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Received for publication: 1.12.2013; Accepted in revised form: 30.1.2014

Nephrol Dial Transplant (2014) 29: 1902–1909
doi: 10.1093/ndt/gfu065
Advance Access publication 7 August 2014

ORIGINAL ARTICLE

Calcium-sensing-related gene mutations in hypercalcaemic hypocalciuric patients as differential diagnosis from primary hyperparathyroidism: detection of two novel inactivating mutations in an Italian population

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ABSTRACT

Background. Inactivating mutations of the calcium-sensing receptor (*CaSR*), of the G-protein subunit α11 (*GNA11*) and of the adaptor-related protein complex 2, sigma 1 subunit (*AP2S1*) genes are responsible for familial hypocalciuric hypercalcaemia (FHH). The aim of this study was to analyse prevalence and pathogenicity of *CaSR*, *GNA11* and *AP2S1* mutations in patients with an FHH phenotype and to compare them with a sample of patients with primary hyperparathyroidism (PHPT) in order to identify the most useful laboratory parameter for a differential diagnosis.
Methods. Patients with an FHH phenotype were studied with polymerase chain reaction amplification and direct sequencing of the entire *CaSR*, *GNA11* and *AP2S1* coding sequences. Novel mutations were introduced in a Myc-tagged human wild-type (WT) CaSR cDNA-expressing vector, and functional assay was performed on human embryonic kidney cells evaluating expression and function of mutated proteins.
Results. Among 16 FHH patients, none had an inactivating *GNA11* or *AP2S1* mutation while 3 (18.8%) carried a CaSR mutation and 10 (62.5%) at least one CaSR polymorphism. Within the latter group, 7 of 10 patients had more than one polymorphism (4.1 ± 2.1 per patient). Two novel CaSR mutations [c.2120A>T (E707V) and c.2320G>A (G774S)] were identified: the E707V mutation prevented *CaSR* expression (western blot), whereas the G774S mutation determined a reduced receptor sensitivity to calcium (IP_3 assay). PHPT patients showed significantly ($P < 0.001$) higher serum calcium, parathyroid hormone, urinary calcium and calcium–creatinine clearance ratio (CCCR) and significantly lower serum phosphate than FHH ones.
Conclusions. FHH should be clearly differentiated by PHPT to avoid unnecessary surgery: CCCR could be a useful screening tool while genetic analysis should include the two novel

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CaSR mutations herein described. The role of multiple polymorphisms deserves further investigation in patients with an FHH phenotype.

Keywords: AP2S1, CaSR, familial hypocalciuric hypercalcemia, GNA11, hypercalcaemia

INTRODUCTION

The differential diagnosis of hypercalcaemia represents a relatively common task in clinical practice and includes mainly primary hyperparathyroidism (PHPT) and familial hypocalciuric hypercalcaemia (FHH). The discovery of inactivating mutations of the calcium-sensing receptor gene (*CaSR*, FHH1, chr 3q21.1) [1, 2], of the G-protein subunit α11 (*GNA11*, FHH2, chr 19p13.3) [3] and of the adaptor-related protein complex 2, sigma 1 subunit (*AP2S1*, FHH3, chr 19q13.32) [4] has greatly changed the diagnostic work-up of hypercalcaemia: the identification of mutations in these genes is needed to avoid diagnostic mistakes with potentially serious consequences, such as unnecessary parathyroidectomy (PTX).

The CaSR is a G-protein-coupled plasma membrane receptor, which is widely expressed in the parathyroid gland and kidney tubule: it plays a key role in maintaining stable extracellular calcium concentrations through its ability to sense small increases in calcium concentrations and consequently inhibit parathyroid hormone (PTH) secretion and increase urine calcium excretion [5]. Inactivating mutations impairing CaSR expression or function determine a generalized resistance to high serum calcium concentrations and have been associated with hypercalcaemia [1]. Homozygous inactivating mutations result in neonatal severe hyperparathyroidism (NSHPT, MIM239200), which is characterized by extreme hypercalcaemia and skeletal changes of unremitting hyperparathyroidism [2]. Heterozygous loss-of-function mutations determine FHH1 (MIM145980), which is characterized usually by mild and asymptomatic hypercalcaemia (ionized calcium within 10% of the upper normal limit), a relatively low calcium excretion and a calcium-clearance to creatinine-clearance ratio (CCCR) typically <0.01 [6]. In FHH, the resetting of calcium homeostasis to a mildly elevated level is not maintained by an increased PTH and the kidney participates in the genesis of hypercalcaemia by avidly reabsorbing calcium [6].

