A G-protein Subunit-α11 Loss-of-Function Mutation, Thr54Met, Causes Familial Hypocalciuric Hypercalcemia Type 2 (FHH2)

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ABSTRACT
Familial hypocalciuric hypercalcemia (FHH) is a genetically heterogeneous disorder with three variants, FHH1 to FHH3. FHH1 is caused by loss-of-function mutations of the calcium-sensing receptor (CaSR), a G-protein coupled receptor that predominantly signals via G-protein subunit alpha-11 (Gαq/11) to regulate calcium homeostasis. FHH2 is the result of loss-of-function mutations in Gαq/11, encoded by GNA11, and to date only two FHH2-associated Gαq/11 missense mutations (Leu135Gln and Ile200del) have been reported. FHH3 is the result of loss-of-function mutations of the adaptor protein-2 σ-subunit (AP2σ), which plays a pivotal role in clathrin-mediated endocytosis. We describe a 65-year-old woman who had hypercalcemia with normal circulating parathyroid hormone concentrations and hypocalciuria, features consistent with FHH, but she did not have CaSR and AP2σ mutations. Mutational analysis of the GNA11 gene was therefore undertaken, using leucocyte DNA, and this identified a novel heterozygous GNA11 mutation (c.161C>T; p.Thr54Met). The effect of the Gαq/11 variant was assessed by homology modeling of the related Gαq protein and by measuring the CaSR-mediated intracellular calcium (Ca2+) responses of HEK293 cells, stably expressing CaSR, to alterations in extracellular calcium (Ca2+) using flow cytometry. Three-dimensional modeling predicted the Thr54Met mutation to be located at the interface between the Gαq/11 helical and GTPase domains, and to likely impair GDP binding and interdomain interactions. Expression of wild-type and the mutant Gαq/11 in HEK293 cells stably expressing CaSR revealed that the Ca2+ responses after stimulation with Ca2+ of the mutant Met54Gαq/11 led to a rightward shift of the concentration-response curve with a significantly (p < 0.01) increased mean half-maximal concentration (EC50) value of 3.88 mM (95% confidence interval [CI] 3.76–4.01 mM), when compared with the wild-type EC50 of 2.94 mM (95% CI 2.81–3.07 mM) consistent with a loss-of-function. Thus, our studies have identified a third Gαq/11 mutation (Thr54Met) causing FHH2 and reveal a critical role for the Gαq/11 interdomain interface in CaSR signaling and Ca2+ homeostasis. © 2016 The Authors. Journal of Bone and Mineral Research published by Wiley Periodicals, Inc. on behalf of American Society for Bone and Mineral Research (ASBMR)

KEY WORDS: DISORDERS OF CALCIUM/PHOSPHATE METABOLISM; PTH/VIT D/FGF23; PARATHYROID-RELATED DISORDERS; CELL/TISSUE SIGNALING – ENDOCRINE PATHWAYS

Introduction
Familial hypocalciuric hypercalcemia (FHH) is characterized by lifelong elevations of serum calcium concentrations in association with normal or mildly raised serum parathyroid hormone (PTH) concentrations in 80% of patients and low urinary calcium excretion (urinary calcium-to-creatinine clearance ratio <0.01) in 80% of patients.1,2 FHH may be inherited as an autosomal dominant condition, and it is a genetically heterogeneous disorder with three recognized variants, FHH1-3. FHH1 (OMIM #145980) is caused by loss-of-function mutations of the calcium-sensing receptor (CaSR), a G-protein coupled receptor (GPCR)3 that initiates activation of the G-protein subunit αq/11 (Gαq/11) family, leading to enhancement of phospholipase C (PLC) activity4 and elevation of inositol 1,4,5-trisphosphate (IP3) with rapid increase in intracellular calcium

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Received in original form December 13, 2015; revised form January 2, 2015; accepted January 4, 2015. Accepted manuscript online January 5, 2016.

