white
Number	163 F	58 F, 64 M	448 F	97 F	387 F	230 F	102 F	1252 F	184 M 137 pre-m F 56 post-m F
Age range (years)	18–35	29–88	66–92	13–21*	18–35	40–70	43–73*	70–85	18–52, 48–65, 18–65
CaSR polymorphism†									
A986S	0.84/0.16	○	○	0.84/0.16	0.85/0.15	0.87/0.13	0.83/0.17	0.83/0.17	0.76/0.24
R990G	0.88/0.12	0.75/0.25	○	○	○	○	○	○	0.93/0.07
Q1011E	0.97/0.03	○	○	○	○	○	○	○	0.96/0.04
(ca) _{12–20}	○	○	0.66/0.34 (A3 allele) 0.41/0.59 (A9 allele)	○	○	○	○	○	○
Intron 4	○	0.72/0.28	○	○	○	○	○	○	○
Correlation									
Ca ionized	+(A986S)	○	○	○	○	○	0	○	+(R990G) –(A986S, Q1011E)
sCa-corrected	+(A986S)	0	○	+(A986S)	+(A986S)	○	0	0	○
sCa-uncorrected	○	○	○	0	○	○	0	0	○
Urine Ca : Cr	○	○	○	○	○	○	○	0	○
PTH	0	+(R990G)	○	0	○	○	0	0	0
BMD									
LS	○	○	○	–(A986S)	○	0	0	○	○
FN	○	○	○	0	○	0	0	○	○
Distal radius	○	○	–(ca) _{12–20}	0	○	○	0	○	○
Hip	○	○	○	0	○	○	0	○	○
Trochanter	○	○	○	○	○	○	0	0	○
Ward's triangle	○	○	○	○	○	○	0	○	○

pre-m/post-m, pre/post-menopausal; M, male; F, female; CaSR, calcium-sensing receptor; +, positive; –, negative; 0, no correlation; ○, not investigated; Ca, calcium; sCa, serum calcium; Cr, creatinine; BMD, bone mineral density; LS, lumbar spine; FN, femoral neck.

*Age range calculated as mean \pm 3 SD from published data.^{14,17}

†Frequencies of common/rare alleles.

CaSR-deleted allele mimic the phenotype of FBHH patients with modest elevations in serum calcium and relative hypocalcaemia, whereas CaSR null mice exhibit a phenotype similar to NSHPT, with severe hypercalcaemia, parathyroid hyperplasia, bone abnormalities and neonatal death.⁶ Moreover, mice with an activating CaSR mutation, which have cataracts and ectopic calcification, show phenotypic similarities to ADHH patients in developing hypocalcaemia, hyperphosphataemia and inappropriately reduced plasma concentrations of PTH.⁷ These studies have clearly established the role of the CaSR and its mutations in calcium homeostasis and bone metabolism. However, the role of CaSR polymorphisms in the control of serum calcium, PTH and bone mineral density (BMD) remains controversial (Table 1). Nine studies in different populations have investigated associations between serum calcium, PTH, BMD and five CaSR polymorphisms that included three in the coding region [Ala986Ser (A986S), Arg990Gly (R990G) and Gln1011Glu (Q1011E)], a $t \rightarrow c$ transition at position –88 bp of intron 4, and a (ca)_{12–20} repeat located within 110 kb of the CaSR.^{11–19} Six of these nine studies reported an association between one or more CaSR polymorphisms and serum

calcium, PTH and BMD, while the remaining three, which studied only the A986S polymorphism (Table 1), did not identify any association. None of these nine studies has investigated the effects of the CaSR coding region polymorphisms on the dose–response curves and the EC₅₀ of the receptor. These CaSR coding region polymorphisms involve evolutionary conserved residues of the intracellular carboxy-terminal domain^{2,20} and the amino acid substitutions involve nonconservative changes. Thus, at codon 986, the nonpolar hydrophobic alanine (A) residue is replaced by the polar but uncharged serine (S) residue; at codon 990, the positively charged arginine (R) residue is replaced by the polar but uncharged glycine (G) residue; and at codon 1011, the polar but uncharged glutamine (Q) residue is replaced by the negatively charged glutamic acid (E) residue. We therefore assessed the functional effects of these three coding region polymorphisms, A986S, R990G and Q1011E, which are located in exon 7 of the CaSR gene. In addition, we investigated for a possible association between these polymorphisms and serum calcium, PTH, 25-hydroxyvitamin D₃ (25OHD₃), 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], urinary calcium excretion and BMD in unselected females.

