A novel germline inactivating mutation in the CASR gene in an Italian kindred affected by familial hypocalciuric hypercalcemia

Alberto Falchetti, Alessia Gozzini, Annalisa Terranepra 1, Laura Soldatii 2, Giuseppe Vezzoli 1, Gigliola Leoncini, Francesca Giusti, Francesco Franceschelli, Laura Masi, Annalisa Tanini, Loredana Cavalli and Maria Luisa Brandi

Unit of Metabolic Bone Diseases, Department of Internal Medicine, University of Florence, Viale Morgagni 85, 50135 Florence, Italy, 1Nephrology and Dialysis Unit, Scientific Institute San Raffaele Hospital, Vita Salute University, Milan, Italy and 2Department of Medicine, Surgery and Dentistry, Università degli Studi di Milano, Milan, Italy

(Correspondence should be addressed to M L Brandi; Email: m.brandi@dmi.unifi.it)

Abstract

Objective: Familial hypocalciuric hypercalcemia (FHH) syndrome is a rare benign condition, inherited as an autosomal dominant trait, in which inactivating mutations of the calcium-sensing receptor (CASR) gene affects the body’s ability to regulate calcium homeostasis. Its outcome is featured by increased levels of serum calcium, moderate hypophosphatemia, and inadequately normal or elevated circulating parathyroid hormone levels. Affected patients are mostly asymptomatic and do not benefit from surgical resection of their mildly enlarged parathyroids.

Design: We evaluated for hypercalcemia an Italian family that was identified via a young adult male proband referred to our center for parathyroidectomy.

Methods: The patients and the family members were evaluated both biochemically and genetically as suspected FHH subjects. An in vitro functional study was performed by site-directed mutagenesis, and CASR activity was monitored by measuring intracellular calcium ([Ca^{2+}]).

Results: The patient had a novel germline heterozygous CASR mutation (c.361_364GATT; p.D121del/fsX122). The mutation caused a premature stop codon at codon 122, exiting a truncated protein. The biochemical phenotype of all family members carrying the heterozygous deletion was concordant with classic FHH syndrome.

Conclusions: Our findings confirm the role of CASR gene mutational analysis to offer a valuable addition for the recognition of FHH in hypercalcemic patients not yet characterized for a positive familial history of hypercalcemia, the only condition that identifies CASR gene mutations in hypercalcemia.

Introduction

Familial hypocalciuric hypercalcemia (FHH) syndrome (OMIM #145980) is a rare benign condition, inherited as an autosomal dominant trait, in which inactivating mutations of the calcium-sensing receptor (CASR) gene affect the body’s ability to regulate calcium homeostasis (1). FHH is featured by increased levels of serum calcium, moderate hypophosphatemia, inadequately normal or elevated circulating parathyroid hormone (PTH) levels (when correlated with serum calcium levels) mildly elevated in 5–10% of cases, and a normal urinary calcium excretion (2). Consequently, FHH is clinically similar to sporadic, nonsyndromic, early diagnosed primary hyperparathyroidism (PHPT).

FHH patients are mostly asymptomatic and do not benefit from surgical resection of their mildly enlarged parathyroids as surgery cannot correct the genetically determined abnormal set-point of calcium-dependent PTH secretion (3, 4). Both FHH-associated hypercalcemia and relative hypocalciuria (urinary calcium/creatinine clearance ratio typically <0.01) are lifelong highly penetrant due to inactivation of CASR protein both in parathyroid glands and in renal tubules (5). Serum magnesium is typically found in the higher range of normal values or modestly elevated (5, 6).

Parathyroid cells respond to the decrease in extracellular calcium concentration through activation of the CASR gene, a cell surface seven-membrane-spanning G protein-coupled receptor, modulating phosphatidylinositol turnover and intracellular calcium amount that ultimately leads to an increase in the secretion of PTH (7). CASR is able to sense small changes in circulating calcium concentration and when activated inhibits PTH secretion and renal tubule calcium reabsorption. The ‘set-point’ of parathyroid cells, defined as that calcium concentration at which PTH secretion is half maximal, is increased in FHH patients. Renal calcium handling is also abnormal in
FHH individuals and even if the magnitude of the PTH suppression by increased serum calcium concentrations is normal, they generally fail in the hypercalciuric response to hypercalcemia (5). In fact, the tubular reabsorption of calcium in FHH is high and remains increased even after an intended or unintended total parathyroidectomy.