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(Ca^{2+})_\text{extracellular}}. These signal transduction events allow the parathyroid CaSR to respond to small fluctuations in the prevailing extracellular calcium concentration ([Ca^{2+}]_\text{extracellular}) by inducing alterations in PTH secretion through mechanisms that likely involve effects on PTH mRNA stability and PTH granule exocytosis from the apical pole of parathyroid cells. Moreover, the kidney CaSR is considered to influence urinary calcium excretion by modulating expression of claudin proteins that mediate the paracellular reabsorption of calcium in the renal thick ascending limb. FHH2 (OMIM #145981) is the result of loss-of-function mutations in the G-protein subunit-α11 (Gα_{11}), encoded by GNA11, and to date only two FHH2-associated Gα_{11} missense mutations have been reported (Fig. 1). These two FHH2-causing Gα_{11} mutations comprise a Leu135Gln missense substitution and an in-frame isoleucine deletion at codon 200 (Ile200del), which are located in the Gα-subunit helical and GTPase domains, respectively. Both of these FHH2-causing Gα_{11} mutations, which are predicted to disrupt G-protein activation, have been shown to impair CaSR signal transduction. FHH3 (OMIM #600740) is caused by loss-of-function mutations of the adaptor protein-2 gene. AP2S1, encoded by GNA11 gene. AP2S1 mutations had been previously excluded.

**Materials and Methods**

**Case report**

The patient, a 65-year-old woman of Indian origin, presented with poor mobility and recurrent falls. She underwent end-onuclease analyses, as described. DNA sequence analyses of the GNA11 gene consists of 7 exons with the start (ATG) and stop (TGA) codons located in exons 1 and 7, respectively. The GTPase domain (encoded by exon 1, 5’ portion of exon 2, 3’ portion of exon 4 and exons 5 to 7) is connected to the helical domain (encoded by the 3’ portion of exon 2, exon 3, and 5’ portion of exon 4) by the linker 1 (L1) and linker 2 (L2) peptides. The locations of the P-loop (P) (red line), three flexible switch regions (S1 to S3) (red line), and the interdomain interface (comprising portions of the α1, αF, α5, β1, P-loop, and L1 peptide motifs) (blue line) are shown below the GNA11 exons. The previously reported loss-of-function Leu135Gln and Ile200del mutations (blue) are located in the helical and GTPase domains, respectively, whereas the Thr54Met mutation (bold), identified by this study, is located at the interdomain interface. Coding regions are shaded gray and untranslated regions are represented by open boxes.
reported three-dimensional structure of Gα11 in complex with the small molecule inhibitor YM-254890 (Protein Data Bank accession no. 3AH8). The Gα11 protein, which shares 90% identity at the amino acid level with Gα11, was used because crystal structures of Gα11 are not available. Molecular modeling was performed using the PyMOL Molecular Graphics System (Version 1.2r3pre, Schrödinger, LL Pymol).

Cell culture and transfection

Wild-type and mutant GNA11 (pBl-CMV2-GNA11) expression constructs were generated as described, and transiently transfected into HEK293 cells stably expressing CaSR (HEK293-CaSR) using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). The bidirectional pBl-CMV2 cloning vector was used because it facilitated the co-expression of Gα11 and GFP, and site-directed mutagenesis was used to generate the mutant GNA11 construct using the Quikchange Lightning Site-directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) and gene-specific primers (Sigma-Aldrich), as described. Cells were maintained in DMEM-Glutamax media (Thermo-Fisher, Waltham, MA, USA) with 10% fetal bovine serum ( Gibco, Thermo-Fisher) and 400 μg/mL genetin (Thermo-Fisher) at 37°C, 5% CO2. Successful transfection was confirmed by visualizing GFP fluorescence using an Eclipse E400 fluorescence microscope with a Y-FL Epi-fluorescence attachment and a trambid 4,6-diamidino-2-phenylindole-FITC-Rhodamine filter, and images captured using a DXM1200C digital camera and NIS Elements software (Nikon, Tokyo, Japan). The expression of Gα11 and CaSR proteins was also determined by Western blot analyses using anti-Gα11 (Santa Cruz Biotechnology, Dallas, TX, USA), anti-GFP (Santa Cruz), anti-calnexin (Millipore, Billerica, MA, USA) or anti-CaSR (AbCam, Cambridge, UK) antibodies. The Western blots were visualized using an Immuno-Star Western C kit (Bio-Rad, Hercules, CA, USA) on a Bio-Rad Chemidoc XRS-1 system.

