Filamin A is reduced and contributes to the CASR sensitivity in human parathyroid tumors

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Abstract

Parathyroid tumors display reduced sensitivity to extracellular calcium ([Ca\textsuperscript{2+}]\textsubscript{o}). [Ca\textsuperscript{2+}]\textsubscript{o} activates calcium-sensing receptor (CASR), which interacts with the scaffold protein filamin A (FLNA). The study aimed to investigate: (1) the FLNA expression in human parathyroid tumors, (2) its effects on the CASR mRNA and protein expression, and (3) on ERK signaling activation, (4) the effect of the carboxy-terminal CASR variants and (5) of the treatment with the CASR agonist R568 on FLNA-mediated ERK phosphorylation in HEK293 cells. Full-length FLNA immunostaining was variably reduced in parathyroid tumors. Immunofluorescence showed that FLNA localized in membrane and cytoplasm and co-localized with CASR in parathyroid adenomas (PAds)-derived cells. Cleaved C-terminus FLNA fragment could also be detected in PAds nuclear protein fractions. In HEK293 cells transfected with 990R-CASR or 990G-CASR variants, silencing of endogenous FLNA reduced CASR mRNA levels and total and membrane-associated CASR proteins. In agreement, FLNA mRNA levels positively correlated with CASR expression in a series of 74 PAds; however, any significant correlation with primary hyperparathyroidism severity could be detected and FLNA transcript levels did not differ between PAds harboring 990R or 990G CASR variants. R568 treatment was efficient in restoring 990R-CASR and 990G-CASR sensitivity to [Ca\textsuperscript{2+}]\textsubscript{o} in the absence of FLNA. In conclusion, FLNA is downregulated in parathyroid tumors and parallels the CASR expression levels. Loss of FLNA reduces CASR mRNA and protein expression levels and the CASR-induced ERK phosphorylation. FLNA is involved in receptor expression, membrane localization and ERK signaling activation of both 990R and 990G CASR variants.

Key Words
- filamin A
- calcium-sensing receptor
- parathyroid tumors
- primary hyperparathyroidism
Introduction

Tumors of the parathyroid glands display reduced sensitivity to extracellular calcium ([Ca^{2+}]_o) resulting in failure in inhibiting parathormone (PTH) synthesis and release. Overall, derangements in calcium sensing result in increased parathyroid cell proliferation. The parathyroid cell sensitivity to [Ca^{2+}]_o is mediated by the calcium-sensing receptor (CASR). CASR is a G-protein-coupled membrane receptor interacting with different intracellular pathways (Breitwieser 2013, Conigrave & Ward 2013): active CASR dimers increase inositol-triphosphate and intracellular calcium concentrations, activate protein kinase C and intracellular calcium oscillations and stimulate mitogen-activated protein kinase (MAPK) cascade through p44/42 extracellular signaling-regulated kinase (ERK) phosphorylation (Breitwieser & Gama 2001, Corbetta et al. 2002, Chakravarti et al. 2012). CASR activates intracellular signaling through direct interaction with Gq/11 protein and the cytoskeletal scaffold protein filamin A (Awata et al. 2001, Hjalm et al. 2001).

Parathyroid tumors are characterized by variable degrees of insensitivity to [Ca^{2+}]_o, sustained by deregulation of key molecular components of CASR-coupled intracellular signaling: CASR mRNA and protein expression is downregulated (Corbetta et al. 2000, Varshney et al. 2013a); the major 990R allele of the CASR gene is associated with higher serum PTH levels (Corbetta et al. 2006) in hyperparathyroid patients and with in vitro lower responsiveness to [Ca^{2+}]_o than the minor CASR 990G allele (Terranegra et al. 2010); Gq/11 protein expression is reduced (Corbetta et al. 2000); CASR-stimulated p44/42 ERK activity is blunted (Corbetta et al. 2002).

Filamin A (FLNA) is a 280kDa protein that contains N-terminal actin-binding domain and consists of 24 repeated domains of approximately 96 amino acids each. FLNA is a scaffolding molecule facilitating protein interaction. The C-terminal domains 14 and 15 of FLNA interact with the carboxyl-terminal portion of CASR (Awata et al. 2001, Hjalm et al. 2001). This interaction stabilizes CASR in membrane and reduces its degradation, thereby facilitating the MAPK signaling (Zhang & Breitwieser 2005). In adult cells, FLNA regulates cell cycle: suppression of FLNA leads to prolongation of the cell cycle, mainly in the M phase and increases phosphorylation of cyclin-dependent kinase 1 (Lian et al. 2012). Moreover, FLNA plays a significant role in cancer development and progression: a number of human cancers overexpress FLNA. By contrast, in human prolactin-secreting pituitary adenomas resistant to dopamine treatment, a tumor model close to human parathyroid tumors, FLNA expression is diminished and involved in dopamine resistance (Peverelli et al. 2012).

Data about FLNA in parathyroid cells are from neonatal bovine parathyroid glands, whose dispersed cells express endogenously FLNA at confocal immunofluorescence microscopy: FLNA exhibits the highest density within the cytoplasm and colocalizes with endogenous CASR proteins (Hjalm et al. 2001). To our knowledge, data about FLNA expression in human parathyroid cells from normal or tumor glands are still lacking.