The cloning of *CaSR* has provided a wealth of information about FHH and NSHPT pathophysiology, and overall >100 inactivating *CaSR* mutations were described in FHH and/or NSHPT [7], including missense and nonsense mutations, insertions and deletion/insertions [8]. Several *CaSR* gene polymorphisms have also been identified, which proved to predict calcium concentrations even in the general population, in the absence of any other symptom or laboratory finding [1].

Elevated extracellular calcium concentrations activate CaSR and in turn the G-protein subunit α11 (GNA11), resulting in accumulation of secondary messengers such as IP_3. This eventually leads to a reduction of circulating PTH levels and to an increase of urinary calcium excretion. As inactivating mutations of *GNA11* have been proven to alter the switch from guanosine diphosphate GDP to guanosine triphosphate (GTP), they decrease CaSR-induced signal transduction leading to type 2 FHH. On the other hand, activating mutations of *GNA11* have been identified in a specific form of autosomal dominant hypocalcaemia (ADH2), producing the opposite effect on the protein function with an overall hyperactivation of the GDP–GTP switch and an increased CaSR sensibility to extracellular calcium [3].

The *AP2S1* gene codes for a key protein of clathrin-coated vesicles, which plays a pivotal role in CaSR internalization. Inactivating mutations of *AP2S1* cause a decrease in the overall CaSR expression on cell membrane, thus reducing cellular sensitivity to extracellular calcium concentrations [4].

While inactivating *CaSR* mutations account for ∼47–56% of FHH cases [9, 10], the prevalence of *GNA11* and *AP2S1* mutations is based on scant data: two *GNA11* mutations out of 11 subjects, one with a familial form (10%) [3] and 11 *AP2S1* mutations out of 50 subjects, of which two as a familial form (22%) have been so far reported [4].

As a molecular diagnosis might be possible in up to 70% of FHH patients, analysis of *CaSR*, *GNA11* and *AP2S1* genes is becoming the cornerstone for the management of hypercalcaemia, particularly for differential diagnosis between FHH and PHPT. The aim of the study was to analyse prevalence, type and potential pathogenicity of *CaSR*, *GNA11* and *AP2S1* mutations in a series of 16 patients with a clinical phenotype compatible with FHH, by analysing the whole coding sequences of these three genes. Moreover, we compared clinical and biochemical features of FHH patients with those of a cohort of PHPT patients in order to identify blood or urinary parameters that could be useful to clinically differentiate these two disorders before molecular diagnosis is performed.

MATERIALS AND METHODS

Inclusion criteria

We included all patients with clinical and biochemical features compatible with FHH followed up at our Nephrology Outpatient Clinic >2 years (January 2010–December 2012). Patients were included as probands for FHH if they fulfilled all of the following criteria:

(i) Moderately elevated serum calcium: total calcium >10.0 mg/dL to <12 mg/dL or ionized calcium >5.4 mg/dL
(ii) Low calcium-clearance to creatinine-clearance ratio (<0.02) by at least two independent urine samples and in the absence of any medication can modify renal calcium clearance (lithium, thiazide diuretics).
(iii) Normal or mildly elevated serum PTH (<219 pg/mL; normal range 17.3–73 pg/mL).

Patients with PHPT followed at our Nephrology Outpatient Clinic over the same period (January 2010–December 2012) were included if they had a histological diagnosis after PTX,

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normocalcaemia following successful neck exploration, or, in cases of clinical contraindications to surgery, the presence of all the following criteria: serum PTH at least four times higher than normal (>292 pg/mL) at repeated assessments and calcaemia persistently >11.5 mg/dL with normal vitamin D levels.

All patients gave their written informed consent to participate in the study and to undergo study procedures, including genetic analyses. The local ethical committee approved the study.

Molecular screening (Phase 1)

Peripheral blood was collected and deoxyribonucleic acid (DNA) extracted following a classic phenol–chloroform protocol. Molecular screening of the entire *CaSR*, *GNA11* and *AP2S1* coding sequences (18 exons–21 amplicons, including exon–intron boundaries) was performed by polymerase chain reaction (PCR) amplification and direct sequencing. Mutations and polymorphisms were confirmed by sequencing in both directions on the original amplicon and on a different PCR product.

Functional testing of novel mutations

cDNA expression vectors and mutagenesis. *CaSR* unknown variants were introduced in a Myc-tagged human WT *CaSR* cDNA-expressing pCDNA3.1 vector (Myc-tagged WT and R66H CaSR vectors were kindly provided by Dr G.N. Hendy, Calcium Research Laboratory, Royal Victoria Hospital and McGill University Health Centre, Montreal, QC, Canada). Briefly, mutagenesis reaction was carried out as previously described [10] with the following primers (p.E707 V, For: 5′-tgtcctcctggtgtttgTggccaagatccccacca-3′ and Rev: 5′-tggtggggatcttggccAcaaacaccaggaggaca-3′; p.G774S, For: 5′-gctccctcatggccctgAgcttcctgatcggctac-3′ and Rev: 5′-gtagccgatcaggaagcTcagggccatgagggagc-3′, mutated bases are in capital). One microlitre of Dpn1 (New England Biolabs) was added to digest parental DNA, and 3 μL used to transform E. cloni 5-alpha chemically competent cells (Lucigen). Colony PCR and sequencing identified the mutated clone. Midipreps were performed with Plasmid Midi Kit (QIAGEN).