Intracellular calcium measurements

The Ca2+ responses of HEK293-CaSR cells expressing wild-type or mutant Gα11 proteins were assessed by a flow cytometry-based assay, as reported. In brief, HEK293-CaSR cells were cultured in T75 flasks and transiently transfected 24 hours later with 16 μg DNA. Forty-eight hours after transfection, the cells were detached, resuspended in calcium (Ca2+)– and magnesium (Mg2+)-free HBSS, and loaded with 1 μg/mL Indo-1-acetoxymethylester (Indo-1-AM) for 1 hour at 37°C. After removal of free dye, cells were resuspended in Ca2+- and Mg2+-free HBSS and maintained at 37°C. Transfected cells, in suspension, were stimulated by sequentially adding Ca2+ to the Ca2+- and Mg2+-free HBSS to increase the [Ca2+]i in a stepwise manner from 0 to 150 mM and then analyzed on a MoFlo modular flow cytometer (Beckman Coulter, Indianapolis, IN, USA) by simultaneous measurements of GFP expression (at 525 nm), Ca2+-bound Indo-1-AM (at 410 nm), and free Indo-1-AM (ie, not bound to Ca2+) (at 485 nm), using a JDSU Xcyte UV laser (Coherent Radiation, Santa Clara, CA, USA), on each cell at each [Ca2+]i as described. The peak mean fluorescence ratio of the Ca2+ transient response after each individual stimulus was measured using Cytomation Summit software (Beckman Coulter) and expressed as a normalized response, as described. Nonlinear regression of concentration-response curves was performed with GraphPad Prism (GraphPad, La Jolla, CA, USA) using the normalized response at each [Ca2+]i for each separate experiment for the determination of EC50 (ie, [Ca2+]i required for 50% of the maximal response) and Hill coefficient values. The maximal signaling response was measured as a fold-change of the peak transient Ca2+· response to the basal Ca2+· response measured at 0 mM [Ca2+]i. The maximal signaling responses for mutant Gα11 proteins were expressed as a percentage of the wild-type Gα11 protein maximal signaling response. The mean EC50 and Hill coefficients obtained from four separate transfection experiments were used for statistical comparison by using the F-test, and alterations in maximal signaling responses assessed using the Mann-Whitney U test.

Results

Identification of a novel Thr54Met Gα11 mutation in a FHH proband

DNA sequence analyses of the GNA11 coding regions and adjacent splice sites (Fig. 1) identified a heterozygous C-to-T transition at nucleotide c.161, in the FHH patient (Figure 2A). This C-to-T transition (ACG to ATG) resulted in a missense substitution, Thr54Met, of the Gα11 protein (Fig. 2B). The sequence alteration also led to the gain of an NspI and loss of a BsiHIK restriction endonuclease site (Fig. 2B), which were used to confirm the presence of the mutation in the patient (Fig. 2C, D). Bioinformatic analyses using SIFT and MutationTasting software predicted the variant to be damaging and likely disease-causing (SIFT score 0, MutationTasting score 0.99). In addition, the absence of this DNA sequence abnormality in >6500 exomes from the NHLBI-ESP cohort and ~60,700 exomes from the ExAC cohort, together with evolutionary conservation of the Thr54 residue in vertebrate Gα-subunit paralogs (Fig. 3A), indicated that the Thr54Met abnormality likely represented a pathogenic GNA11 mutation rather than a benign polymorphic variant.

Structural characterization of the Thr54Met Gα11 mutant protein

The Thr54Met mutation is located within the Gα11 α-1 helix (Fig. 1 and Fig. 3A, B), which comprises part of the interface at which the GTPase and helical domains interact to bind GDP and GTP. In contrast, the previously reported FHH2-causing Ile200Leu and Leu135Gln mutations, which affect the GTPase and helical domains of Gα11, respectively, are situated away from the guanine-nucleotide binding site (Fig. 1 and Fig. 3B). The Thr54 Gα11 residue is located next to the phosphate-binding loop (P-loop) (Fig. 3A), which is a highly conserved nucleotide-binding peptide motif that plays a critical role in binding GDP. Three-dimensional homology modeling of the Gα11 protein revealed the wild-type Thr54 residue to form polar contacts with the ribose and β-phosphate moieties of GDP within the interdomain interface (Fig. 3B, C) and to interact with the α-F helix of the helical domain, which also mediates GDP binding (Fig. 3C). These findings are consistent with the reported role of the α helix as a structural hub that mediates interactions between the GTPase and helical domains to ensure GDP binding, thereby maintaining the Gα-subunit in an inactive conformation. The Gα11 Met54 mutant is predicted to disrupt these interdomain contacts and alter GDP binding (Fig. 3C).