Materials and methods

Human subjects

One hundred and ten dizygotic female twin pairs (age range 40–76 years) of Caucasian origin from the UK Adult Twin Registry,²¹ in whom zygosity had been determined by a standardized questionnaire and DNA fingerprinting, were studied.²² The research was approved by the St Thomas' Hospital Ethical Committee and all subjects provided written consent.

Clinical and biochemical analyses

Serum calcium, albumin and creatinine and second void early morning urinary calcium and creatinine concentrations were measured using assays (Vitros; Johnson and Johnson Diagnostics, Rochester, NY, USA) as described previously.²³ Serum calcium concentrations were corrected for albumin using the following equation: $CCa (Alb) = Ca + 0.016 (41 - Alb)$, where Ca, Alb and CCa (Alb) are serum total calcium (mmol/l), albumin (g/l) and serum calcium corrected for albumin (mmol/l), respectively. Serum intact PTH was measured using a two-site chemiluminometric immunoassay (MagicLite Intact PTH; Chiron Diagnostics, South Notwood, MA, USA). Serum 25OHD₃ was measured by a specific radioimmunoassay (RIA) (INCSTAR 25-hydroxyvitamin D RIA kit, Incstar Corp., Stillwater, MN, USA) and serum 1,25(OH)₂D₃ was measured using a quantitative RIA (Nichols Institute Diagnostics Radioceptor Assay; Nichols Institute, San Juan Capistrano, CA, USA) as described.²³ BMD was measured at the lumbar spine, left forearm and left total hip using dual-energy X-ray absorptiometry on a Hologic QDR-2000 (Hologic, Waltham, MA, USA), as reported.^{24,25}

Genotyping for CaSR polymorphisms

Leucocyte DNA was extracted and used with CaSR exon 7 specific primers (forward 5'-CAGAAGGTCATCTTTGGCAGCGCA-3'; and reverse 5'-TCTTCCTCAGAGAAAGGAGTCTGG-3') for polymerase chain reaction (PCR) amplification, described as previously.²⁶ The DNA sequences of the 443-bp PCR products that encompassed the polymorphic codons 986, 990 and 1011 were then determined by the use of *Taq* polymerase cycle sequencing and a semiautomated detection system (ABI 373XL sequencer, PE Applied Biosystems, Foster City, CA, USA).²⁶

Statistical analyses

The allele and haplotype frequencies were computed with the software package ZAPLO.²⁷ This calculates the frequencies under the assumption of Hardy–Weinberg equilibrium (HWE) at each locus and allows for the relatedness of the genotypes. ZAPLO also uses an E–M algorithm to complete the haplotype frequencies with the additional assumption that the loci have a zero recombination fraction between them. This is a valid assumption as the three CaSR polymorphisms of codons 986, 990 and 1011 are all contained within a 25-bp region. Estimates of linkage disequilibrium (LD) were derived from the calculated haplotype frequencies. The sample of dizygotic

pairs provides orthogonal information on a phenotype–genotype association from between and within the twin pairs. This enables a test of population stratification prior to a test of association that would use all the association information. Tests of association were carried out in STATA using the regress and cluster options with scaled and centred dummy variables representing the marker genotypes. These robust regression methods allowed for the non-independence of siblings as compared to singletons and adjusted the levels of significance accordingly. The reparameterization of the marker data into dummy variables was conducted under the assumption of a dominant penetrance model for allele action. This combines the groups with heterozygous and rare homozygous genotypes into one group and ensures that the regression analysis does not give undue influence to the very few individuals with rare homozygous genotypes. The dominant penetrance model was also assumed when testing for population stratification.