Most FHH patients are identified by chance and the recognition of mild hypercalcemia in first-degree relatives supports the diagnosis. Easy fatigue, weakness, thought disturbance, or polydipsia is generally less common and less severe than in typical PHPT patients mainly consist of chondrocalcinosis (usually clinically silent), premature vascular calcification (8, 9), and pancreatitis (10). However, acute pancreatitis (clinically silent), premature vascular calcification (8, 9), and pancreatitis (10). However, acute pancreatitis has also been reported in isolated cases (11).

Unfortunately, despite what has been just described, most asymptomatic FHH subjects are still misdiagnosed as affected by sporadic PHPT and addressed to undergo neck surgery, exposing them to the potentially related metabolic and clinical assessment before neck surgery. The previous biochemical findings (Table 1), leading to the initial diagnosis of PHPT, consisted of a moderate hypercalcemia (10.8 mg/dl) in the presence of a slightly increased serum PTH (7.7 pmol/l). However, the young age of the subject prompted us to investigate the possibility of FHH.

The pattern of biochemical investigations routinely used confirmed the suspicion of hypocalciuric hypercalcemia (Table 1). An accurate clinical familial history was completed and the pedigree (FHH-01) was drawn (Fig. 1), and after having obtained informed consent, as formulated by the Local Ethics Committee of the University of Florence, a mutation analysis of the CASR gene was performed in the II-2 proband.

After genetic counseling, the other members from the family (father (I-1), mother (I-2), sister (II-1), and brother (II-3; Fig. 1)) underwent DNA tests. All subjects except I-1 accepted to be clinically screened for calcium phosphate metabolism (Table 1).

### DNA testing

Blood-EDTA samples were collected from I-1, I-2, II-1, II-2, and II-3 to search for the CASR gene germline mutation detected in II-2. CASR mutational analysis was also performed on 120 normal individuals (60 males and 60 females) whose ages ranged from 50 to 85 years.

---

**Materials and methods**

**Family members**

The proband was a 32-year-old male diagnosed as affected by PHPT by an outside outpatient clinic and parathyroidectomy was already planned for him. He was referred to our center to receive an adequate metabolic and clinical assessment before neck surgery.

### Table 1: Biochemical features of members from the family FHH-01.

<table>
<thead>
<tr>
<th>Referring range</th>
<th>II-2 mean ± S.D.</th>
<th>II-3 mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl/(Cr.Cl)</td>
<td>10.5 ± 0.1</td>
<td>131 ± 29.7</td>
</tr>
<tr>
<td>CaCl/(Cr.Cl)</td>
<td>10.6 ± 0.2</td>
<td>21 ± 0.1</td>
</tr>
<tr>
<td>s-Ca (mg/dl; 8.2–10.7)</td>
<td>2.5 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>u-Ca (mg/dl; 2.5–5.0)</td>
<td>2.4 ± 0.4</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>s-P (mg/dl; 75–125)</td>
<td>78.2 ± 12.3</td>
<td>42 ± 6.4</td>
</tr>
<tr>
<td>u-P (mg/24 h; 400–1000)</td>
<td>114.4 ± 24.0</td>
<td>130 ± 138</td>
</tr>
<tr>
<td>s-Mg (mg/dl; 1.8–2.8)</td>
<td>7.1 ± 0.1</td>
<td>7.2 ± 0.1</td>
</tr>
<tr>
<td>u-Mg (mg/24 h; 75–125)</td>
<td>7.1 ± 0.2</td>
<td>7.2 ± 0.1</td>
</tr>
<tr>
<td>s-Cr, (mg/dl; 0.6–1.5)</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.0</td>
</tr>
<tr>
<td>u-Cr, (mg/24 h; 0.8–1.8)</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Cr.Cl. (ml/min; 70–140)</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.0</td>
</tr>
<tr>
<td>s-Total proteins (g/dl; 8–10.6)</td>
<td>7.1 ± 0.1</td>
<td>7.2 ± 0.1</td>
</tr>
<tr>
<td>s-PTH (pmol/l; 1.3–7.6)</td>
<td>6.1 ± 1.7</td>
<td>6.7 ± 0.2</td>
</tr>
<tr>
<td>s-PH (pmol/l; 1.3–7.6)</td>
<td>7.2 ± 1.1</td>
<td>6.7 ± 0.2</td>
</tr>
<tr>
<td>i-Ca (mmol/l; 1.15–1.30)</td>
<td>1.3 ± 0.1</td>
<td>1.3 ± 0.0</td>
</tr>
<tr>
<td>Pyridinoline (nmol/nmol Cr.; 2.3–5.4)</td>
<td>4.4 ± 1.0</td>
<td>6.5 ± 0.3</td>
</tr>
</tbody>
</table>

