A novel mutation in the calcium-sensing receptor responsible for autosomal dominant hypocalcemia in a family with two uncommon parathyroid hormone polymorphisms

D Álvarez-Hernández, Í Santamaría, M Rodríguez-García, P Iglesias¹, R Delgado-Lillo² and J B Cannata-Andía

Bone and Mineral Research Unit, Instituto Reina Sofía de Investigación, Hospital Universitario Central de Asturias, c/Julián Clavería s/n, 33006 Oviedo, Spain

¹Endocrinology Unit, Hospital General de Segovia, Segovia, Spain

²Nephrology Unit, Clínica Ruber, Madrid, Spain

(Requests for offprints should be addressed to Í Santamaría; Email: isr@hca.es)

Abstract

A novel missense activating mutation in the extracellular calcium-sensing receptor (CaSR) is reported in this work. It was identified in three related subjects with the phenotypic features of autosomal dominant hypocalcemia (ADH). The proband, a 27-year-old woman, diagnosed as having hypoparathyroidism at 7 years of age and a history of seizures, showed the highest penetrance of the mutation. The remaining two affected members presented asymptomatic chronic hypocalcemia despite severe hypoparathyroidism associated with high levels of serum phosphate and calcium urinary excretion. The missense mutation (Glu⁶⁰⁴Lys) affected an amino acid residue in the C terminus of the cysteine-rich domain of the extracellular amino-terminal domain, which seems to be required for the coupling of ligand binding to the activation of intracellular signaling pathways. This genetic change cosegregated with hypocalcemia in all the individuals where the mutation was found. As parathyroid hormone (PTH) secretion is the regulatory target of the CaSR, polymorphism analysis of the PTH gene was carried out. PTH polymorphisms were analyzed in the kindred studied. Affected members for the Glu⁶⁰⁴Lys CaSR mutation which also carried the uncommon PTH alleles showed higher penetrance of the mutation, with more severe autosomal dominant hypocalcemia. These results suggested that the PTH gene could act as a modifier locus of ADH, affecting the penetrance of the activating CaSR mutation described.

Journal of Molecular Endocrinology (2003) **31**, 255–262

Introduction

Autosomal dominant hypocalcemia (ADH) is a rare hereditary syndrome characterized by low serum parathyroid hormone (PTH) levels despite hypocalcemia and relative hypercalciuria, leading to nephrolithiasis and nephrocalcinosis, especially after active vitamin D treatment (Winter *et al.* 1983, De Campo *et al.* 1988). Mutations responsible for a gain-of-function in the calcium-sensing receptor (CaSR) gene and able to inhibit PTH secretion and renal calcium reabsorption are the cause of ADH

(Pollak *et al.* 1994, Baron *et al.* 1996). The clinical presentation of ADH varies from asymptomatic hypocalcemia to neonatal hypocalcemic seizures even in families with the same CaSR gene mutation.

The CaSR was first identified in bovine parathyroid cells and was later found to be expressed in the kidney and other tissues (Brown *et al.* 1995b). The human CaSR encodes a polypeptide of 1078 amino acids organized into three domains: a very large (612 amino acids) extracellular domain at the amino-terminus, a

transmembrane domain with seven membrane-spanning helices characteristic of G protein-coupled receptors and an intracellular (216 amino acids) carboxyl-terminal tail (Brown *et al.* 1993, 1995a, Aida *et al.* 1995, Garrett *et al.* 1995, Brown 2000). Mutations in this CaSR involving loss of function cause familial benign hypercalcemia, also known as familial hypocalciuric hypercalcemia; those who have this autosomal disorder, generally asymptomatic, have life-long elevations of serum calcium concentrations, together with a low urinary excretion of calcium (Pollak *et al.* 1993). On the other hand, heterozygous gain-of-function (activating) mutations are associated with the reverse phenotype – autosomal dominant hypocalcemia – as described previously. To date, 40 inactivating and 25 activating mutations have been identified (Hendy *et al.* 2000). These are mostly missense mutations, unevenly distributed throughout the coding region of the CaSR but clustered in two regions: within the first 300 amino acids of the extracellular domain and from amino acids 520 to 881 in or near the transmembrane domain. Residues in the first 300 amino acids of the extracellular domain have been shown to be important for calcium binding to the CaSR and/or dimerization of the receptor (Brauner-Osborne *et al.* 1999, Zhang *et al.* 2001).

