CASE REPORT

A novel mutation in the calcium-sensing receptor in a French family with familial hypocalciuric hypercalcaemia

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Abstract

Objective: The calcium-sensing receptor (CASR) has an important role in calcium homeostasis by controlling PTH secretion and renal calcium handling. Inactivating mutations in the CASR gene (HGNC ID: 1514) cause familial hypocalciuric hypercalcaemia (FHH). We present a case of FHH patient to describe a novel mutation in the CASR.

Subjects and methods: A 34-year-old patient was referred because of recurrent hypercalcaemia after resection of two hyperplastic parathyroids. Extensive evaluation found elevated PTH and low calcium/creatinine clearance ratio. One of her three children had high serum calcium concentrations. Genetic studies were performed by PCR amplification of CASR coding exons and direct sequencing of PCR products. Transient transfection of the wild-type (WT) CASR and the mutant CASR into COS-7 was performed to assess functional impact of the mutation and the capacity of either protein to mediate increases in cellular levels of inositol phosphates (IPs).

Results: CASR sequencing found a previously undescribed heterozygous base substitution, determining a change of threonine to isoleucine at codon 550 (p.T550I) in the sixth exon. In contrast to those transfected with WT CASR, which showed a five- to eightfold increase in total IPs at high levels of calcium, COS-7 cells transfected with the (p.T550I) mutant showed no increase confirming to the inactivating nature of the mutation. COS-7 cells co-transfected with the WT and the (p.T550I) mutant showed an intermediate response suggesting a possible dominant negative effect.

Conclusion: This case report presents a not-yet-described mutation in the cysteine-rich region of the CASR extracellular domain, a mutation with a possible dominant negative effect.

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Introduction

Calcium is a ubiquitous cation and its homeostasis is tightly controlled. One of the most important factors in controlling calcium homeostasis is the cell surface calcium-sensing receptor (CASR), a member of family C of the seven transmembrane G-protein-coupled receptor superfamily. Being expressed predominantly in the parathyroid chief cells, the CASR recognises small changes in extracellular calcium and responds by affecting PTH secretion, PTH gene expression and parathyroid cellular proliferation (1). Moreover, it is also expressed in the cells lining the renal tubule, where its actions depend on the region of the nephron in which it is located, in the calcitonin-secreting C-cells, and in several other organs (2).

The human CASR is composed of 1078 amino acid residues and has three structural domains

(<http://www.casrdb.mcgill.ca>): a large extracellular domain (ECD) of 612 residues, a transmembrane domain (TMD) of 250 amino acids and an intracellular, C-terminal domain of 216 amino acids. The ECD contains 11 N-linked glycosylation sites and a bilobed Venus-flytrap-like domain that is important for ligand binding (3). Glycosylation is required for cell surface expression. The most abundant species on the cell surface and the functional form are dimers (4). The human CASR is encoded by Exons 2–7 of the CASR gene (HGNC ID: 1514) that is located on chromosome 3q13.3–21, while Exon 1A and B encode alternative 5' UTR (5, 6).

Upon ligand binding, stimulated CASR is coupled to Gq, causing activation of phospholipase C (PLC) which breaks down phosphatidylinositol 4,5-bisphosphate to form 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate. The increasing intracellular calcium concentrations and phospholipid metabolites such as

Figure 1 Pathology of the hyperplastic parathyroid gland with a few adipocyte nests among the principal cells.

DAG would activate protein kinase C. The CASR also activates PLA2, PLD and other protein kinases such as the MAP kinases (7). Stimulated CASR also binds to Gi, inhibiting cAMP formation.

Familial hypocalciuric hypercalcaemia (FHH) is an autosomal dominant disorder with characteristics such as moderately elevated serum calcium concentrations, low urinary calcium excretion and inappropriately normal or mildly elevated PTH. Hypercalcaemia in FHH, usually within 10% of the upper limit of normal (8), is generally asymptomatic and benign without complications of chronic hypercalcaemia, even though some affected patients may experience chondrocalcinosis and rarely gallstones and acute pancreatitis (9). A total of 80% of FHH patients have a calcium/creatinine clearance ratio of <0.01 (10). Although serum magnesium concentration might be within the upper part of the normal range, vitamin D levels are normal. The majority of FHH cases are caused by inactivating heterozygous mutations in the *CASR* gene, a condition referred to as FHH type I. FHH is benign and does not require treatment. However, the most important objective is to differentiate FHH from primary hyperparathyroidism (PHPT) to avoid unnecessary parathyroidectomy that was observed in 23% of FHH patients in one study (11).