Ca, calcium; P, phosphate; Mg, magnesium; s, serum; u, urine; bAP, bone alkaline phosphatase; i, ionized; Cr., creatinine; Cl., clearance.

www.eje-online.org
bacterial clones (wild-type and deleted) were mixed in
cultures for screening by means of PCR from bacterial white colonies
clones containing the wild-type or mutated allele were
isolated using a plasmid containing the wild-type allele as a vector in DH5α Competent Cells (Takara Bio, Inc.,
Otsu, Shiga, Japan) was used as host strain. Positive
Escherichia coli
amplified PCR products were directly cloned into the
plasmid to construct a mutant plasmid. The deletion was
produced by PCR amplification using two overlapping
primers, only one containing the target mutation
site (forward mutagenic primer: TGCTCAAAACAAAA-
TT/CTTGTGAACCTTGATGAC; reverse primer: ATTTTG-
TTTGTAGGCAACAAAACTTCAGGGTGG). The Platinum
Taq DNA Polymerase High Fidelity (Invitrogen) was
used for 20 amplification cycles. Mutant plasmids were
amplified in E. coli. DH-5α strain, and DNA was extracted with the alkaline lysis procedure.

**Biochemical investigations**

Patients I-2, II-2, and II-3 did accept to undergo biochemical screening at our center. Subject II-1, non-
mutant, accepted to undergo the baseline tests in 2006.

In particular, subject I-2 has been screened from 2002 through 2010; II-2, the proband, from 1999 to
2001 in external laboratories and from 2002 through 2010 at our center; and II-3 from 2005 through 2006
(Table 1). The results of the biochemical screening performed in all individuals are reported in Table 1.

**Instrumental investigations**

Subjects I-2 and II-2 underwent bone mass evaluation (bone mineral density (BMD)) by Dual X-ray absorptio-
metry (DXA) scans (Hologic QDR-4500A, Waltham,
MA, USA): I-2 at left femur (LF) in 2002 and LF and
lumbar spine (LS) in 2008; II-2 at LS and LF in 2005
and 2008. I-2 has also been investigated by total
abdomen and right shoulder ultrasounds (US) to verify
the clinical suspect of gall bladder stones and calcifica-
tions at the soft tissues of the right shoulder respectively.

Moreover, in patient I-2, the presence of a degener-
ative maculopathy with visual impairment has also
been investigated by eye evolution and fluoroangiogra-
phy. In January 2009, after performing LS and LF DXA
scans, she underwent lumbar-sacral nuclear magnetic
resonance (NMR).

**Site-directed mutagenesis of the human CASR and transient receptor expression**

The human wild-type CASR cDNA, cloned in pCR3.1
plasmid (Invitrogen), was kindly provided by Dr Jianxin
Hu (NIH, Bethesda, MD, USA).

*In vitro* site-directed mutagenesis was directly applied
on wild-type CASR plasmid to construct a mutant
receptor containing the four-nucleotide deletion (briefly
referred to as Δ121) using the Gene Tailor Site-Directed
Mutagenesis System (Invitrogen). The deletion was
produced by PCR amplification using two overlapping
primers, only one containing the target mutation
site (forward mutagenic primer: TGCTCAAAACAAAA-
TT/CTTGTGAACCTTGATGAC; reverse primer: ATTTTG-
TTTGTAGGCAACAAAACTTCAGGGTGG). The Platinum
Taq DNA Polymerase High Fidelity (Invitrogen) was
used for 20 amplification cycles. Mutant plasmids were
amplified in E. coli. DH-5α strain, and DNA was extracted with the alkaline lysis procedure.

Mutation was confirmed by automated DNA sequen-
cing of entire CASR cDNA using ABI PRISM 3100
Genetic Analyzer with the Big-Dye Terminator V1.1 Kit
(Applied Biosystem, Foster City, CA, USA).