PTH secretion is the regulatory target of the CaSR. Several polymorphisms have been described in the gene encoding PTH, which may influence not only the secretion rate of the hormone in response to the CaSR signaling but also the efficiency of the hormone, as has been shown in bone metabolism studies (Katsumata *et al.* 2002) and familial isolated hypoparathyroidism (FIH) (Miric & Levine 1992). This syndrome, characterized by hypocalcemia and hyperphosphatemia due to an inherited deficient secretion of biologically active parathormone, has been related to PTH gene mutations and polymorphisms (Ahn *et al.* 1986, Sunthornthepvarakul *et al.* 1999). A mutation in the transcription factor GCMB has also been associated with FIH (Ding *et al.* 2001). Three major polymorphisms and two mutations have been described in the PTH gene; one of the polymorphisms is located in intron 1, only 15 nucleotides from the start codon (Yamamoto *et al.* 1997) whilst the remaining two are located in intron 2 and exon 3 (Mullersman *et al.* 1992). A mutation in the signal peptide of the parathyroid hormone gene has

Figure 1 Pedigree of the studied kindred. The arrow indicates the proband. Solid symbols are individuals with the Glu⁶⁰⁴Lys mutation.

also been described (Sunthornthepvarakul *et al.* 1999), along with a donor splice site mutation in the first nucleotide of intron 2 (Parkinson *et al.* 1993). Although all five genetic variations have been studied in relation to FIH syndrome, no conclusive general results have been defined, thus pointing out the variability of the causes of this condition (Ahn *et al.* 1986).

As previously stated, the penetrance of CaSR-activating mutations is extremely variable. To date, no relationship has been found either between activating changes and polymorphisms of the CaSR gene with pathological expression of the mutations (Kanazawa *et al.* 2000) or with the location of those changes within the CaSR gene (Nagase *et al.* 2002). In this work we report a novel activating mutation (Glu⁶⁰⁴Lys) in a Spanish kindred suffering autosomal dominant hypocalcemia that may be modulated by polymorphisms of the PTH gene and thus affecting its clinical presentation.

Materials and methods

Clinical subjects

The proband (subject II.3; Fig. 1) was born in 1976 and was diagnosed as having idiopathic hypoparathyroidism at 7 years of age after presenting with seizures. She has been clinically monitored to date. Studies were also conducted on the other four members of her family. The absence of the Glu⁶⁰⁴Lys mutation was verified in 100 unrelated Spaniards by RFLP. The study was approved by the Hospital Universitario Central de Asturias Clinical Ethics Committee.

Table 1 Oligonucleotides designed for the amplification and sequencing of the DNA fragments used in this work. Primers for the CaSR gene: in those coding regions too large to sequence in one row (exons 4 and 7) internal primers were designed for sequencing. Primers for the PTH gene are also shown

Gene	Location	Primers (5' to 3')
CaSR	Exon 2	Forward: CTGCAGGGAGTGAACTGCTCC Reverse: GTGGGGGCAATAAGCTTCAGCAT
	Exon 3	Forward: GGGCTCTGTACAGAGCATGCC Reverse: TGGTAAACCGTATGGCTATTGGG
	Exon 4	Forward: ACAGCCTGGAGGCTCACTCAG Reverse: TGCCTGGAGTTGCAGCCCAAC
	Exon 4–internal-1	Forward: TCTCCAGTGGCCCAGATCTTGA Reverse: GTGGGGGCAATAAGCTTCAGCAT
	Exon 4–internal-2	Forward: TAGCAACAGCTCGACAGCCTTC Reverse: GTGGGGGCAATAAGCTTCAGCAT
	Exon 5	Forward: GTGCAGGGCACAGCCTACCTA Reverse: TTCCTGCCCCTGGTGGAGACA
	Exon 6	Forward: GAAGAGACAGTAGGGCTGGCC Reverse: CCAGGCCTCCGAAGTGGACC
	Exon 7	Forward: GTCTGTGCCACACAATAACTCAC Reverse: CCAAGAAACCTCTCTGCATTCTC
	Exon 7–internal-1	Forward: CTGCATCTCATGCATCCTGGTG Reverse: CCAAGAAACCTCTCTGCATTCTC
	Exon 7–internal-2	Forward: TCATTCCAGCCTATGCCAGCAC Reverse: CCAAGAAACCTCTCTGCATTCTC
	Exon 7–internal-3	Forward: AGCAGCAACGATCTCAGCAGCA Reverse: CCAAGAAACCTCTCTGCATTCTC
PTH	Intron 2 polymorphism	Forward: ATTAGTTAGTATTGCATTCTGT Reverse: AACAGTACTTACTTAACAGATT
	<i>Bst</i> BI, <i>Drall</i> polymorphisms	Forward: ATTAGTTAGTATTGCATTCTGT Reverse: TTGAATTAGCAGCATGTATTG

Genomic DNA isolation

High molecular weight nuclear DNA was isolated from peripheral blood leukocytes of the proband, the other four family members and 100 normal subjects by standard procedures (Sambrook *et al.* 1989).

