Identification and functional characterization of loss-of-function mutations of the calcium-sensing receptor in four Italian kindreds with familial hypocalciuric hypercalcemia

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Abstract

Objective: Identification and characterization of calcium-sensing receptor (CASR) mutations in four unrelated Italian kindreds with familial hypocalciuric hypercalcemia.


Methods: Direct sequencing of CASR gene in genomic DNA. Studies of CASR-mediated increases in cytosolic calcium concentration $[\text{Ca}^{2+}]_i$ in CASR-transfected COS-7 cells in vitro.

Results: Four unreported heterozygous CASR mutations were identified, including three missense (H595Y, P748H, and C765W) and one splice site (IVS2 $C\rightarrow G$) mutation. The H595Y, P748H, and C765W mutant receptors, although expressed at normal levels on the cell surface, showed a reduced response in $[\text{Ca}^{2+}]_i$ relative to the wildtype (WT) CASR to increasing extracellular calcium concentrations. Cotransfection experiments showed that the H595Y and P748H mutants did not affect the apparent affinity of the WT CASR for calcium, suggesting that they do not exert a dominant-negative effect. On the other hand, the co-transfected C765W mutant decreased the maximum response of the WT CASR to calcium, suggesting that it may reduce the effective concentration of the normal CASR on the cell surface or impair its maximal signaling capacity.

Conclusions: Four CASR mutations were identified. The reduced functional responses to extracellular calcium and normal expression of the mutant receptors suggest that conformational changes account for altered CASR activity. Moreover, a reduced complement of normal CASRs in these heterozygous patients, perhaps combined with a mutant receptor-induced decrease in maximal activity of the WT receptor, may contribute to defective calcium-sensing in vivo.

Introduction

Familial hypocalciuric hypercalcemia (FHH) is a rare disorder inherited as an autosomal dominant trait with a high penetrance of over 90%, characterized by lifelong, mild to moderate asymptomatic hypercalcemia, relative hypocalciuria, and inappropriately normal PTH levels (1). Patients with FHH exhibit mild to moderate resistance of the parathyroid glands to the inhibitory effects of $\text{Ca}^{2+}$ on PTH secretion, resulting in an increase in the ‘set-point’ of $\text{Ca}^{2+}$-regulated PTH secretion (2).

The cloning of the calcium-sensing receptor (CASR) gene has permitted clarification of the molecular basis of FHH. The CASR is a G-protein-coupled receptor that senses small perturbations in the level of extracellular $\text{Ca}^{2+}$, $[\text{Ca}^{2+}]_o$, and modulates the functions of parathyroid and kidney appropriately so as to normalize $[\text{Ca}^{2+}]_o$. On the cell surface, the CASR forms disulfide-linked dimers through cysteine residues within its extracellular domain and the dimerization has functional implications (3).

Heterozygous loss-of-function mutations, mainly scattered throughout the extracellular domain of the CASR, are responsible for most cases of FHH. The majority of mutations are missense (4–6), with a few nonsense (7–9) and splice site mutations (10) as well as an Alu insertion in one family (11, 12). Functional in vitro studies have shed light on the mechanisms responsible for the resistance of the parathyroid gland and kidney to calcium. Some mutations produce receptors that do not reach the cell surface and,
therefore, the number of active, cell surface receptors is \( \sim 50\% \) of that in normal subjects. This interpretation has been supported by the \( \sim 50\% \) reduction in the expression of the normal CASR in the parathyroid glands and kidneys of mice heterozygous for knockout of the CASR gene (13). Other mutations produce receptors that reach the cell surface and exert a dominant-negative action on the wildtype (WT) CASR. About one-third of FHH kindreds do not have mutations in the coding region or splice sites of the CASR gene, and other causes of hypocalciuric hypercalcemia, such as autoantibodies to the CASR, resulting in a condition called autoimmune hypocalciuric hypercalcemia (14, 15), or abnormalities at one of the other two FHH loci on chromosome 19 have been identified (16, 17).

We describe four unrelated Italian kindreds with FHH caused by mutations of the CASR gene and characterize the functional properties of three of these mutant receptors.

