HAL
open science

A homozygous inactivating calcium-sensing receptor mutation, Pro339Thr, is associated with isolated primary hyperparathyroidism: correlation between location of mutations and severity of hypercalcaemia

Fadil Mohammed Hannan, M. Andrew Nesbit, Paul Christie, Willy Lissens, Bart Vanderschueren, Marie Bex, Roger Bouillon, Rajesh V Thakker

To cite this version:

Fadil Mohammed Hannan, M. Andrew Nesbit, Paul Christie, Willy Lissens, Bart Vanderschueren, et al.. A homozygous inactivating calcium-sensing receptor mutation, Pro339Thr, is associated with isolated primary hyperparathyroidism: correlation between location of mutations and severity of hypercalcaemia. Clinical Endocrinology, 2010, 73 (6), pp.715. 10.1111/j.1365-2265.2010.03870.x . hal-00593440

HAL Id: hal-00593440

https://hal.science/hal-00593440v1

Submitted on 16 May 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

CLINICAL ENDOCRINOLOGY

A homozygous inactivating calcium-sensing receptor mutation, Pro339Thr, is associated with isolated primary hyperparathyroidism: correlation between location of mutations and severity of hypercalcaemia

Journal:	*Clinical Endocrinology*
Manuscript ID:	CEN-2010-000276.R1
Manuscript Type/Office:	1 Original Article - UK/Europe
Date Submitted by the Author:	26-Jul-2010
Complete List of Authors:	Hannan, Fadil; University of Oxford, Academic Endocrine Unit, Nuffield Department of Clinical Medicine Nesbit, M. Andrew; University of Oxford, Academic Endocrine Unit, Nuffield Department of Clinical Medicine Christie, Paul; University of Oxford, Academic Endocrine Unit, Nuffield Department of Clinical Medicine Lissens, Willy; Vrije Universiteit Brussel, Center for Medical Genetics Vanderschueren, Bart; Katholieke Universiteit Leuven, Clinic and Laboratory of Experimental Medicine and Endocrinology Bex, Marie; Katholieke Universiteit Leuven, Clinic and Laboratory of Experimental Medicine and Endocrinology Bouillon, Roger; Katholieke Universiteit Leuven, Clinic and Laboratory of Experimental Medicine and Endocrinology Thakker, Rajesh; University of Oxford, Nuffield Department of Clinical Medicine
Key Words:	familial, parathyroid disorder, extracellular domain, hypercalciuria, kidney stones

SCHOLARONE™
Manuscripts

A homozygous inactivating calcium-sensing receptor mutation, Pro339Thr, is associated with isolated primary hyperparathyroidism: correlation between location of mutations and severity of hypercalcaemia

Short Title: CaSR mutation in hyperparathyroidism

Authors: Fadil M. Hannan*†, M. Andrew Nesbit*†, Paul T. Christie*, Willy Lissens‡, Bart Vanderschueren§, Marie Bex§, Roger Bouillon§¶, Rajesh V. Thakker*¶

†FMH and MAN contributed equally to the study. ¶RB and RVT contributed equally to the study.

Departments: *Academic Endocrine Unit, Nuffield Department of Clinical Medicine, University of Oxford, United Kingdom. ‡Center for Medical Genetics, Vrije Universiteit Brussel, Brussels, Belgium. §Clinic and Laboratory of Experimental Medicine and Endocrinology, Katholieke Universiteit Leuven, Leuven, Belgium.

Correspondence: Professor R. V. Thakker, Academic Endocrine Unit, Nuffield Department of Clinical Medicine, University of Oxford, Oxford Centre for Diabetes, Endocrinology and Metabolism (OCDEM), Churchill Hospital, Headington, Oxford, OX3 7LJ, UK. Tel: +44(0)1865 857501; Fax: +44(0)1865 857502; E-mail: rajesh.thakker@ndm.ox.ac.uk.

Key words: familial, parathyroid disorder, extracellular domain, hypercalciuria, kidney stones

Acknowledgements: This work was supported by the Medical Research Council (MRC), UK (grant number G9825289/2004). FMH was an MRC Clinical Research Training Fellow. The authors have no conflict of interest and nothing to declare.

Manuscript word count: 3250 words

For Peer Review

Summary

Background: Inactivating mutations of the calcium-sensing receptor (CaSR), a G-protein coupled receptor with extracellular (ECD), transmembrane (TMD) and intracellular (ICD) domains, cause familial hypocalciuric hypercalcaemia, neonatal severe primary hyperparathyroidism, and occasionally primary hyperparathyroidism in adults.

Objective: To investigate a patient with typical symptomatic primary hyperparathyroidism for CaSR abnormalities.

Patient and Design: A 51-year old woman with primary hyperparathyroidism was investigated for CaSR abnormalities as her severe hypercalcaemia (3.75 mmol/l) persisted after the removal of 2 large parathyroid adenomas and she was the daughter of normocalcaemic consanguineous parents. Following informed consent, *CASR* mutational analysis was undertaken using leukocyte DNA. Wild-type and mutant CaSR constructs were expressed in human embryonic kidney (HEK) 293 cells and assessed by measuring their intracellular calcium responses to changes in extracellular calcium. Clinical data were pooled with previous studies to search for genotype-phenotype correlations.

Results: The proband was homozygous for a Pro339Thr CaSR missense mutation, located in the ECD, and her normocalcaemic relatives were heterozygous. The mutant Thr339 CaSR had a rightward shift in its dose-response curve with a significantly higher EC_{50} = 3.18 mM ± 0.19 compared to the wild-type EC_{50} = 2.16 mM ± 0.1 (p <0.01), consistent with a loss-of-function mutation. An analysis of CaSR mutations in patients with primary hyperparathyroidism revealed that those of the ECD were associated with a significantly greater hypercalcaemia that was less likely to be corrected after removal of the parathyroid tumours.