**Table 1**

<table>
<thead>
<tr>
<th>Generation</th>
<th>Subject</th>
<th>Age (years)</th>
<th>Gender</th>
<th>CASR Mutation</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>I-1</td>
<td>72</td>
<td>Male</td>
<td>野生型</td>
<td>FHH-01</td>
</tr>
<tr>
<td></td>
<td>I-2</td>
<td>40</td>
<td>Male</td>
<td>5′UTR deletion</td>
<td>FHH-01</td>
</tr>
<tr>
<td></td>
<td>II-1</td>
<td>70</td>
<td>Male</td>
<td>野生型</td>
<td>FHH-01</td>
</tr>
<tr>
<td></td>
<td>II-2</td>
<td>37</td>
<td>Male</td>
<td>5′UTR deletion</td>
<td>FHH-01</td>
</tr>
<tr>
<td></td>
<td>II-3</td>
<td>42</td>
<td>Male</td>
<td>野生型</td>
<td>FHH-01</td>
</tr>
</tbody>
</table>

Genomic DNA was extracted from peripheral whole
blood using the QIAamp DNA Blood Mini Kit according
to the manufacturer’s instructions (Qiagen).

Each of the six coding exons (exons 2–7) and part of
the flanking introns of the CASR gene were amplified in
separate tubes by PCR using appropriate primer sets
(available upon request; MWG-Biotech, Ebersberg,
Germany). PCR reactions were performed in a Bio-Rad
iCycler system thermocycler (Bio-Rad) according to an
already described protocol (12) and the products were
purified, cycle sequencing, sequencing products
purification, and sequencing analysis were performed
according to the literature (12). The obtained forward
and reverse DNA sequences were compared with the
genomic reference sequence of CASR gene published on
the NCBI database (sequence accession number and
version: NC_000003.11).

Following identification of the heterozygous causative
mutation in exon 3, amplification of exon 3 from the
genomic DNA was repeated. The Taq polymerase-
amplified PCR products were directly cloned into the
blue/white selection vector pCRII using TA Cloning Kit
Dual Promoter (Invitrogen Corp., Carlsbad, CA, USA)
according to the manufacturer’s instructions. TaKaRa
Escherichia coli DH5α Competent Cells (Takara Bio, Inc.,
Otsu, Shiga, Japan) was used as host strain. Positive
clones containing the wild-type or mutated allele were
screened by means of PCR from bacterial white colonies
and then sequenced. Amplified exon 3 from different
bacterial clones (wild-type and deleted) were mixed in
similar concentrations and then denatured and renatu-
ed (7 min at 94 °C and 15 min at 42 °C) to be visualized by agarose gel electrophoresis.

**Figure 1** The pedigree of FHH-01 is depicted. On the left side, Roman numbers I and II indicate the generation. Black symbols (squares and circles) indicate subjects carrying heterozygous mutation in CASR gene. Open square and open circle represents non-mutant subjects. Letter P and the arrow indicate the proband. Under each subject, the corresponding current age is reported.

Subjects I-2 and II-2 underwent bone mass evaluation (bone mineral density (BMD)) by Dual X-ray absorptio-
metry (DXA) scans (Hologic QDR-4500A, Waltham,
MA, USA): I-2 at left femur (LF) in 2002 and LF and
lumbar spine (LS) in 2008; II-2 at LS and LF in 2005
and 2008. I-2 has also been investigated by total
abdomen and right shoulder ultrasounds (US) to verify
the clinical suspect of gall bladder stones and calcifica-
tions at the soft tissues of the right shoulder respectively.

Moreover, in patient I-2, the presence of a degener-
ative maculopathy with visual impairment has also
been investigated by eye evolution and fluoroangiogra-
phy. In January 2009, after performing LS and LF DXA
scans, she underwent lumbar-sacral nuclear magnetic
resonance (NMR).

The human wild-type CASR cDNA, cloned in pCR3.1
plasmid (Invitrogen), was kindly provided by Dr Jianxin
Hu (NIH, Bethesda, MD, USA).

