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Case Report

A novel missense variant of the calcium-sensing receptor gene associated with familial hypocalciuric hypercalcemia

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Abstract

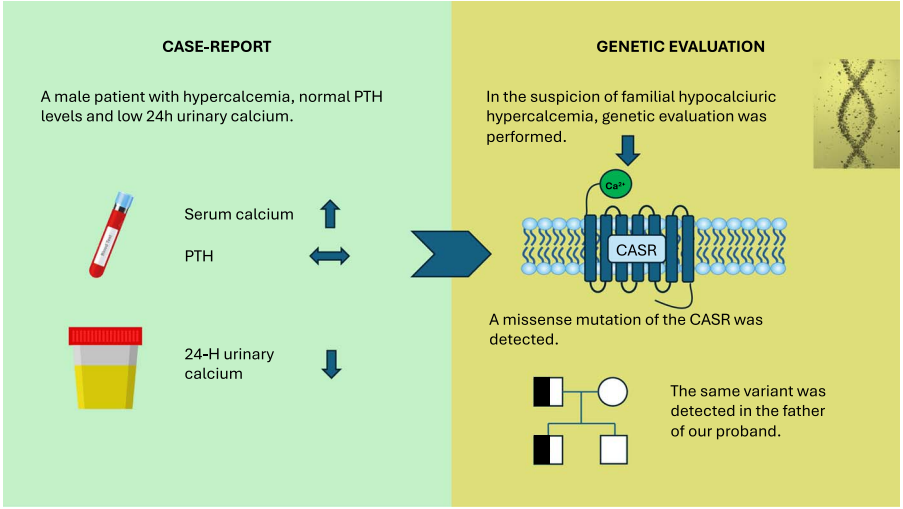
Familial hypocalciuric hypercalcemia (FHH) is a rare genetic disorder caused by inactivating variants of the calcium-sensing receptor gene (*CASR*), *GNA11*, and *AP2S1* genes, which lead to decreased intracellular receptor activity. Typical biochemical features include hypercalcemia and hypocalciuria, while PTH serum levels may be inappropriately normal or high. Despite the finding of hypercalcemia, the disorder has a benign course and patients usually do not require any therapeutical intervention. We describe a man with FHH caused by a previously unreported missense variant, c.496A>C, p.(Ser166Arg), located in exon 4 of *CASR*. The same variant was subsequently found in the father who presented with asymptomatic hypercalcemia. The variant was classified as "likely pathogenic." In silico analysis predicted that the variant destabilizes for the tertiary structure of the protein and may induce conformational changes. An in vitro functional assay on HEK293-transfected cells demonstrated that the variant likely disrupts calcium binding and transport by CaSR. The present case expands the mutational spectrum of *CASR* and reinforces the clinical utility of multidisciplinary assessment for adults presenting with unexplained hypercalcemia.

Keywords: Familial hypocalciuric hypercalcemia, calcium-sensing receptor, hyperparathyroidism, Asymptomatic hypercalcemia, Parathyroid, Genetic

Lay Summary

Familial hypocalciuric hypercalcemia (FHH) is a rare genetic disorder, caused by inactivating variants of the calcium-sensing receptor gene, which determine general calcium hyposensitivity. It is characterized by life-long hypercalcemia and relative hypocalciuria. It is a benign condition, as affected patients are usually asymptomatic and do not require treatment of any kind. We describe a young man, affected by FHH, caused by a previously unreported pathogenic change of the CaSR gene.