Conclusions: A CaSR missense mutation causing a loss-of-receptor-function can cause symptomatic primary hyperparathyroidism in adulthood.

For Peer Review

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 (ECD) that binds ionized calcium, magnesium, amino acids and other compounds; seven transmembrane domains (TMDs) that are characteristic of all GPCRs; and an intracellular domain (ICD) that has a role in cell surface expression of the CaSR [2]. 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 (IP_3) and rapid release of calcium ions from intracellular stores ($[Ca2+]_i$), which is followed by an influx of $[Ca2+]_o$ ions [3]. These intracellular events mediate a decrease in the rate of PTH secretion from the parathyroid chief cell and a reduction in renal tubular calcium reabsorption. In addition, CaSR activation via the G_i-protein, stimulates the extracellular signal-regulated kinase (ERK)1/2 and the mitogen activated protein (MAP) kinase cascade [4]. The CaSR is also expressed at sites other than the parathyroids and kidneys, and its role in these tissues in man remains to be elucidated [2].

The key role of the CaSR in the regulation of $[Ca2+]_o$ homeostasis has been demonstrated by the identification of CaSR mutations in human disorders. Inactivating CaSR mutations result in familial hypocalciuric hypercalcaemia (FHH) and neonatal severe primary hyperparathyroidism (NSHPT), whereas activating CaSR mutations result in autosomal dominant hypocalcaemia with hypercalciuria (ADHH) and Bartter syndrome type V [2,5]. FHH is characterised biochemically by lifelong elevation of serum calcium concentrations and is associated with inappropriately low urinary calcium excretion and normal or mildly elevated circulating PTH concentrations [2,5]. Individuals with FHH are usually asymptomatic and the disorder is considered benign. NSHPT is a life-threatening disorder associated with severe

For Peer Review

hypercalcaemia, hypotonia, bone demineralisation, fractures and respiratory distress [2,5,6] FHH individuals are heterozygous for loss-of-function CaSR mutations [6], whilst NSHPT patients may be either heterozygous [7], homozygous [6], or compound heterozygous for loss-of-function CaSR mutations [8]. Occasionally, patients who present after the neonatal period and have features such as hypercalcaemia without a low urine calcium excretion [9-11] which are inconsistent with FHH, may also have CaSR mutations. To date 18 patients from 10 families with these CaSR abnormalities, which are all missense mutations have been reported (Table 1) [9-15]. These patients had elevated or inappropriately normal serum PTH concentrations [9-15] and the majority were found to have parathyroid adenomata or hyperplasia[9-15], whose removal following surgery resulted in a lowering of serum calcium concentrations[10-15]. Thus, these patients fulfill the criteria of primary hyperparathyroidism in having hypercalcaemia in association with normal or increased urinary calcium excretion and elevated or inappropriately normal circulating PTH concentrations, together with abnormalities of the parathyroid glands that include adenomata or hyperplasia (Table 1) [16]. However, these patients with primary hyperparathyroidism are unusual in having CaSR mutations, which are more typically associated with FHH [2,5]. We report a patient with primary hyperparathyroidism who has a missense CaSR mutation, Pro339Thr, that involved a residue in the ECD of the CaSR and was associated with a loss-of-function.

For Peer Review

Materials and methods

Patient and family

A 51-year old woman, who had hypertension and type 2 diabetes, treated with an angiotensin II receptor antagonist and insulin, respectively, presented with bilateral renal calculi. Investigations revealed her to have severe hypercalcaemia (corrected total serum calcium = 3.75 mmol/l (normal = 2.10-2.60 mmol/l)) with a urine calcium: creatinine clearance ratio = 0.024, and to have an elevated serum PTH concentration of 65 pg/ml (normal for PTH (1-84) assay < 30 pg/ml) [17], findings that are consistent with primary hyperparathyroidism. Previous serum calcium estimations were not available, and it was thus not possible to ascertain the duration and precise age of onset of the hypercalcaemia. The urine calcium: creatinine clearance ratio was calculated using the formula UCa*PCr/PCa*UCr, where UCa is the urinary concentration of calcium (mmol/l), PCa is the plasma concentration of calcium (mmol/l), UCr is the urinary concentration of creatinine (mmol/l), and PCr is the plasma concentration of creatinine (mmol/l), and in FHH this value is < 0.01, but in normal individuals it is > 0.02. A DEXA scan revealed osteoporosis at the femoral neck with a T-score of -3.14, and an ultrasound scan of her neck identified a left sided 10 x 4 x 15 mm lesion, that was consistent with a parathyroid adenoma. Surgical neck exploration was undertaken and a left superior parathyroid adenoma was excised. However, she remained hypercalcaemic, and one month later she underwent a second parathyroidectomy with the excision of a right superior parathyroid adenoma. Following this, there was a modest improvement in the severity of the hypercalcaemia although her corrected serum calcium remained elevated (corrected total serum calcium = 3.00 mmol/l). She was noted to have consanguineous parents, who were first cousins, and the persistent hypercalcaemia despite parathyroidectomies, raised the possibility of an underlying CaSR mutation. Her family members (mother, 2 daughters and grandson, Fig. 1) were assessed and found to be normocalcaemic, and not to suffer from kidney stones, osteoporosis or diabetes mellitus.