In the present study, we investigated: (1) the FLNA expression in parathyroid adenomas and carcinomas samples compared to normal glands from normocalcemic subjects; (2) the effects of FLNA loss on CASR expression, (3) on CASR-activated ERK signaling and (4) in presence of the CASR agonist R568 in HEK293 cells transiently transfected with CASR; (5) whether the 990G-CASR variant expression and function were differently affected by FLNA loss with respect to the 990R variant.

Methods

Sample collection

Formalin-fixed paraffin-embedded sections from 4 normal parathyroid glands incidentally removed during thyroid surgery of normocalcemic patients, 17 parathyroid tumors (10 typical parathyroid adenomas and 7 parathyroid carcinomas) from patients with primary hyperparathyroidism (PHPT) were collected and analyzed by immunohistochemistry. Fresh tissue samples from 3 parathyroid adenomas (PAd) were collected, fragmented and the dispersed cells were cultured for immunofluorescence experiments.

Immunohistochemistry (IHC)

Archival normal and tumor parathyroid samples (6 normal parathyroid glands incidentally removed from normocalcemic patients during surgery for thyroid diseases, 10 sporadic benign PAd and 7 parathyroid carcinomas (PCa) from patients with PHPT) were used for immunohistochemistry after tissues morphology was assessed by haematoxylin and eosin staining. Histological diagnosis of parathyroid carcinoma was established...
according to WHO published guidelines (Bondeson et al. 2004). Briefly, IHC was performed using an anti-human full-length FLNA antibody (1:200; Abnova Corporation, Taipei City, Taiwan) as previously described (Peverelli et al. 2012). Slides with absence of the primary antibody were included as negative controls. Percentage of positive cells was calculated considering at least 400 cells in the main representative high-power field, as previously described (Lania et al. 2004); blinded scoring of the cells was performed by an experienced pathologist (S.E.).

**Immunofluorescence (IF)**

Human PAd-derived cells (n=3) cultured for 48h and subconfluent HEK293 cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 and blocked in 1% BSA-PBS for 2h. Then, cells were incubated with primary antibodies against human full-length FLNA (mouse monoclonal H00002316-M01; Abnova Corp.) and human CASR (rabbit polyclonal PA1-934; Affinity Bioreagents, Golden, CO, USA), overnight at 4°C, washed thrice in PBS and followed by incubation with secondary antibodies conjugated with FITC or DyLight549 (1:100; Jackson Immuno Research). Nuclei were stained with Hoechst 33342 (1:500 dilution). For negative controls, PBS was used instead of primary antibodies to exclude unspecific binding of secondary antibody. Images were captured using a fluorescent confocal microscope (TCS SP2, Leica) and a digital camera.

**Nuclear cleaved FLNA fragment expression in parathyroid tissues**

Cells were homogenized using a Nuclear Extract kit (Active Motif, Carlsbad, CA, USA) following the manufacturer’s instructions to obtain both cytoplasmic and nuclear protein fractions. The kit provide protease and phosphatase inhibitors cocktail for the cell lysis and protein extraction procedures. Samples (40μg proteins) were denatured with loading dye and β-mercaptoethanol for 10min at 95°C. Proteins were separated on 10% w/v SDS-PAGE, and antigens were revealed by a primary antibody that recognized the C-terminal (90–100 kDa) calpain cleavage fragment of Filamin A (Millipore). Histone H3 was used as internal control (Abcam). Specific protein bands were detected using SuperSignal West Pico enhanced chemiluminescence system (Pierce).

**Quantitative real-time PCR**

Total RNA was extracted from frozen human PAd samples using TRIzol reagent (Thermo Fisher Scientific) following manufacturer’s instructions. One microgram of RNA was digested with DNase I (Thermo Fisher Scientific) to remove genomic DNA contamination and reverse-transcribed using the iScript cDNA Synthesis kit (BioRad). CASR and FLNA expression levels were estimated by quantitative real-time PCR using specific primers: CASR gene (Forward: 5′-ATGCCAAGAGGGGGAAGACTCTT-3′; Reverse: 5′-TCAGGACACTCCACACCTCAAGG-3′); FLNA gene (Forward: 5′-CAGTGCTATGCGCTGTAT-3′; Reverse: 5′-CCACCTTGTACATGCCATCG-3′) and housekeeping GAPDH gene (Forward: 5′-CTCATGACTCAGTCCATGCCC-3′ and Reverse: 5′-CAGTGCTATGCGCTGTAT-3′). Real-time PCR was performed with 7500 Fast Real-Time PCR Systems (Applied Biosystems, Life Technologies) with 100ng of cDNA as template and SYBR Premix Ex Taq II (Takara Bio Inc). Specific temperature of primers annealing were 57°C for CASR gene, 65°C for FLNA gene or 60°C for GAPDH. Mean cycle threshold values of triplicate samples were used for analysis. Data were analyzed by 7500 Fast System SDS Software. CASR and FLNA mRNA quantities were normalized using the housekeeping GAPDH expression levels, and data were expressed as CASR/GAPDH and FLNA/GAPDH mRNA ratios.

**Culture of parathyroid adenomas (PAd)-derived and human embryonic kidney (HEK293) cells**

Samples from human parathyroid adenomas (PAd) were cut into fragments less than 1 mm³, washed with PBS and partially digested with 2mg/mL collagenase type I (Worthington, Lakewood, NJ, USA). After digestion, tissue fragments were filtered with a cell strainer (100μm Nylon, BD Falcon, Milan, Italy). Cells derived from human PAd and human embryonic kidney (HEK293) cells were routinely grown in DMEM medium, supplemented with 10% FBS, 2mM l-glutamine and 1% penicillin/streptomycin (all from Gibco-Invitrogen, ThermoFischer Scientific) under standard culture conditions (5% CO₂, 37°C).