*In vitro* site-directed mutagenesis was directly applied
on wild-type CASR plasmid to construct a mutant
receptor containing the four-nucleotide deletion (briefly
referred to as Δ121) using the Gene Tailor Site-Directed
Mutagenesis System (Invitrogen). The deletion was
produced by PCR amplification using two overlapping
primers, only one containing the target mutation
site (forward mutagenic primer: TGCTCAAAACAAAA-
TT/CTTGTGAACCTTGATGAC; reverse primer: ATTTTG-
TTTGTAGGCAACAAAACTTCAGGGTGG). The Platinum
Taq DNA Polymerase High Fidelity (Invitrogen) was
used for 20 amplification cycles. Mutant plasmids were
amplified in E. coli. DH-5α strain, and DNA was extracted with the alkaline lysis procedure.

Mutation was confirmed by automated DNA sequen-
cing of entire CASR cDNA using ABI PRISM 3100
Genetic Analyzer with the Big-Dye Terminator V1.1 Kit
(Applied Biosystem, Foster City, CA, USA).

www.eje-online.org
Human embryonic kidney cells (HEK-293; Cell Bank, IST, Genoa, Italy) were grown in DMEM medium (Sigma Chemicals), supplemented with 10% FBS and 1% penicillin/streptomycin (Euroclone, Devon, UK). Transfection was performed using 10 μg plasmid DNA for each transfection in a 25 cm² flask of HEK-293 cells. DNA was diluted in OptiMEM (Invitrogen); Lipofectamine (Invitrogen) was incubated with OptiMEM for 5 min at room temperature. Then, DNA was gently mixed with Lipofectamine and the mixture was incubated at room temperature for 30 min. The DNA–Lipofectamine complex was further diluted in OptiMEM and was added to 80% confluent HEK-293 cells plated in 25 cm² flasks. After 5–6 h of incubation, 2.5 ml complete DMEM was added. The medium was refreshed 24 h after transfection. Cotransfection of wild-type and mutant receptor was carried out as described before with 5 μg of each plasmid. The expressed CASR protein was assayed 48 h after the initiation of transient transfection on immunoblots stained with anti-CASR MAB to detect total immunoreactive species.

**Western blotting**

Transiently transfected cells were lysed with 0.1 M NaOH and scraped from flasks. The viscose solution was passed through a needle of a syringe and transferred into microcentrifuge tubes. Proteins were dissolved in Laemmli sample buffer (final concentration 3% SDS and 2% mercaptoethanol). Samples (150 mg proteins/well) were separated on Laemmli gel (acrylamide gradient 7–15%) and electrophoretically transferred on PVDF membrane. The blots were blocked in a blocking solution (5% milk in PBS) and then incubated overnight at 4 °C with primary rabbit anti-CASR polyclonal antibody, PA1-934, diluted 1:500 in the blocking solution (Affinity Bioreagents, Golden, CO, USA). The same membranes were incubated with polyclonal anti-actin antibody (Sigma Chemicals) overnight at 4 °C with a 1:1000 dilution. Both blots were incubated for 1 h at room temperature in HRP-conjugated goat anti-rabbit secondary antibodies diluted 1:2000. The specific protein bands were detected using an enhanced chemiluminescence system.

**Measurement of [Ca²⁺]_i**

Transfected HEK-293 cells were trypsinized and counted for each experiment. About 5 × 10⁵ cells/ml were used. The samples were loaded with Fura-2 AM (Sigma Chemicals; 1 μM final concentration, 37 °C, 45 min) in a bath solution (123 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM glucose, 25 mM HEPES, 0.5 mM CaCl₂, pH 7.4 at 37 °C, and 285 mosm/l). At the end of incubation, cells were washed using the bath solution and transferred into a thermostated quartz cuvette containing the bath solution. Extracellular calcium concentration [Ca²⁺]_o was increased stepwise by 1 mM (from 0.5 to 12 mM). The fluorescence measurements were carried out with the luminescence spectrometer LS 50B (Perkin-Elmer, Norwalk, CT, USA) at 37 °C under magnetic stirring and were recorded at 340 and 380 nm excitation light with 510 nm emission. The data were analyzed by WinLab Software package (Perkin-Elmer) that allows for a fluorescence lecture every 1.9 s. The intracellular calcium concentration [Ca²⁺]_i was calculated from fluorescence ratio (340/380 excitation) as described previously (13, 14).