Cell culture and transfection. Human embryonic kidney (HEK293) cells (ECACC) were cultured and transfected with WT or mutant vector using Lipofectamine 2000 Transfection Reagent (Invitrogen) as previously described [10, 11].

Western blot. Forty-eight hours after transfection, total cell proteins were extracted in RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, 1%, Nonidet P-40, 0.1% sodium dodecyl sulphate (SDS), 0.5% sodium deoxycholate, pH 8.0) supplemented with one tablet/10 mL of PhosStop and complete ethylenediaminetetraacetic acid (both from Roche). Fifty micrograms were loaded onto a 6% SDS polyacrylamide gel, and proteins were electrotransferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA), blotted overnight at 4°C with rabbit anti-Myc monoclonal antibody (Cell Signaling Technology, 1 : 800 in blocking buffer) and for 1 h at room temperature with the horseradish peroxidase-conjugated goat antirabbit IgG antibody (Cell Signaling Technology, 1 : 300 in blocking buffer) as secondary antibody. Membrane was stripped with Re-Blot Solution (Millipore) and reblotted with β-tubulin rabbit monoclonal antibody (Cell Signaling Technology, 1 : 1000 in blocking buffer) as loading control.

CaSR intact cell surface enzyme-linked immunosorbent assay. Forty thousand HEK293 cells were seeded in 96 wells and transfected in quadruplicate with WT and mutant vectors. After 48 h from the transfection, cells were fixed for 20 min with 4% paraformaldehyde at room temperature (RT) and washed with 1× phosphate-buffered saline (PBS). After incubation with c-Myc rabbit monoclonal antibody [Cell Signaling Technology, 1 : 800 in 1× PBS, 0.1% bovine serum albumin (BSA), 3 h at RT], cells were washed with 1× PBS and peroxidase-conjugated goat antimouse antibody was added (Cell Signaling Technology, 1 : 3000 in 1% PBS, 0.1% BSA, 1 h at RT). Cells were washed with 1× PBS, and 100 μL of 3,3′,5,5′-tetramethylbenzidine liquid substrate (Sigma-Aldrich) was added for 25 min at room temperature. The reaction was stopped with 100 μL 1 N H_2SO_4 and the optical density (OD) read at 450 nm with an ELISA reader instrument. Controls included HEK293 cells transiently transfected with empty vector, non-transfected cells or omission of the anti-CaSR (c-Myc) antibody.

IP3 assay. A modified protocol for measurement of IP1 [11] was set-up (IpOne Cell ELISA Assay Kit, CISBIO Bioassay, Paris). Sixty thousand HEK293 cells were seeded in 96 wells and transfected in eight replicates for each vector. After 48 h from the transfection, stimulation buffer (HEPES 10 mM, $MgCl_2$ 0.5 mM, KCl 4.2 mM, NaCl 146 mM, Glucose 5.5 mM and LiCl 50 mM, pH 7,4) differently supplemented with two calcium concentrations (4 and 10 mM) was added for 1 h at 37°C, in quadruplicate for each vector. Then, the cells were lysed and the assay performed following the manufacturer's instructions. Controls included cells expressing the previously characterized inactivating (R66H) *CaSR* mutation and cells not transfected. The ODs at 450/620 nm were calculated as previously described [11].

Clinical and biochemical comparison of FHH and PHTP (Phase 2)

The following serum and urine parameters were evaluated to compare the two subgroups of patients with FHH and PHPT: serum calcium, phosphate, PTH, urine calcium excretion, calcium clearance, calcium to creatinine clearance and calcium-clearance to creatinine-clearance ratio (CCCR).

Statistical analysis

Data are expressed as mean ± standard deviation (SD) from triplicates experimental replicates and a P value of <0.01 was considered to be statistically significant. Comparisons between groups have been carried out with Wilcoxon test or χ^2 test as appropriate. All analyses were performed using SAS Release 9.1 (SAS Institute, Cary, NC).