Subjects and methods

Families

All probands and participating family members underwent genetic and biochemical studies, which included the measurement of fasting serum total and ionized \( \text{Ca}^{2+} \), PTH, creatinine, phosphate, and magnesium, as well as 24-hour urinary calcium and urinary calcium to creatinine clearance ratio. Family history was negative for hypercalcemia, peptic ulcer, kidney stones, or skeletal manifestations in all cases. Our internal review board approved the study, and written informed consent was obtained from all patients who participated in the study.

Family A The proband (II-3, Fig. 1A) is a 30-year-old man with mild hypercalcemia (2.63 mmol/l) diagnosed at 27 years of age during routine investigation for a left testicular carcinoma, which was successfully treated by surgery and chemotherapy. Serum PTH was normal (25 ng/l). Serum calcium and PTH were stable thereafter and the patient remains in good health.

Family B The proband (II-1, Fig. 1D) is a 72-year-old woman in whom primary hyperparathyroidism (PHPT) was diagnosed elsewhere (serum calcium 2.68 mmol/l; PTH 68 ng/l). She underwent neck exploration, but no enlarged parathyroid glands were found.

Methods

Genetic analysis of the CASR gene Leukocyte DNA was isolated using standard methods. Exons 2–7 and adjacent splice sites of the CASR were amplified, and purified PCR products sequenced as described (18, 19). The DNA sequences of both strands were determined using an autosequencer (ABI PRISM 310. Genetic Analyzer, PE Applied Biosystems, Foster city, CA, USA).

Sequence alignment The alignment was made using the Clustal W service at http://www.ebi.ac.uk/clusalw/index.html.

Site-directed mutagenesis The Quick Change Site-Directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was used. For each mutation, the primers were complementary with the mutant sequence placed in the middle. The primers were annealed to the template CASR cDNA (HuPCaR4, kindly provided by Prof. Brown).

Transient expression of the WT and mutated CASRs in COS-7 cells COS-7 cells were propagated in DMEM as described (19). The cells were plated in Petri dishes and 24 h later transfected with CASR cDNA (625 and 800 ng for intracellular calcium measurement and immunocytochemical studies respectively) using Lipofectamine 2000 transfection reagent (Invitrogen). For co-expression experiments, equal amounts (625 ng) of WT and mutant CASR cDNAs were mixed and transfected as above.

Functional studies Cells were studied 48 h after transfection. Cells transfected with pcDNA3 alone were used as controls. Triplicate dishes were used, and each experiment was repeated at least four times.
Intracellular calcium \([Ca^{2+}]_{i}\) measurement COS-7 cells were detached from Petri dishes by trypsinization and loaded for 30 min at 37 °C with fura-2/AM (2 μM), as described (20, 21). Cells were pelleted, resuspended in Ca\(^{2+}\)- and Mg\(^{2+}\)-free HBSS/HEPES, and transferred to a quartz thermostatted cuvette (37 °C). Extracellular Ca\(^{2+}\) concentration \([Ca^{2+}]_{o}\) was varied by adding CaCl\(_2\). The fluorescence intensity was quantified with a LS-50B Luminescence Spectrometer (Perkin–Elmer, Norwalk, CT, USA) at 340 nm (F340) and 380 nm (F380 nm), and emission wavelength was monitored at 510 nm. The photomultiplier was coupled to a personal computer equipped with the Perkin–Elmer FL WinLab software for data acquisition. An increment of \([Ca^{2+}]_{i}\) induces an increase in F340 and a decrease in F380, resulting in an increase in F340/F380. Results (mean ± S.E.M.) are expressed as a percentage of the maximal response obtained for the WT or each mutant receptor. The EC\(_{50}\) (concentration producing half the maximum effect) was determined by non-linear regression curve analysis of the dose–response curves using the software GraphPad Prism (GraphPad Software, La Jolla, CA, USA). The \(E_{\text{max}}\) (percentage of the maximal CASR WT response) was also calculated for each mutant. Differences in EC\(_{50}\) and \(E_{\text{max}}\) values among dose–response curves were calculated with the F test and ANOVA followed by the Newman–Keuls multiple comparison post-test respectively.