For Peer Review

Mutational analysis of the CaSR was undertaken after obtaining informed consent using protocols approved by the local ethics committee.

DNA sequence analysis of the CASR gene

Leukocyte DNA was extracted from venous blood samples and used with *CASR* gene-specific primers (details available on request) for PCR amplification of the exons and intron-exon boundaries, as previously described [7]. The DNA sequences of both strands were determined, as described [18]. DNA sequence abnormalities were confirmed by restriction endonuclease analysis (New England Biolabs) as described [19,20], and demonstrated to be absent as common polymorphisms in the DNA from 55 unaffected, unrelated individuals.

Functional expression of the CASR mutation

The previously reported wild-type pEGFP-N1-CaSR construct [21] was used to generate a mutant construct by site-directed mutagenesis (QuikChange, Stratagene, La Jolla, CA, USA) [22]. The wild-type and mutant CaSR constructs were transiently transfected into human embryonic kidney cells (HEK293, American Type Culture Collection Catalogue no. CRL1573) by using Lipofectamine Plus (Invitrogen), as described [23]. 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) [22]. The wild-type and mutant CaSRs were functionally assessed by measuring the alterations in $[Ca^{2+}]_i$ in response to changes in $[Ca^{2+}]_o$, as described previously [21]. 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-acetoxymethylester (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. Flow-assisted cell sorting was performed with a Cytomation MoFlo flow cytometer (Dako-Cytomation, Carpenteria, CA)

For Peer Review

equipped with an argon laser (Coherent Radiation, Palo Alto, CA), as described [21]. Baseline fluorescence ratio was measured for 2 min, the fluorescence ratio versus time recorded, and data collected for 2 min at each $[Ca^{2+}]_o$. Cytomation summit software was used to determine the peak mean fluorescence ratio of the transient response after each individual stimulus expressed as a normalized response, as previously reported [23]. The EC_{50} (i.e., $[Ca^{2+}]_o$ required for 50% of the maximal response) for each normalised concentration–response curve was determined. The mean EC_{50} was calculated and statistical analysis performed using the Mann Whitney U test. Expression of CaSR–EGFP fusion proteins was also confirmed by Western blot analysis of cellular protein extract using a monoclonal CaSR antibody (ADD, NPS Pharmaceuticals) [24].

Statistical analysis

The data previously reported from 7 studies [9-15] in patients with primary hyperparathyroidism and CaSR mutations were pooled with the results of this study. Patients in these reports [9-15] were defined as having primary hyperparathyroidism on the basis of hypercalcaemia occurring in association with elevated or inappropriately normal circulating PTH concentrations together with abnormalities of the parathyroid glands that included parathyroid adenomata or hyperplasia (Table 1). However, parathyroid hyperplasia has been reported in some patients with FHH [25] and thus it is important to note that in all 9 of the 11 probands, from whom data were reported, that the post-parathyroidectomy serum calcium concentrations were lower than those pre-operatively (Table 1), consistent with a diagnosis of parathyroid overactivity due to primary hyperparathyroidism. The occurrence of kidney stones and/or osteoporosis, which occur more frequently in patients with primary hyperparathyroidism [16] was not an inclusion criteria for the patients in these studies as these phenotypic features can occur in the absence of primary hyperparathyroidism, and indeed 45% of the probands did not have these phenotypic features (Table 1). The serum calcium and serum PTH concentrations associated with CaSR extracellular domain (ECD) mutations in

For Peer Review

primary hyperparathyroidism patients were compared with those of the transmembrane (TMD) and intracellular domain (ICD) CaSR mutations using the Mann Whitney U test. ECD mutations reported as being homozygous [10,15] (Table 1), were analysed as a separate group to the heterozygous ECD mutations. Mutations reported [11] (Table 1) without stating the occurrence of homozygosity or heterozygosity, were considered as occurring in the homozygous state if there was consanguinuity; otherwise they were considered to occur in the heterozygous state for the purposes of this analysis. The ionized calcium reported in the patient with the Leu11Ser mutation (number 1, Table 1) was converted to a total serum calcium concentration by doubling the value. The reported circulating PTH concentrations had been measured using different assays, which had different normal ranges, and these were therefore expressed as a percentage of the upper limit of normal (ULN) for the particular assay used, so as to facilitate pooling of the data for the analysis. The outcomes following parathyroidectomy in the patients with ECD CaSR mutations versus those with TMD and ICD mutations were analysed using a Chi square test.

For Peer Review

Results

Identification of a missense CASR mutation

DNA sequence analysis of the entire 3,236-bp coding region and intron-exon boundaries of the *CASR* gene in the proband (individual II-2, Fig. 1) revealed a C → A transversion (CCC to ACC) at nucleotide c.1015 that resulted in a missense mutation, Pro339Thr, located in the ECD of the receptor. This sequence alteration resulted in the loss of a *Fok*I restriction enzyme site, which was used to confirm its presence in the proband. This revealed that the proband was homozygous for the Pro339Thr mutation and that her mother, 2 daughters and grandson were heterozygous for the Pro339Thr missense mutation. However, it was not possible to demonstrate that the father (individual I-1, Fig. 1), who was deceased, was heterozygous for the Pro339Thr mutation, and thus it is possible that the father could have a deletion of the CaSR gene and hence the patient (individual II-2, Fig. 1) could possibly be a compound heterozygote, in harbouring a CaSR deletion and the Pro339Thr mutation, rather than being homozygous for the Pro339Thr CaSR mutation. The former possibility of the patient being a compound heterozygote is unlikely as it would require two independent and different CaSR mutations to have occurred in the same family, as the parents are first cousins. Thus, it seems more likely the patient (individual II-2, Fig. 1) is homozygous for the Pro339Thr CaSR mutation. Furthermore, the absence of this DNA sequence abnormality in 110 alleles from 55 unrelated normal individuals indicated that this abnormality did not represent a common sequence polymorphism that may be expected to occur in over 1% of the population. Moreover, the Pro residue at codon 339 is evolutionary conserved in vertebrate species (data not shown), indicating that the substitution of a non-polar Pro residue for a polar Thr residue is likely to be of functional significance.

