Identification of a G-Protein Subunit-α11 Gain-of-Function Mutation, Val340Met, in a Family With Autosomal Dominant Hypocalcemia Type 2 (ADH2)

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ABSTRACT

Autosomal dominant hypocalcemia (ADH) is characterized by hypocalcemia, inappropriately low serum parathyroid hormone concentrations and hypercalciuria. ADH is genetically heterogeneous with ADH type 1 (ADH1), the predominant form, being caused by germline gain-of-function mutations of the G-protein coupled calcium-sensing receptor (CaSR), and ADH2 caused by germline gain-of-function mutations of G-protein subunit α11 (Gα11). To date Gα11 mutations causing ADH2 have been reported in only five probands. We investigated a multigenerational nonconsanguineous family, from Iran, with ADH and keratoconus which are not known to be associated, for causative mutations by whole-exome sequencing in two individuals with hypoparathyroidism, of whom one also had keratoconus, followed by cosegregation analysis of variants. This identified a novel heterozygous germline Val340Met Gα11 mutation in both individuals, and this was also present in the other two relatives with hypocalcemia that were tested. Three-dimensional modeling revealed the Val340Met mutation to likely alter the conformation of the C-terminal α5 helix, which may affect G-protein coupled receptor binding and G-protein activation. In vitro functional expression of wild-type (Val340) and mutant (Met340) Gα11 proteins in HEK293 cells stably expressing the CaSR, demonstrated that the intracellular calcium responses following stimulation with extracellular calcium, of the mutant Met340 Gα11 led to a leftward shift of the concentration-response curve with a significantly (p < 0.0001) reduced mean half-maximal concentration (EC50) value of 2.44 mM (95% CI, 2.31 to 2.77 mM) when compared to the wild-type EC50 of 3.14 mM (95% CI, 3.03 to 3.26 mM), consistent with a gain-of-function mutation. A novel His403Gln variant in transforming growth factor, beta-induced (TGFBI), that may be causing keratoconus was also identified, indicating likely digenic inheritance of keratoconus and ADH2 in this family. In conclusion, our identification of a novel germline gain-of-function Gα11 mutation, Val340Met, causing ADH2 demonstrates the importance of the Gα11 C-terminal region for G-protein function and CaSR signal transduction. © 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: WHOLE-EXOME SEQUENCING; G-PROTEIN; CALCIUM; HYPOPARATHYROIDISM; KERATOCONUS

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Introduction

Autosomal dominant hypocalcemia (ADH) is a disorder of systemic calcium homeostasis that is associated with enhanced sensitivity of the calcium-sensing receptor (CaSR) to extracellular calcium (Ca\(^{2+}\)) concentrations.\(^{1-3}\) The CaSR is a guanine-nucleotide binding protein (G-protein)-coupled receptor (GPCR) that plays a pivotal role in Ca\(^{2+}\) homeostasis by transducing increases in the prevailing Ca\(^{2+}\) concentration into multiple signaling cascades that include G\(_{q/11}\)-mediated activation of phospholipase C (PLC), which in the parathyroid glands and kidneys induces rapid increases in intracellular calcium (Ca\(^{2+}\)) that lead to decreased parathyroid hormone (PTH) secretion and increased urinary calcium excretion, respectively.\(^{2,4,5}\) ADH is a genetically heterogeneous disorder most commonly caused by germline gain-of-function mutations of the CaSR, which is encoded by the CASR gene on chromosome 3q21.1, and this is referred to as ADH type 1 (ADH1; OMIM #601198).\(^{1,2,5}\) However, some ADH patients and families have recently been shown to harbor germline mutations of G-protein subunit-\(\alpha\)11 (G\(_{\text{a11}}\)), which is encoded by the GNA11 gene (Fig. 1) on chromosome 19p13.3,\(^{3,6,7}\) and referred to as ADH type 2 (ADH2; OMIM #615361).\(^{3,9}\) These ADH-associated G\(_{\text{a11}}\) mutations have been demonstrated to enhance CaSR-mediated signaling in cellular studies, consistent with a gain-of-function.\(^{3,7}\) ADH1 patients have calcitropic phenotypes, such as hypocalcemia with inappropriately low or normal PTH concentrations and a relative hypercalcemia that is characterized by urinary calcium to creatinine ratios that are within or above the reference range,\(^{1,8,9}\) and mice with a gain-of-function CaSR mutation, that are representative of ADH1, have been reported to also have non-calcitropic phenotypes such as cataracts.\(^{10}\) Although these features are similar to hypoparathyroidism, ADH1 is considered to represent a distinct disease entity from hypoparathyroidism, because affected individuals generally have PTH concentrations that are detectable and within the reference range.\(^{1,8}\)