Fluorescence immunocytochemistry and confocal microscopy Cells were harvested after incubation at 37 °C for 5 min with trypsin (Gibco), and about 10\(^5\) cells were seeded on six-well Petri dishes containing a coverslip and used at 90% confluence. Cells were fixed in 4% paraformaldehyde. Cells were permeabilized in 0.2% Triton X-100 in PBS for 15 min and incubated with blocking solution (PBS containing 5% normal goat

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**Figure 1** Left panels. Pedigrees and phenotypes of the members of the four kindreds with FHH. Circles, women; squares, male; filled symbols, affected by FHH; quartered symbols, individuals submitted to complete or partial clinical assessment: upper right filled quarter indicates hypercalcemia; lower right filled quarter indicates urinary Ca/Cr clearance ratio ≤0.01; dotted symbols, unknown with regard to phenotype and genotype; slashed symbols, deceased; arrow, proband. *Subjects submitted to DNA testing. Right panels. DNA sequences of wildtype and mutated CASRs. The sequence of the sense strand is shown.
In the proband of family A, a novel heterozygous base substitution C to T was detected, producing a change of histidine to tyrosine at codon 595 (H595Y) of exon 7. This mutation was located in the extracellular domain of the CASR. The same heterozygous mutation was found in two other affected members of the family (I-2 and II-2).

In family B, there was a heterozygous C to A substitution in exon 7 causing a change of proline to histidine (P748H). This mutation was located in the second extracellular loop. Direct sequencing of the region of interest of exon 7 revealed the same heterozygous mutation in another affected family member (III-2).

In the proband of family C, a heterozygous C to G substitution, producing a change of cysteine to tryptophan at codon 765 (C765W) of exon 7, was detected. This mutation, also located in the second extracellular loop, led to the loss of a BglII site providing a convenient diagnostic test to confirm the presence of the mutation in the proband and other family members.

In the proband of family D, there was a heterozygous G to C substitution in the donor splice site of intron 2 (IVS2+1G>C). This mutation would lead to a disruption of the splice site preventing proper excision of the intron.

**Functional studies**

**Effects on [Ca^{2+}]_i** As shown in Fig. 2(A and B), COS-7 cells transfected with WT CASR had a threshold for a response to [Ca^{2+}]_i between 0.5 and 1.0 mM. In addition, increments in [Ca^{2+}]_i from the threshold level produced rapid followed by sustained increases of basal fluorescence (F340/F380) with a sigmoidal concentration–response curve (EC50 = 2.11 ± 0.03 mM; E_{max} = 128.3 ± 0.9%; Fig. 2D). As expected, the increase of F340/F380 after exposure of the cells to the maximal [Ca^{2+}]_i (10 mM) was due to an increase of F340 and a decrease of F380, thus representing a specific increase of [Ca^{2+}]_i (Fig. 2B). In particular, in COS-7 cells transfected with WT CASR, [Ca^{2+}]_i increased from a baseline value of ~ 60 ± 4 up to ~100 ± 6 nM, while no change in [Ca^{2+}]_i was observed in non-transfected COS-7 cells (Fig. 2C), indicating the specific involvement of CASR in our [Ca^{2+}]_i responses.

As shown in Fig. 2E and Table 2, the P748H and H595Y mutants had concentration–response curves shifted to the right with lower maximal responses relative to that of WT. By contrast, the co-transfected WT/P748H and WT/H595Y had the same behavior as the WT receptor. The C765W mutant had a concentration–response curve shifted to the right and the co-transfected WT/C765W had the same EC50 as the WT. The maximal responses of C765W and co-transfected WT/C765W, however, relative to that of WT were both lower. The co-transfection results, if reflective of what happens in vivo, would suggest that the mutated receptors do not exert a dominant-negative effect manifested by an increase in EC50, but in the case of
Table 1 Clinical, biochemical, and genetic findings in the subjects who participated in the study.