For Peer Review

Functional characterization of the mutant Pro339Thr CaSR

The wild-type Pro339 and mutant Thr339 CaSRs were expressed in HEK293 cells. Fluorescence microscopy of HEK293 cells transfected with the wild-type or mutant CaSR constructs showed similar expression patterns with fluorescence in the cytoplasm and at the plasma membrane (data not shown). Western blot analysis, using the ADD anti-CaSR antibody, and total cell protein extracts from these transfected HEK293 cells confirmed the expression of the EGFP-tagged wild-type and mutant CaSRs (data not shown). The responses of the wild-type and mutant CaSRs to alterations in $[Ca2+]_o$ were assessed by measurements of $[Ca2+]_i$ (Fig. 2). The mutant Thr339 CaSR showed a significant rightward shift in its concentration–response curve when compared with the wild-type Pro339 CaSR, thereby demonstrating that the mutant CaSR is activated by a higher $[Ca^{2+}]_o$ than the wild-type, which is consistent with it being an inactivating mutation of the *CASR* gene (Fig. 2). Indeed, the mutant Thr339 CaSR was found to have a significantly ($p < 0.01$) raised EC_{50} (3.18 mM ± 0.19) when compared with the EC_{50} of the wild-type (2.16 mM ± 0.1). Co-transfection of the wild-type Pro339 and mutant Thr339 CaSRs, to mimic the situation in the heterozygous individuals I-2, III-1, III-2 and IV-1 (Fig. 1), yielded an EC_{50} that was significantly higher (2.80 mM ± 0.16, $p < 0.05$) than that of the wild-type CaSR (Fig. 2), but was not significantly different to that of the mutant Thr339 CaSR.

Analysis of CaSR mutation location and clinical features in patients with primary hyperparathyroidism

The Pro339Thr CaSR mutation identified in the patient with primary hyperparathyroidism (Fig. 1a) is located in the ECD of the CaSR (Fig. 1d, Table 1). An examination of the other 10 CaSR missense mutations reported in patients with primary hyperparathyroidism or hypercalcaemia that had an onset after infancy revealed that > 70% of these 10 CaSR mutations are also located in the CaSR ECD [9-13,15] (Table 1). We pooled the clinical data

For Peer Review

reported from the patients with these 9 mutations with those from the proband of our study, and undertook an analysis that sought for correlations between the locations of the mutations (genotype) and the clinical features (phenotype). This revealed that the CaSR mutations located in the ECD were associated with a more severe hypercalcaemia than those located in the TMDs and ICD (Fig. 3a). Thus, the homozygote and heterozygote CaSR mutations within the ECD were associated with a mean serum calcium of 3.83 ± 0.20 mmol/l and 3.06 ± 0.07 mmol/l, respectively, which were significantly higher than the mean serum calcium of 2.81 ± 0.03 mmol/l associated with the mutations located in the TMDs and ICD. In addition, serum PTH concentrations associated with the homozygous ECD CaSR mutations were significantly higher than those of the TMD and ICD mutations (Fig. 3b). There were nine hypercalcaemic individuals from the family with the Phe881Leu CaSR mutation (mutation 11, Table 1), and a re-analysis using the serum total calcium concentration of the proband alone revealed that the mean serum total calcium remained significantly different between the ECD and TMD/ICD groups. There was no significant difference in the mean serum PTH concentrations between the ECD and TMD/ICD groups, when the circulating PTH concentrations from the proband alone were used for analysis. An analysis of the location of the CaSR mutation and indices of urinary calcium excretion revealed an absence of any correlations (data not shown). In addition, parathyroidectomy successfully resolved the hypercalcaemia in patients with CaSR mutations in the TMDs or ICD, but this was significantly less successful in those patients who had a CaSR mutation in the ECD (82% versus 17%, $p < 0.05$). Six of the eleven families had the occurrence of renal calculi and/or osteoporosis but the presence of these phenotypic features did not correlate with the locations of the CaSR mutations (Table 1).

For Peer Review

Discussion

Our study reports on the unusual occurrence of a missense CaSR mutation in a patient with primary hyperparathyroidism. The patient, who suffered from osteoporosis and renal calculi, had marked hypercalcaemia with raised serum PTH concentrations and a calcium clearance: creatinine clearance ratio > 0.02. These features are consistent with primary hyperparathyroidism but not with FHH, which is typically benign, asymptomatic and associated with a calcium clearance: creatinine clearance ratio < 0.01 [2,5]. The missense, Pro339Thr, CaSR mutation resulted in a loss-of-function (Fig. 2). The proband was shown to be homozygous for the mutation (Fig. 1). Homozygous inactivating CaSR mutations usually present soon after birth with NSHPT [6,8,26], and the identification of the Pro339Thr homozygous mutation in adulthood is unusual and indicates that this may be leading to a milder inhibition of CaSR function compared with mutations associated with NSHPT. Indeed, our functional expression studies have revealed that the mutant Thr339 CaSR resulted in an EC_{50} that was only < 50% greater than that of the wild-type Pro339 CaSR, whereas, NSHPT mutant CaSRs have been reported to be associated with EC_{50} values that are at least 2-fold greater than that of the wild-type receptor [23,26,27]. There have been 3 other reported homozygous CaSR mutations (Leu13Pro, Gln27Arg, Pro39Ala) that were associated with hypercalcaemia occurring after infancy (Table 1) [10,15,28], and the Gln27Arg and Pro39Ala CaSR mutations when functionally expressed also showed a milder increase in EC_{50} [10,28]. The milder loss-of-function associated with these homozygous CaSR mutations may provide a possible explanation for primary hyperparathyroidism or severe hypercalcaemia presenting after infancy. The association of a CaSR mutation with hypercalcaemia only in the homozygous state has been reported previously with a homozygous mutation that resulted in NSHPT, while the heterozygous parents were normocalcaemic [7]. Although, the majority of CaSR mutations are heterozygous and alter receptor function in a dominant-negative manner, it appears that some mutations such as the Pro339Thr need to be present on both CaSR alleles before a disturbance in calcium homeostasis develops. The occurrence of primary