Furthermore, ADH1 patients may also develop a Bartter-like syndrome characterized by hypokalemic alkalosis, renal salt wasting, and hyperreninemic hyperaldosteronism,\(^{11,12}\) and the use of active vitamin D metabolites to treat symptomatic ADH1 patients may result in the development of marked hypercalciuria, nephrocalcinosis, nephrolithiasis, and renal impairment.\(^{1,9}\) In contrast to ADH1, the phenotypic spectrum of ADH2 has not been fully elucidated, especially because only five ADH2 probands with G\(_{\text{a11}}\) mutations (Fig. 1) have been reported to date.\(^{3,6,7}\) Thus, it remains to be established whether ADH2 patients with germline gain-of-function G\(_{\text{a11}}\) mutations are susceptible to hypercalcemia, particularly when treated with active vitamin D preparations, or at risk of a Bartter-like syndrome. Moreover, G\(_{\text{a11}}\) is a widely expressed protein that mediates the biological effects of GPCRs in a range of tissues,\(^{13}\) and it is currently unknown whether patients with ADH2 may harbor additional calcitropic and non-calcitropic phenotypes. We ascertained a family with ADH and keratoconus, a non-calcitropic disorder of the cornea, and hypothesized that either a single genetic abnormality may be causing ADH and keratoconus, or that ADH and keratoconus may be due to two different genetic abnormalities; ie, digenic inheritance. To explore these hypotheses, we undertook whole-exome sequencing (WES) analysis of two relatives, both of whom had hypocalcemia, and one of whom also had keratoconus, to identify the causative variant(s).

**Patients and Methods**

**Patients**

The proband (individual I.4; Fig. 2A) is a 66-year-old male, from Iran, who was diagnosed with hypocalcemia at the age of 16 years following a childhood history of intermittent muscle cramps. His serum adjusted-calcium concentrations have ranged from 1.82 to 1.95 mmol/L (normal range = 2.20 to 2.60 mmol/L), in association with mild hyperphosphatemia (serum phosphate = 1.42 to 1.81 mmol/L; normal range = 0.80 to 1.45 mmol/L), normal serum creatinine concentrations of 72 to 80 \(\mu\)mol/L (normal range = 44 to 133 \(\mu\)mol/L), low/normal serum PTH concentrations of 4.0 to 18.7 pg/mL (normal range = 12 to 65 pg/mL), a normal 25-hydroxyvitamin D concentration of 18.7 ng/mL (normal range = 10 to 50 ng/mL), and a normal 1,25-dihydroxvitamin D concentration of 25.7 pg/mL (normal range = 16 to 45 pg/mL). He had been treated with oral calcium and active vitamin D preparations, and developed non-obstructive bilateral nephrolithiasis in association with urinary calcium values ranging from 4.3 to 10.2 mmol/24 hours (normal range = 2.5 to 7.5 mmol/24 hours). He was found to have mild bilateral cataracts at the age of 62 years. His family history revealed that 11 relatives (7 males and 4 females) over three generations had hypocalcemia, with symptoms such as seizures, muscle cramps, and tetany. Affected relatives also had low circulating PTH concentrations, and relative or absolute hypercalcemia (Fig. 2A), and these findings supported a diagnosis of ADH. Five hypocalcemic family members (3 males and 2 females) and one normocalcemic family member also had keratoconus, a corneal disorder, which in individuals II.3, II.5, and II.7 (Fig. 2A) had developed between the ages of 30 and 34 years.\(^{14}\) There was no consanguinity and the inheritance of hypocalcemia from father to son in individuals; eg, from I.1 to II.1 and II.2; I.4 to II.5; and II.5 to III.1 (Fig. 2A), indicated that hypocalcemia was transmitted as an autosomal dominant
disorder. Similarly, the inheritance of keratoconus from father to son in individuals II.5 to III.1 (Fig. 2A), may be because keratoconus is transmitted as an autosomal dominant disorder, but the occurrence of keratoconus in the children (II.3, II.5 and II.7; and II.8) of unaffected parents (I.4 and I.5; and I.8 and I.9, respectively, Fig. 2A) indicates an autosomal recessive inheritance or autosomal dominant inheritance with reduced penetrance. To investigate the cause of the hypocalcemia and keratoconus in this family, venous blood samples were collected from four available patients, of whom two had hypocalcemia only (individuals I.4 and II.4), and two others had both hypocalcemia and keratoconus (individuals II.3 and II.5; Fig. 2A). Leukocyte DNA was extracted using the Gentra PureGene blood kit (Qiagen, Crawley, UK). CaSR mutations, which would cause ADH1, and mutations of three genes, GCMB, PTH, and AIRE1, which are known to cause hypoparathyroidism had been previously excluded by an accredited genetics diagnostic laboratory. Informed consent was obtained from all participants included in the study, using protocols approved by the UK Multicentre Research Ethics Committee (MREC/02/2/93) and the Hospital Research Ethics in Israel.