Graphical Abstract



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Introduction

Familial hypocalciuric hypercalcemia (FHH) is an autosomal dominant inherited disorder caused by deleterious variants of three different genes. FHH type 1 (FHH1) is the most common genetic form and is associated with inactivating variants in the gene *CASR*, which encodes for the calcium-sensing receptor (CaSR). CaSR is expressed on parathyroid cells, regulates calcium levels, and, consequently, PTH synthesis and release.[1] In a few cases, FHH derives from deleterious variants in the G-protein subunit α11 gene (*GNA11*), encoding the GNa-11 protein implicated in downstream CaSR signal transduction (FHH2), or in the adaptor-related protein complex 2 (AP2), sigma 1 subunit gene (*AP2S1*) that encodes the clathrin-associated AP2S1 protein involved in CaSR endocitosis (FHH3).[2] Loss-of-function (LoF) alterations in these genes increase the set-point for calcium sensing and consequently shift suppression of PTH secretion to a higher threshold of serum calcium concentration. Consequently, serum calcium exceeds the normal range, whereas serum PTH is inappropriately normal or mildly elevated, as the mechanism of negative feedback is compromised. Moreover, LoF variants lead to an increase in renal threshold for calcium tubular reabsorption, resulting in a lower renal calcium excretion than what is expected from the enhanced filtered load of calcium.[1,3,4] The differential diagnosis between FHH and other causes of hypercalcemia, mainly primary hyperparathyroidism (PHP), may be challenging, owing to variability in presentation and significant overlap, but it appears crucial for prognosis and correct treatment.

Familial hypocalciuric hypercalcemia remains largely underdiagnosed due to its frequently benign clinical course. Despite the rarity of the condition, clinicians should be advised to screen for FHH in patients with suggestive biochemical data (asymptomatic hypercalcemia and hypocalciuria) to prevent superfluous tests and unnecessary surgical treatment. Hence, the characterization of novel deleterious variants responsible for FHH could be helpful in the diagnosis of new cases and could lead to a better understanding of genotype-phenotype correlations in this condition.

Case presentation

We report a man with FHH caused by a previously unreported deleterious missense change in *CASR* to contribute to the current knowledge on the clinical and molecular variability of this condition. In a 20-yr-old man, hypercalcemia was incidentally identified by routine laboratory assessment. At first evaluation, he did not complain of any symptom related to hypercalcemia and did not show any related clinical sign. In his past history, there was no mention of nephrolithiasis and/or nephrocalcinosis, as well as renal failure. Family history was negative for endocrine disorders.

Methods

Investigation

At entry in our outpatient clinic, serum total and ionized calcium were high; PTH was within the normal range, while 25OHD levels were sufficient (Table 1). Biochemical data attested moderately elevated levels of both total and ionized serum calcium in association with normal levels of PTH in multiple determinations. Bone remodeling markers (alkaline phosphatase, osteocalcin, C-terminal telopeptide or CTX) were within normal range (Table 1). The evaluation of urinary parameters on a 24 h sample demonstrated low levels of 24-h urinary calcium (48 mg/24 h; n.v. 100-300 mg/24 h). The patient denied assumption of any medication that could potentially alter calcium metabolism/clearance, but declared to have reduced dietary calcium intake, on the advice of is general practitioner. At re-evaluation of urinary parameters on a 24 h sample, 24-h-urinary calcium was confirmed low (48 mg/24 h; n.v. 100-300). C calcium to-creatinine (Ca/Cr) clearance ratio (CCCR) was less than 0.01 in both determinations (Table 1). BMD assessed by DXA was in the average for his age and neither nephrolithiasis nor nephrocalcinosis was documented at abdominal ultrasound examination. Neck ultrasonography was negative for parathyroid adenoma/hyperplasia. All the previous findings raised the suspicion of FHH, and familial screening and confirmatory genetic test was performed. By evaluating the patient's family (Figure 1A), the father was found to have asymptomatic hypercalcemia and low 24-h-urinary calcium, while the mother and his brother had normal serum calcium and PTH levels, as well as normal 24-h-urinary calcium (Table 2). Unfortunately, other key relatives, such as paternal grandparents and uncle, had died from unrelated causes, and no information regarding calcemia values was available.

3D modeling

Three in silico prediction tools (FoldX,[7] PremPS[8] and CUPSAT[9]) were used to investigate the protein thermodynamic stability changes upon variation. Starting from protein tertiary structure, these tools predict $\Delta\Delta G = \Delta G_{mutant} - \Delta G_{wild\text{-}type}$, where ΔG is the difference between the free energies of the folded/unfolded states: in this setting, a point aminoacidic change with $\Delta\Delta G > 0$ suggests destabilization. The tertiary structure of the extracellular domain of human CaSR (hCaSR-ECD; residues 20-541) was retrieved from Protein Data Bank (ID: 5FBK, chain A). Missing loop (aa 361-391) was built with MODELLER.[10]