Functional expression of CaSR polymorphisms

Functional studies were performed using a pEGFP-N2–CaSR construct, as reported previously.⁷ The A986S, R990G and Q1011E polymorphisms, and the CaSR mutant Y129 associated with ADHH,⁹ were introduced by site-directed mutagenesis (QuikChange, Stratagene, La Jolla, CA, USA).²⁸ The wild-type, mutant and polymorphic CaSRs and an empty pEGFP vector were transiently transfected into human embryonic kidney cells (HEK293, American Type Culture Collection Catalogue no. CRL1573) by using Lipofectamine Plus (Invitrogen), as described.¹⁰ The empty pEGFP vector and ADHH-associated CaSR mutant⁹ were used as controls. Cells were visualized by a fluorescence microscope (Zeiss, Axiovert S-100) with an epifluorescence filter and images captured using Openlab software (Improvision Inc. Lexington, MA, USA).²⁹ Expression of CaSR–EGFP fusion proteins was also confirmed by Western blot analysis of cellular protein extract using an ADD monoclonal CaSR antibody directed at amino acids 214–235 (NPS Pharmaceuticals).³⁰ The wild-type, mutant and three polymorphic CaSRs, and the pEGFP vector, were functionally assessed by measuring the alterations in $[Ca^{2+}]_i$ in response to changes in $[Ca^{2+}]_o$, as described previously.⁷ Forty-eight hours post-transfection, the cells were harvested, washed in calcium- and magnesium-free Hanks' balanced salt solution (HBSS) (Invitrogen), and loaded with 1 µg/ml indo-1-acetoxymethyl ester (Molecular Probes) for 1 h at 37 °C. After removal of free dye, the cells were resuspended in 1 ml of calcium and magnesium-free HBSS and maintained at 37 °C. Fluorescence-activated cell sorting (FACS) was performed with a cytometer (FACS Vantage SE, Becton Dickinson, San Jose, CA, USA) equipped with an argon laser, as described.³¹ Baseline fluorescence ratio was measured for 2 min, the fluorescence ratio vs. time was recorded, and data collected for 2 min at each $[Ca^{2+}]_o$, as described.⁷ MultiTime software (Phoenix Flow Systems) was used to determine the peak mean fluorescence ratio of the transient response after each individual stimulus and normalized response $[Ca^{2+}]_o$ curves were generated.¹⁰ The EC₅₀ (i.e. $[Ca^{2+}]_o$ required for 50% of the maximal response) for each normalized concentration–response curve was determined, and the mean EC₅₀ for four separate transfection experiments was used for statistical comparison by using Student's *t*-test.

Table 2. Clinical and biochemical findings in the dizygotic twins

Phenotype	N	Mean	SD	Reference range
Age (years)	220	55.9	7.8	–
BMI (kg/m ²)	214	25.1	4.5	18–25
Serum				
Ca (mmol/l)	220	2.38	0.17	2.2–2.6
Albumin (g/l)	220	41.8	3.2	35–46
CCa (Alb) (mmol/l)	220	2.37	0.14	2.2–2.6
Cr	220	76.5	12.0	70–150
PTH (ng/l)	79	36.0	16.2	10–65
25OHD ₃ (nmol/l)	81	70.8	33.9	25–120
1,25(OH) ₂ D ₃ (ng/l)	66	38.1	16.7	20–50
Urine				
Ca (mmol/l)	188	3.94	1.83	NA
Cr (mmol/l)	188	8.65	2.60	NA
Ca : Cr ratio	188	0.45	0.32	< 0.57
BMD				
LS (g/cm ²)	218	0.984	0.163	0.82–1.15
Left total hip (g/cm ²)	215	0.896	0.128	0.77–1.02
Left forearm (g/cm ²)	204	0.543	0.06	0.48–0.6

N, number of individuals; BMI, body mass index; Ca, total calcium; CCa (Alb), calcium corrected for albumin of 41 g/l; Cr, creatinine; 25OHD₃, 25-hydroxyvitamin D₃; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; Ca : Cr ratio, calcium : creatinine ratio on morning urine specimen; BMD, bone mineral density; LS, total lumbar spine; NA, not applicable.