Amplification, sequence and confirmation of the CaSR mutation

In the proband, all protein-coding exons (2–7) of the CaSR were amplified by PCR using the primer pairs detailed in Table 1. PCR was performed in 30 cycles using the following steps: denaturation at 95 °C for 30 s, annealing at 64 °C for 30 s and extension at 72 °C for 90 s on a volume of 20 µl. The PCR reactions were carried out in a GeneAmp 9700 PCR system from Applied

Biosystems (Foster City, CA, USA). The amplification products were purified and nucleotide sequences of both strands were determined by direct sequencing with an Applied Biosystems 310A automatic sequencer.

After finding the Glu⁶⁰⁴Lys mutation in the proband, exon 7 of the CaSR gene of other family members and control subjects was amplified by PCR using the primer pair 5'-GTCTGTGCCACACAATAACTCAC-3' and 5'-CCAAGAAACCTCTCTGCATTCTC-3'. As the mutation, a replacement of a guanine by an adenine, destroys a recognition site for TaqI (TCGA), this endonuclease was used to digest the amplified 431 bp fragment. The absence of the mutation generates two fragments of 310 and 121 bp, detected by electrophoresis on a 1.2% agarose gel (Fig. 2A). These experiments were carried out at least twice

and the results were reproducible. Each experiment contained the affected patient's DNA which was heterozygous for the mutation to ensure that all samples were completely digested by restriction enzyme.

PTH polymorphisms analysis

PTH gene polymorphisms were studied by PCR amplification (primers reproduced in Table 1) and restriction or direct sequencing analysis was carried out in all the family members. PCR conditions were: denaturation at 95 °C for 30 s, annealing at 54 °C for 15 s and extension at 72 °C for 30 s on a volume of 20 µl. All three polymorphisms described to date were analyzed. For intron 1 polymorphism, a 186 bp DNA fragment was amplified and sequenced. The remaining two PTH gene polymorphisms, located in intron 2 and exon 3 disrupt restriction sites *Bst*BI and *Dra*II respectively; they were determined by amplification of a 609 bp fragment and restriction analysis with the appropriate enzymes. In the case of *Bst*BI, the presence of the restriction site (denoted as B) produces two DNA fragments of 383 and 226 bp, while the presence of the *Dra*II restriction site (denoted as D) produces two fragments of 434 and 175 bp.

Results

DNA sequence analysis of the proband showed a heterozygous single base G→A missense mutation in position 2182 of exon 7 (GenBank U20759), resulting in the amino acid substitution of a glutamic acid by a lysine. The mutation found (Glu⁶⁰⁴Lys), lies at the C terminus of the Cys-rich domain of the extracellular head, only nine residues from the first transmembrane helix.

Once the mutation was found by sequencing the proband's coding regions of the CaSR gene, and taking into account that the G→A transition destroys a *Taq*I recognizing sequence, this restriction site was used to analyze the other members of

the family and 100 unrelated DNA controls. Figure 2A shows that the mutation analysis of the CaSR found three affected members of the family that was studied: I.1, II.1 and the proband II.3. The results are thus consistent with the autosomal dominant inheritance pattern in ADH. None of the unrelated controls was positive for the genetic change, thus confirming its mutation character. Although members I.1 and II.3 were currently in treatment (being the only ones suspected of carrying the mutation), II.1 was also found to possess the genetic change in the CaSR gene. II.1 was undiagnosed, but the analytical results confirmed low calcium serum levels (calcium 6.5 mg/dl), thus confirming the autosomal dominant hypocalcemia determined by the Glu⁶⁰⁴Lys change.