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RESULTS

Molecular screening

Overall, 16 patients with an FHH phenotype (64.1 ± 17.6 years, male: 10/16 = 62.5%) met the inclusion criteria and underwent molecular screening of the entire *CaSR*, *GNA11* and *AP2S1* coding sequences: 13 of them (13/16 = 81.3%) turned out to carry either CaSR mutations ($n = 3$) or polymorphisms ($n = 10$; Table 1). None of the patients carried an inactivating GNA11 or AP2S1 mutation, but molecular screening led to the identification of several previously identified polymorphisms (Table 2). Seven patients (43.8%) had more than one *CaSR* polymorphism, with a mean of 4.1 ± 2.1 (min–max range: 2–7) polymorphisms per patient; the most common ones were the exon 3 IVS3+260C>T and the exon 6 IVS5−89C>T polymorphism each found in four patients.

Most patients had moderate chronic kidney disease (CKD), with an estimated glomerular filtration rate (eGFR) of 54.9 ± 29.9 mL/min/1.73m^2, hypercalcaemia (10.6 ± 0.35 mg/dL), hypocalciuria and a CCCR of 0.0062 ± 0.0048. Nephrolithiasis was relatively common (3/16 = 18.8%), particularly in patients with *CaSR* mutations (2/3 = 66.7%). Moreover, patients with an inactivating *CaSR* mutation were younger, had a better renal function and had a lower urinary phosphate-to-creatinine clearance ratio (although non-statistically significant, Table 1). The clinical features of patients with inactivating CaSR mutations are separately outlined in Table 3.

Genetic analysis. The screening led to the identification of several polymorphisms (Table 2) and two coding variants (Table 2), c.2120A>T (E707V) and c.2320G>A (G774S), neither of which has so far been reported in the Mutation Discovery Database (http://www.mutationdiscovery.com/md/MD.com/home_page.jsp, last access: September 2013), or in the 1000 genomes database (http://www.ncbi.nlm.nih.gov/variation/tools/1000genomes, last access: September 2013), thus ruling out the possibility that these variants are polymorphisms.

Moreover, Patient #6 (novel mutation G774S, male, age at the diagnosis 70 years) had no children and his parents were deceased at the time of FHH diagnosis; he had two brothers and four sisters, one of whom had a mild hypercalcaemia with normal calciuria, but refused genetic testing. Patient #11 (novel mutation E707V, female, age at diagnosis 46 years) had two daughters with normal serum and urinary calcium; her parents were deceased at the time of diagnosis and her two brothers had normal serum calcium.

Western blot. With respect to the fully glycosylated 160 kDa and immature glycosylated 140 kDa species, the G774S mutant protein exhibited almost the same pattern as the WT *CaSR,* with a lower expression of the 140 kDa form. On the contrary in the E707V variant both forms were underexpressed and the 160 kDa expression was almost undetectable, as the R66H inactivating mutant control (Figure 1).

Cell ELISA assay. Consistently, the expression of the G774S mutant protein appeared similar to that of the WT, while the E707V was almost totally unexpressed (Figure 2).

IP_3 assay. Taking into account that the EC_{50} of the WT CaSR protein is in the range of 3.1–4.7 mM [11], we deduced that the G774S variant impaired the signalling response of the receptor at basal level of calcium, while at higher level the activation is similar to that of the WT. Moreover, the E707V variants were characterized by a lower signalling activity for both calcium concentrations, as also compared with the R66H inactivating control (Figure 3).

Table 1. Main laboratory and clinical feature of the patients screened for FHH

Parameter	Overall ($n = 16$)	Gene mutations ($n = 3$)	Multiple polymorphisms[a] ($n = 7$)	One polymorphism ($n = 3$)	No gene alteration ($n = 3$)	Normal value
Age at referral (years)	64.1 ± 17.6	43.0 ± 29.0	63.56 ± 11.6	76.3 ± 6.4	74.3 ± 4.2	
Gender male (%)	10 (62.5)	2 (66.6)	3 (42.8)	3 (100)	3 (100)	
eGFR (mL/min/1.73 m^2)[b]	54.87 ± 29.86	106 ± 28.28	55.14 ± 26.3	33.33 ± 13.01	41.67 ± 5.13	90–125
Serum ionized calcium (mg/dL)	5.31 ± 0.35	5.67 ± 0.04	5.29 ± 0.45	–	5.23 ± 0.15	4.6–5.4
Serum phosphate (mg/dL)	2.79 ± 0.39	2.67 ± 0.47	2.73 ± 0.38	3.03 ± 0.49	2.83 ± 0.32	2.7–4.5
Serum PTH[c] (pg/mL)	116.86 ± 59.39	106.47 ± 32.33	93.4 ± 53.55	173.67 ± 44.61	125.17 ± 88.21	17.3–73
Serum vitamin D (ng/mL)	21.83 ± 19.68	17.6 ± 4.53	12.62 ± 8.29	59.9 ± 17.11	–	30–100
24-h Urinary calcium (mg)	68.56 ± 46.2	61.1 ± 41.7	83.17 ± 37.46	74.53 ± 84.53	35.97 ± 20.96	100–300
Calcium clearance (mL/min)	0.37 ± 0.28	0.42 ± 0.29	0.49 ± 0.33	0.15 ± 0.12	0.25 ± 0.14	1–3
CCCR[d]	0.0062 ± 0.0048	0.0058 ± 0.0015	0.0081 ± 0.0062	0.0035 ± 0.0028	0.0046 ± 0.0029	0.02–0.03
24-h Urinary phosphate (mg)	695.71 ± 199.83	625.77 ± 149.67	755.63 ± 211.21	785.5 ± 206.85	536.03 ± 173.84	400–1300
Phosphate-to-creatinine clearance ratio (%)	30.46 ± 12.06	19.5 ± 9.19	32.39 ± 12.59	39.0 ± 11.79	22.74 ± 8.39	20–25
Nephrolithiasis (%)	3 (18.8)	2 (66.6)	1 (33.3)	0 (0)	0 (0)	
Need for urological surgery (%)	1 (6.3)	0 (0)	1 (33.3)	0 (0)	0 (0)	
Family history of nephrolithiasis (%)	4 (25)	1 (33.3)	0 (0)	3 (42.8)	0 (0)	
Family history of renal failure (%)	4 (25)	0 (0)	1 (33.3)	2 (28.5)	1 (33.3)	

