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CASE REPORT

Mutations of calcium-sensing receptor gene: two novel mutations and overview of impact on calcium homeostasis

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Abstract

Objective: Genetic disorders of calcium metabolism arise in a familial or sporadic setting. The calcium-sensing receptor (CASR) plays a key role in maintaining calcium homeostasis and study of the *CASR* gene can be clinically useful in determining etiology and appropriate therapeutic approaches. We report two cases of novel *CASR* gene mutations that illustrate the varying clinical presentations and discuss these in terms of the current understanding of CASR function.

Patients and methods: A 16-year-old patient had mild hypercalcemia associated with low-normal urinary calcium excretion and normal-to-high parathyroid hormone (PTH) levels. Because of negative family history, familial hypocalciuric hypercalcemia was originally excluded. The second patient was a 54-year-old man with symptomatic hypocalcemia, hyperphosphatemia, low PTH, and mild hypercalciuria. Familial investigation revealed the same phenotype in the patient's sister. The coding region of the *CASR* gene was sequenced in both probands and their available first-degree relatives.

Results: The first patient had a novel heterozygous inactivating *CASR* mutation in exon 4, which predicted a p.A423K change; genetic analysis was negative in the parents. The second patient had a novel heterozygous activating *CASR* mutation in exon 6, which predicted a p.E556K change; the affected sister of the proband was also positive.

Conclusions: We reported two novel heterozygous mutations of the *CASR* gene, an inactivating mutation in exon 4 and the first activating mutation reported to date in exon 6. These cases illustrate the importance of genetic testing of *CASR* gene to aid correct diagnosis and to assist in clinical management.

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Introduction

Extracellular ionized calcium (Ca^{2+}) values are maintained within a very narrow range as a result of a complex homeostatic system that regulates cell signaling, bone metabolism, neural and muscle function. Parathyroid hormone (PTH) plays the key role in modulating ionized levels via its actions on renal tubular cells and bone resorption, while it is also crucial for intestinal absorption of calcium through its regulation of enzymatic activation of vitamin D (1–3). PTH synthesis and secretion is finely regulated by the calcium-sensing receptor (CASR) on parathyroid gland cells. The CASR senses minor fluctuations in ionized calcium levels and can rapidly modulate PTH appropriately to maintain optimal circulating Ca^{2+} (4, 5).

Inborn disorders of calcium metabolism causing hypo- and hyper-calcemia have multiple etiologies. Hypoparathyroidism occurs in the setting of several congenital syndromes such as Di George, Kenny-Caffey, and Barakat syndromes or as part of polyglandular autoimmune disorders (6); there are also isolated, sporadic or familial genetic diseases. Familial forms of hypoparathyroidism can be related to mutations of the *PTH* gene (7, 8), the *CASR* gene (9), and the 'glial cells missing' gene (10), with a X-linked form of the disease also being described (11). Genetic conditions related to hyperparathyroidism include multiple endocrine neoplasia types 1 (MEN1) and 2 (MEN2), familial hypocalciuric hypercalcemia (FHH), hyperparathyroidism-jaw tumor syndrome and familial isolated hyperparathyroidism (12).

Mutations in the *CASR* gene may cause hypo- or hypercalcemia. The CASR belongs to the subfamily C of the G protein-coupled receptors (13) and is organized in three major structural domains: a large amino-terminal extracellular domain (ECD), the typical seven transmembrane domains (TMD), and a cytoplasmic carboxy-terminal tail (14). The Venus flytrap (VFT) model has been proposed to illustrate the CASR–ligand interaction (15), showing two possible conformations: an open

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conformational status in which the ligand is bound to a low-affinity pocket in the ECD and a closed conformational status in which the ligand binds a second high-affinity domain. The bound N-terminal segment interacts with the membrane-associated domain to generate a signal. Molecular studies indicated that calcium interacts with polar residues in the binding pockets in the ECD of the receptor, emphasizing the role of eight residues (Ser 147, Ser 170, Asp 190, Gln 193, Tyr 218, Phe 270, Ser 296, and Glu 297) in calcium coordination (15, 16).