Next-generation sequencing

Proband's genomic DNA was extracted from peripheral blood leucocytes and analyzed by a customized next-generation sequencing (NGS) multigene panel (Agilent Technologies), designed for genes known to be associated with calcium, parathormone, and phosphorus altered metabolism (*AIP*, *AIRE*, *AP2S1*, *CASR*, *CDC73*, *CDKN1A*, *CDKN2*, *CDKN2B*, *CDKN2C*, *CDKN1B*, *CLDN19*, *CYP24A1*, *FAM111A*, *FGFR1*, *FLCN1*, *GATA3*, *GNA11*, *GCM2*, *GNAS*, *MAX*, *MEN1*, *PRUNE2*, *PTH*, *RET*, *SEMA3D*, *SLC34A1*, *TBCE*, and *TBX1*) according to manufacturer's instructions. Sequencing was performed on a NextSeq 500 platform (Illumina) using the MidOutput flow cells (300 cycles), with a minimum coverage of 200×. FastQC files were checked, reads were trimmed and mapped, and variants were annotated by the Alissa Align & Call bioinformatics pipeline (Agilent Technologies). Annotated variants were filtered and interpreted by an implemented variant triage system by Alissa Interpret pipeline (Agilent Technologies). Variants were filtered according to their minor allele frequency (MAF < 0.005) and according to the following criteria: (i) variants reported as pathogenic/likely pathogenic in ClinVar without conflicting evidence of data, (ii) nonsense/frameshift variants in genes previously described as disease-causing by haploinsufficiency/loss of function, (iii) variants affecting D/A splice sites, (iv) synonym/deep

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Table 1. Biochemical data of our index patient.[a]

	First evaluation	Second evaluation	Normal range
Calcium	**11.7 mg/dL**	**11.3 mg/dL**	8.2-10.4
Albumine corrected calcium	**11.7 mg/dL**	**11.3 mg/dL**	8.2-10.4
Ionized calcium	1.4 mmol/L	1.4 mmol/L	1.10-1.30
Magnesium	2.4 mg/dL	2.2 mg/dL	1.5-3.8
Phosphate	4.1 mg/dL	2.9 mg/dL	2.5-4.6
PTH[b]	38.5 pg/mL	36.2 pg/mL	8-76
Vitamin D	**31 ng/mL**	**32.4 ng/mL**	30
Osteocalcin	**20**	24.5 ng/mL	11.3-37
CTX	**0.481**	0.540 ug/L	0.115-0.748
Alkaline phosphatase	57 U/L	52 U/L	40-150
24-h urine calcium	**43 mg/24 h**	**48 mg/24 h**	100-300
24-h urine phosphate	591 mg/24 h	648 mg/24 h	400-1000
Ca/Cr clearance ratio (CCCR)[c]	**<0.01**	**<0.01**	>0.02

CTX, C-terminal telopeptide. [a]The patient underwent several determinations of both serum and urinary parameters during diagnostic work-up and subsequent follow-up. For sake of simplicity, illustrative values at entry and at a second evaluation were reported. Boldface values indicate abnormality. In Italics, normal values. / = not determined at first evaluation. [b]Serum intact PTH was measured using a solid-phase, two-site chemiluminescent enzyme labeled immunometric assay (Immulite 2000). [c]Calcium-to-creatinine clearance ratio. A CCCR value below the threshold of 0.02 is considered suggestive for FHH.