**Statistical analysis**

The mean EC₅₀, which is extracellular calcium concentration giving half maximal response, for wild-type and mutant receptor was calculated from all individual experiments (n = 4) and expressed as s.e.m. of the mean. The EC₅₀ values were compared using Mann–Whitney U test.

**Results**

**DNA analysis**

After comparison with the wild-type genomic CASR sequence, a novel germline heterozygous 4 bp deletion of nucleotides 361–364 (GATT) in exon 3 (coding DNA reference sequence on NCBI database NM_000388.3:c.361_364delGATT or c.361_364del4; protein reference sequence on NCBI database NP_000379.2:p.Asp121fs or p.Asp121LeufsX) was detected in I-2, II-2, and II-3 subjects (Fig. 1). The mutation was inherited by I-2, with I-1 exhibiting wild-type sequence. The deletion of a nonmultiple of three nucleotide bases changed the reading frame for the entire subsequent sequence, impairing the function of the resulting protein, if it is formed. Indeed, the mutation causes a frameshift after Ile120 that results in a UGA (opal) termination codon, two codons downstream of the mutation site (Fig. 2A). In fact, two bands appeared after separation of DNA from two different PCR products. This deletion introducing a premature stop codon in the gene sequence causes a loss-of-function mutation, deleting all the functional parts of the protein and it has not been previously described (http://www.casrdb.mcgill.ca). This sequence variation could not be found in a sample of 120 normal chromosomes.

The presence of the wild-type allele in the homozygous state was indicated by a single PCR product of 364 bp. Instead, an abnormal pattern of migration of the PCR-amplified products containing exon 3 was detected for each heterozygous member of the family (Fig. 2B). In fact, two bands appeared after separation by electrophoresis on a standard 2% agarose gel stained with ethidium bromide due to homoduplex and heteroduplex formation between the long and short
alleles, which were 364 and 360 bp respectively. Annealing of mutated to wild-type DNA alleles gives duplexes with one or more mismatched (heteroduplexes) running slower than homoduplexes. Base mismatching causes the double helix to take on a conformation that retards its mobility during electrophoresis.

Moreover, the sequence data obtained when CASR exon 3 PCR products from the proband were individually cloned in pCR II vectors and sequenced using M13 forward primer more clearly revealed the presence of the wild-type and mutated alleles, the latest with a deletion of four bases (5’-GATT-3’; Fig. 2C). The sequence data of clones were in agreement with those obtained by direct sequencing of the PCR products from heterozygous individuals.

After a denaturation/renaturation step, the mixture of exon 3 PCR products separately amplified from wild-type and mutated alleles was electrophoresed on ethidium bromide-stained agarose gel. Homoduplexes and heteroduplexes were detected with the same electrophoretic migration pattern observed for PCR products from heterozygous individuals.

Biochemical/clinical investigations
The results of biochemical screening and corresponding normal range values are detailed in Table 1. The results are expressed as mean values ± S.D. of measurements made in the years outlined earlier, but for patient II-1 who had only one evaluation.

I-2 She also suffered from arterial hypertension and esophageal hiatus hernia. Moreover, she was mildly hypercalcemic, with PTH values in the upper part of the normal range and inappropriate low urinary calcium excretion levels. Her u-Ca/Cr clearance ratio was <0.001 (Table 1).

II-1 She was found to be normocalcemic, with normal PTH levels and normal urinary calcium excretion. Her u-Ca/Cr clearance ratio was >0.01 ng/ml (Table 1).

II-2 (proband) He was originally diagnosed as affected by sporadic PHPT due to the presence of hypercalcemia, with slightly increased PTH levels, inappropriate urinary calcium excretion levels, and u-Ca/Cr clearance...
ratio < 0.001 (Table 1). His pituitary/gonadal axis evaluation (FSH, LH, and serum total testosterone) did not show any abnormality (data not shown).

**II-3** He was found to be normocalcemic with PTH levels in the upper part of the normal range, inappropriate urinary calcium excretion levels, and u-Ca/Cr. clearance ratio < 0.001 (Table 1).

All the mutant subjects (I-2, II-2, and II-3) also exhibited serum magnesium values in the upper part of the normal range, while the average of their 25-hydroxyvitamin D circulating levels was adequate.