**CASP gene transfection and FLNA silencing**

HEK293 cells were plated in MW6 plates at a density of 10⁵ cells/well in complete medium. When cells reached
70–90% confluence, they were simultaneously silenced for FLNA, with predesigned small interfering RNA (siRNA) (Stealth RNAi HSS103734, Invitrogen), and transiently transfected with plasmid encoding for wild-type CASR (990R-CASR), obtained by site-directed mutagenesis as previously described (Terranegra et al. 2010), or 990G CASR, kindly provided by Dr Jianxin Hu (NIH). Transfection, silencing and cotransfection were performed with 10, 5 and 12 μL Lipofectamine 2000 (Invitrogen), respectively, 4 μg CASR plasmid and 500 pmol of FLNA siRNA in OptiMEM serum-free medium (Invitrogen) for each well. To obtain the best efficiency of FLNA silencing, three different human FLNA siRNAs were tested. Preliminary experiments to determine the optimal concentration of siRNA and the FLNA silencing kinetics were performed. As negative control in each experiment, medium GC duplex stealth RNAi negative control duplex human (control siRNA) was used as indicated by manufacture instructions (Invitrogen). The growth medium was changed to starvation medium (serum-free medium supplemented with 0.2% BSA and 1% penicillin/streptomycin) 24 h after cotransfection. Gene expression and activity were tested 72 h after transfection.

**Total and membrane CASR and FLNA protein quantifications**

HEK293 cells, plated in MW6 plates, were trypsinized, and the pellets were lysed with ice-cold lysis buffer (150 mM NaCl, 10 mM Tris–HCl pH 7.2, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA, 1 mM PMSF, 10 mM benzamidine, 2 μg/mL leupeptin, 0.1 mM Na-orthovanadate). The lysates were centrifuged and the supernatants were recollected to quantify proteins by BCA protein assay kit (Pierce). The membrane proteins extraction was performed with Mem-Per plus kit by manufacture instructions (Thermo Fisher Scientific, Life Technologies Italia). All samples (10 μg proteins/well) were denatured with loading dye buffer and β-mercaptoethanol for 10 min at 95°C to obtain proteins for FLNA and ERK analysis or with denaturing buffer: 7 M urea, 2 M thiourea, 65 mM DTT, 5× Laemmli sample buffer (final concentration 2% SDS) for 30 min at room temperature for the analysis of the CASR dimeric (250 kDa) and monomeric (130–150 kDa) receptor isoforms. Iodoacetamide (130 mM) was added to block DTT for 30 min at room temperature. Proteins were separated by 8% w/v SDS-PAGE, and then electrotransferred to PVDF membrane. The blots were blocked in a blocking solution (5% milk in TBST or 5% BSA in TBST) and then incubated overnight at 4°C with different antibodies: 1:5000 dilution monoclonal CASR antibody (Affinity Bioreagents, Golden, CO, USA), 1:10,000 dilution monoclonal anti-GAPDH antibody (Abnova Corporation), 1:1000 dilution polyclonal anti-β-actin antibody (Sigma Chemicals) and 1:1000 dilution anti-Na/K-ATPase antibody (Cell Signaling), for 2 h at room temperature. The FLNA expression levels were assessed in each experiment with 1:5000 dilution monoclonal full-length FLNA antibody (Abnova Corporation). Membranes were incubated in the blocking solution with secondary horseradish peroxidase-coupled anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Specific protein bands were detected using SuperSignal West Pico enhanced chemiluminescence system (Pierce). Experiments were repeated at least thrice. The band intensities, corresponding to the levels of protein expression, were measured by ImageJ software.

**Phosphorylated ERK1/2 quantification**

Serum-starved transfected HEK293 cells were stimulated with increasing concentrations of calcium chloride (CaCl₂; 0.5, 1.0, 3.0 and 5.0 mM) with or in the absence of the calcimimetic R568 (0.01 μM; kindly provided by Amgen Inc.) for 10 min at 37°C in saline solution PSS (NaCl 125 mM, KCl 4 mM, HEPES 20 mM, d-Glucose 0.1%, NaH₂PO₄ 0.8 mM, MgCl₂ 1 mM, pH 7.45). Incubation was stopped by placing the cells on ice. The PSS was removed and the cells were treated with 50 μL ice-cold lysis buffer as described previously, supplemented with complete phosphatase inhibitor cocktail (Roche Diagnostics Spa). Samples (20 μg proteins/well) were denatured with loading dye and β-mercaptoethanol for 10 min at 95°C. Proteins were separated on 10% w/v SDS-PAGE and analysis of ERK1/2 activation was performed by western blot with 1:1000 and 1:2000 dilution polyclonal anti-p44/42 ERK antibodies, respectively (Cell Signaling Technology). Specific protein bands were detected by a chemiluminescent method as described previously for CASR and FLNA proteins quantification. Experiments were repeated at least thrice.