The human *CASR* gene is located on chromosome 3 (17) and contains six coding exons (18), from exons 2 to 7. The amino-terminal ECD is encoded by the exons 2–6 and the beginning of exon 7, while the TMD and the cytoplasmic tail are encoded by exon 7.

Over the past decade around 150 inactivating mutations and 70 activating mutations have been described. Inactivating mutations have been described in the context of FHH and neonatal severe primary hyperparathyroidism (NSHPT). Conversely, activating mutations have been related to autosomal dominant hypocalcemia (ADH) and type V Bartter syndrome.

This study describes two clinical cases of disordered calcium homeostasis caused by novel *CASR* mutations in the context of current understanding of the role of the CASR in calcium homeostasis and disease.

Patients and methods

Case 1

A 16-year-old patient (Table 1) was referred to the University Hospital of Liège for investigation of hypercalcemia noted during a routinely biochemical evaluation for abdominal pain. Repeated calcium measurements confirmed moderate hypercalcemia (mean value: 2.96 mmol/l; normal range: 2.15–2.60). Hypercalcemia was associated with low-normal phosphate levels, normal vitamin D status (serum 25-hydroxyvitamin D: 37 ng/ml) and mildly elevated PTH. The 24 h urinary calcium excretion (24 h CaUr) was high-normal and the calcium/creatinine clearance ratio (CCR) was low on repeated measurement (0.009–0.02). As previously reported (19), a CCR <0.01 has been suggested as a cut off value in patients with FHH in the absence of other factors that would lower urinary calcium excretion, whereas a CCR >0.02 supports hyperparathyroidism. Both ultrasound and scintigraphy revealed no parathyroid gland abnormality. An oral calcium-loading test was performed. PTH, calcium and creatinine were measured 1 h before and 1, 2, and 3 h after calcium load. The increase in serum calcium levels from 2.95 at baseline to 3.23 mmol/l was associated with partial (40%) suppression of PTH (from 60 to 34 pg/ml, Fig. 1). These results suggested that PTH secretion was not completely autonomous, which possibly suggested primary hyperparathyroidism (20). Conversely, biochemical evaluation of both parents showed no serum or urine abnormality. On the basis of hypercalcemia and slightly elevated PTH levels in the proband and normal serum calcium in his parents, FHH was originally excluded and primary hyperparathyroidism was suspected.

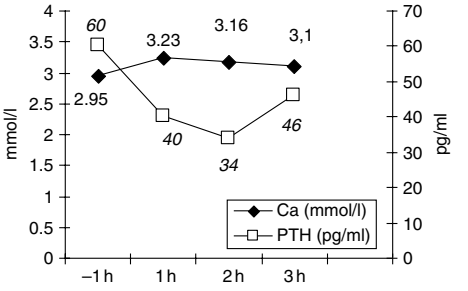

Figure 1 Clinical case 1. Changes in serum calcium (Ca) and PTH during oral calcium-loading test: the increase in Ca levels from 2.95 to 3.23 mmol/l was associated with a 40% PTH suppression, supporting a possible diagnosis of primary hyperparathyroidism.

Taking into account the negative long-term outcome of primary hyperparathyroidism (21–23) and the age of the patient, a surgical exploration of the parathyroid glands was recommended. This showed normal anatomic position, with the superior glands being slightly increased in size and the inferior ones being clearly hyperplasic. The inferior glands were resected surgically and histological analysis confirmed the diagnosis of parathyroid hyperplasia.

Postoperatively, serum calcium levels were still slightly increased with persisting inappropriately elevated PTH (69 pg/ml) and normal serum phosphorus. Due to the persistent biochemical abnormalities, genetic analysis of the *CASR* gene was undertaken in all family members.

Nucleotide sequencing from the proband identified a novel mutation from GCA to AAA at codon 423 in exon 4 of the *CASR*, resulting in a conversion of alanine to lysine. DNA extraction and sequencing in both parents revealed no mutation in *CASR* gene, and as molecular analysis using microsatellite markers (24) indicated that the paternity was as stated, a diagnosis of a *de novo* heterozygous *CASR* mutation was made.