**Instrumental investigations**

**I-2 Bone mineral density.** She was osteopenic at LF neck, 0.604 g/cm² (T-score: −2.21 S.D.) in 2002, whereas in 2008, the LF neck BMD became osteoporotic, 0.641 g/cm² (T-score: −2.8 S.D.). A clear osteoporotic result has also been observed at LS level in 2008 (not performed in 2002): L2–L4, 0.690 g/cm² (T-score −4.3 S.D.).

**Lumbar-sacral NMR.** A lumbar right convex scoliosis, stress fracture at S1 vertebral body, and multiple discs dehydration were reported.

**Total abdomen US.** The presence of a mobile small (8 mm) gall bladder stone and cholecystitis and an angiolipoma of the left kidney was reported.

**Right shoulder US.** Presence of degenerative aspects in this joint and the surrounding structures with some fine microcalcifications of soft tissues was reported. The final diagnosis was of a second-degree subacromial conflict syndrome.

**Eyes visit and fluoroangiography.** Both second-degree hypertensive retinopathy and hyperfluorescence at the interpapillo-macular level have been diagnosed in the left eye.

**II-2 Bone mineral density.** In 2001, he exhibited a slight osteopenia at LS (L2–L4), 0.969 g/cm² (T-score: −1.32 S.D.), and a normal LF neck BMD, 0.879 g/cm² (T-score −0.37 S.D.). Then, in 2005, LS BMD was 1.027 g/cm² (T-score −1.4 S.D.) and LF neck BMD scored 0.924 g/cm² (T-score 0.0 S.D.). Finally, in 2008, a decreased, osteopenic BMD at LS level was observed, 1.012 g/cm² (T-score −1.9 S.D.), whereas a stable condition of normality was still reported at LF neck, 1.029 g/cm² (T-score −0.3 S.D.).

**Site-directed mutagenesis and transient receptor expression**

The sequencing showed a shorter CASR molecule (four-nucleotide deletion) and no other mutations were produced.

We compared the receptor expression at protein level in the wild type (WT)-CASP-transfected cells with that in the Δ121-CASR-transfected and WT/Δ121 cotransfected cells. Antibody detected a ~140 kDa band for both WT-CASR-transfected and WT/Δ121 cotransfected cells (with no appreciable differences of expression), while no expression was detected for Δ121-CASR-transfected cells (Fig. 3A).

**Functional characterization of Δ121-CASR using the intact cell [Ca²⁺]ᵢ stimulated assay**

Measurements of [Ca²⁺]ᵢ responses in HEK-293 cells transfected with the WT receptor showed an EC₅₀ for
Discussion

Inactivating mutations in the human CASR gene cause both FHH and its homozygous form, the neonatal severe hyperparathyroidism (NSHPT) (15). In FHH and NSHPT subjects, probably mutations in the amino acid coding region are detected in more than 99% of cases.

We found a novel germline heterozygous frameshift mutation at codon 121 in a hypercalcemic subject and his family members. Asp121 is located in the extra-cellular calcium-binding domain of the receptor, phylogenetically well conserved through many species. This frameshift mutation caused a premature stop codon at codon 122, exiting a truncated protein, completely disrupting the function of the resulting protein and likely to be pathogenic. Its presence, in the heterozygous state, was detectable by agarose gel electrophoresis as heteroduplex artifact, because the deletion of 4 bp from one allele in the heterozygotes creates PCR artifact products with an aberrant deletion of 4 bp from one allele in the heterozygotes.

The biochemical phenotype of the mutated family members was concordant with the classical expression reported in FHH patients. In particular, all of them had a calcium clearance/creatinine clearance ratio <0.001 whereas nonmutant II-1 exhibited a ratio >0.01.

An inappropriately lower 24 h urinary calcium excretion was observed in subject I-2 when compared with subjects II-2 and II-3. As the coexistence of mutated and normal CASR in parathyroid tissue and in renal tubular cells is supposed to exist in all heterozygous subjects, it may not provide an alternative explanation for the absence of a clear hypocalciuria in II-2 and II-3 with respect to I-2. Consequently, other unidentified genetic (e.g. modifying genes) and/or nongenetic factors (e.g. diet, physical activity, and age) could account for such a difference.

Average serum PTH levels were within the normal range in all the mutants, even if subject II-2, with higher average values of serum calcium, showed PTH values higher than the upper normal range.

Adequate daily supplementation of cholecalciferol, 750–1000 IU, has been provided to all the subjects when needed. The average levels of bAP were within the referring range in all heterozygous subjects.