They have been divided by the presence of inactivating mutations or known polymorphisms of the CaSR gene. Values are expressed as mean ± SD.

[a]More than one CaSR polymorphism detected.

[b]eGFR, by CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) formula.

[c]Intact parathormone.

[d]Calcium-clearance to creatinine-clearance ratio.

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Table 2. CaSR, GNA11 and AP2S1 polymorphisms detected in patients without inactivating mutations

Patient	CaSR polymorphism	GNA11 polymorphism	AP2S1 polymorphism
#1	Ex3 IVS3+238del2 Ex6 IVS5−89C>T Ex7 A986S	–	–
#2	Ex3 IVS3+260C>T Ex3 IVS3+298T>C Ex3 IVS3+301T>C Ex3 IVS3+313A>G	Ex4 IVS4+54C>T	–
#3	Ex3 IVS3+260C>T Ex3 IVS3+298T>C Ex3 IVS3+301T>C Ex3 IVS3+313A>G	–	Ex3 IVS2−32T>G
#4	Ex6 IVS6+16T>C	Ex2 IVS2+18T>G	–
#5	Ex7 E870E	Ex2 IVS2+18T>G Ex4 IVS4+54C>T	–
#7	–	Ex2 IVS2+18T>G Ex2 IVS2+30C>T	–
#8	Ex3 IVS3+238del2 Ex7 A986S	Ex3 IVS3−23G>C Ex4 IVS4+54C>T	Ex3 IVS2−32T>G Ex3 IVS3+5G>A
#9	–	Ex2 IVS2+18T>G	Ex3 IVS2−94C>A Ex3 IVS2−32T>G
#10	Ex3 IVS3+260C>T Ex3 IVS3+298T>C Ex3 IVS3+301T>C Ex3 IVS3+313A>G Ex3 IVS3+238del2 Ex6 IVS5−89C>T Ex6 IVS6+16T>C	Ex4 IVS4+54C>T	Ex1 IVS1+34insG Ex3 IVS2−32T>G Ex3 IVS3+5G>A
#12	Ex3 IVS3+260C>T Ex3 IVS3+298T>C Ex3 IVS3+301T>C Ex3 IVS3+313A>G Ex3 IVS3+238del2 Ex6 IVS6+16T>C Ex7 A986S	–	–
#13	Ex6 IVS5−89C>T Ex6 IVS6+16T>C	–	–
#14	–	–	–
#15	Ex6 IVS6+16T>C	–	–

Ex, exon.

Clinical and biochemical comparison of PHTP and patients with an FHH phenotype

The comparison between PHPT and patients screened for FHH shows similar demographic characteristics while PHPT had a slightly better renal function (Table 4), higher mean calcium (11.9 ± 0.98 versus 10.6 ± 0.35), lower mean phosphate (1.89 ± 0.59 versus 2.79 ± 0.39) and higher mean PTH (433 ± 518 versus 116.9 ± 59.4). However, these variables significantly overlapped between the two groups. Urinary calcium and CCCR were higher in PHPT, with only two PHPT patients (12.5%) showing a CCCR of <0.001. As for clinical manifestations, nephrolithiasis and related surgery were less common in FHH than in PHPT patients (Table 4).