Functional characterization of the Thr54Met Gα11 mutant protein

To determine the effects of the predicted changes in Gα11 structure (Fig. 3B, C) on CaSR-mediated signaling, Ca2+·
responses to alterations in $[\text{Ca}^{2+}]_o$ were assessed in HEK293-CaSR cells that were transiently transfected with either the pBI-CMV2 empty vector or pBI-CMV2 expressing the wild-type (Thr54) or mutant (Met54) Ga11 proteins. The $\text{Ca}^{2+}$i responses of cells expressing the Met54 Ga11 mutant were also compared with cells transiently transfected with the reported FHH2-associated Gln135 Ga11 mutant protein. (11) Expression of CaSR, Ga11 and GFP was confirmed by fluorescence microscopy and/or Western blot analyses (Fig. 4A, B). Calnexin was used as a loading control in Western blot analyses, and Ga11 expression was demonstrated to be similar in cells transiently transfected with wild-type or mutant Ga11 proteins and greater than that of cells transfected with the empty pBI-CMV2 vector (Fig. 4B). The $\text{Ca}^{2+}$i responses in wild-type and mutant Ga11-expressing cells were shown to increase in a dose-dependent manner after stimulation with increasing concentrations of $\text{Ca}^{2+}$o between 0–15 mM. However, exposure to a significantly greater $[\text{Ca}^{2+}]_o$ was required to achieve half-maximal (EC50) $\text{Ca}^{2+}$i responses for cells expressing either the Met54 or Gln135 mutant Ga11 proteins compared with wild-type-expressing cells (Fig. 4C, D). Thus, the Met54 or Gln135 mutant-expressing cells showed rightward shifts in the concentration-response curves, with significantly elevated mean EC50 values ($p<0.01$) of 3.88 mM (95% confidence interval [CI] 3.76–4.01 mM) and 3.65 mM (95% CI 3.57–3.74 mM), respectively, compared with 2.94 mM (95% CI 2.81–3.07 mM) for wild-type expressing cells and consistent with the Ga11 mutants leading to an impairment of CaSR signal transduction (Fig. 4C, D). The Hill coefficients did not significantly differ between wild-type and mutant Ga11-expressing cells (Fig. 4E). However, cells expressing the Met54 mutant had significantly reduced maximal signaling responses compared with cells expressing either wild-type or Gln135 mutant Ga11 proteins ($p<0.05$) (Fig. 4F).

**Discussion**

Our studies have identified a novel heterozygous germline GNA11 mutation in a patient with FHH, which resulted in an impairment of $\text{Ca}^{2+}$i signaling similar to the loss-of-function previously reported for the FHH2-associated Leu135Gln and Ile200del GNA11 mutations. (11) The Thr54Met mutation represents only the third loss-of-function GNA11 mutation to be reported, and thus these findings provide further support for a critical role of the Ga11 protein in parathyroid gland function and $\text{Ca}^{2+}$i homeostasis, and highlight the importance of GNA11 gene analyses in FHH patients that do not harbor CASR or AP2S1 mutations. The Thr54Met, Leu135Gln, and Ile200del loss-of-function GNA11 mutations are all associated with a mild FHH phenotype characterized by serum adjusted-calcium concentrations <2.80 mmol/L, and these clinical findings are in keeping with our in vitro studies that have shown FHH2-associated

![Diagram](image-url)
mutations to induce only minor disturbances of CaSR signal transduction.\(^\text{(11)}\) Indeed, the FHH2 mutants were associated with around a 30% increase in the EC\(_{50}\) values of HEK293-CaSR cells used in this study, whereas CaSR mutations leading to FHH1 generally cause a >50% increase in the EC\(_{50}\) value.\(^\text{(12,27)}\) The milder shift in the Ca\(^{2+}\) set point of cells expressing FHH2-associated G\(_{\alpha q}\) mutants indicates that the CaSR may promote Ca\(^{2+}\)\(^{\text{max}}\) signaling by G\(_{\alpha 11}\)-independent mechanisms, such as via the related G\(_{\alpha q}\) protein. Indeed, reported studies that selectively ablated G\(_{\alpha q}\) and/or G\(_{\alpha 11}\) in the parathyroid glands of mice have highlighted the importance of both of these G-proteins for CaSR function,\(^\text{(28,29)}\) and in the setting of FHH1, CaSR mutations likely impair Ca\(^{2+}\)\(^{\text{max}}\) responses via both G\(_{\alpha 11}\) and G\(_{\alpha q}\); thus leading to a greater loss-of-function than G\(_{\alpha 11}\) mutations that cause FHH2.