Exome capture and DNA sequence analysis
Exome capture was performed in individual I.4, who was affected with hypocalcemia, and in individual II.5, who was affected with both hypocalcemia and keratoconus (Fig. 2A), using the SeqCap EZ Human Exome Library v2.0 (Roche NimbleGen, Madison, WI, USA), and DNA sequences determined using a 100-bp paired-end read protocol on an Illumina HiSeq2000 platform. A minimum of 20 × vertical read depth was obtained for >88% of the coding exome, as specified by the consensus coding sequence (CCDS) project in both individuals. Reads were aligned to the Human Reference Genome 37d5 (hs37d5) reference genome using Stampy and variant calling of single nucleotide variants (SNVs) and short insertions and deletions (indels) was undertaken using Platypus (v0.5.1). Analysis of coding variants was undertaken using Ingenuity Variant Analysis. To search for variants causing hypocalcemia and keratoconus under autosomal dominant inheritance models, variants with a minor allele frequency (MAF) >5% within all populations of 100 genomes data (April 2012 phase 1 call set [v3 update]) and from the National Heart, Lung, and Blood Institute (NHLBI) exome sequencing project (ESP), Seattle, WA (http://evs.gs.washington.edu/EVS/), were excluded. To search for variants causing keratoconus under an autosomal recessive inheritance model, variants with a MAF >5% were not excluded. The pathogenicity of variants was inferred from several criteria: allele frequency within the NHLBI exome sequencing project and the Exome Aggregation Consortium (ExAC) (Cambridge, MA, USA; http://exac.broadinstitute.org/), amino acid conservation, physicochemical alterations in amino acid substitutions, splice site prediction algorithms (NNSPLICE [http://www.fruitfly.org/seq_tools/splice.html], MaxEntScan [http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html], GeneSplicer [http://www.cbcb.umd.edu/software/GeneSplicer/gene_spl.shtml], and
Protein sequence alignments and three-dimensional modeling

Amino acid sequences of Gα11-subunit paralogs and transforming growth factor, beta-induced (TGFβI) orthologs were aligned using the Clustal Omega program (European Bioinformatics Institute, the European Molecular Biology Laboratory). The crystal structure of Gαq in complex with the GTP analogue guanosine diphosphate (GDP)-AlF₄ has been determined (Protein Data Bank [PDB] accession number 3OHM), and this was used to model Gα11, which has 90% identity with Gαq at the amino acid level, using the PyMOL Molecular Graphics System (version 1.2r3pre; Schrodinger, LLC).

Cell culture and transfection

The generation of wild-type and mutant Leu341 pBl-CMV2-GNA11 expression constructs have been reported. Site-directed mutagenesis was used to generate the Val to Met mutation at codon 340 in the pBl-CMV2-GNA11 construct using the Quikchange Lightning Site-directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). Cells were maintained in DMEM-Glutamax media using Lipofectamine 2000 (LifeTechnologies, Carlsbad, CA, USA) and 400 μg/mL geneticin (Thermo-Fisher, Waltham, MA, USA) at 37°C, 5% CO₂. Successful transfection was confirmed by visualizing GFP fluorescence using an Eclipse E400 fluorescence microscope with a Y-FL Epifluorescence attachment and a triband 46-diamidino-2-phenylindole-FITC-Rhodamine filter, and images captured using a DXM1200C digital camera and NIS Elements software (Nikon).(3,21) The expression of Gα11, GFP, calnexin, and CaSR proteins was also determined by Western blot analysis 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 Immuno-Star WesternC kit (BioRad, Hercules, CA, USA) on a BioRad Chemidoc XRS+ system.(3)