For Peer Review

hyperparathyroidism or severe hypercalcaemia after infancy in individuals with CaSR mutations may be due to other genetic factors that modify the severity of the hypercalcaemic phenotype. For example, the Glu297Lys mutation has been reported in association with primary hyperparathyroidism in one patient [13], and FHH in another patient [6]. In addition, it is interesting to note that the Pro339Thr mutation, when present in her heterozygous children and mother, was associated with normocalcaemia. However, our functional studies that co-expressed the mutant Thr339 and wild-type Pro339 CaSRs, to mimic the heterozygous state, revealed a rightward shift of the CaSR dose-response curve when compared to wild-type (Fig. 2). This discrepancy between the *in vitro* and *in vivo* findings suggests that the parathyroid proliferation that leads to tumour proliferation and hypercalcaemia may involve signalling pathways other than intracellular calcium in the parathyroid cells. These other pathways may involve the MAP kinase cascade. The MAP kinases are serine/threonine specific protein kinases that are expressed widely, and this includes the parathyroid glands [29]. Activation of the CaSR has been demonstrated to stimulate the MAP kinase cascade [4], which in the parathyroid is considered to inhibit cell proliferation. Consistent with this, in parathyroid adenomas, MAP kinase activity has been found to be reduced [30]. These findings suggest the possibility that the Pro339Thr CaSR mutation in the homozygous state, may result in inhibition of the MAP kinase cascade and stimulation of parathyroid cell proliferation, but that this does not occur in the heterozygous state.

Finally, our analysis of the 11 CaSR missense mutations identified in patients with primary hyperparathyroidism and severe hypercalcaemia that arises after infancy, indicates that those CaSR mutations located in the ECD are associated with more severe hypercalcaemia which is recalcitrant to treatment by parathyroidectomy, because of possible multiple gland involvement. These findings may have some clinical utility in identifying those patients (i.e. with CaSR ECD mutations) in whom minimal invasive surgery should perhaps be avoided but instead a full neck exploration undertaken, as they are more likely to have multi-gland disease that is associated with a greater severity of hypercalcaemia.

For Peer Review

Figure 1

Identification of the CaSR missense mutation, Pro339Thr, by restriction enzyme analysis. (a) DNA sequence analysis of the proband (individual II-2, arrowed) revealed a C→A transversion at codon 339, thus altering the wild-type (WT) sequence CCC, encoding a proline residue to the mutant (m) sequence ACC, encoding a threonine residue. The C→A transversion resulted in the loss of a *FokI* restriction enzyme site (CATC/C). (b) The proband with primary hyperparathyroidism was homozygous for the mutant alleles, while her normocalcaemic mother (individual I-2), 2 daughters (individuals III-1 and III-2), and grandson (individual IV-1) were heterozygous in having wild-type and mutant alleles. The absence of this Pro339Thr in 110 alleles from 55 unrelated normal individuals (N1-N3 shown) indicates that it is not a common sequence polymorphism. (c) PCR amplification and *FokI* restriction enzyme digestion resulted in 2 products of 305 bp and 82 bp from the wild-type allele, but only one product of 387 bp from the mutant allele. Individuals are represented as males (square), female (circle), unaffected (open circle) and affected (filled circle). S is the size marker - 100bp ladder; and the arrows represent the location of PCR primers. (d) Schematic representation of the CaSR to illustrate the locations of the 11 mutations (Table 1) identified in patients with primary hyperparathyroidism. All of the 11 mutations are missense, and 8 are located in the extracellular domain (ECD), 2 in the transmembrane domain (TMD), and 1 in the intracellular domain (ICD). The numbers refer to the mutations in Table 1. The Pro339Thr mutation identified in this study is mutation number 8 and is located in the ECD.

For Peer Review

Figure 2

Functional expression in HEK293 cells of the wild-type (WT, Pro339) and mutant (Thr339) CaSRs. HEK293 cells were transiently transfected with wild-type or the mutant CaSR-EGFP construct or with empty EGFP vector. Live cells loaded with indo-1 acetoxymethylester, emitting fluorescence at 525 nm, and hence containing transfected CaSR were selected by fluorescence-activated cell sorting and the $[Ca^{2+}]_o$-evoked increases 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 shown on the y axis (mean ± SEM of a minimum of 4 separate transfections). The EC_{50} of the mutant CaSR was significantly higher ($p < 0.01$) than that of the wild-type CaSR. The higher EC_{50} and the rightward shift of the concentration–response curve of the mutant CaSR indicate that this mutation confers a loss of function. Co-transfection of wild-type and mutant CaSRs (WT and Pro339Thr) resulted in an EC_{50} that was that was significantly higher ($p < 0.05$) than that of the wild-type CaSR, but not significantly different from that of the mutant receptor, indicating a likely dominant-negative effect of the mutant CaSR in this *in vitro* assay.