While I.1 and II.1 were asymptomatic, and only low levels of serum calcium and PTH have determined their medical treatment, II.3, on the other hand, was diagnosed with idiopathic hypoparathyroidism at 7 years of age after suffering tetanias caused by hypocalcemia (calcium 6 mg/dl; intact PTH (iPTH) < 1 pg/ml). Treatment with vitamin D produced a toxic hypercalcemia episode (calcium 15.7 mg/dl), probably due to D3 hypervitaminosis. Lithotripsy was required to eliminate a calcium oxalate kidney stone at 14 years of age and a digestive hemorrhagic episode was treated at 19 years of age.

I.1 was diagnosed with hypocalcemia (calcium 6.5 mg/dl; iPTH 6 pg/ml) after a routine analysis at 54 years of age and has been on calcium, vitamin D, magnesium, potassium and thiazide treatment without an improvement in the calcemic levels. A review of his clinical records showed an isolated episode of loss of consciousness at 10 years of age, while lithiasis in both kidneys, along with calcification of his cerebral falx and several smooth tissues, are a good indication of a long and severe penetrance of the pathology.

II.1 had remained undiagnosed until the genetic study showed the presence of the CaSR-activating

Figure 2 Genetic analysis of the proband's family. (A) Analysis of the Glu⁶⁰⁴Lys mutation in all members of the family. The mutation destroys a *Taq*I restriction site present in exon 7 of the CaSR gene. I.1, II.1 and II.3 are heterozygous for the mutation. (B) Analysis of the PTH polymorphisms. A 609 bp DNA fragment partially comprising intron 2 and exon 3 was amplified. Restriction analysis with *Bst*BI and *Dra*II is shown in the upper images, with uppercase letters B and D denoting presence of those restriction sites. For the intron 1 polymorphism, a 186 bp was amplified and sequenced. The partial sequence comprising the genetic change (G to A) is shown in the lower image.

mutation. The biochemical parameters revealed hypocalcemia, low levels of serum PTH and hyperphosphatemia, but none of the related pathological consequences found in both his father and younger sister.

Neither I.2 nor II.2 showed any anomalies in their biochemical parameters. Both parents of I.1 are deceased and therefore unavailable for the mutation analysis, but as none had a history of hypocalcemia, it is possible to speculate that the CaSR dominant mutation appeared *de novo* in I.1.

In order to explain the differences in the clinical presentation among the affected members, a study of the PTH gene variations was performed. The results, shown in Fig. 2B, demonstrated that I.1, II.2 and II.3 presented variation for the polymorphism located 15 nucleotides from the initiation codon (intron 1) and for the *BstBI* restriction site. All family members were homozygous for the silent polymorphism located in the *DraII* site of exon 3. Analysis of the signal peptide of the preproparathyroid hormone gene and the donor splice site mutations was also carried out, showing that none of them were present in the kindred (data not shown).

Discussion

The present study demonstrates a novel missense activating mutation (Glu⁶⁰⁴Lys) affecting the CaSR in a Spanish kindred with ADH. The proband has been suffering severe hypocalcemia and hypercalciuria since childhood, while the other affected members of this family showed asymptomatic hypocalcemia with hyperphosphatemia, relative hypercalciuria and low levels of PTH. As these results and the autosomal dominant pattern of inheritance are suggestive of ADH, the molecular studies necessary to establish the diagnosis were conducted. The results suggested that the Glu⁶⁰⁴Lys mutation caused ADH in this family, as the mutation was found in all three hypocalcemic members but in none of the normocalcemic members of the family or in 100 control subjects.

Interestingly, this mutation has also been recently described in an Australian kindred during the elaboration of this work (Tan *et al.* 2003). Functional analysis of the Glu⁶⁰⁴Lys mutation confirmed that the mutation significantly increased the sensitivity to Ca²⁺, with values comparable

with those for other activating mutations (EC₅₀ values for extracellular Ca²⁺: wild-type, 4.4 mM; E604K mutant, 3.6 mM) (Tan *et al.* 2003). To date, 25 activating mutations in the CaSR have been reported, and with the exception of a large deletion in the carboxyl-terminal tail, all are missense mutations (Hendy *et al.* 2000). Their location within the CaSR gene is variable as 14 are localized in the extracellular domain (Pollak *et al.* 1994, Baron *et al.* 1996, Pearce *et al.* 1996, De Luca *et al.* 1997, Mancilla *et al.* 1998, Okazaki *et al.* 1999, Conley *et al.* 2000, Hendy *et al.* 2000, Tan *et al.* 2003), six in the transmembrane helices (Baron *et al.* 1996, De Luca *et al.* 1997, Watanabe *et al.* 1998a, Hendy *et al.* 2000) and two in the extracellular loops within the transmembrane domain (Baron *et al.* 1996, Hendy *et al.* 2000). Although some authors have pointed out that mutations in the transmembrane helices might produce more severe hypocalcemia than those present in the extracellular domain (Watanabe *et al.* 1998b), no significant relationship has been further demonstrated between mutation location and penetrance of the mutation. The Glu⁶⁰⁴Lys-activating mutation lies in the Cys-rich domain of the extracellular head, which seems to be required for the coupling of ligand binding to the activation of intracellular signaling pathways (Hu *et al.* 2000). The activating effect of Glu⁶⁰⁴Lys might therefore arise from a sensitizing effect on signal transmission (Tan *et al.* 2003).