<table>
<thead>
<tr>
<th>Families</th>
<th>Age (year) at diagnosis/sex</th>
<th>Total calcium (mmol/l)</th>
<th>Ionized calcium (mmol/l)</th>
<th>PTH (ng/l)</th>
<th>25OHD (nmol/l)</th>
<th>Phosphate (mmol/l)</th>
<th>Magnesium (mmol/l)</th>
<th>Urinary calcium (mmol/24 h)</th>
<th>Urinary Ca/Cr clearance ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CASR genotype</th>
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<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II-3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30/M</td>
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<td>1.43</td>
<td>18</td>
<td>61.9</td>
<td>1.06</td>
<td>0.82</td>
<td>3.1</td>
<td>0.007</td>
<td>H595Y</td>
</tr>
<tr>
<td>I-1</td>
<td>55/F</td>
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<td>–</td>
<td>–</td>
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<td>54</td>
<td>–</td>
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</tr>
<tr>
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<td></td>
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<td></td>
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<td></td>
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<tr>
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<td>80.9</td>
<td>–</td>
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<td>1.47</td>
<td>30</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>C</td>
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<tr>
<td>II-2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>61/F</td>
<td>2.70</td>
<td>1.47</td>
<td>71</td>
<td>27.7</td>
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<td>0.95</td>
<td>2.1</td>
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<tr>
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<td>–</td>
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<td>–</td>
<td>–</td>
<td>–</td>
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<td>ND</td>
</tr>
<tr>
<td>III-1</td>
<td>33/F</td>
<td>2.70</td>
<td>–</td>
<td>54</td>
<td>–</td>
<td>0.90</td>
<td>–</td>
<td>4.8</td>
<td>0.006</td>
<td>ND</td>
</tr>
<tr>
<td>D</td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II-1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>71/F</td>
<td>2.53</td>
<td>1.37</td>
<td>88</td>
<td>42</td>
<td>1.06</td>
<td>0.95</td>
<td>1.7</td>
<td>0.004</td>
<td>IVS2 + 1G&gt;C</td>
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</table>

Normal range 2.05–2.55 1.12–1.32 10–65 62–150 0.87–1.45 0.70–1.07 ♀ < 6.3; ♂ < 7.5

F, female; M, male.

<sup><sup>a</sup>U<sub>Ca</sub>x S<sub>Cr</sub>/U<sub>Cr</sub>, where U<sub>Ca</sub>, U<sub>Cr</sub>, S<sub>Ca</sub> and S<sub>Cr</sub> are the 24-h urinary excretions of calcium and creatinine and the serum total calcium and creatinine concentration, respectively. Values below 0.01 are typically found in patients with FHH.</sup>

<sup><sup>b</sup>Proband.</sup>

<sup><sup>c</sup>NA, Not available.</sup>

<sup><sup>d</sup>ND, not done because of patient refusal.</sup>
C765W reduce the maximal activation of the WT receptor (Table 2). The CASR gene mutated in the donor splice site in family D could not be tested because the splice product(s), if any, formed in vivo as a result of this mutation are unknown.

**Fluorescence immunocytochemistry and confocal microscopy** Experiments were performed using non-permeabilized cells to identify cell surface staining (positive staining indicates normal receptor processing; negative staining indicates trapping within the cell) and permeabilized cells to identify the receptor trapped within the cell, Fig. 3.

Non-permeabilized cells transfected with the WT receptor showed strong staining at the cell surface. Permeabilization of these cells revealed additional perinuclear staining. Non-permeabilized cells transfected with H595Y, C765W, and P748H mutants showed a pattern similar to that of the WT, indicating normal CASR trafficking from endoplasmic reticulum to the membrane. In permeabilized cells transfected with the H595Y, P748H, and C765W mutants, perinuclear staining associated with the Golgi apparatus and the endoplasmic reticulum similar to that of the WT cells was observed.