For Peer Review

Figure 3

Analysis of serum calcium and PTH concentrations reported in patients with CaSR mutations and primary hyperparathyroidism. (a) Serum total calcium concentrations and (b) serum PTH concentrations obtained from this study and previous reports (Table 1) are shown. Data were available from 9 members of the family with the Phe881Leu mutation (number 11, Table 1), and those from the proband and each of the hypercalcaemic relatives are shown as 11a and 11, respectively. Patients with heterozygous or homozygous ECD CaSR mutations had greater elevations in serum calcium concentrations than those with TMD or ICD CaSR mutations, and the serum PTH concentrations in those with homozygous ECD CaSR mutations were significantly higher than those with heterozygous TMD or ICD CaSR mutations. Mean values for the respective groups are indicated by horizontal solid lines. The normal serum calcium range is indicated by the horizontal broken lines and the serum PTH concentrations are represented as the % of the upper limit of normal (ULN) for the assay that was used. Number adjacent to each dot represents the associated mutation (Table 1). $*p < 0.05$, $**p < 0.01$.

For Peer Review

References

1. Brown, E.M., Gamba, G., Riccardi, D., et al. (1993) Cloning and characterization of an extracellular Ca(2+)-sensing receptor from bovine parathyroid. *Nature*, **366,** 575-580.
2. Chattopadhyay, N.,Brown, E.M. (2006) Role of calcium-sensing receptor in mineral ion metabolism and inherited disorders of calcium-sensing. *Mol Genet Metab*, **89,** 189-202.
3. Hofer, A.M.,Brown, E.M. (2003) Extracellular calcium sensing and signalling. *Nat Rev Mol Cell Biol*, **4,** 530-538.
4. Kifor, O., MacLeod, R.J., Diaz, R., et al. (2001) Regulation of MAP kinase by calcium-sensing receptor in bovine parathyroid and CaR-transfected HEK293 cells. *Am J Physiol Renal Physiol*, **280,** F291-302.
5. Thakker, R.V. (2004) Diseases associated with the extracellular calcium-sensing receptor. *Cell Calcium*, **35,** 275-282.
6. Pollak, M.R., Brown, E.M., Chou, Y.H., et al. (1993) Mutations in the human Ca(2+)-sensing receptor gene cause familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. *Cell*, **75,** 1297-1303.
7. Pearce, S.H., Trump, D., Wooding, C., et al. (1995) Calcium-sensing receptor mutations in familial benign hypercalcemia and neonatal hyperparathyroidism. *J Clin Invest*, **96,** 2683-2692.
8. Kobayashi, M., Tanaka, H., Tsuzuki, K., et al. (1997) Two novel missense mutations in calcium-sensing receptor gene associated with neonatal severe hyperparathyroidism. *J Clin Endocrinol Metab*, **82,** 2716-2719.
9. Pidasheva, S., Canaff, L., Simonds, W.F., et al. (2005) Impaired cotranslational processing of the calcium-sensing receptor due to signal peptide missense mutations in familial hypocalciuric hypercalcemia. *Hum Mol Genet*, **14,** 1679-1690.

For Peer Review

10. Chikatsu, N., Fukumoto, S., Suzawa, M., et al. (1999) An adult patient with severe hypercalcaemia and hypocalciuria due to a novel homozygous inactivating mutation of calcium-sensing receptor. *Clin Endocrinol (Oxf)*, **50,** 537-543.

11. Warner, J., Epstein, M., Sweet, A., et al. (2004) Genetic testing in familial isolated hyperparathyroidism: unexpected results and their implications. *J Med Genet*, **41,** 155-160.

12. Guarnieri, V., Canaff, L., Yun, F.H., et al. (2010) Calcium-sensing receptor (CASR) mutations in hypercalcemic states: studies from a single endocrine clinic over three years. *J Clin Endocrinol Metab*, **95,** 1819-1829.

13. Brachet, C., Boros, E., Tenoutasse, S., et al. (2009) Association of parathyroid adenoma and familial hypocalciuric hypercalcaemia in a teenager. *Eur J Endocrinol*, **161,** 207-210.

14. Carling, T., Szabo, E., Bai, M., et al. (2000) Familial hypercalcemia and hypercalciuria caused by a novel mutation in the cytoplasmic tail of the calcium receptor. *J Clin Endocrinol Metab*, **85,** 2042-2047.

15. Miyashiro, K., Kunii, I., Manna, T.D., et al. (2004) Severe hypercalcemia in a 9-year-old Brazilian girl due to a novel inactivating mutation of the calcium-sensing receptor. *J Clin Endocrinol Metab*, **89,** 5936-5941.

16. Fraser, W.D. (2009) Hyperparathyroidism. *Lancet*, **374,** 145-158.

17. Bouillon, R., Coopmans, W., Degroote, D.E., et al. (1990) Immunoradiometric assay of parathyrin with polyclonal and monoclonal region-specific antibodies. *Clin Chem*, **36,** 271-276.

18. Hannan, F.M., Nesbit, M.A., Christie, P.T., et al. (2008) Familial isolated primary hyperparathyroidism caused by mutations of the MEN1 gene. *Nat Clin Pract Endocrinol Metab*, **4,** 53-58.

19. Lloyd, S.E., Pearce, S.H., Fisher, S.E., et al. (1996) A common molecular basis for three inherited kidney stone diseases. *Nature*, **379,** 445-449.