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 plated in T75 flasks and transiently transfected 24 hours later with 16 μg DNA. At 48 hours posttransfection, cells were detached, resuspended in calcium (Ca²⁺)-free and magnesium (Mg²⁺)-free Hanks buffered saline solution (HBSS) and loaded with 1 μg/mL Indo-1-acetoxyethylster (Indo-1-AM) for 1 hour at 37°C. After removal of free dye, the cells were resuspended in Ca²⁺,free and Mg²⁺-free HBSS and maintained at 37°C. Transfected cells, in suspension, were analyzed by flow cytometry on a MoFlo modular flow cytometer (Beckman Coulter, Indianapolis, IN, USA) and data on the Ca²⁺, responses to alterations in Ca²⁺, collected from all cells that expressed GFP, by simultaneous measurements of GFP expression (at 525 nm), Ca²⁺,bound Indo-1AM (at 410 nm), and free Indo-1AM (ie, not bound to Ca²⁺,) (at 485 nm), using a JDSU Xcyte laser (Coherent Radiation, Santa Clara, CA, USA), on each cell at each Ca²⁺, concentration [Ca²⁺,]₀ as described. Assays were performed in four biological replicates for each of the expression constructs. The peak mean fluorescence ratio of the 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 using the normalized response at each [Ca²⁺,]₀ for each separate experiment for the determination of the EC50 (ie, [Ca²⁺,]₀ required for 50% of the maximal response). Data is presented as mean with confidence interval or mean ± standard error of the mean (SE). Statistical analysis was performed using the F-test. A value of p < 0.05 was considered significant for all analyses.

Results

Identification of a novel Val340Met Gα11 mutation

Exome capture and high-throughput sequence analysis of genomic DNA from both affected individuals, one with hypocalcemia (individual I.4) and the other with hypocalcemia and keratoconus (individual II.5) (Fig. 2A) resulted in the detection of >5000 non-dbSNP variants in both individuals. The exclusion of variants with a MAF of >5% resulted in the identification of 104 novel variants in 103 genes, of which three variants were excluded as sequence artifacts as they occurred within trinucleotide repeats. The only variant identified in a gene previously associated with hypocalcemia, was a heterozygous G->A transition at nucleotide c.1018 (c.1018G->A), located in exon 7 of the GNA11 gene (Fig. 1). The variant was confirmed by Sanger DNA sequencing to be present in both patients affected with hypocalcemia (individuals I.4 and II.5) and in two other family members (individuals II.3 and II.4) with hypocalcemia from whom samples were available (Fig. 2B). This G->A transition (GTG to ATG) resulted in a missense substitution, Val340Met, of the encoded Gα11 protein (Fig. 2C). The absence of this DNA sequence abnormality in >60,000 exomes from the Exome Aggregation Consortium (ExAC), together with evolutionary conservation of the Val340 residue in vertebrate Gα-subunit paralogs (Fig. 3A) indicated that the Val340Met abnormality likely represented a pathogenic GNA11 mutation rather than a benign polymorphic variant.

Identification of candidate variant for keratoconus

Whole-exome sequencing revealed a novel heterozygous c.1209T>G transversion in TGFβI, encoding a variant His403Gln in TGFβI, which was present in both individuals (I.4 and II.5, Supporting Fig. 1A, Supporting Table 1). The TGFβI His403Gln variant is not present in ExAC, although a different variant in the same amino acid, His403Tyr, is present in only 3 out of >60,000 individuals. The His403 residue is also evolutionarily conserved in TGFβI orthologs (Supporting Fig. 1B). The TGFβI variant was confirmed by Sanger DNA sequence analysis in the siblings, II.3 and II.5, who had hypocalcemia and keratoconus, but its absence in the sister II.4, who has hypocalcemia only, indicates that this TGFβI variant is not involved in the etiology of hypocalcemia (Supporting Fig. 1A). Moreover, the presence of
this TGFBI variant in the father (individual I.4), who was affected with hypocalcemia only, indicates likely nonpenetrance of the mutant allele (Supporting Fig. 1).

Structural characterization of the Val340Met Ga11 mutant protein

To evaluate whether the Val340Met Ga11 variant represents a pathogenic mutation and the cause of hypocalcemia in this family, structural and functional studies were undertaken. The crystal structure of Ga11A, which shares 90% amino acid identity with Ga11B,20 was used to predict the effects of the Val340Met mutation. The mutated Val340 residue is located within the a5 helix of the GTPase domain of Ga11 (Fig. 3, C), and next to the Phe341 residue, which has been reported to be associated with a gain-of-function Phe341Leu mutation (Figs. 1 and 3B) causing ADH2.13 The a5 helix is located at the C-terminus of Ga-subunits and plays a critical role in GPCR binding and G-protein activation.23,24 Indeed, the C-terminal portion of the a5 helix, which is known as the interface module, directly binds to the transmembrane domain and intracellular loops of activated GPCRs,24 whereas the N-terminal portion, which is known as the transmission module, facilitates conformational changes in Ga-subunit structure that lead to guanine nucleotide exchange and Ga-subunit activation.23 The Val340 residue (red) is predicted to form non-polar interactions with the Phe272 and His327 residues (cyan),26 which are located in the b5 and b6 strands, respectively. The introduction of a mutant Met340 residue (red, space filling model) is predicted to sterically hinder His327 (cyan, stick model), thereby altering the conformation of the a5 helix and/or b6 strand.