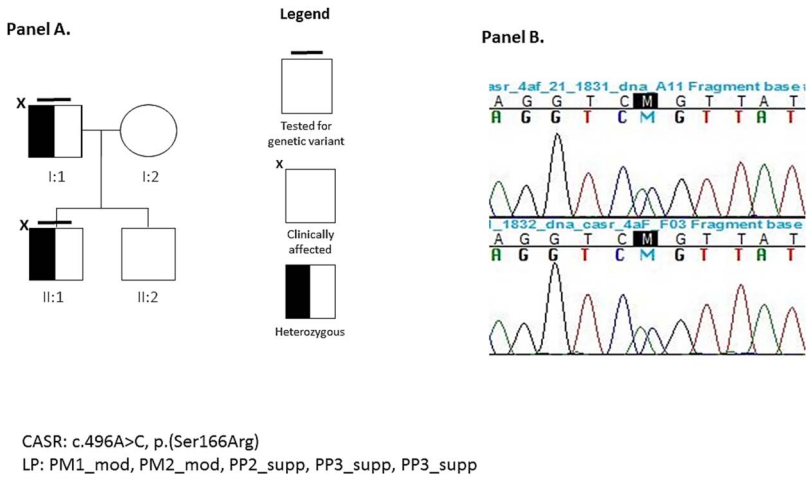

Figure 1. (A) Family tree of our index patient. (B) Sequencing electropherogram from our index patient harboring a heterozygous variation, c.496A>C, p.(Ser166Arg), in exon 4 of *CaSR*.

Table 2. Available biochemical data of the parents and relative of our index patient.

	Father	Mother	Brother	Normal range
Calcium	**11.2**	**9.6 mg/dL**	**9.8 mg/dL**	8.2-10.4
Albumine corrected calcium	**11.2 mg/dL**	**9.6 mg/dL**	**9.9 mg/dL**	8.2-10.4
Ionized calcium	1.4 mmol/L	1.1 mmol/L	1.2 mmol/L	1.10-1.30
phosphate	3.7 mg/dL	3.09 mg/dL	3.2 mg/dL	2.5-4.6
PTH	31.5 pg/mL	48.2 pg/mL	39.7 pg/mL	8-76
Vitamin D	40 ng/mL	31.94 ng/mL	33 ng/mL	>30
Alkaline phosphatase	67 U/L	52 U/L	48 U/L	40-150
24-h urine calcium	**52 mg/24 h**	**205 mg/24 h**	**170 mg/24 h**	100-300
24-h urine phosphate	622 mg/24 h	890 mg/24 h	640 mg/24 h	400-1000
Ca/Cr clearance ratio (CCCR)**	**<0.01**	**0.034**	**0.037**	>0.02

The most relevant data are highlighted in bold.

intronic variants predicted to affect the splicing, (v) small in-frame/indel variants, and (vi) private/very rare missense variants with a REVEL score ≥ 0.644.

cDNA expression vectors

At the residue S166, ClinVar (https://www.ncbi.nlm.nih.gov/clinvar) reports three aminoacidic changes: S166I, S166G, and S166N. We decided to test our S166R along with the S166G (that was previously published by our colleagues in 2009[5]). Both the S166R and S166G variants were introduced in a Myc tagged human WT CaSR cDNA expressing pCDNA3.1 vector (a kind gift of Prof. G.N. Hendy and Dr. Lucie Canaff, McGill University, Montreal) with classic single-site mutagenesis reactions with the following primers (S166R For: 5′-tacattccccaggtcCgttatgcctcctcca-3′ and Rev: 5′-tggaggaggcataacGgacctggggaatgta-3′ and S166G For: 5′-tacattccccaggtcGgttatgcctcctcca-3′ and Rev: 5′-tggaggaggcataacCgacctggggaatgta-3′, mutated bases are in capital). After digestion of the parental DNA with Dpn I (Promega) and transformation into DH5alpha Chemically Competent

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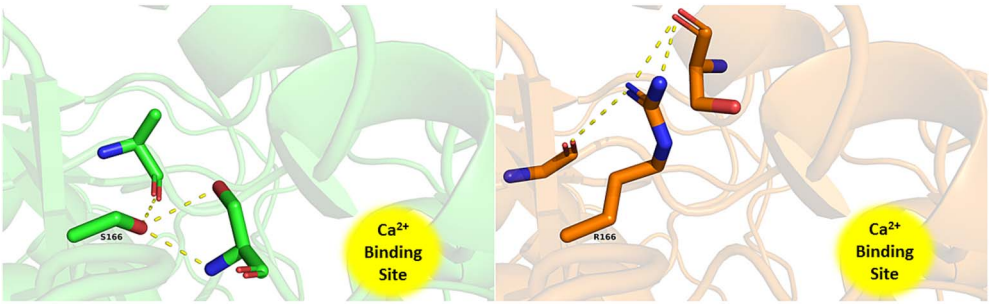

Figure 2. Graphical representation of the pathogenic variant site in human CaSR WT (on the left) and S166R mutant (on the right).