For Peer Review

20. Pearce, S.H., Williamson, C., Kifor, O., et al. (1996) A familial syndrome of hypocalcemia with hypercalciuria due to mutations in the calcium-sensing receptor. *N Engl J Med*, **335,** 1115-1122.

21. Hough, T.A., Bogani, D., Cheeseman, M.T., et al. (2004) Activating calcium-sensing receptor mutation in the mouse is associated with cataracts and ectopic calcification. *Proc Natl Acad Sci U S A*, **101,** 13566-13571.

22. Nesbit, M.A., Bowl, M.R., Harding, B., et al. (2004) Characterization of GATA3 mutations in the hypoparathyroidism, deafness, and renal dysplasia (HDR) syndrome. *J Biol Chem*, **279,** 22624-22634.

23. Pearce, S.H., Bai, M., Quinn, S.J., et al. (1996) Functional characterization of calcium-sensing receptor mutations expressed in human embryonic kidney cells. *J Clin Invest*, **98,** 1860-1866.

24. Goldsmith, P.K., Fan, G., Miller, J.L., et al. (1997) Monoclonal antibodies against synthetic peptides corresponding to the extracellular domain of the human Ca2+ receptor: characterization and use in studying concanavalin A inhibition. *J Bone Miner Res*, **12,** 1780-1788.

25. Thorgeirsson, U., Costa, J., Marx, S.J. (1981) The parathyroid glands in familial hypocalciuric hypercalcemia. *Hum Pathol*, **12,** 229-237.

26. Pidasheva, S., Grant, M., Canaff, L., et al. (2006) Calcium-sensing receptor dimerizes in the endoplasmic reticulum: biochemical and biophysical characterization of CASR mutants retained intracellularly. *Hum Mol Genet*, **15,** 2200-2209.

27. Bai, M., Pearce, S.H., Kifor, O., et al. (1997) In vivo and in vitro characterization of neonatal hyperparathyroidism resulting from a de novo, heterozygous mutation in the Ca2+-sensing receptor gene: normal maternal calcium homeostasis as a cause of secondary hyperparathyroidism in familial benign hypocalciuric hypercalcemia. *J Clin Invest*, **99,** 88-96.

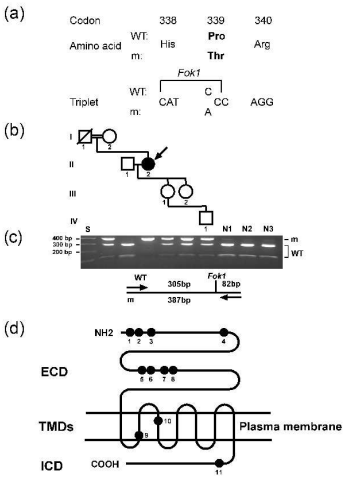
For Peer Review

28. Aida, K., Koishi, S., Inoue, M., et al. (1995) Familial hypocalciuric hypercalcemia associated with mutation in the human Ca(2+)-sensing receptor gene. *J Clin Endocrinol Metab*, **80,** 2594-2598.

29. Kuida, K.,Boucher, D.M. (2004) Functions of MAP kinases: insights from gene-targeting studies. *J Biochem*, **135,** 653-656.

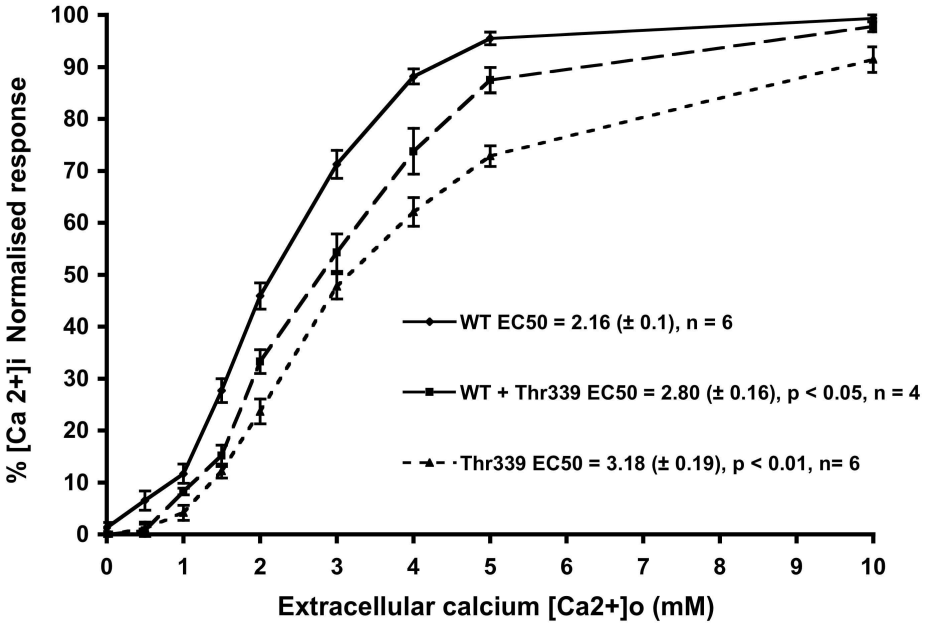
30. Corbetta, S., Lania, A., Filopanti, M., et al. (2002) Mitogen-activated protein kinase cascade in human normal and tumoral parathyroid cells. *J Clin Endocrinol Metab*, **87,** 2201-2205.