Results

Clinical characteristics

The 220 females had a mean age (\pm SD) of 55.9 (\pm 7.8) years and a mean body mass index (BMI) (\pm SD) of 25.1 (\pm 4.5) kg/m². Serum calcium, albumin, PTH, 25OHD₃, 1,25(OH)₂D₃, urinary calcium and creatinine, and BMD were normally distributed in the 110 female twin pairs, and the means (\pm SD) are detailed in Table 2. These values did not differ significantly from those expected from a normal population of women in this age group.^{24,25,32}

CaSR genotypes

The frequencies of the CaSR polymorphisms A986/S986, R990/G990 and Q1011/E1011 in 110 unrelated individuals from the 110 dizygotic pairs were found to be 0.89/0.11, 0.93/0.07 and 0.96/0.04, respectively. The frequencies of these respective alleles in another 55 unrelated normal individuals of British origin were found to be 0.88/0.12, 0.93/0.07 and 0.96/0.04. Thus, these allelic frequencies were similar in the twin and singleton populations. In addition, these allelic frequencies were similar to those reported in populations from Canada, Sweden, Hungary, New Zealand, Australia, Italy and Japan (Table 1). The allele frequencies of the three CaSR polymorphisms revealed no evidence of LD ($P = 0.82$ – 0.87) or departure from the HWE ($P = 0.4$ – 0.67). Evidence of population stratification was not detected for any phenotype.

Association studies

The phenotypes of serum total calcium, albumin, calcium corrected for albumin, PTH, 25OHD₃, 1,25(OH)₂D₃, urinary calcium, urinary creatinine, urinary calcium : creatinine ratio and BMD for the total lumbar spine, left total hip and left forearm obtained from the 220 individuals (i.e. 110 dizygotic twin pairs) for each of the nine genotypes at codons 986, 990 and 1011 were compared (Table 3) (total calcium, albumin, urinary calcium, urinary creatinine; data not shown). This revealed that the mean values of these phenotypes were similar in all the different genotype groups (Table 3). Furthermore, tests of association revealed an absence of an association ($P = 0.08$ – 0.99) between each of the three CaSR coding region polymorphisms and these phenotypic assessments of calcium homeostasis and bone metabolism (Table 3). We did not proceed to performing a further analysis using CaSR haplotypes as the analysis of the individual genotypes had not yielded a significant association with any phenotypes.

Functional characterization of CaSR coding region polymorphisms

Functional expression of the wild-type and the three polymorphic CaSRs (A986S, R990G and Q1011E) was undertaken by using HEK293 cells (Fig. 1). The previously reported CaSR mutant, Y129, in a family with ADHH⁹ was used as a control, together with the empty pEGFP vector, in these functional studies. The wild-type, mutant and polymorphic CaSRs were all expressed mainly at the plasma membrane and in the cytoplasm but not in the nucleus (Fig. 1a). Western blot analysis confirmed expression of the wild-type, mutant and polymorphic CaSRs as 167-kDa proteins (Fig. 1b), which consisted of the 140-kDa CaSR tagged with a 27-kDa-enhanced GFP (EGFP). A functional characterization of the wild-type and mutant, Y129, CaSRs, as assessed by the alteration in $[Ca^{2+}]_i$ in response to $[Ca^{2+}]_o$, revealed a marked leftwards shift in the dose–response and a significantly ($P < 0.01$) lower EC₅₀ (mean \pm SEM) of 0.98 ± 0.26 mM for the mutant CaSR, when compared to that of the wild-type (mean EC₅₀ = 2.74 ± 0.29 mM). This is consistent with the previously reported association of this mutation with ADHH.⁹ However, a similar functional characterization of the wild-type and polymorphic CaSRs revealed that all three polymorphic CaSRs had dose–response curves that were indistinguishable from that of the wild-type (Fig. 1c). Moreover, the three polymorphic CaSRs had EC₅₀ values that did not differ significantly from each other; the EC₅₀ (mean \pm SEM) values of the A986S, R990G and Q1011E polymorphisms were 3.09 ± 0.34 , 2.99 ± 0.23 and 2.96 ± 0.30 mM, respectively, and these were not significantly different from that of the wild-type CaSR, which had an EC₅₀ value of 2.74 ± 0.29 mM. Thus, these results indicate that the CaSR polymorphisms at codons 986, 990 and 1011 do not lead to altered set-points of the CaSR.