Case history

A 34-year-old female patient was referred for hypercalcaemia. She complained of musculoskeletal pain and her general practitioner had requested laboratory evaluations that revealed hypercalcaemia. She had no previously relevant medical history, she was not receiving any medication and her family history was unremarkable. Initial laboratory evaluation showed hypercalcaemia at 2.77 mmol/l (fluctuating between 2.77 and 2.87 mmol/l on repeat analyses; Normal: 2.2–2.6), albumin 35 g/l, normal creatinine ($47 \mu\text{mol/l} = 5.4 \text{ mg/l}$)

and low phosphorus levels (between 0.64 and 0.8 mmol/l; Normal: 0.8–1.5); 24 h urinary calcium excretion was found to be 4.1 mmol (165 mg; Normal: 100–300 mg/day). PTH was inappropriately normal at 38 pg/ml (Normal: 12–60) and 41 pg/ml (Normal: 10–55) when repeated in another laboratory. A cervical echography was consistent with hyperplasia of the left lower parathyroid gland (7 mm) and the right lower parathyroid gland (10 mm). PHPT was diagnosed and surgical exploration of the neck was performed. During surgery, the surgeon removed both upper parathyroid glands that were clearly enlarged ($10 \times 6 \times 5 \text{ mm}$ on the right and $10 \times 5 \times 5 \text{ mm}$ on the left). The two lower parathyroid glands were considered to be normal on neck exploration and were left in place. Pathological examination revealed principal cell hyperplasia in both parathyroid glands with only rare adipose cells (Figs 1 and 2).

Intraoperative PTH monitoring showed that PTH levels decreased from 63 pg/ml (Normal: 10–55) to 5.5 pg/ml at 20 min post surgery. However, 2 days later PTH levels returned to 39 pg/ml and hypercalcaemia persisted (2.82 mmol/l). Therefore, the patient was transferred to our department where sustained hypercalcaemia (2.77 mmol/l) was confirmed, with elevated PTH levels at 61 pg/ml (Normal: 10–55), undetectable PTHrP and a calcium/creatinine clearance ratio of 0.009. Her 25 OH-vitamin D levels were within the normal range. Extensive investigations for other causes of hypercalcaemia were negative. FHH was suspected and evaluation of her first-degree relatives (three sons) was carried out, revealing mild hypercalcaemia (2.82 mmol/l) in her middle son (13 years of age) while her oldest (16 years) and youngest (9 years) sons had normal calcium levels (Table 1). Repeated analyses confirmed that the middle son was hypercalcaemic, whereas phosphorus levels were low (1.07 mmol/l, Normal: 1.3–1.85) and 24 h urinary calcium was 3.15 mmol. Therefore, FHH was diagnosed and genetic studies of the *CASR* gene were performed in the proband, her affected son and the youngest son with normal calcium blood levels. Her husband was previously deceased in a traffic accident. According to our institutional ethics committee and after obtaining informed consent from the patient for her own case and for her sons, genetic studies of the *CASR* gene were carried out by PCR amplification of coding exons and direct sequencing of PCR products. In the proband, a

Figure 2 Normal parathyroid (for comparison with that of our patient).

Table 1 Biochemical values in the affected proband and her family.

	Proband	First son (16 years)	Middle son (13 years)	Last son (9 years)
P calcium levels mmol/l (2.2–2.6)	2.84/2.75	2.42	2.97/2.82	2.53/2.40
P phosphorus levels mmol/l (0.8–1.5)	0.77/0.86		1.07/0.89	1.44
U calcium levels mg/day (100–300)	162		126	
U creatinine levels g/day	1.06		0.78	

novel heterozygous base C to T substitution was detected, determining a change of threonine to isoleucine at codon 550 (p.T550I; Fig. 3). This mutation was located in the sixth exon, i.e. the ECD of the CASR (Fig. 4). Direct sequencing of the region of interest of Exon 6 revealed the same mutation in the affected son. On the contrary, the unaffected son did not carry the (p.T550I) mutation (Fig. 5). Repeat calcium, phosphorus and PTH measurements in the propositus 10 years later found: calcium level 2.82 mmol/l, phosphorus level 0.96 mmol/l and PTH level 26 pg/ml (*n*: 5–39).

Functional studies

Functional studies in COS-7 cells transiently transfected with wild-type (WT) and mutant receptor were performed as described previously (12). Briefly, COS-7 cells were transfected with 625 ng of cDNA by the diethylaminoethyl-dextran method followed by a 2 min 10% dimethyl sulfoxide shock after 3 h. For co-expression experiments, equal amounts (625 ng) of WT and mutant CASR cDNA were mixed and used to transfect COS-7 cells. To explore the functional impact of the (p.T550I) mutation, we compared its capacity to mediate calcium-elicited PLC-dependent increases in cellular levels of inositol phosphates (IPs) with that of the WT receptor when transfected in COS-7 cells. Expression of the WT receptor conferred five to eightfold increases in total IPs at high levels of calcium, with an EC₅₀ value for a calcium level of 3.8 mM. Non-transfected cells showed no changes in IPs at any calcium concentration tested, while the (p.T550I) mutant resulted in no response in IPs after stimulation with calcium. In Figure 6, the calcium dose-response curve of the co-expressed receptors (WT/p.T550I) was lower than that of the WT receptor alone, suggesting that the mutant receptor interferes with the function of the WT receptor.