**Genotyping**

Genomic DNA was extracted from 74 frozen human PAdS tissues samples using TRIzol reagent (Invitrogen) following manufacturer's instructions. CASR R990G SNP genotyping was performed by a specific TaqMan SNP genotyping.
genotyping assay (C_7504854_20, Life Technologies Ltd) with the Applied Biosystems 7500 Fast Real-Time PCR Systems. Amplification was performed in 8 μL final volume with 20 ng of genomic DNA at the following conditions: 95°C for 20 s, and 40 cycles each of 95°C for 3 s and 60°C for 30 s. SNP variation was assessed by means of the allelic discrimination assay employing the Applied Biosystems Software Package SDS 2.1.

PHPT patients

We collected the clinical and biochemical data of the 74 patients with primary hyperparathyroidism (PHPT) (57 females, 17 males, age 59.7 ± 14.2 years), whose surgically removed PAds were analyzed. PHPT was diagnosed when hypercalcemia (serum calcium >10.2 mg/dL and/or ionized calcium >1.30 mmol/L) and elevated or inappropriately normal serum PTH level occurred. Exclusion criteria were diagnosis of familial hypocalciuric hypercalcemia (detection of calcium-to-creatine clearance ratio >0.01 following vitamin D deficiency correction (Marcocci et al. 2015)), previously established diagnosis of chronic kidney disease, hyperthyroidism, pregnancy, glucocorticoids, bisphosphonates, diuretics and calcimimetic treatments. All patients were Caucasians. Patients underwent clinical and laboratory evaluation, including: (1) personal and family medical history; (2) physical examination including arterial blood pressure, weight and height measurement; (3) fasting biochemical evaluation including total and ionized calcium, phosphate, intact PTH, 25-hydroxyvitaminD3, creatinine; a 24-h urine collection was obtained from all patients for urinary calcium and phosphate excretion; and (4) imaging evaluation including lumbar and femoral dual-energy X-ray absorptiometry (DEXA), vertebral spine X-ray and ultrasound kidney examination. Osteoporosis was diagnosed in 50% and kidney stones in 30% of PHPT patients. Biochemical and hormonal parameters were assayed by routine methods. All participants gave their informed consent after full explanation of the purpose and nature of all used procedures; the protocol study was approved by the local ethics committee.

Statistical analysis

Results are expressed as mean ± standard error of the mean (S.E.M.) or median and range interquartile. Analysis of variance was performed by ANOVA or Wilcoxon rank-sum tests. Correlation between FLNA and CASR expression levels in PAds was analyzed by linear regression analysis. Differences between two groups were tested by Student’s t-test or Mann–Whitney test. A P value <0.05 was considered statistically significant.

Results

FLNA protein expression in human normal and tumor parathyroids

In normal parathyroid glands (n=6), most epithelial parathyroid cells showed intense staining for full-length FLNA in the cytoplasm and at membrane level (Fig. 1, panel A, a). Endothelial cells lining the vessels showed intense staining for FLNA (arrows in Fig. 1, panel A, a, b and c) and were considered as internal positive controls. A pattern of staining similar to that in normal parathyroid glands was detected in sporadic parathyroid adenomas samples (PAds, n=10), though the amount of FLNA-expressing cells was variably reduced among the samples (Fig. 1, panel A, b and c) ranging from 50% to 10% of tumor parathyroid epithelial cells. Remarkably, the FLNA-expressing cells were <10% or absent in parathyroid carcinomas (PCas, n=7) (Fig. 1, panel A, d). Therefore, a subset of PAds and the most PCas showed a variable loss of FLNA-expressing cells (Fig. 1, panel B). By immunofluorescence on PAds-derived cultured single cells, full-length FLNA was detected at membrane level and in the cytoplasm of a subset of tumor parathyroid cells, where it co-localized with CASR with a membrane and peripheral cytoplasmic distribution (Fig. 1, panel C). Indeed, immunoblotting-fractioned proteins from HEK293 cells and PAds, using a primary antibody against the C-terminal calpain cleaved fragment of FLNA, detected a specific band of 100 kDa in the nuclear protein fractions. An additional band of 270 kDa corresponding to the full-length FLNA was also visualized in all samples (Fig. 1, panel D); the nuclear accumulation of full-length FLNA, though detected by IHC in some tumor cells (data not shown), may be related to the sensitivity of the immunoblotting on fractioned proteins.

Effect of FLNA gene silencing on CASR expression levels

The effect of FLNA loss on CASR expression was investigated in 990R-CASR-transiently transfected HEK293 cells silenced for FLNA by siRNA technique. As shown in Fig. 2, panel A, in HEK293 cells, 72-h FLNA silencing consistently decreased endogenous FLNA protein expression (about 70–80%), compared to control siRNA transfected cells. The siRNA efficiency was not affected by cotransfection of the CASR plasmids (Fig. 2, panel A).
Loss of FLNA significantly reduced the total and membrane 990R-CASR protein levels (Fig. 2, panels B and C). Cotransfection of the 990R-CASR plasmid and control siRNA did not affect the 990R-CASR expression levels both in the total and in the membrane protein fractions, which were similar to those detected in HEK293 cells transfected with the 990R-CASR plasmid alone (Fig. 2, panels B and C).