Cells (Invitrogen), mutated clones were amplified (GenElute Plasmid Miniprep Kit, Sigma-Aldrich) and correctness verified by colony PCR and Sanger sequencing.

Cell culture, transfection, and western blot

Human embryonic kidney (HEK293) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), and supplemented with FBS (both from GIBCO) 10% and penicillin/streptomycin 1% (Sigma Aldrich) at 37 °C with CO_2 5%.

Expression of the WT and mutant receptor

HEK293 cells were plated in six-well culture dishes at a density of 7.5×10^5 cells/well and then transfected with Myc-tagged CaSR WT and mutant vectors using Lipofectamine 3000 (Invitrogen), according to the manufacturer's instructions. After 48 h, total cell proteins were extracted in RIPA buffer [150 mM NaCl, 50 mM Tris-HCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, pH 8.0] supplemented with one tablet/10 mL of PhosStop and Complete EDTA, phosphatase and protease inhibitors (both from Roche-Sigma Aldrich). About 60 μg of protein was separated on a 7.5% SDS-PAGE and transferred to PVDF membrane (BioRad).

Membranes were incubated overnight at 4 °C with primary antibodies: anti-Myc (Roche, 1:500 in blocking solution) and, as reference, anti-β-actin (Santa Cruz Biotechnology, 1:500 in blocking solution). Following this, membranes were incubated for 1 h at room temperature with horseradish-peroxidase-conjugated anti-mouse IgG antibody (BioRad). The chemiluminescence reaction was performed (Pierce ECL, Thermo Fisher), and the ChemiDoc Imaging Systems (BioRad) was used for detection. Corresponding Image-Lab software was then used for acquisition and densitometry evaluation.

MAP kinase assay

HEK293 cells were seeded in six wells plates and transfected (F351V mutant as inactivating control[6]) as previously described. After 36 h, cells were switched to a DMEM/F12 with minimal $MgCl_2$ and $CaCl_2$ concentration (0.5 mM final). After other 12 h, cells were stimulated with $CaCl_2$ for 5 min (4 or 10 mM, final concentration). Whole-cell protein extracts were prepared with RIPA buffer as described above. About 60 μg of proteins were loaded onto a 10% SDS-PAGE and electrotrasferred (Transblot, Biorad). Immunoblotting was done overnight at 4 °C with Phospho p42/44 and Total p42/44 rabbit monoclonal antibodies (both 1:1000 in blocking solution, both from Cell Signaling Technology) and, as internal control, β-actin mouse monoclonal antibody (1:500 in blocking solution, Santa Cruz Biotechnology). Immunoblotting with secondary antibody was carried out at room temperature for 1 h with the horseradish-peroxidase-conjugated goat anti-rabbit or mouse IgG antibody (both from BioRad). Chemiluminescent reaction and acquisition were performed as above described.

Statistical analysis

For MAPK assay, expression of phospho-42/44 and total-p42/44 is normalized to actin expression. Results are expressed as mean and SD derived from triplicate experiments. A p-value $<.05$ was considered for statistical significance.

Results

Variant interpretation

The analysis put the *CASR* (NM_000388.4) c.496A>C, p.(Ser166Arg) missense variant at the top of the list (Figure 1B). The variant falls in exon 4 owing a high rate of deleterious variants and making part of the key Venus-Fly Trap like domain, (residues 22-528,[4] PM1_Moderate); it is absent in public databases (gnomAD v4.1.0, PM2_Moderate); at the same codon, a different amino acid change was previously identified and classified as "pathogenic" (PM5_Moderate); it involves a gene in which missense variants are frequently deleterious (PP2_Supporting); the variant was scored with a REVEL of 0.95 (PP3_Supporting), and the phenotype was considered specific for CASR (PP4_Supporting). Finally, in vitro analysis supported the deleterious effect of the identified variant by comparing it to other previously characterized variants (see below; PS3_Moderate). According to the American College of Medical Genetics and Genomics (ACMG) guidelines, the variant was classified as "pathogenic." The variant resulted inherited from the biochemically affected father.