Discussion

In this case of FHH, we found a previously undescribed mutation at codon 550 (p.T550I) in Exon 6 of the CASR gene (Fig. 3). Inactivating mutations in the CASR cause an impairment of function according to the following mechanisms (6): i) mutations that affect cell surface expression of the receptor: either impaired biosynthesis or defective trafficking of the receptor from the endoplasmic reticulum to the cell membrane. ii) Mutations

affecting function of the mutant receptor with relatively normal cell surface expression: these mutations modify the ability of the receptor to bind the ligand or to couple to G proteins. iii) Mutations that cause increased degradation of the receptor: these mutations generally lead to a decrease in cell surface expression of the mutant receptor. However, one could imagine that some of these mutations confer normal cell surface expression with less stable dimers that are more easily ubiquitinated and degraded.

Several of these mutations have a dominant negative effect, which reduces the function of the normal CASR encoded by the WT allele upon dimerisation with the abnormal CASR.

Mutations occur through the entire sequence of the protein, with some clustering in the first half of the ECD (amino acids 137–250), the terminal part of the ECD (amino acids 549–595) and parts of the TMD. Missense mutations are the most frequent types of mutation (13). Our family has a novel missense mutation in the cysteine-rich region of the ECD domain that is important for signal transduction from the ligand-binding sites in the ECD to the TMD (14). The threonine to isoleucine substitution and, consequently, the introduction of a hydrophobic rather than a hydrophilic residue might induce conformational changes in the CASR protein. Moreover, the threonine residue at codon 550 is highly conserved between species (www.ebi.ac.uk/clustalw) and therefore its substitution might impair the receptor function. Functional studies confirmed the inactivating nature of

Figure 3 Automated sequence analysis of CASR heterozygous mutant (T550I) in the proband (both alleles are sequenced).

Figure 4 Calcium-sensing receptor structure and mutations (Adapted from D'Souza-Li *et al.* (3) and reproduced with permission from <http://www.casrdb.mcgill.ca/> © 2003 DeBelle – CASRdb); blue, polymorphism; green, inactivating mutations; black, two inactivating mutations at the same site; red, activating mutations.

this mutation, as increasing extracellular calcium concentrations did not stimulate IP₃ production in COS-7 cells transfected with the (p.T550I) mutant. Moreover, the mutation co-segregated with the hypercalcaemia trait in the family, which confirmed the diagnosis of FHH.

The less than normal response found when the COS-7 cells were co-transfected with the WT and the (p.T550I) mutant suggested that this mutant had a dominant negative effect. Another explanation for the less than normal response was suggested by Hauache *et al.* (15) who proposed that two intact cysteine-rich regions are

required for functional complementation in every dimer. Thus, the site of the mutation in the cysteine-rich region could affect signal transmission in the heterodimers formed by the WT and the (p.T550I) mutant. It should be noted that we did not study the cell surface expression of the (p.T550I) mutant, as low cell surface expression could also lead to a less than normal response. However, the cysteine-rich domain may not be crucial for cell surface expression and some studies have found that cysteine-rich domain deletion mutants still possess some degree of cell surface expression (16).

Our patient had two enlarged parathyroid glands that were found to be hyperplastic on microscopic examination. Moreover, the resected glands showed principal cell hyperplasia without adipose cells. Interestingly, parathyroid hyperplasia has been previously described in patients with FHH, but this hyperplasia was in the form of simultaneous hyperplasia of fat and parathyroid principal cells called 'lipohyperplasia' (17). However, this was not the case in our patient or for those reported by Cetani *et al.* (18). Parathyroid hyperplasia has also been described by Carling *et al.* (19) in several members of an atypical FHH family with relative hypercalciuria. Whether parathyroid hyperplasia is related to mutation in the CASR gene or not is not clear. It is worth noting that mice heterozygous for inactivating mutations of the *Casr*, a murine model of FHH, develop parathyroid hyperplasia. Moreover, the fact that patients with neonatal severe HPT (NSHPT) have parathyroid hyperplasia indicates such a relationship between inactivating mutations of CASR and parathyroid hyperplasia.

In hypercalcaemic patients, it can sometimes be clinically or biologically difficult to distinguish between PHPT and FHH. CASR testing, with functional analysis, provides valuable evidence in the differential diagnosis of hypercalcaemic states, as recently published (20). New technologies such as denaturing HPLC provide a rapid and effective tool to screen for CASR mutations (21).

In conclusion, we present this case to describe a novel mutation in the CASR gene with functional studies, to discuss the possibility of parathyroid hyperplasia, the mechanism of which is confusing, and to emphasise the well-known fact that differentiating FHH from PHPT

Figure 5 Pedigree and phenotype of the members of our patient's family. Circles, women; boxes, male; slashed symbols, deceased family members; arrow, proband; open symbols, refer to unaffected family members.

Figure 6 Effects of increasing concentrations of calcium on IPs levels in COS-7 cells transiently transfected with WT *CASR*, *CASR* mutant (T550I) and T550I/WT *CASR*.

might be difficult, especially nowadays when asymptomatic PHPT is increasing in frequency.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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