**Discussion**

We describe four FHH families with four previously unreported CASR mutations. The clinical features of the probands and affected family members were consistent with the diagnosis of FHH. Indeed, all had mild asymptomatic hypercalcemia associated with normal or slightly elevated serum PTH values. The mildly elevated serum PTH levels in the probands of families C and D led to the initial mistaken diagnosis of sporadic PHPT and therefore to parathyroid surgery. A mildly enlarged parathyroid gland was removed in the former and was histologically scored as a hyperplastic gland, possibly a parathyroid adenoma. The microscopic

### Table 2

<table>
<thead>
<tr>
<th>Clone</th>
<th>EC50 (mM)</th>
<th>P</th>
<th>E&lt;sub&gt;max&lt;/sub&gt; (% WT)</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Wildtype (WT)</td>
<td>2.11±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>–</td>
<td>100</td>
<td>–</td>
</tr>
<tr>
<td>H595Y</td>
<td>2.64±0.05</td>
<td>&lt;0.001</td>
<td>69.4±3.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WT/H595Y</td>
<td>2.31±0.04</td>
<td>NS</td>
<td>103.8±4.3</td>
<td>NS</td>
</tr>
<tr>
<td>P748H</td>
<td>3.87±0.03</td>
<td>&lt;0.001</td>
<td>72.3±3.1</td>
<td>&lt;0.001</td>
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<tr>
<td>WT/P748H</td>
<td>2.29±0.04</td>
<td>NS</td>
<td>104.1±4.9</td>
<td>NS</td>
</tr>
<tr>
<td>C765W</td>
<td>3.20±0.01</td>
<td>&lt;0.001</td>
<td>61.2±2.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WT/C765W</td>
<td>2.23±0.05</td>
<td>NS</td>
<td>66.2±3.6</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

WT, wildtype.

<sup>a</sup>Compared to WT.

<sup>b</sup>Values are means ± S.E.M. of at least four different experiments.
appearance was not consistent with the so-called ‘lipohyperplasia’, which has been reported in several parathyroid glands of FHH patients, where, at variance with what we found in our case, concurrent hyperplasia of fat and parathyroid chief cells have been observed in some cases (22, 23). Thus, we believe that the resected parathyroid gland was pathological. Whether the anatomic lesion was truly hyperplasia or an adenoma remains unclear. The normal macroscopic appearance of the other parathyroid glands at neck exploration might favor the latter diagnosis. On the other hand, in the absence of the histological demonstration that the apparently unaffected glands were normal, diffuse hyperplasia cannot be excluded. Serum calcium levels were unchanged after surgery suggesting that abnormal calcium-regulated PTH secretion by the enlarged parathyroid gland was readily taken over by the remaining, presumably functionally abnormal, parathyroid glands. At variance with our case, the removal of a large parathyroid adenoma, which was associated with marked symptomatic hypercalcemia in a subject with FHH, was followed by an initial rapid decrease in serum calcium to normal levels, followed by a recurrence of mild hypercalcemia characteristic of FHH (24). Parathyroid adenomas have also been reported in several members of an atypical FHH family described by Carling and coworkers (25).

The question of whether the finding of a parathyroid adenoma in subjects with FHH is coincidental or secondary to the CASR mutations remains to be established. However, somatic inactivating mutations of the CASR gene are not present in sporadic parathyroid adenomas (18, 26), suggesting that co-occurrence by chance of PHPT and FHH, although very rare, accounts for the association in occasional cases. By contrast, a causal role of a CASR mutation in the development of parathyroid adenomas has been proposed in the kindred described by Carling et al. (25). Indeed, in this family all abnormal glands displayed allelic losses at loci commonly involved in sporadic parathyroid tumors, and the authors suggested that the germline CASR mutation may increase the susceptibility to secondary genetic hits, at least with this specific mutation in the receptor’s C-terminal tail (27).

Molecular analysis showed that all four kindreds carried unreported CASR mutations. Three of them (H595Y, P748H, and C765W) were missense and involved amino acids highly conserved between species. The fourth was a splice site mutation in intron 2 (IVS2+1G>C) and, to our knowledge, is the second splice site mutation identified in the CASR gene. The first mutation (IVS2-1G>T) was identified in a family with FHH (10). This mutation resulted in exon 3 skipping, causing a shift in the reading frame of exon 4 and the introduction of a premature stop codon leading to a predicted truncated protein of 153 amino acids. The splice site mutation IVS2+1G>C described herein would most likely give little or no CASR mRNA or partially spliced mRNA and probably no protein. None of the four mutations we describe herein was located in the so-called cysteine-rich domain, which plays a critical role in the interaction between the ‘Venus fly-trap’ domain and the seven transmembrane domains (28, 29).