In order to explain the clinical differences between family members affected with the activating dominant mutation Glu⁶⁰⁴Lys, polymorphisms of the PTH gene were also analyzed. Only two out of the three PTH polymorphisms were informative, showing that I.1, II.2 and II.3 were heterozygous for a nucleotide variation in both *BstBI* and intron 1 polymorphisms. I.1 and II.3 were also carriers of the Glu⁶⁰⁴Lys and those with major ADH penetrance. Although none of the *BstBI* and intron 1 polymorphisms have been associated with drastic effects on PTH expression or efficiency, translated as FIH presentation, both have been associated with differences in bone mineral density, bone dimension and osteoporosis (Almahroos *et al.* 1987, Gong *et al.* 1999, Hosoi *et al.* 1999, Gohda *et al.* 2002). I.1 and II.3, carrying both the CaSR mutation and the less favorable allelic form of each polymorphism, are likely to have their CaSR-related condition worsened due to PTH deficiency.

Thus, considering both the CaSR mutation and the polymorphic changes in the PTH gene, it might be speculated that the PTH gene acts as a modifier locus of the ADH condition. Further studies in kindreds presenting this activating mutation or other diseases related to mutations of the CaSR will be required to confirm this hypothesis.

Acknowledgements

We would like to thank Pelayo González for his support in the sequencing process and Diego García-Manso and Saleh Tuwajri for supplying HEK-293 cells for the activation analysis. D A-H is a recipient of a Fundación para la Investigación Científica aplicada y la Tecnología scholarship. I S is a recipient of a Research Contract from Fondo de Investigaciones Sanitarias (FIS 00/3161).