**FLNA and CASR mRNA expression levels in PAds**

Loss of FLNA-induced CASR protein reduction might be due to reduced receptor stabilization with consequent increased degradation and/or by inhibition of CASR gene expression. The FLNA full-length and cleaved fragments can regulate gene expression directly or indirectly through interaction with a number of intracellular pathways (Savoy & Ghosh 2013). We observed that FLNA silencing also reduced
Filamin A in human parathyroid tumors

CASR mRNA levels in 990R-CASR-HEK293 cells (Fig. 3, panel A). HEK293 cells do not express endogenous CASR gene and protein and the 990R-CASR-HEK293 cell model was generated by transfecting a CASR plasmid lacking the endogenous regulatory region. Therefore, loss of FLNA-induced CASR inhibition could not be a direct nuclear effect.

We further investigated FLNA and CASR mRNA expression levels in tissue samples from 74 sporadic PAds surgically removed from patients with a clinical and hormonal diagnosis of PHPT. FLNA and CASR mRNA levels, reported as a ratio with GAPDH mRNA, were highly variable among PAds samples and CASR mRNA levels positively correlated with FLNA mRNA levels ($r^2 = 0.223$, $P < 0.0001$) (Fig. 3, panel B), suggesting a potential modulation of the CASR gene transcript by FLNA. Nonetheless, FLNA and CASR mRNA levels of PAds did not show any significant correlation with the PHPT severity (Table 1): FLNA and CASR mRNA levels of PAds associated with severe PHPT, defined as clinical diagnosis of kidney stones and/or osteoporosis and/or osteoporotic fractures and/or serum calcium >1 mg/dL above the upper limit of the normal range, were similar to those detected in PAds associated with mild PHPT (Marcocci et al. 2015).

However, CASR and FLNA protein expression levels, detected by western blot analysis, did not show significant

Figure 2 Effects of FLNA silencing in CASR-transfected HEK293 cells. Mean expression levels quantified by densitometry of the indicated proteins in every cell preparations (left diagrams); representative western blot of the experiments (on the right). (A) Endogenous FLNA silencing was efficient in HEK293 cells co-transfected with the empty vector, the 990R-CASR and the 990G-CASR; the representative western blot shows the full-length FLNA protein of 280 kDa; GAPDH was used as internal control. (B) Endogenous FLNA silencing induced a down-regulation of both 990R-CASR and 990G-CASR proteins in total protein extractions, while the FLNA control siRNA did not affect the CASR expression levels; the representative western blot shows the CASR monomeric isoforms of 140–160 kDa; α-actin was used as internal control. (C) Endogenous FLNA silencing induced a down-regulation of both 990R-CASR and 990G-CASR proteins in membrane protein fractions, while the FLNA control siRNA did not affect the CASR expression levels; the representative western blot shows the CASR monomeric isoforms of 140–160 kDa; Na/K-ATPase was used as internal control. *$P < 0.001$; **$P < 0.05$; Ctrl siRNA, control FLNA siRNA.
correlation (Fig. 3A and B); though there were tumors (PAD1 and PAD6) with low FLNA levels and low CASR levels or high FLNA levels and high CASR levels (PAD2), other tumors (PAD3 and PAD4) had low FLNA levels and consistent CASR expression levels or high FLNA levels and low CASR levels (PAD5).

Effect of the 990G CASR allele on FLNA and CASR mRNA expression

Genotyping for the CASR 990 single nucleotide variants of the PAd samples identified 68 tumors harboring the major allele 990R and 6 tumors harboring the minor allele 990G of the CASR gene. PAdS harboring the 990G

<table>
<thead>
<tr>
<th>Criteria for PHPT severity are according Marcocci et al. (2015).</th>
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<tr>
<td>aP &lt; 0.0046; bP = 0.0003.</td>
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Table 1 Comparisons of biochemical parameters and tumor gene expressions between severe and mild PHPT patients.

<table>
<thead>
<tr>
<th></th>
<th>Severe PHPT</th>
<th>Mild PHPT</th>
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<tbody>
<tr>
<td>No. patients</td>
<td>52</td>
<td>22</td>
</tr>
<tr>
<td>Serum ionized calcium (mmol/L)</td>
<td>1.57 (1.47–1.67)</td>
<td>1.46 (1.36–1.53)</td>
</tr>
<tr>
<td>Serum alb-corr calcium (mg/dL)</td>
<td>11.6 (11.0–12.3)</td>
<td>10.9 (10.7–11.2)</td>
</tr>
<tr>
<td>Serum phosphate (mg/dL)</td>
<td>201.5 (130.0–319.0)</td>
<td>125.0 (104.0–254.4)</td>
</tr>
<tr>
<td>Kidney stones (%)</td>
<td>2.25 (1.88–2.6)</td>
<td>2.40 (2.13–2.73)</td>
</tr>
<tr>
<td>Osteoporosis (%)</td>
<td>41.7</td>
<td>0</td>
</tr>
<tr>
<td>Mean Qty FLNA</td>
<td>0.07 (0.04–0.19)</td>
<td>0.08 (0.04–0.12)</td>
</tr>
<tr>
<td>Mean Qty CASR</td>
<td>0.74 (0.41–1.08)</td>
<td>0.84 (0.48–1.46)</td>
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CASR variant were associated with mean serum intact PTH levels lower than those in PAds harboring the 990R variant (171.0 ± 24.9 vs 328.1 ± 44.0pg/mL, P=0.003), whereas mean serum albumin-corrected calcium levels were similar (11.3 ± 0.4 vs 11.6 ± 0.1mg/dL; P=0.76). No significant difference could be detected in the PAds harboring the 990R compared with PAds harboring the 990G variants in the median expression levels of the FLNA (median, range interquartile: 0.08, 0.04–0.15 vs 0.09, 0.06–0.31; P=0.33; Fig. 4, panel C) and CASR genes (median, range interquartile: 0.68, 0.43–0.96 vs 0.84, 0.56–2.98; P=0.14; Fig. 4, panel D).