Discussion

Our results are the first to reveal that the three CaSR polymorphisms 986S, 990G and 1011E, which are located in the coding region, do not alter the set-point of the receptor (Fig. 1). Indeed, all these CaSR polymorphisms have EC₅₀ values that are similar to the wild-type

Table 3. Phenotypes for each genotype at codons 986, 990 and 1011, obtained from the 220 individuals

		Codon 986			Codon 990			Codon 1011				
		N	Mean	SD	N	Mean	SD	N	Mean	SD		
<i>Serum</i>												
CCa (Alb) (mmol/l)	AA	170	2.36	0.14	RR	192	2.37	0.14	QQ	202	2.37	0.14
	AS	46	2.41	0.12	RG	28	2.33	0.12	QE	18	2.37	0.13
	SS	4	2.35	0.24	GG	2	2.55	0.04	EE	0	–	–
PTH (ng/l)	AA	61	38.0	17.2	RR	67	34.2	14.5	QQ	76	36.2	16.3
	AS	18	29.4	10	RG	10	40.5	19	QE	3	34.5	11.3
	SS	0	–	–	GG	2	73.3	0.21	EE	0	–	–
25OHD ₃ (nmol/l)	AA	61	66.5	36.2	RR	69	73.1	34.7	QQ	78	71.5	34.3
	AS	15	84.1	21.3	RG	10	64.4	24.5	QE	3	65.7	36.8
	SS	0	0	0	GG	2	24.5	1.6	EE	0	–	–
1,25(OH) ₂ D ₃ (ng/l)	AA	51	38.3	18.6	RR	56	38.6	17.8	QQ	65	38.1	16.8
	AS	15	37.3	7.7	RG	10	34.9	8	QE	0	–	–
	SS	0	–	–	GG	0	–	–	EE	0	–	–
<i>Urine</i>												
Ca : Cr ratio	AA	141	0.44	0.15	RR	167	0.45	0.17	QQ	172	0.45	0.14
	AS	43	0.46	0.13	RG	19	0.37	0.32	QE	16	0.37	0.62
	SS	4	0.44	0.5	GG	2	0.67	1.2	EE	0	–	–
<i>BMD</i>												
LS (g/cm ²)	AA	170	0.986	0.161	RR	191	0.989	0.166	QQ	202	0.984	0.16
	AS	44	0.98	0.167	RG	28	0.969	0.141	QE	18	1.014	0.168
	SS	4	0.879	0.178	GG	2	0.814	0.003	EE	0	–	–
Left total hip (g/cm ²)	AA	168	0.889	0.126	RR	189	0.907	0.13	QQ	199	0.897	0.129
	AS	43	0.925	0.139	RG	27	0.835	0.117	QE	18	0.913	0.126
	SS	4	0.87	0.019	GG	2	0.857	0.004	EE	0	–	–
Left forearm (g/cm ²)	AA	158	0.54	0.058	RR	181	0.544	0.058	QQ	188	0.545	0.059
	AS	43	0.558	0.059	RG	24	0.54	0.061	QE	18	0.541	0.045
	SS	3	0.492	0.081	GG	2	0.45	0.014	EE	0	–	–