Table 1 Clinical case 1: patient profile at study entry.

	Before surgery	After surgery	Reference range
Mean repeated Ca (mmol/l)	2.96	2.61	2.15–2.60
Mean repeated Ca^{2+} (mmol/l)	1.56	–	1.00–1.30
PO_4^{3-} (mg/l)	25	25	23–47
PTH (pg/ml)	66	69	12–58
25-Hydroxyvitamin D (ng/ml)	37	39	>32
Creatinine (mg/l)	6.5	7.4	4.5–12.1
24 h CaUr (mmol/24 h)	7.86	–	2.50–8.00
CCR	0.02	–	1.00–2.60

Ca, serum calcium; Ca^{2+}, ionized calcium; PO_4^{3-}, phosphate; PTH, parathyroid hormone; 24 h CaUr, 24 h urine calcium; CCR, calcium/creatinine clearance ratio.

Table 2 Clinical case 2: patient profile at study entry.

	Value	Reference range
Mean repeated Ca (mmol/l)	1.96	2.15–2.60
Mean repeated Ca^{2+} (mmol/l)	1.00	1.00–1.30
PO_4^{3-} (mg/l)	40	23–47
PTH (pg/ml)	<12	12–58
Creatinine (mg/l)	8.4	4.5–12.1
24 h CaUr (mmol/24 h)	6.24	2.50–8.00
CCR	0.013	1.00–2.60

Ca, serum calcium; Ca^{2+}, ionized calcium; PO_4^{3-}, phosphate; PTH, parathyroid hormone; 24 h CaUr, 24 h urine calcium; CCR, calcium/creatinine clearance ratio.

Case 2

A 54-year-old man had hypocalcemia associated with low PTH. In the previous 6 months, he had received supplemental calcium carbonate (1000 mg daily) and cholecalciferol (25 000 IU weekly) with serum calcium remaining below the normal range. One month before presentation he suffered a first *grand mal* epileptic seizure. Computed tomography of the brain revealed calcification in the basal ganglia and in the frontal cerebral cortex. Biochemical evaluation confirmed the presence of hypocalcemia in three consecutive determinations: 1.91, 1.96, and 1.68 mmol/l, with mean ionized Ca^{2+} being 1.0 mmol/l. Hypocalcemia was accompanied by low PTH levels (<12 pg/ml), normal serum 25-OH vitamin D and serum phosphorus of 40 mg/l. The 24 h CaUr was within the normal range and CCR was 0.013. The patient profile at study entry is shown in Table 2. Renal ultrasound showed normal renal parenchymal structure without signs of nephrocalcinosis and/or nephrolithiasis.

The family history was positive for a similar clinical picture in the patient's sister, who had received oral calcium and vitamin D supplementation for some years without biochemical normalization of calcium. Laboratory analyses in the sister revealed a serum calcium of 1.95 mmol/l, a low PTH (1.0 pg/ml), and a high-normal 24 h CaUr and CCR of 0.026. Kidney ultrasound did not reveal parenchymal abnormalities, whereas the brain computerized tomography showed calcification in the basal ganglia. The patient's father was deceased; his mother and his son had normal serum and urine biochemical tests.

Given the clinical history and familial nature of the condition, genetic analysis of the *CASR* was performed. In the proband a novel heterozygous mutation from GAG to AAG at codon 556 in exon 6 of the *CASR* gene, was found which resulted in a change from glutamic acid to lysine (E556K).

The mutation was also present in the affected sister, while the asymptomatic mother and son of the proband had normal *CASR* sequencing (Fig. 2).

Assays

Calcium, phosphorus, and creatinine were measured by automated laboratory methods. The urinary calcium and creatinine measurements were done on 24 h urine samples. CCR was calculated with the following formula: (U(Ca)×P(Crea)/P(Ca)×U(Crea)), where U stands for urine, P for plasma, Crea for creatinine, and Ca for calcium. PTH was measured by the direct immunochemiluminescent sandwich assay (Liaison, DiaSorin, MN, USA). The sensitivity of the assay was 1 ng/l. The intra-assay coefficient of variation (CV) was 1.7–3.7% and the inter-assay CV was 2.6–5.9%.