Functional characterization of the CASR mutations was performed by transfecting WT and mutated CASRs into COS-7 cells. All mutations caused substantially reduced maximal responses to $[\text{Ca}^{2+}]_o$. These results are consistent with the inactivating nature of these CASR mutations. Although these mutations only

Figure 3 Expression of wildtype and mutant CASRs in transfected COS-7 cells using fluorescence immunocytochemistry and confocal microscopy. Examples of fields of non-permeabilized (upper panel) and permeabilized (lower panel) cells. Magnification: 600×.
modestly altered the EC$_{50}$ for extracellular calcium-evoked increases in [Ca$^{2+}$]$_{o}$, compared with other mutations (30–32), the combination of reduced maximal responses and increased EC$_{50}$s produced significantly impaired functional responses in vitro and were clearly associated with defective [Ca$^{2+}$]$_{o}$-sensing in vivo.

To further investigate the mechanisms responsible for the loss of function of mutated CASRs, fluorescence immunocytochemistry was performed on COS-7 cells transiently transfected with WT and mutated CASR cDNAs. These studies showed that all mutants were expressed at levels comparable with that of the WT both in non-permeabilized and permeabilized cells, as assessed by fluorescence intensity. These data suggest that the mutated receptors were properly trafficked to the cell surface. Thus, the loss of function of these receptors is likely due to conformational changes that decrease their responses to [Ca$^{2+}$]$_{o}$ without affecting their immunoreactivity, rather than to a reduced receptor number on the cell surface. In this regard, it is worth noting that all three of these mutations were located in the extracellular domain. The proline to histidine substitution at codon 748 and of cysteine to tryptophan at codon 765 and, consequently, the introduction of the aromatic side chains of histidine and tryptophan might alter the polarity of the protein. Moreover, a single mutation from the small and flexible amino acids, proline and cysteine to the larger amino acids, histidine and tryptophan, could also induce conformational changes of the respective receptor proteins.

As our patients harbored heterozygous CASR mutations, co-transfection of COS-7 cells with both WT and mutated receptors was performed to mimic in vitro heterozygosity. The EC$_{50}$ [Ca$^{2+}$]$_{o}$ and the $E_{\text{max}}$ of WT/H595Y and WT/P748H were similar to those of the WT receptor suggesting that these mutant receptors, which have a pattern of protein expression similar to that of the wildtype, do not exert a dominant-negative effect. Because patients belonging to kindreds A and B have a mild degree of hypercalcemia, it is conceivable that the mutated CASRs, being less sensitive to extracellular calcium, are only minimally activated at ambient blood calcium levels in vivo, and CASR signaling is limited to the reduced complement of remaining normal receptors. Indeed, the biochemical profile of these patients is similar to that observed in the heterozygous CASR knock-out mice (13). These mice have about half of the normal amount of CASR in parathyroid and kidney (haploinsufficiency), and this results in hypocalciuric hypercalcemia of a mild degree. On the other hand, cells co-transfected with the WT and C765W, which is also expressed at the cell surface in a manner similar to that of the WT CASR, showed a decreased $E_{\text{max}}$ compared with WT but a similar EC$_{50}$ [Ca$^{2+}$]$_{o}$, suggesting that it may reduce the effective concentration of the functional WT CASR on the cell surface or otherwise impair the maximal signaling capacity of the normal CASR.

In conclusion, we have identified unreported heterozygous loss-of-function mutations in the extracellular domain of the CASR gene in four Italian families with FHH. Functional analysis of three mutant CASRs provided an explanation for the clinical phenotype in the patients. A reduced response to calcium stimulation and the finding of normal cell surface expression of the mutant receptors suggest that conformational changes account for altered CASR activity. Moreover, a reduced complement of normal CASRs in these heterozygous patients, perhaps combined in one case with a mutant receptor-induced decrease in maximal activity of the WT receptor, may contribute to defective calcium-sensing in vivo.

**Declaration of interest**

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

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**References**


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