Effect of FLNA gene silencing on the CASR-stimulated p44/42 ERK phosphorylation

To evaluate the effect of the loss of FLNA expression on the CASR-activated signaling, we measured the levels of phosphorylated p44/42 ERK in 990R-CASR cells after FLNA silencing. After 72-h incubation with FLNA siRNA or control siRNA, 990R-CASR was activated by increasing extracellular calcium concentrations ([Ca\textsuperscript{2+}])\textsubscript{o}, from 0.5 to 1.0, 3.0 and 5.0mM, as shown in Fig. 5, panel A. Loss of FLNA expression in 990R-CASR HEK293 cells did not significantly alter ERK phosphorylation levels at 0.5 and 1.0 mM [Ca\textsuperscript{2+}]\textsubscript{o}, whereas at 3.0 and 5.0 mM [Ca\textsuperscript{2+}]\textsubscript{o}, it significantly decreased the 990R-CASR activation-induced phospho-ERK levels compared to those detected in 990R-CASR HEK293 cells transfected with the control siRNA (0.75 ± 0.06 vs 0.92 ± 0.08; P=0.006 at 3.0mM [Ca\textsuperscript{2+}]\textsubscript{o}, 0.71 ± 0.24 vs 1.21 ± 0.38; P=0.02 at 5.0mM [Ca\textsuperscript{2+}]\textsubscript{o}) (Fig. 5, panel A). The incubation with the potent calcimimetic RS68 0.01μM blunted the effect of FLNA loss on the 990R-CASR-induced phosphorylation of ERK at any [Ca\textsuperscript{2+}]\textsubscript{o} (Fig. 5, panel B).

Effects of the 990G CASR allele on FLNA-modulated CASR protein expression and CASR-mediated ERK phosphorylation

In HEK293 cells co-transfected with either 990R-CASR and 990G-CASR and siRNA control, we observed that the amount of 990G-CASR in the membrane protein fraction was significantly higher than that of 990R-CASR (5.3 ± 0.25 vs 4.4 ± 0.1, P=0.03) (Fig. 2, panel C). Loss of FLNA in 990G-CASR-HEK293 cells reduced the total and membrane 990G-CASR protein levels at an extent similar to that observed in the 990R-CASR transfected HEK293 cells (Fig. 2, panels B and C). In HEK293 cells transfected with the 990G-CASR and endogenous FLNA, 990G-CASR activation by increasing [Ca\textsuperscript{2+}]\textsubscript{o}-stimulated ERK phosphorylation at higher levels than that observed in 990R-CASR-expressing cells, which gained the statistical significance at 5.0mM [Ca\textsuperscript{2+}]\textsubscript{o} (2.80 ± 0.68 vs 1.21 ± 0.38; P=0.02), in agreement with previous report (Terranegra et al. 2010) (Fig. 5, panel A). Loss of FLNA by treatment with siRNA reduced 990G-CASR-induced phospho-ERK levels with a significant difference at 5.0mM [Ca\textsuperscript{2+}]\textsubscript{o} (1.33 ± 0.43 vs 2.80 ± 0.68; P=0.04) (Fig. 5, panel A). Finally, treatment of 990G-CASR-HEK293 cells with 0.01μM RS68 blunted the effect of FLNA loss as observed in 990R-CASR-expressing cells, though in the presence of FLNA, 990G-CASR-expressing cells showed higher [Ca\textsuperscript{2+}]\textsubscript{o}-induced phospho-ERK levels than 990R-CASR-expressing cells reaching statistical significance at 3.0mM and 5.0mM [Ca\textsuperscript{2+}]\textsubscript{o} (3.10 ± 0.34 vs 1.94 ± 0.38 and 3.13 ± 0.20 vs 2.48 ± 0.03, respectively; P=0.04) (Fig. 5, panel B), in agreement with previous report (Terranegra et al. 2010).
in association with membrane, whereas FLNA-expressing cells were variably reduced in parathyroid adenomas with tumor samples displaying an immunostaining pattern similar to that in normal glands and samples with a proportion of FLNA-expressing cells less than 10%. FLNA-expressing cells were definitely reduced in all the parathyroid carcinomas samples. Loss of FLNA expression has been reported in prolactin-secreting pituitary tumors (Peverelli et al. 2012), where it is related to dopamine resistance, at variance with the increased expression detected in a variety of human cancers. The role of FLNA in tumorigenesis is complex and related to its subcellular localization: as scaffold protein, FLNA interacts with receptors localized in membranes; as cytoplasmic protein, FLNA functions in various growth signaling pathways, such as vascular endothelial growth factor, R-Ras and integrin signaling; as nuclear active cleaved fragment, it interacts with transcription factors and nuclear receptors (Savoy & Ghosh 2013). In human parathyroid tumor cells, full-length FLNA was localized in membrane and in cytoplasm, similar to what was reported in bovine parathyroid cells (Hjälm et al. 2001); in addition, cleaved FLNA fragment could be detected in the nuclear protein fractions, suggesting FLNA potential involvement in multiple regulatory pathways in parathyroid tumor cells.