DISCUSSION

The aim of this study was to analyse the prevalence of *CaSR*, *GNA11* and *AP2S1* genetic variations in a cohort of Italian patients with FHH without hyperparathyroidism and to compare biochemical parameters of FHH and PHPT patients in order to identify the most useful discriminating clinical features. The main results are the relatively high frequency of *CaSR* alterations (mutations and multiple polymorphisms) among FHH probands and the identification of two novel inactivating *CaSR* mutations (E707V and G774S).

As to the first result, we found an unexpectedly high prevalence of patients carrying multiple *CaSR* polymorphisms: seven patients carry at least two polymorphisms, which may not be 'pathogenic' by themselves but might partially reduce CaSR activity when present in combination [7, 12, 13]; however, the impact of multiple polymorphisms on FHH phenotype deserves more functional studies. Moreover, biochemical data of these probands' family members are currently available in only four families and show an autosomal dominant transmission of hypercalcaemia in two of them. Therefore, wider studies are necessary to extend genetic analyses to all family members in order to study the transmission of multiple FHH-related *CaSR* polymorphisms. In three patients, we found a specific polymorphism that has already been associated with hypercalcaemia (exon 7, A986S) [12]; in two of them, it was associated with other polymorphisms, and in the third one with a *CaSR* mutation. Interestingly, the biochemical phenotype of patients without any polymorphism is not substantially different from those with a single polymorphism, suggesting that at least in part of these cases CaSR inactivation may be caused by an autoimmune mechanism, as widely reported in the past decade [14–16].

Clinically, 4 out of 16 FHH-phenotype patients had previously suffered from one or more episodes of nephrolithiasis (two mutated patients, one patient with a single polymorphism and one patient without any genetic alterations), thus reflecting the well-known but complex relationship between *CaSR* mutations and nephrolithiasis. While on one hand activating mutations and polymorphisms such as R990G predispose to renal stones through hypercalciuria [17, 18], on the other hand inactivating mutations could facilitate stone formation through hyperphosphaturia, impaired distal acidification and impaired dilution of urine. The latter two effects are an expected consequence of the inhibition of the CaSR physiological effects on proton secretion and vasopressin response at the collecting duct level [19]. However, further studies are needed to dissect the mechanisms of nephrolithiasis in patients with either activating or inactivating *CaSR* mutations.

Moreover, apart from stone-related mild and sporadic events, clinical symptoms and events were few or absent even in patients carrying an inactivating mutation, therefore, confirming the benign clinical course of FHH and hypocalciuric hypercalcaemia, especially if compared with PHPT.

As to the two novel *CaSR* mutations herein described, the mutation at residue 707 is located at the intracellular loop 2 (residues 701–724): only one naturally occurring inactivating mutation has been identified so far in this domain (W718X) [20]. The other novel mutation at the residue 774 is located into the transmembrane domain 5 (TM5, residues 770–792): four mutations of this domain have so far been associated with either ADH or FHH [21–24]. In this study, we report

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Table 3. Detailed description of the three patients carrying a CaSR inactivating mutation

Parameter	Overall Mean ± SD	Patient #6	Patient #11	Patient #16	Normal range
Age at referral (years)	43.0 ± 29.0	70	46	13	
Gender male (%)	2 (66.6)	M	F	M	
eGFR (mL/min/1.73m^2)[a]	106 ± 28.3	86	126	ND	*90–125*
Serum ionized calcium (mg/dL)	5.67 ± 0.04	5.37	5.64	5.7	*4.6–5.4*
Serum phosphate (mg/dL)	2.67 ± 0.47	2.5	2.3	3.2	*2.7–4.5*
Serum PTH (pg/mL)[b]	106.5 ± 32.3	138	73.4	108	*14.4–63*
Serum vitamin D (ng/mL)	17.6 ± 4.53	22	20.8	14.4	*30–100*
24-h Urinary calcium (mg)	61.1 ± 41.7	80	90	13.3	*100–300*
Calcium clearance (mL/min)	0.42 ± 0.29	0.58	0.59	0.09	*1–3*
CCCR[c]	0.0058 ± 0.0015	0.0069	0.0047	ND	*0.01–0.03*
24-h Urinary phosphate (mg)	625.77 ± 149.67	798	552	527.3	*400–1300*
Urinary phosphate-to creatinine clearance ratio (%)	19.5 ± 9.19	26	13	ND	*20–25*
CaSR inactivating mutation		*Ex7* c.2320G>A (G774S)	*Ex7* c2120A>T (E707V)	*Ex4a* c.653A>G (Y218C)	
CaSR polymorphisms		No	*Ex3 IVS3+238del2* *Ex6 IVS5−89C>T* *Ex7 A986S*	No	
GNA11 polymorphisms		No	*Ex4* IVS4+54C>T	No	
AP2S1 polymorphisms		No	No	No	

Values are expressed as mean ± SD; Ex: exon.
[a]eGFR, as by CKD-EPI formula.
[b]Intact parathormone.
[c]Calcium-clearance to creatinine-clearance ratio.