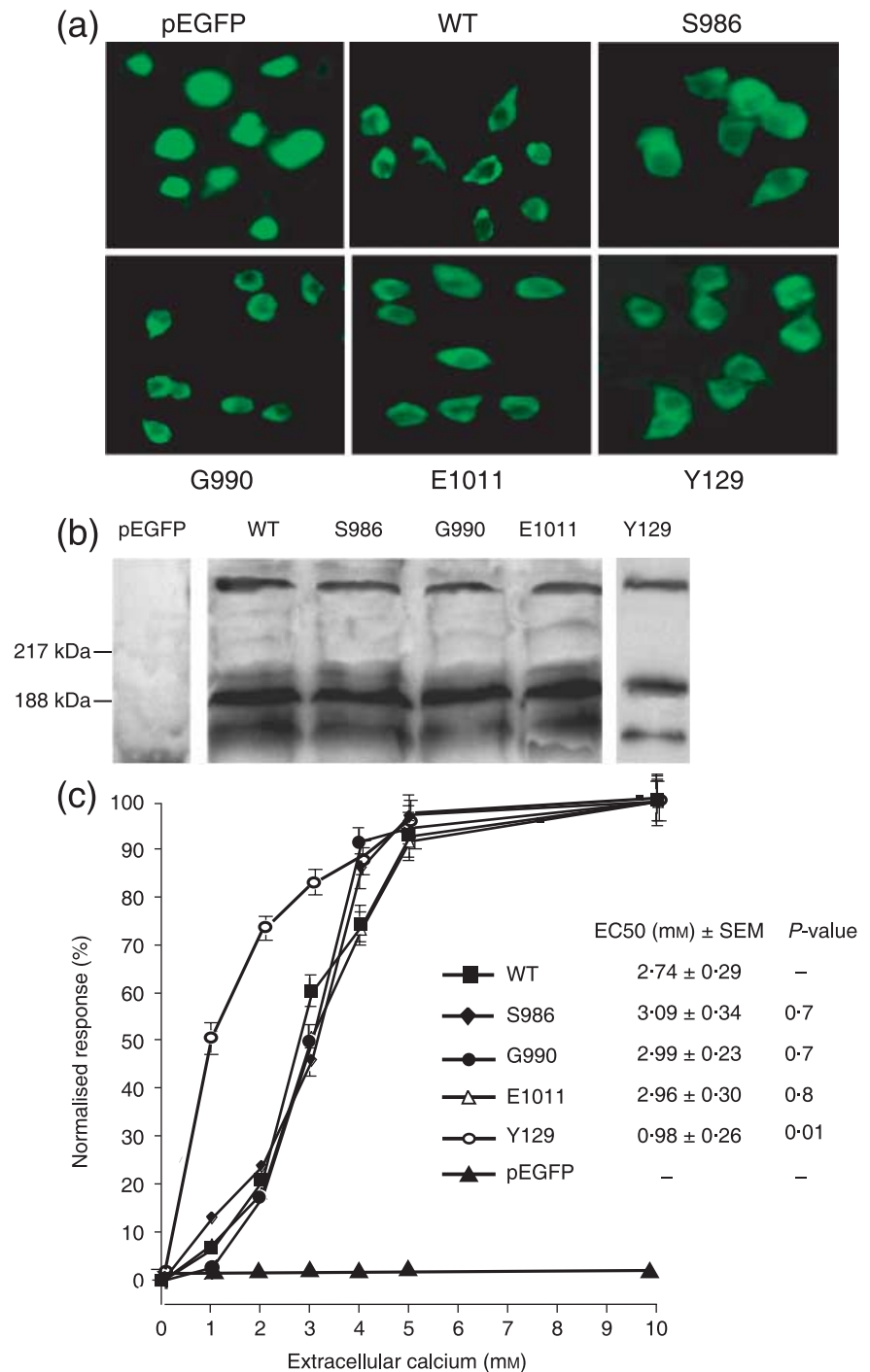
N, number of individuals [0 indicates that there were no available samples from individuals in that genotypic group, and hence there are no mean or SD values (indicated by –)]; CCa (Alb), calcium corrected for albumin of 41 g/l; 25OHD₃, 25-hydroxyvitamin D₃; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; Ca : Cr ratio, calcium : creatinine ratio on morning urine specimen; BMD, bone mineral density; LS, total lumbar spine.