DNA sequencing

Both probands and first-degree relatives provided the written informed consent for the genetic analysis, and the local ethics committee authorized the study. DNA was extracted from peripheral blood sample on EDTA by standard phenol–chloroform method. DNA sequencing procedure is detailed in the Supplemental Material (see section on supplementary data given at the end of this article).

Discussion

This study first describes two novel heterozygous mutations of the *CASR* gene, a two nucleotide inactivating A423K mutation in codon 4 and the activating E556K mutation in codon 6.

Over the past decade more than 220 mutations of the *CASR* gene have been described. Inactivating mutations are generally related to FHH and NSHPT. On the other hand, activating mutations have been described in the context of ADH and type V Bartter syndrome

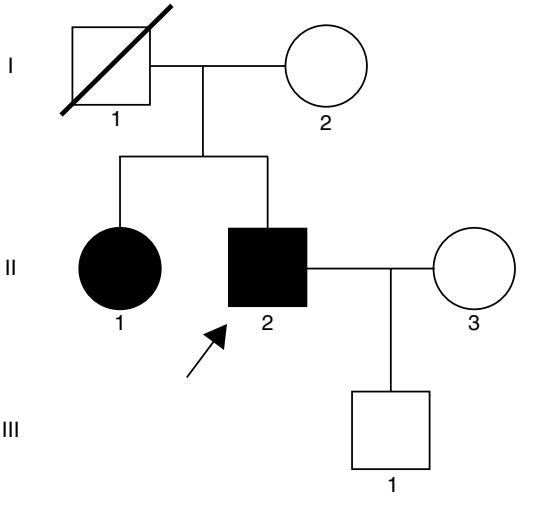

Figure 2 Clinical case 2. Pedigree of the patient's family. The arrow shows index patient and the filled symbols indicated affected family members with a heterozygous *CASR* gene mutation.

(see http://www.casrdb.mcgill.ca). Taking into account the last 6 years, about 36 *CASR* gene mutations have been described (25–38), with 34 being inactivating and two activating. Among them, most have been found in exons 4 and 7.

Efforts have been done to clarify the impact of these genetic alterations on receptor function. An *in vitro* functional study of the mutated receptor showed ligand-dependent changes in receptor affinity (26). Inactivating mutations led either to total loss of function and inability to bind calcium or to a lack of expression at the membrane (26). At molecular level this could be the result of an abnormal glycosylation, crucial disulfide bond formation, and agonist-induced dimer conformational changes or altered G-protein coupling (27). The majority of inactivating mutations are missense. Whether such missense mutations are more closely related to increased serum calcium levels than truncating mutations remains speculative (28, 39).

Almost all activating mutations affect loop 2 of the VFT domain and TMD 5, 6, and 7 (26). Activating mutations are thought to increase receptor sensitivity by facilitating ligand-induced VFT closure or dimer rotation. Recent research points out the importance of TMD 6 helix motion in receptor activation (39, 40). Particularly, the TMD A843E mutation leads to a constitutive activation of the CASR, independent of calcium levels and ECD structure, probably locking the TMD in an active conformation (41). Moreover, the activating E297D and the inactivating E297K mutations have been described in codon 297, suggesting its crucial role in ligand binding and receptor activation (15, 18).