References

- Ahn TG, Antonarakis SE, Kronenberg HM, Igarashi T & Levine MA 1986 Familial isolated hypoparathyroidism: a molecular genetic analysis of 8 families with 23 affected persons. *Medicine* **65** 73–81.
- Aida K, Koishi S, Tawata M & Onaya T 1995 Molecular cloning of a putative Ca(2+)-sensing receptor cDNA from human kidney. *Biochemical and Biophysical Research Communications* **214** 524–529.
- Almahroos GM, Docherty K, Fletcher JA, Webb T & Heath DA 1987 Studies of the parathyroid hormone gene in normal subjects, and in subjects with primary hyperparathyroidism and familial benign hypercalcaemia. *Journal of Endocrinology* **115** 183–186.
- Baron J, Winer KK, Yanovski JA, Cunningham AW, Laue L, Zimmerman D & Cutler GB Jr 1996 Mutations in the Ca(2+)-sensing receptor gene cause autosomal dominant and sporadic hypoparathyroidism. *Human Molecular Genetics* **5** 601–606.
- Brauner-Osborne H, Jensen AA, Sheppard PO, O'Hara P & Krogsgaard-Larsen P 1999 The agonist-binding domain of the calcium-sensing receptor is located at the amino-terminal domain. *Journal of Biological Chemistry* **274** 18382–18386.
- Brown EM 2000 G protein-coupled, extracellular Ca2+ (Ca2+(o))-sensing receptor enables Ca2+(o) to function as a versatile extracellular first messenger. *Cell Biochemistry and Biophysics* **33** 63–95.
- Brown EM, Gamba G, Riccardi D, Lombardi M, Butters R, Kifor O, Sun A, Hediger MA, Lytton J & Hebert SC 1993 Cloning and characterization of an extracellular Ca(2+)-sensing receptor from bovine parathyroid. *Nature* **366** 575–580.
- Brown EM, Pollak M, Chou YH, Seidman CE, Seidman JG & Hebert SC 1995a The cloning of extracellular Ca(2+)-sensing receptors from parathyroid and kidney: molecular mechanisms of extracellular Ca(2+)-sensing. *Journal of Nutrition* **125** 1965S–1970S.
- Brown EM, Pollak M, Chou YH, Seidman CE, Seidman JG & Hebert SC 1995b Cloning and functional characterization of extracellular Ca(2+)-sensing receptors from parathyroid and kidney. *Bone* **17** 7S–11S.
- Conley YP, Finegold DN, Peters DG, Cook JS, Oppenheim DS & Ferrell RE 2000 Three novel activating mutations in the calcium-sensing receptor responsible for autosomal dominant hypocalcemia. *Molecular Genetics and Metabolism* **71** 591–598.
- De Campo C, Piscopello L, Noacco C, Da Col P, Englaro GC & Benedetti A 1988 Primary familial hypoparathyroidism with an autosomal dominant mode of inheritance. *Journal of Endocrinological Investigation* **11** 91–96.
- De Luca F, Ray K, Mancilla EE, Fan GF, Winer KK, Gore P, Spiegel AM & Baron J 1997 Sporadic hypoparathyroidism caused by *de novo* gain-of-function mutations of the Ca(2+)-sensing receptor. *Journal of Clinical Endocrinology and Metabolism* **82** 2710–2715.
- Ding C, Buckingham B & Levine MA 2001 Familial isolated hypoparathyroidism caused by a mutation in the gene for the transcription factor GCMB. *Journal of Clinical Investigation* **108** 1215–1220.
- Garrett JE, Capuano IV, Hammerland LG, Hung BC, Brown EM, Hebert SC, Nemeth EF & Fuller F 1995 Molecular cloning and functional expression of human parathyroid calcium receptor cDNAs. *Journal of Biological Chemistry* **270** 12919–12925.
- Gohda T, Shou I, Fukui M, Funabiki K, Horikoshi S, Shirato I & Tomino Y 2002 Parathyroid hormone gene polymorphism and secondary hyperparathyroidism in hemodialysis patients. *American Journal of Kidney Disease* **39** 1255–1260.
- Gong G, Johnson ML, Barger-Lux MJ & Heaney RP 1999 Association of bone dimensions with a parathyroid hormone gene polymorphism in women. *Osteoporosis International* **9** 307–311.
- Hendy GN, D'Souza-Li L, Yang B, Canaff L & Cole DE 2000 Mutations of the calcium-sensing receptor (CASR) in familial hypocalciuric hypercalcemia, neonatal severe hyperparathyroidism, and autosomal dominant hypocalcemia. *Human Mutations* **16** 281–296.
- Hosoi T, Miyao M, Inoue S, Hoshino S, Shiraki M, Orimo H & Ouchi Y 1999 Association study of parathyroid hormone gene polymorphism and bone mineral density in Japanese postmenopausal women. *Calcified Tissue International* **64** 205–208.
- Hu J, Hauache O & Spiegel AM 2000 Human Ca2+ receptor cysteine-rich domain. Analysis of function of mutant and chimeric receptors. *Journal of Biological Chemistry* **275** 16382–16389.
- Kanazawa H, Tanaka H, Kodama S, Moriwake A, Kobayashi M & Seino Y 2000 The effect of calcium-sensing receptor gene polymorphisms on serum calcium levels: a familial hypocalciuric hypercalcemia family without mutation in the calcium-sensing receptor gene. *Endocrine Journal* **47** 29–35.
- Katsumata K, Nishizawa K, Unno A, Fujita Y & Tokita A 2002 Association of gene polymorphisms and bone density in Japanese girls. *Journal of Bone Mineral Metabolism* **20** 164–169.
- Mancilla EE, De Luca F & Baron J 1998 Activating mutations of the Ca2+-sensing receptor. *Molecular Genetics and Metabolism* **64** 198–204.
- Miric A & Levine MA 1992 Analysis of the preproPTH gene by denaturing gradient gel electrophoresis in familial isolated hypoparathyroidism. *Journal of Clinical Endocrinology and Metabolism* **74** 509–516.
- Mullersman JE, Shields JJ & Saha BK 1992 Characterization of two novel polymorphisms at the human parathyroid hormone gene locus. *Human Genetics* **88** 589–592.
- Nagase T, Murakami T, Tsukada T, Kitamura R, Chikatsu N, Takeo H, Takata N, Yasuda H, Fukumoto S, Tanaka Y, Nagata N, Yamaguchi K, Akatsu T & Yamamoto M 2002 A family of autosomal dominant hypocalcemia with a positive correlation between serum calcium and magnesium: identification of a novel gain of function mutation (Ser(820)Phe) in the calcium-sensing receptor. *Journal of Clinical Endocrinology and Metabolism* **87** 2681–2687.
- Okazaki R, Chikatsu N, Nakatsu M, Takeuchi Y, Ajima M, Miki J, Fujita T, Arai M, Totsuka Y, Tanaka K & Fukumoto S 1999 A novel activating mutation in calcium-sensing receptor gene