CaSR, which consists of A986/R990/Q1011. Thus, these three CaSR polymorphisms are markedly different from the missense mutations that are associated with FBHH and NSHPT, and with ADHH, which alter the set-point of the receptor and are associated with significantly higher and lower EC₅₀ values, respectively.¹⁰ The missense mutations associated with FBHH, NSHPT and ADHH result in major disturbances in calcium homeostasis and bone metabolism. For example, FBHH, which is due to heterozygous loss-of-function CaSR mutations, is characterized by hypercalcaemia in association with normal or mildly elevated serum PTH concentrations and an inappropriately low urinary calcium excretion. Serum concentrations of 25OHD₃ and 1,25(OH)₂D₃ are normal and there are no reported abnormalities of BMD.^{33,34} By contrast, in NSHPT, which may be due to homozygous loss-of-function CaSR mutations, bone undermineralization occurs in association with hypercalcaemia.⁴ Gain-of-function mutations result in ADHH (Fig. 1), which is characterized by mild hypocalcaemia with low-normal serum PTH concentrations.⁵ In addition, patients with ADHH have an elevated urinary calcium excretion.⁵ The BMD in ADHH patients has been reported to be either normal or increased.⁵ However, the three CaSR polymorphisms A986S, R990G and Q1011E, which result in amino

acid changes that resemble the missense changes seen in FBHH, NSHPT and ADHH, are not associated with alterations in the EC₅₀ values of the receptor (Fig. 1), and thus on this basis would not be expected to result in marked changes in serum calcium, PTH or urinary calcium excretion. Indeed, this is the case in our study, where we found no association between any of the CaSR coding region polymorphisms and indices of calcium homeostasis and BMD (Table 3). Thus, statistically significant associations between each of the three CaSR polymorphisms, A986S, R990G and Q1011E, and serum calcium corrected for albumin, serum PTH; serum 25OHD₃; serum 1,25(OH)₂D₃; urinary calcium and creatinine ratios; and BMD at the lumbar spine, forearm and hip were not found. However, our study, which focused only on investigating the three known CaSR polymorphisms of the coding region, does not represent an exhaustive analysis of the gene. Such an analysis would include intronic polymorphisms and also low frequency single nucleotide polymorphisms (SNPs) obtained from detailed resequencing.

Our results showing a lack of association between the three CaSR polymorphisms of the coding region and serum calcium, serum PTH and BMD are in agreement with those of three other studies in Australian, New Zealand and Hungarian populations.^{16–18} All of

Fig. 1 Functional expression in HEK293 cells of the wild-type, mutant Y129 and three polymorphic coding region CaSRs. HEK293 cells were transiently transfected with wild-type, WT (A986/R990/Q1011) or the three CaSR-EGFP constructs, which yielded the S986, G990 and E1011 polymorphic variants. In addition, a CaSR mutant, Y129, construct, which was associated with ADHH,⁹ and the pEGFP vector alone (pEGFP) were used as controls and similarly transfected. (a) Fluorescence microscopy was used to confirm successful transfection. HEK293 cells transfected with WT, mutant Y129 or polymorphic CaSRs showed similar expression patterns with fluorescence in the cytoplasm and at the plasma membrane, but not in the nucleus. Cells transfected with pEGFP alone showed a uniform pattern, whereas untransfected cells had no fluorescence (data not shown). (b) Western blot analysis of total cell protein extracts from HEK293 cells transfected with WT, mutant Y129 or polymorphic CaSRs by using an ADD monoclonal CaSR antibody confirmed the expression of EGFP-tagged CaSR (167 kDa), which was not present in the control cells transfected with the pEGFP vector only. (c) Single, live cells loaded with indo-1-acetomethylester emitting fluorescence at 525 nm, and hence containing transfected CaSR, were selected by FACS and the $[Ca^{2+}]_o$ -evoked increase in $[Ca^{2+}]_i$ were measured. The increments in $[Ca^{2+}]_o$ from 0 to 10 mM are shown on the *x* axis, and the $[Ca^{2+}]_i$ response, which was measured as a percentage of the maximum normalized response, is on the *y* axis (mean \pm SEM of four estimations). Transfection with pEGFP vector alone evoked no increases in $[Ca^{2+}]_i$, in keeping with an absence of endogenous CaSRs in HEK293 cells. The EC_{50} of the ADHH-associated mutant Y129 CaSR was significantly ($P < 0.01$) lower than that of the wild-type, whereas the EC_{50} of each of the polymorphic CaSRs, S986, G990 and E1011, was similar to that of the wild-type (A986/R990/Q1011).