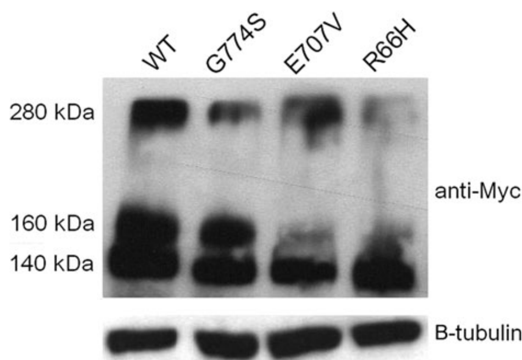

FIGURE 1: Western blot of Myc-tagged CaSR on crude protein extract. The arrows indicate the different forms of the protein in terms of maturation: the fully mature glycosylated form is at 160 kDa, the immature non-glycosylated form is at 140 kDa. Lower panel: WT on the reference protein, β-tubulin. The assay showed that the E707V mutant protein is lower expressed as is the inactivating control (R66H) while the G774S protein appeared expressed similarly to the WT.

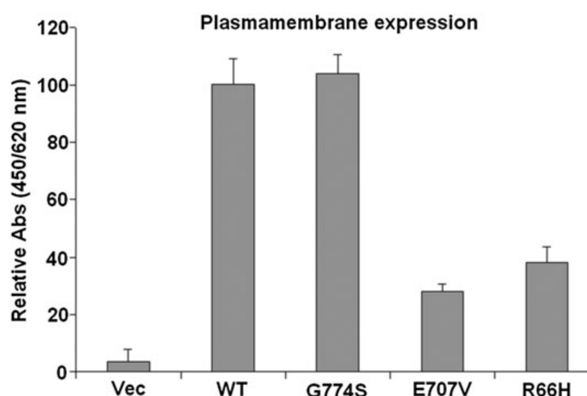

FIGURE 2: CaSR cell surface expression ELISA assay shows that the E707V mutant protein is not expressed at the plasma membrane while the G774S is subject to a normal trafficking process as the WT. Values shown are the means (±SEM) of three replicate experiments. *P < 0.05 compared with the WT.

functional assays showing that the two novel *CaSR* variants (E707V and G774S) impair the activity of the receptor, thus acting as inactivating *CaSR* mutations, in keeping with the clinical suspicion. Unfortunately, both affected individuals have relatively small pedigrees, in particular the parents of both probands were already deceased when FHH was diagnosed: as FHH does not have major clinical symptoms, its diagnosis relies on laboratory findings and thus cannot be excluded in an asymptomatic relative until serum and urinary calcium are measured, which is not possible in these two probands' parents. Therefore, we cannot determine if in these cases these mutations have been transmitted by one of their parents or if they arose '*de novo*'.

With regard to the GNA11 and AP2S1 genes, we found only eight patients with one or more *GNA11* or *AP2S1* polymorphisms and none with a known inactivating mutation (Table 2). Even if *GNA11-AP2S1* combined polymorphisms could possibly explain the hypocalciuric hypercalcaemia in patients without *CaSR* polymorphisms (such as Patient #7 and #9), functional studies are needed to confirm this hypothesis.

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Moreover, as 'non-pathogenetic' *CaSR* variants and single-nucleotide polymorphisms are very common in the general population and *CaSR* mutations have a higher prevalence in FHH with respect to those of *GNA11* and *AP2S1* taken together (67 versus 30%), the clinical suspicion of FHH should be confirmed as a first step by the sequencing of at least the *CaSR* gene.

Lastly, the comparison between FHH and PHTP patients showed several significantly different laboratory parameters, with a small area of overlap: this is true for serum calcium, phosphate, PTH and both urinary calcium and phosphate, but the best discriminating parameter was the CCCR.

The main limits of this study are its relatively small sample size and the lack of genetic analysis of family members, as we just started it.

In conclusion, in the presence of moderate hypercalcaemia, the discrimination between PHPT and FHH, including genetic analyses, is crucial to avoid useless PTX. Inactivating mutations of *CaSR*, *GNA11* or *AP2S1* should be considered even in sporadic cases, as '*de novo*' mutation may be relatively common and family history may be silent. In a small cohort of patients with an FHH phenotype, we identified three *CaSR* mutations (two of them never reported before) and a high prevalence of multiple polymorphisms: our ongoing project is to extend the study to family members including non-affected ones, in order to analyse association between combined *CaSR* polymorphisms and FHH. Due to the low frequency of mutations, the impact of *GNA11* and *AP2S1* genetic analysis in the FHH patient management needs to be verified in larger studies and might be useful only in patients with a clearly defined FHH who do not carry any *CaSR* mutation. As to probands with multiple polymorphisms, current evidence suggests that many of these variants are not pathogenic, but we cannot exclude that some variant combinations exert an impact on the laboratory phenotype. On the other hand an autoimmune pathogenesis of *CaSR* inactivation could be responsible for FHH in cases without any polymorphism or with a single polymorphism.