- associated with a family of autosomal dominant hypocalcemia. *Journal of Clinical Endocrinology and Metabolism* **84** 363–366.
- Parkinson DB, Shaw NJ, Himsworth RL & Thakker RV 1993 Parathyroid hormone gene analysis in autosomal hypoparathyroidism using an intragenic tetranucleotide (AAAT)_n polymorphism. *Human Genetics* **91** 281–284.
- Pearce SH, Williamson C, Kifor O, Bai M, Coulthard MG, Davies M, Lewis-Barned N, McCredie D, Powell H, Kendall-Taylor P, Brown EM & Thakker RV 1996 A familial syndrome of hypocalcemia with hypercalciuria due to mutations in the calcium-sensing receptor. *New England Journal of Medicine* **335** 1115–1122.
- Pollak MR, Brown EM, Chou YH, Hebert SC, Marx SJ, Steinmann B, Levi T, Seidman CE & Seidman JG 1993 Mutations in the human Ca(2+)-sensing receptor gene cause familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. *Cell* **75** 1297–1303.
- Pollak MR, Brown EM, Estep HL, McLaine PN, Kifor O, Park J, Hebert SC, Seidman CE & Seidman JG 1994 Autosomal dominant hypocalcaemia caused by a Ca(2+)-sensing receptor gene mutation. *Nature Genetics* **8** 303–307.
- Sambrook J, Fritsch EF & Maniatis T 1989 *Molecular Cloning: A Laboratory Manual*, edn 2. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
- Sunthornthepvarakul T, Churesigaew S & Ngowngarmratana S 1999 A novel mutation of the signal peptide of the preproparathyroid hormone gene associated with autosomal recessive familial isolated hypoparathyroidism. *Journal of Clinical Endocrinology and Metabolism* **84** 3792–3796.
- Tan YM, Cardinal J, Franks AH, Mun HC, Lewis N, Harris LB, Prins JB & Conigrave AD 2003 Autosomal dominant hypocalcemia: a novel activating mutation (E604K) in the cysteine-rich domain of the calcium-sensing receptor. *Journal of Clinical Endocrinology and Metabolism* **88** 605–610.
- Watanabe T, Bai M, Lane CR, Matsumoto S, Minamitani K, Minagawa M, Niimi H, Brown EM & Yasuda T 1998a Familial hypoparathyroidism: identification of a novel gain of function mutation in transmembrane domain 5 of the calcium-sensing receptor. *Journal of Clinical Endocrinology and Metabolism* **83** 2497–2502.
- Watanabe T, Mochizuki H, Kohda N, Minamitani K, Minagawa M, Yasuda T & Niimi H 1998b Autosomal dominant familial hypoparathyroidism and sensorineural deafness without renal dysplasia. *European Journal of Endocrinology* **139** 631–634.
- Winter WE, Silverstein JH, Maclaren NK, Riley WJ & Chiaro JJ 1983 Autosomal dominant hypoparathyroidism with variable, age-dependent severity. *Journal of Pediatrics* **103** 387–390.
- Yamamoto T, Yoshimasa Y, Koshiyama H, Sugiyama K, Okigaki M, Yamada T, Ishii H, Yagura T, Fujimoto K, Ohnishi T, Koh T, Hino M, Kurahachi H, Yoshimasa T & Nakao K 1997 Lack of mutations of preproparathyroid hormone gene in three kindreds with familial isolated hypoparathyroidism. *Endocrine Journal* **44** 175–180.
- Zhang Z, Sun S, Quinn SJ, Brown EM & Bai M 2001 The extracellular calcium-sensing receptor dimerizes through multiple types of intermolecular interactions. *Journal of Biological Chemistry* **276** 5316–5322.

Received 6 May 2003

Accepted 5 June 2003

Made available as an

Accepted preprint on 6 June 2003