Parathyroid tumors are characterized by extracellular calcium insensitivity (Corbetta et al. 2000) and loss of CASR expression: compared with normal parathyroid glands, both CASR mRNA and protein are downregulated in parathyroid adenomas (Yano et al. 2003, Kawata et al. 2006), carcinomas (Haven et al. 2004, Wittenveen et al. 2011) and primary and secondary hyperplasia (Martin-Salvago et al. 2003, Latus et al. 2013, Varshney et al. 2013a). CASR gene mutations have been rarely identified in sporadic parathyroid tumors (Guarnieri et al. 2010, Frank-Raue et al. 2011) and CASR gene deregulation is not sustained by promoter hypermethylation (Varshney et al. 2013b). Therefore, alternative mechanisms might induce CASR mRNA and protein downregulation in parathyroid tumor cells. It has been reported in HEK293 cells stably transfected with CASR that FLNA interaction with CASR carboxyl-terminal tail (Awata et al. 2001) is required for CASR stabilization through attenuation of its degradation (Zhang & Breitwieser 2005). We tested the hypothesis that downregulation of CASR expression might be related to loss of FLNA in parathyroid tumors. We performed in vitro studies to investigate the effect of FLNA loss on CASR expression levels. Experiments were realized in HEK293 cells transiently transfected with 990R-CASR because

Discussion

In the present study, the scaffold protein filamin A (FLNA) was firstly shown to be downregulated in human parathyroid tumors. Most cells in normal parathyroid glands expressed full-length FLNA protein in cytoplasm and

Figure 5
Effect of FLNA silencing in CASR-activated ERK signaling in CASR-transfected HEK293 cells. (A) In HEK293 cells transfected with the 990G-CASR and endogenous FLNA (black dashed line), 990G-CASR activation by increasing [Ca2+]i stimulated ERK phosphorylation at higher levels than that observed in 990R-CASR-expressing cells (black continuous line), reaching the statistical significance at 5.0 mM [Ca2+]i. Loss of FLNA by treatment with siRNA reduced 990G-CASR-induced phospho-ERK levels (grey dashed line) with a significant difference at 5.0 mM [Ca2+]i. §§Ctrl siRNA + 990G-CASR vs Ctrl siRNA + 990R-CASR, P=0.02. (B) Treatment of 990G-CASR-expressing HEK293 cells with increasing [Ca2+]i concentrations in presence of 0.01 μM R568 overrode the FLNA loss as observed in 990R-CASR-expressing cells (grey dashed line), though, in presence of FLNA, 990G-CASR-expressing cells (black dashed line) showed higher [Ca2+]i-induced phospho-ERK levels than 990R-CASR-expressing cells (black continuous line) reaching statistically significance at 5.0 mM [Ca2+]i. *Ctrl siRNA + 990G-CASR vs Ctrl siRNA + 990R-CASR, P=0.02; *Ctrl siRNA + 990G-CASR vs Ctrl siRNA + 990G-CASR, P=0.04; *Ctrl siRNA + 990R-CASR vs FLNA siRNA + 990R-CASR, P=0.02. (B) Treatment of 990G-CASR-expressing HEK293 cells with increasing [Ca2+]i concentrations in presence of 0.01 μM R568 overrode the FLNA loss as observed in 990R-CASR-expressing cells (grey dashed line).
lack of suitable human parathyroid cells; a robust cell system has been developed, where unspecific effects of CASR plasmids and FLNA siRNA cotransfection were ruled out. Loss of FLNA significantly reduced the 990R-CASR expression levels both in the total protein pools and in the membrane protein fractions, suggesting an involvement of FLNA in stabilization of 990R-CASR proteins in HEK293 cells. Indeed, loss of FLNA also reduced CASR mRNA levels, suggesting that FLNA might be active on CASR gene regulation, likely at post-transcriptional level (Savoy et al. 2015). In parathyroid adenomas, FLNA gene expression levels positively correlated with CASR gene expression levels, suggesting a FLNA-mediated modulation of CASR gene transcription; this hypothesis is further supported by the detection in the parathyroid adenomas nuclear protein fractions of cleaved FLNA fragments accumulation, which has been demonstrated to directly regulate gene transcription (Savoy & Ghosh 2013). FLNA and/or CASR mRNA levels did not correlate with PHPT severity, suggesting that expression of these molecules is not a major determinant of the clinical phenotype associated with parathyroid tumors. Indeed, FLNA and CASR protein expression levels in parathyroid adenomas showed different patterns ranging from tumors with either low or high FLNA and CASR expression levels to tumors with discordant FLNA and CASR expression, in line with the wide range of reduced sensitivity to extracellular calcium characterizing the parathyroid tumors.

We further tested the hypothesis that FLNA is involved in the regulation of the CASR-mediated ERK activation and, firstly, evaluated the effect of the CASR agonist R568 when FLNA was reduced. FLNA interaction with CASR facilitates ERK phosphorylation (Zhang & Breitwieser 2005) and CASR activation increases ERK phosphorylation in human normal and tumor parathyroid cells (Corbetta et al. 2002). In line with the previous report (Zhang & Breitwieser 2005), in HEK293 cells transiently transfected with 990R-CASR, FLNA gene silencing reduced the [Ca\(^{2+}\)]\(_i\)-induced ERK phosphorylation. Treatment with R568 blunted the effect of FLNA loss, consistent with the stabilizing effect of R568 on CASR membrane expression demonstrated in previous study (Miedlich et al. 2004, Huang & Breitwieser 2007).