Predicted effect of the variant

$\Delta\Delta G$ values predicted by FoldX, PremPS, and CUPSAT (respectively +23.7, +1.6 and +1.1 Kcal/mol) suggest that the S166R is destabilizes and might cause protein dysfunction by reducing the levels of folded hCaSR-ECD, critical for receptor dimerization and activation. Moreover, the aminoacidic change falls near a well-known Ca^{2+} binding site[11,12] and a visual inspection of the variation site suggests that potential mutation-induced conformational changes, led by a different network of polar contacts, might lead to the disruption of calcium binding and transport (Figure 2).

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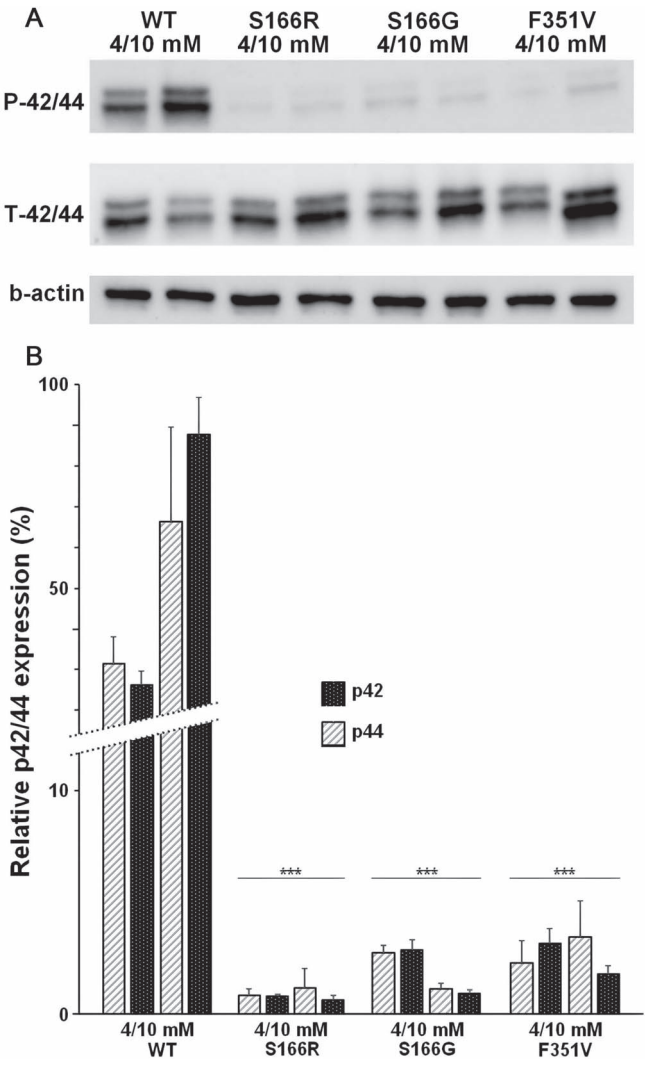

Figure 3. A representative western blot was shown. (A) Signaling activity of WT, S166R, S166G, and F351V mutated proteins in terms of level of phosphorylated (top) and total (bottom) p42/44 proteins. At higher concentration (Ca^{2+}, 10 mM) the activity induced by the mutants appeared faint compared with the WT. (B) Plot of the signaling activities. Values were normalized with respect to the WT at 10 mM.

Expression of CASR WT and mutants

The western blot performed on crude lysates showed that the S116R has a similar pattern of the WT for the 140 immature glycosylated band and for the dimers (280-300 kDa) while the fully glycosylated 160 kDa band showed a down-expression. Conversely, a strong down-expression was evident for the S166G either for the 140 kDa and dimers and mostly for the 160 kDa (Figure 3A and B).

p42/44 levels

Compared to the WT, the MAPK kinase assay revealed that both the S166R and S166G aminoacidic changes dramatically impaired the phosphorylation of p42/44 proteins even at higher Ca^{2+} stimulation (10 mM) (Figure 4), in agreement with the pathogenic destabilizing effect presumed by the in silico modeling.