For Peer Review

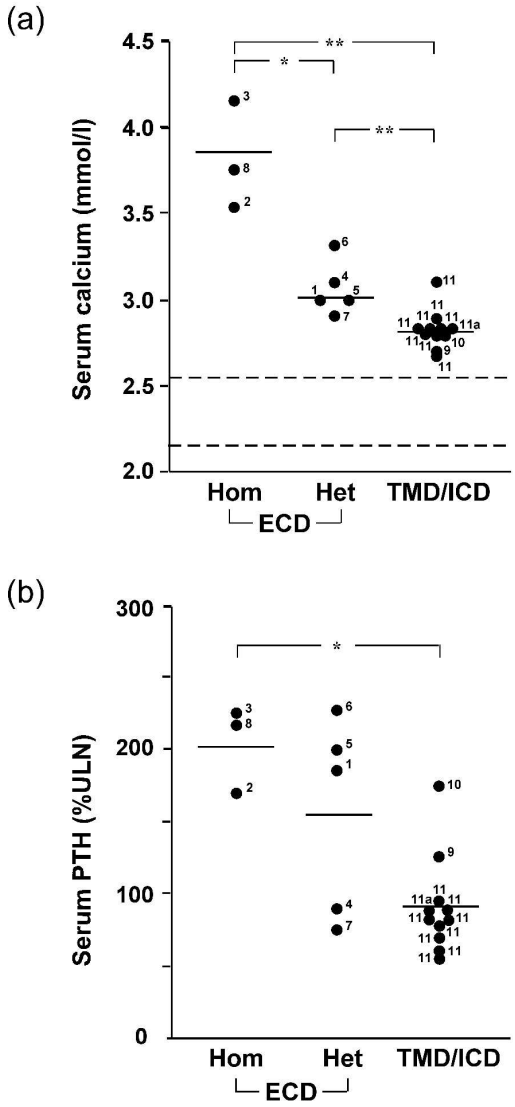


492x263mm (450 x 450 DPI)

eer Review


% [Ca 2+]i Normalised response
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
Extracellular calcium [Ca2+]o (mM)
WT EC50 = 2.16 (± 0.1), n = 6
WT + Thr339 EC50 = 2.80 (± 0.16), p < 0.05, n = 4
Thr339 EC50 = 3.18 (± 0.19), p < 0.01, n= 6

Review



139x266mm (600 x 600 DPI)

Table 1. Summary of clinical findings and CaSR mutations from this and ten previous reports associated with primary hyperparathyroidism or severe hypercalcaemia occurring after infancy.

				At diagnosis							
Mutation (number)	**Genotype [a]**	**Exon**	**Gender [b]**	**Age (years)**	**Clinical features**	**Serum calcium (mmol/l) [c]**	**Serum PTH**	**Urine calcium excretion**	**Serum calcium (mmol/l) [c] post-parathyroidectomy**	**Parathyroid histology [n]**	**Reference [o]**
Extracellular domain											
Leu11Ser (1)	Het	2	M	43	Renal calculi	1.50 [d]	122 [e]	7.5 [l]	NK	H	(9)
Leu13Pro (2)	Hom	2	F	9	Headache, vomiting, low body weight	3.53	110 [e]	0.005 [m]	2.85	H	(15)
Gln27Arg (3)	Hom	2	F	26	Low body weight	4.20	119 [f]	0.0086 [m]	2.67	H	(10)
Thr100Iso (4)	NK	3	M	45	Pancreatitis	3.10	6.5 [g]	0.29 [n]	2.86	Ad	(11)
Ile283Thr (5)	Het	4	F	56	Renal calculi, Osteoporosis	2.98	143 [h]	0.025 [m]	2.45	A	(12)
Glu297Lys (6)	Het	4	M	16	Abdominal pain, polyuria, polydipsia	3.28	211 [i]	0.005 [m]	2.70	A	(13)
Lys336del (7)	NK	4	F	53	Renal calculi	2.90	5.7 [g]	8.5 [l]	NK	NK	(11)
Pro339Thr (8)	Hom	4	F	50	Renal calculi, osteoporosis	3.75	65 [j]	0.024 [m]	3.0	A	Fig. 1 [p]
Transmembrane domain											
Leu650Pro (9)	NK	7	F	58	Asymptomatic	2.71	9.6 [g]	0.19 [n]	2.13	H	(11)
Val689Met (10)	NK	7	F	64	Osteoporosis	2.77	13.3 [g]	0.5 [n]	2.54	H	(11)
Intracellular domain											
Phe881Leu (11)	Het	7	6M, 3F	23-65	Renal calculi	2.70-3.11	31-51 [k]	0.005-0.16 [m]	2.14-2.66	7H, 2A	(14)

[a] Genotype resulting in hypercalcaemia; Het- heterozygous, Hom – homozygous, NK = not known; [b] gender of affected individuals; M = Male, F = Female; [c] normal serum total calcium range, 2.1-2.6 mmol/l; [d] normal serum ionized calcium range, 1.15-1.28 mmol/l; normal PTH reference ranges, [e] 10–65 pg/ml, [f] 13–53 pg/ml, [g] 1.3-7.6 pmol/l, [h] 10–72 pg/ml, [i] 10–93 pg/ml, [j] 0–30 pg/ml, [k] 12–55 pg/ml; [l] 24-hr urine calcium (mmol/24hr); [m] Urine calcium: creatinine clearance ratio (normal > 0.02); [n] Urine calcium: creatinine ratio (normal 0.06-0.6 mmol/mmol); [n] Parathyroid histology, A – adenoma, H – hyperplasia, Ad - tumour admixed with adipose tissue; [o] Reference number of report, [p] This report.

For Peer Review