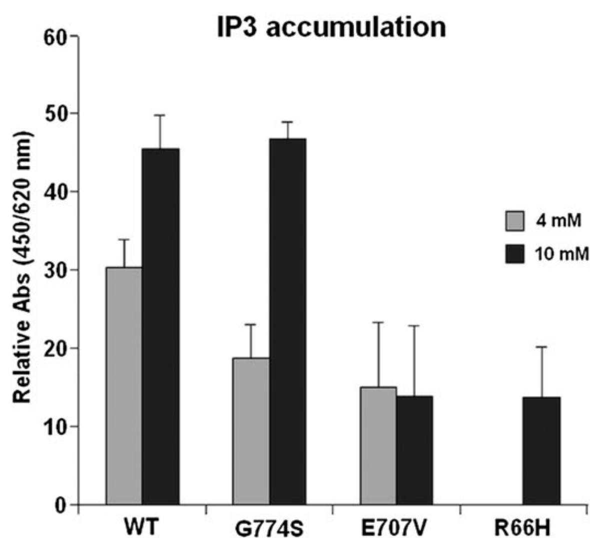

FIGURE 3: IP3 assays with two different calcium concentrations (4 and 10 mM) used to stimulate the receptor. For both mutations the level of activity at 4 mM [Ca^{2+}] is below the WT, thus proving that the two variants impaired receptor signalling. The values have been normalized with respect to the lowest value (4 mM [Ca^{2+}] for the fully inactive R66H mutated form, see Materials and Methods).

ACKNOWLEDGEMENTS

This research was supported by the 'Ricerca Corrente 2010/2012' funding granted by the Italian Ministry of Health and by

Table 4. Comparison of the main clinical and laboratory parameters of patients with PHPT and patients with an FHH phenotype (other)

Features	Normal value	PHPT (*n* = 13)	Other (*n* = 16)	P
Age at referral (years)		62 ± 15	64.13 ± 17.64	0.669
Gender male (%)		6 (46.1)	10 (62.5)	0.409
eGFR (mL/min/1.73m^2)[a]	90–125	77.08 ± 28.5	54.87 ± 29.86	0.056
Serum ionized calcium (mg/dL)	4.6–5.4	6.18 ± 0.57	5.31 ± 0.35	<0.001
Serum phosphate (mg/dL)	2.7–4.5	1.89 ± 0.59	2.79 ± 0.39	<0.001
Serum PTH (pg/mL)[b]	14.4–63	433.77 ± 518.6	116.86 ± 59.39	<0.001
Serum vitamin D (ng/mL)	30–100	10.76 ± 7.74	21.83 ± 19.68	0.046
24-h Urinary calcium (mg)	100–300	261.11 ± 118.75	68.56 ± 46.2	<0.001
Calcium clearance (mL/min)	1–3	1.55 ± 0.46	0.37 ± 0.28	<0.001
CCCR[c]	0.01–0.03	0.0175 ± 0.0085	0.0062 ± 0.0048	0.001
24-h Urinary phosphate (mg)	400–1300	826.49 ± 526.89	695.71 ± 199.83	0.447
Urinary phosphate-to-creatinine clearance ratio (%)	20–25	36.96 ± 16.11	30.46 ± 12.06	0.25
Episodes of nephrolithiasis (%)		11 (84.6)	3 (18.8)	<0.001
Need of urological surgery (%)		8 (61.5)	1 (6.3)	<0.001
Familial nephrolithiasis (%)		5 (38.5)	4 (25)	0.004
Familial renal failure (%)		0 (0)	4 (25)	<0.001
Surgical PTX (%)		7 (53.8)	–	

Values are expressed as mean ± SD.
[a]eGFR, as by CKD-EPI formula.
[b]Intact parathormone.
[c]Calcium-clearance to creatinine-clearance ratio.

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the '5 × 1000' voluntary contributions devolved to the Medical Genetics Laboratory, IRCCS Casa Sollievo della Sofferenza Hospital, San Giovanni Rotondo (FG), Italy. The sponsor (Italian Ministry of Health) did not directly participate to the study design or planning, nor in the collection or interpretation of data. The grant was awarded to carry out a research programme on the genetics of CaSR and CaSR-related genes.

CONFLICT OF INTEREST STATEMENT

None declared.

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Received for publication: 17.12.2013; Accepted in revised form: 26.2.2014

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