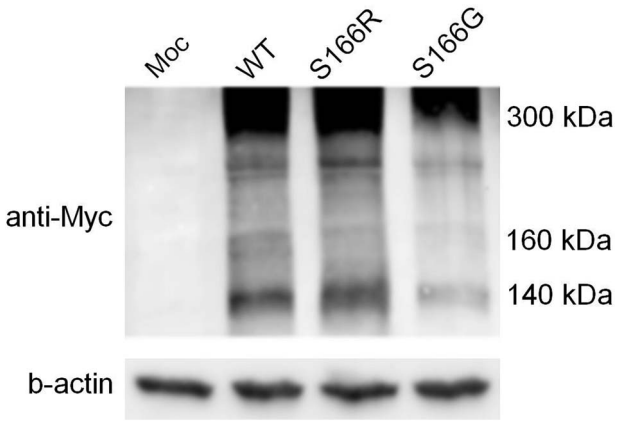

Figure 4. Expression of the WT and mutant CASR receptors. Both the mutants showed a downexpression of one (160 kDa for the S166R) or both the bands (140 and 160 kDa for the S166G), compared to the WT.

Discussion

Familial hypocalciuric hypercalcemia is characterized by life-long hypercalcemia, usually of mild-to-moderate degree and non-progressive through the years. Unlike other causes of hypercalcemia that are often associated with significant morbidity and mortality, and require prompt adequate treatment, FHH generally has a benign prognosis. Most patients are completely symptomless, while a few show moderate-to-severe hypercalcemia and complain of hypercalcemic symptoms, such as polyuria, polydipsia, muscle weakness, asthenia, arthralgias, or may develop more severe complications (eg, pancreatitis, chondrocalcinosis).[10] Usually, there is no bone impairment, as the bone mass appears preserved in comparison with the general population. Occasionally, people with FHH3 may show low BMD, and develop cognitive dysfunctions, not reported in the other two nosological entities (FHH1 and FHH2).[2]

Differential diagnosis of other conditions causing hypercalcemia, mainly PHP, is mandatory to prevent unwarranted surgery that is not beneficial in FHH. However, discriminating between FHH and PHP can be challenging, as the two conditions have overlapping biochemical or clinical features. Determination of calcium excretion in 24-h urine represents the most useful tool for discriminating between PHP and FHH, as it is typically low in FHH and high in PHP. The CCCR is <0.01 in >80% cases of FHH, while it is >0.02 in most but not all PHP patients.[2] Nevertheless, the presence of renal impairment may result in false CCCR and makes more difficult to distinguish the two conditions. Also, family history should be investigated, and, whenever possible, screening for elevated calcium levels in first degree relatives should be carried out.[1] In our patient, the young age, the low CCCR, and the finding of hypercalcemia in his father supported the hypothesis of FHH, which was confirmed by molecular testing. In this patient and his father, we found a novel missense *CASR* variant, c.496A>C, p.(Ser166Arg), classified as "pathogenic" according to the ACMG criteria. The in vitro functional assays confirmed the in silico predictions by showing that the variant affects the expression of the receptor and strongly impairs the ability to modulate MAPK-mediated intracellular signaling.

This case reinforces the fact that the number of *CASR* deleterious variants is increasing and the clinical spectrum

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of the disease is widening, which contributes to the high variability of clinical presentation.

In conclusion, given the difficulty of discriminating between FHH and PHP, diagnosis of FHH cannot stand on suggestive biochemical data only, but it should be confirmed by genetic testing. Several different disease-causing variants have been described so far and their number is increasing. A multidisciplinary approach that also includes Medical Genetics resources is crucial, as molecular testing can lead to the identification of novel variants, not described so far but potentially hereditary, that may present difficulties in their clinical interpretation, and genetic counseling is always recommended.

Author contributions

Martina Laganà (Writing—original draft), Anna Maria Grieco (Conceptualization, Investigation), Vito Guarnieri (Conceptualization, Investigation), Marco Castori (Conceptualization, Investigation), Riccardo Pracella (Conceptualization, Investigation), Salvatore Giovinazzo (Writing—original draft), Salvatore Cannavò (Conceptualization, Data curation), and Rosaria M. Ruggeri (Conceptualization, Data curation)

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Conflicts of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Data availability

The data that support the findings of this study are available from the authors, upon reasonable request.

Patient consent

Written informed consent for publication of their clinical details and/or clinical images was obtained from the patient/parent/guardian/relative of the patient.

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