The G\(_{\alpha 11}\)-subunit consists of a Ras-like GTPase domain that binds GDP and GTP, and a smaller helical domain that acts as a clasp to secure these bound guanine-nucleotides.\(^\text{(30)}\) Three-dimensional modeling indicated the Thr54Met mutation to be located at the interdomain interface, which represents a highly conserved and critical region containing the P-loop motif that binds GDP\(^\text{(23,24)}\) and also facilitates interactions between the helical and GTPase domains that maintain G\(_{\alpha}\)-subunits in an inactive GDP-bound conformation.\(^\text{(22)}\) The Thr54Met mutation likely alters GDP binding, but in contrast to the other reported G\(_{\alpha 11}\) mutations (Arg60Cys, Arg60Leu, and Arg181Gln),\(^\text{(11,31,32)}\) which are also located at the interdomain interface (Fig. 3B, C) and cause G\(_{\alpha 11}\) gain-of-function that is associated with the clinical disorder of autosomal dominant hypocalcemia type-2 (ADH2), the Thr54Met G\(_{\alpha 11}\) mutation causes loss-of-function and FHH2. Thus, it seems that mutations involving the G\(_{\alpha 11}\) interdomain interface may result in G\(_{\alpha 11}\) loss-of-function or gain-of-function. Crystal structures of G\(_{\alpha 11}\) proteins are not available to evaluate the structure-function effects of the Thr54Met mutation at the interdomain interface; however, the introduction of the mutant Met54 residue may sterically impair G-protein function, as highlighted by a previous crystallography study of a loss-of-function G-protein alpha-i (G\(_{\alpha i}\)) P-loop mutation, which revealed the mutant G\(_{\alpha i}\) residue to sterically hinder conformational changes of the flexible “switch” regions during G\(_{\alpha}\)-subunit activation.\(^\text{(33)}\) Moreover, interdomain interface mutations that disrupt guanine-nucleotide binding may also result in an “empty-pocket” mutant G\(_{\alpha}\)-subunit that exerts dominant-negative effects by binding and sequestering partner GPCRs.\(^\text{(34)}\) These findings may also help to provide an explanation for the observed differences in the maximal signaling responses of the FHH2-causing Met54 and Gln135 G\(_{\alpha 11}\) mutants (Fig. 4F). Thus, the Met54 G\(_{\alpha 11}\) mutant, but not the Gln135 G\(_{\alpha 11}\) mutant, led to a significant reduction in the maximal signaling response of CaSR-expressing cells (Fig. 4F), even though both G\(_{\alpha 11}\) mutants increase the Ca\(^{2+}\)\(^{\text{set point}}\) of CaSR-expressing cells to a similar degree, as illustrated by their EC\(_{50}\) values (Fig. 4D). The maximal signaling response of a GPCR is influenced by the ability of the receptor to couple with its cognate G-protein,\(^\text{(35)}\) and thus it is possible that the Met54 G\(_{\alpha 11}\) mutant impairs coupling and/or dissociation of G\(_{\alpha 11}\) from the CaSR by influencing guanine-nucleotide binding at the
interdomain interface, whereas the Gln135 Gα11 mutant, which is located in the Gα11 helical domain and not predicted to influence CaSR-Gα11 coupling, may potentially diminish CaSR signal transduction by influencing the interaction of Gα11 with downstream effectors.

In conclusion, we have identified a novel Gα11 loss-of-function mutation, Thr54Met, that causes FHH2 and which provides new insights into the critical role of the Gα11 interdomain interface in CaSR signaling.

**Disclosures**

All authors state that they have no conflicts of interest.
Acknowledgments

This work was supported by the United Kingdom Medical Research Council (MRC) program grant G1000467/2010 (to CMG, MAN, and RVT), a Wellcome Trust Senior Investigator Award (to RVT); National Institute for Health Research (NIHR) Oxford Biomedical Research Centre Programme (to RVT); and NIHR Senior Investigator Award (to RVT). The GO Exome Sequencing Project of the National Heart, Lung, and Blood Institute and its ongoing studies provided and produced exome variant calls for comparison (the Lung GO Sequencing Project [HL-102923], the Seattle GO Sequencing Project [HL-102925], and the Heart GO Sequencing Project [HL-103010]).

Authors’ roles: Study design: CMG and RVT; study conduct: CMG, TC, and NR; data collection: AQ and FMH; data analyses and interpretation: CMG, FMH, and RVT; manuscript preparation: CMG, TC, FMH, NR, AQ, MAN, and RVT.

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