these studies investigated adult, healthy women who were postmenopausal. The findings from these three reports^{16–18} and our study (Table 3) differ from those of six other studies that found associations between CaSR polymorphisms and serum calcium, serum PTH and BMD in Canadian, Italian, Swedish and Japanese populations (Table 1). These conflicting results may be due in part to previous small sample sizes, differences in the populations together with their health status, sex and age, and the exact CaSR polymorphism that was investigated (Table 1). For example, the only study to report a correlation between a CaSR polymorphism, which consisted of only

the R990G, and serum PTH was based on haemodialysis patients (48% females and 52% males) in Japan.¹² Another study, in Japanese postmenopausal women, reported an association between forearm BMD and a noncoding CaSR polymorphism that involved a (*ca*)_{12–20} repeat located within 110 kb of the CaSR.¹³ Three studies have investigated adolescent¹⁴ or premenopausal women^{11,15} from Swedish¹⁴ or Canadian^{11,15} populations and reported that the A986S polymorphism is associated with elevated serum calcium corrected for albumin^{11,14,15} and reduced BMD,¹⁴ and one study, which consisted of 49% adult males, 36% premenopausal women and 15%

postmenopausal women from Southeastern Italy, reported an association between serum calcium and all three CaSR polymorphisms of the coding region.¹⁹ Although the allelic frequencies of the CaSR polymorphisms in the different populations are similar (Table 1) to those found in our study, it is important to note that small, but significant, pairwise LD between the A986S and R990G and the A986S and Q1011E polymorphisms has been observed in Canadian and Italian populations^{11,19} but not in other populations, including that of our study. These differences in the characteristics and size of the populations make it difficult to perform detailed comparisons. Moreover, in any 'negative' result, the power of the study is a crucial issue. To detect a locus that corresponds to 10% or 5% of the genetic variance at $P = 0.01$, our study of the 220 females from the twin registry had a power of 0.98 and 0.76, respectively, assuming a nominal sib phenotype correlation of 0.1 and a marker frequency of 0.2.³⁵ Thus, our study was sufficiently powered to detect effects that would explain up to 5% of the variance, but would not exclude effects at the level of 1%. However, it is important to note that the previous 'positive' studies^{11–15,19} have all been of a similar or smaller size, and hence of a similar power to that of our study, thereby indicating that any observed effects, if real, would have been large. Hence, based on our data and a review of the published data, it would seem unlikely that these CaSR coding region polymorphisms play a major role in calcium homeostasis and BMD.

We chose to perform our study in dizygotic twins for the following reasons. First, basing the study on the twin pairs has the advantage of minimizing the effects of environmental and other genetic factors. Second, a previous study of this twin population has established a high degree of heritability for plasma calcium concentrations, which follow a Gaussian distribution in healthy individuals.^{23,36,37} Third, a previous report of dizygotic twins has established that the results from such studies (e.g. of BMD) do not differ from those obtained in single individuals.³⁸ We also chose to investigate a homogeneous population and limited the study to postmenopausal women as this is the group that is most frequently affected with disorders of calcium homeostasis, such as primary hyperparathyroidism and osteoporosis.^{39,40} Our results show that the CaSR polymorphisms of codons 986, 990 and 1011 do not significantly influence: serum calcium corrected for albumin; serum PTH; serum 25OHD₃; serum 1,25(OH)₂D₃; urinary calcium : creatinine ratios; and BMD at the total lumbar spine, forearm and total hip. These findings of a lack of association are supported by detecting an absence of functional differences in the dose–response curve of the CaSR polymorphisms, which have similar EC₅₀ values (Fig. 1).

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