FHH was first described as a heritable disorder of mineral metabolism (42), inducing asymptomatic hypercalcemia associated with hypocalciuria (43) and inappropriate PTH levels (44). The presence of a normal PTH in these patients is a direct consequence of an elevated set point for calcium-regulated PTH secretion (45). FHH is a benign autosomal dominant disease and affected individuals usually do not show complications related to hypercalcemia (46). Thus, medical and/or surgical treatments are generally not recommended. In our first case, a 16-year-old patient had the clinical phenotype of mild hypercalcemia associated with high PTH levels and a high-normal 24 h calcium excretion value. Basal biochemical investigation of both this patient and his parents, followed by oral calcium loading and imaging, could not settle a clear initial diagnosis. The discovery of parathyroid hyperplasia but with a negative outcome of surgery led us to perform genetic analysis of the *CASR* gene. Study in both parents was negative and the family relationship was confirmed, which indicates that the A423K mutation likely occurred *de novo*, making this kindred technically not a FHH family. However, analysis was performed on somatic cells and did not exclude the possibility of germline mosaicism as previously reported in the case of an activating *CASR* mutation (46). This clinical case confirms that differential diagnosis of mild hypercalcemia associated with normal-to-high PTH can be difficult and is still a challenge for endocrinologists. This is in line with a previous study reporting that in 23% of patients with undiagnosed mutations in the *CASR* gene, surgical exploration of the neck was undertaken as part of the diagnostic workup (26). Several reports in the literature showed that atypical presentation of FHH is not a rare finding. Hypercalcemia associated with hypercalciuria, high PTH levels and even nephrolithiasis (10, 47–50) could suggest hyperparathyroidism. Moreover, such patients are less likely to achieve normocalcemia after parathyroid surgery (4), as in our first clinical case.

In inactivating *CASR* mutations a clear genotype–phenotype relation is lacking. The first described mutation F881L, associated with atypical FHH-like phenotype (50), is located in the region encoding the C-terminal tail. Thereafter, Rus *et al*. (51) described two novel mutations with atypical phenotype affecting amino acids in the C-terminal tail: Q926R and D1005N. Several other mutations (V268del-11X273, E250K, T100I, L650P, V689M, and 1008delAAG) have been associated with atypical presentations, including severe hypercalcemia, hypercalciuria with or without nephrolithiasis and/or nephrocalcinosis, kindreds with affected members displaying either hypercalciuria or hypocalciuria, and normal calcium levels after surgery and pancreatitis (10, 49). Interestingly, R886P, close to F881L, has been associated to a typical phenotype (10).

In the second clinical case, we have described a family with autosomal dominant hypoparathyroidism due to a novel activating missense mutation in the *CASR*: E556K in exon 6. To the best of our knowledge this is the first activating mutation found in exon 6, all previously described mutations being inactivating. Codon 556 is situated within the Cys-rich domain involved in the signaling between the VFT domain and the TMD7. The strong correlation between phenotype (symptomatic hypocalcemia, low PTH and relative hypercalciuria) and genotype supported that this mutation is pathogenic. The patient presented, albeit at a relatively advanced age for the clinical condition, with symptomatic hypoparathyroidism that had caused an epileptic seizure and was associated with brain calcification. Importantly, the patient had not suffered renal sequelae, which represents an important morbidity in hypoparathyroidism, particularly in patients with his genetic etiology of autosomal dominant hypoparathyroidism. Treatment in these cases is particularly challenging compared with other hypoparathyroid patients, as the enhanced sensitivity of the mutant CASR in the kidney to activation by calcium promotes hypercalciuria, which is worsened by therapeutic intake of calcium and vitamin D to alleviate symptoms (52). Care must be taken to not over treat with supplemental calcium, vitamin D or vitamin D analogs/metabolites, as the aim

is balancing symptom control with renal calcium handling, and a low-normal serum calcium is preferable to achieve this end.

Our clinical cases clearly exemplify that all first-degree relatives of patients with calcium disorders and inappropriate PTH levels should have calcium levels examined after a detailed family history is obtained. DNA sequencing is becoming more affordable, can lead to accurate diagnosis and should therefore be carried out in members of PTH-related families with abnormalities in serum and urine excretion calcium, also including young patients and atypical cases.

In conclusion, we have described two novel mutations of the *CASR* gene. A423K is the first inactivating mutation described in exon 4 and E556K is the first activating mutation described in exon 6. It is noteworthy that *CASR* gene mutations can be associated with symptomatic hyper- or hypocalcemia or atypical clinical presentation.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/EJE-11-0121.

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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