FLNA interacts with the CASR carboxyl-terminal tail at the domain between 972 and 997 amino acid residues (Awata et al. 2001, Hjalm et al. 2001). In this region, single nucleotide polymorphic variants have been identified and extensively investigated for the association with phenotype (Cetani et al. 2002, Mingione et al. 2015, Vezzoli et al. 2015) and with the sensitivity to CASR agonist treatment in PHPT cohorts (Vezzoli et al. 2015). Just inside the FLNA binding region, a non-conservative CASR polymorphism, Arg990Gly (R990G), induces a gain of function in the receptor activity (Vezzoli et al. 2002, Scillitani et al. 2004, Corbetta et al. 2006). We investigated whether 990G allele affects the interaction of CASR C-terminus with FLNA testing the effect of FLNA loss on CASR-induced ERK phosphorylation in HEK293 cells transfected with 990G-CASR. Similar to what was observed with 990R-CASR, FLNA loss reduced 990G-CASR mRNA in 990G-CASR transfected cells. Parathyroid tumors harboring the 990G-CASR variant do not show significant differences in FLNA and CASR mRNA expression levels compared with tumors harboring the more frequent 990R-CASR variant. Loss of FLNA also reduced 990G-CASR protein levels in both total and membrane protein fractions, though in the presence of endogenous FLNA, expression of 990G-CASR in cell membrane was slightly higher than that of the 990R-CASR. Moreover, analyzing the effect of FLNA loss on 990G-CASR- and 990R-CASR-induced ERK phosphorylation, three conditions characterized by increasing sensitivity of CASR-mediated ERK activation could be identified: the less sensitive condition was determined by loss of FLNA associated with the 990R allele, whereas the most sensitive condition was provided by co-expression of FLNA and 990G allele. The conditions characterized by loss of FLNA associated with the 990G allele, and conserved FLNA associated with the 990R allele, displayed a variable reduced sensitivity. Nonetheless, as observed with 990R-CASR, R568 treatment was efficient in rescuing 990G-CASR sensitivity to [Ca\(^{2+}\)]\(_i\), in the absence of FLNA.

In conclusion, FLNA is variably downregulated in parathyroid tumors both at mRNA and protein levels. Therefore, parathyroid tumor cells are characterized by receptor and post-receptor defects, namely loss of CASR (Martin-Salvago et al. 2003, Yano et al. 2003, Haven et al. 2004, Kawata et al. 2006, Wittenween et al. 2011, Latus et al. 2013, Varshney et al. 2013a), Gq/11 protein (Corbetta et al. 2000) and FLNA. Data about loss of FLNA in 990R-CASR-transfected HEK293 cells confirmed previously reported results: reduction of CASR protein and of CASR-induced ERK phosphorylation. We further extended the investigation to the FLNA effect on CASR mRNA expression, finding that it is affected and therefore suggesting an additional FLNA-related deregulation, also supported by cleaved FLNA nuclear localization. Though the 990G allele was associated with increased
sensitivity to $[Ca^{2+}]_o$, FLNA is required for receptor protein expression and ERK signaling activation. Treatment with R568 agonist is effective in the presence of different CASR and FLNA deregulated conditions.

Admittedly, our conclusions were limited by the fact that functional experiments were performed in HEK293 cells and could not be replicated in human normal or tumor parathyroid cells. It should also be considered that FLNA has been reported to interact with over 90 proteins, which indicates the numerous pathways that FLNA can affect (Stossel et al. 2001). Therefore, in parathyroid tumors with loss of FLNA, intracellular pathways other than ERK signaling might be impaired and contribute to the tumor phenotype.

**Declaration of interest**

The authors declare that they have no competing interests, with the exception of Laura Soldati who received funding from Amgen, USA.

**Funding**

The study was supported by Amgen, USA, IRCCS Policlinico San Donato and IRCCS Istituto Ortopedico Galeazzi Ricerca Corrente (L4080) funds.

**Authors’ contribution statement**

M A carried out the experiments in HEK293 cells and CASR genotyping; V C performed immunofluorescence on PAdS-derived cells; F S and V V carried out immunohistochemistry for FLNA on human parathyroid sections; G V performed RNA extraction from human parathyroid tumors and real-time PCR gene quantification; S A collected clinical data from PHPT patients; V L, B G and B E collected fresh parathyroid tumor biopsies during surgery for PCR gene quantification; S A collected clinical data from PHPT patients; V performed RNA extraction from human parathyroid tumors and real-time PCR gene quantification; S L and S C conceived the project, collected and reviewed experimental data, discussed the results and wrote the manuscript. All authors read and approved the final manuscript.

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Haven CJ, Van Puijenbroek M, Karperien M, Fleuren GJ & Morreau H 2004 Differential expression of the calcium sensing receptor and CASR genotyping; S A collected clinical data from PHPT patients; V performed RNA extraction from human parathyroid tumors and real-time PCR gene quantification; S L and S C conceived the project, collected and reviewed experimental data, discussed the results and wrote the manuscript. All authors read and approved the final manuscript.

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Received in final form 13 November 2016
Accepted 21 November 2016
Accepted Preprint published online 21 November 2016