I-2 and II-2 mutants, but not II-3, accepted to perform BMD evaluation. Subject I-2 was osteoporotic whereas subject II-2 showed a mild osteopenia, despite his young age and the fact that he has been performing daily regular physical activity. Osteoporosis has been reported in some FHH families (16), although it seems to be an unusual feature of the disorder. In fact, FHH has been associated with increased PTH and AP levels when compared with normal controls, but not with bone loss irrespective of the severity of the CASR mutations (17).

The overall clinical features reported in the post-menopausal mother (I-2) correlated with a compromised quality of life (osteoporosis, lumbar right convex scoliosis, stress fracture of S1 vertebral body, multiple discs dehydration, gall bladder stone, cholecystitis, left kidney angiomylipoma, hypertension, and hypertensive retinopathy). All these features cannot be directly correlated with FHH, even if disorders such as gallstones have been reported in isolated cases (11).

In fact, even if it has been reported that marked differences in plasma calcium and PTH levels may depend on the CASR gene mutation, an accurate collection of a familial and personal history of the patients together with an accurate physical examination would make possible the recognition of clinical phenotypes segregating with the CASR gene mutations. Indeed, in FHH patients, the need for a very detailed clinical data collection is urgently felt. This would make possible in the future to describe clinical entities, possibly associated with this disease, such as psychiatric symptoms (18).

Our findings support the role of CASR DNA test as a valuable supplement to the clinical diagnosis of FHH in hypercalcemic patients. Today, CASR genetic test may be used for diagnostic testing in the symptomatic individual (proband) and in asymptomomatic subjects (19). The problem is still how to suspect the FHI syndrome. Certainly, a positive family history of hypercalcemia is still the most relevant predictive factor for CASR gene mutations (12), whereas clinical and biochemical parameters do not reliably distinguish between PHPT and FHH (20). However, familial hypercalcemia is not often reported in the history of the patients, mostly if asymptomatic. One reason to suspect a hereditary hyperparathyroid syndrome is certainly the young age of the subject, as it happened in this case.

The possibility to avoid any unuseful parathyroid surgery is very important because cinacalcet can today show efficacy in patients with hypercalcemia due to inactivating mutations of the CASR (18, 21, 22).

In conclusion, FHH syndrome should be suspected when a young subject is diagnosed with PHPT without any family history of this syndrome. The possibility to use CASR genetic screening is certainly recommended when no PHPT syndromic disorders are suspected and

\[ \text{Ca}^{2+} \text{, of 3.6} \pm 0.3 \text{ mM (n=4). Figure 3B shows that HEK-293 transfected with empty vector had no calcium response. The } \Delta 121 \text{ mutant also had no calcium response with } 12 \text{ mM } \text{Ca}^{2+} \text{.} \]

HEK-293 cotransfected with equal amounts of WT and \( \Delta 121 \) mutant CASR cDNA showed a sensitivity to \( \text{Ca}^{2+} \), intermediate between that of cells transfected with mutant or the WT cDNA alone, with an EC\textsubscript{50} of 10.5 ± 0.6 mM for \( \text{Ca}^{2+} \) (n=4; \( P=0.02 \) vs HEK-293 cells transfected with the WT receptor; Mann–Whitney U test; Fig. 3B).
other asymptomatic or paucisymptomatic hypercalcemic subjects are recognized in the family. In the future, common shared clinical protocols could make possible the recognition of clinical signs or symptoms, beside hypercalcemia, recognizable as typical of FHH.

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.

Funding

This paper was supported by an unrestricted grant from F. R. R. M. O. Fondazione Raffaella Becagli (to M L Brandi). The research was supported by grants from the Italian Ministry of University and Research (attributed to L Soldati: FIRST and Cofin 02) and from San Raffaele Hospital.

Acknowledgements

We thank Dr Sergio Salardi, Prassis Research Institute, Milan, for his help with the western blot analysis; Dr Marie Louise Syren, Pediatric Institute, University of Milan, for DNA sequencing; and Drs J Hu and M Spiegel for providing us with the original human CASR plasmid. Identification of novel germline inactivating mutation of CASR and its functional analysis are reported. DNA test was helpful to avoid useless neck surgery.

References


Received 9 November 2011

Revised version received 3 February 2012

Accepted 7 February 2012.