Fig. 3. Predicted effects of the Val340Met mutation on the Ga11 protein. (A) Multiple protein sequence alignment of residues comprising the a5 helix of Ga-paralogs. The wild-type Val340 (V) and mutant Met340 (M) residues are shown in bold. Conserved residues are shaded gray. (B) Overall three-dimensional structure of the Ga11 protein. The Ga-subunit helical (blue) and GTPase (green) domains are connected by the L1 and L2 peptides (gray). The four Ga11 residues, which have been previously reported to be mutated in ADH2,13,20 are shown in magenta. The mutated Arg60 and Arg181 residues are located at the interdomain interface (red dashed line), which forms a pocket for the binding of guanine nucleotides (yellow). The GTPase domain is the location of the mutated Ser211 residue (a2 helix), and mutated Val340 (red) and Phe341 residues (a5 helix). (C) Close-up view of the a5 helix, which comprises a C-terminal interface module (pink) that interacts with partner GPCRs and an N-terminal transmission module (purple), which interacts with the surrounding b1-b6 strands to facilitate conformational changes that lead to guanine nucleotide exchange and Ga-subunit activation.23 The wild-type Val340 residue (red) is predicted to form non-polar interactions with the Phe272 and His327 residues (cyan),26 which are located in the b5 and b6 strands, respectively. (D) The introduction of a mutant Met340 residue (red, space filling model) is predicted to sterically hinder His327 (cyan, stick model), thereby altering the conformation of the a5 helix and/or b6 strand.
Functional characterization of the Val340Met Gα11 mutant protein

To determine the effects of these predicted changes in Gα11 structure (Fig. 3) on CaSR-mediated signaling, bidirectional pBI-CMV2-GNA11 expression constructs containing wild-type Val340, mutant Met340, or the previously reported gain-of-function Leu341 mutant GNA11 cDNA, or vector containing the GFP reporter gene alone were transiently transfected into HEK293 cells stably expressing the CaSR. Expression of CaSR, Gα11, and GFP was confirmed by fluorescence microscopy and/or Western blot analysis (Fig. 4A, B). Calnexin was used as a

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**Fig. 4.** Functional characterization of wild-type and ADH2-associated mutant Gα11 proteins. (A) Fluorescence microscopy of HEK293 cells stably expressing CaSR (HEK293-CaSR) and transiently transfected with wild-type (WT) or ADH2-associated mutant (m) (Met340 and Leu341) pBI-CMV2-GNA11 constructs, or with vector (V) only. Detection of GFP in these cells indicates successful transfection and expression of these constructs. Bar indicates 10 μm. (B) Western blot analysis of whole-cell lysates using antibodies to CaSR, calnexin, Gα11, and GFP. Transient transfection of WT or ADH2-associated mutant constructs resulted in overexpression of Gα11 when normalized to calnexin expression. (C) Concentration-response curves showing normalized Ca2+ responses to changes in [Ca2+]o of HEK293-CaSR cells transfected with WT or ADH2-associated Gα11 mutants. The Ca2+ responses are shown as the mean ± SEM of 4 independent transfections. The ADH2-associated Gα11 mutants (Met340 and Leu341) led to a leftward shift of the concentration-response curves (blue and red, respectively) when compared with WT Gα11 (black), which harbors Val and Phe residues at codons 340 and 341, respectively. (D) The Met340 and Leu341 mutants (blue and red bars, respectively) were associated with significantly decreased EC50 values compared to cells expressing WT Gα11 (black bar). **p < 0.0001.

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loading control in Western blot analyses, and G\textsubscript{\alpha11} expression was demonstrated to be similar in cells transiently transfected with wild-type or mutant G\textsubscript{\alpha11} proteins, and greater than that of cells transfected with the empty pBl-CMV2 vector (Fig. 4B). The responses of [Ca\textsuperscript{2+}]\textsubscript{i} to alterations in [Ca\textsuperscript{2+}]\textsubscript{c} in cells expressing the different G\textsubscript{NA11} vectors were assessed by flow cytometry. The Ca\textsuperscript{2+}\textsubscript{i} responses in wild-type and mutant G\textsubscript{\alpha11}-expressing cells were shown to increase in a dose-dependent manner following stimulation with increasing concentrations of Ca\textsuperscript{2+} between 0 and 15 mM. However, expression of the Met340 and Leu341 mutant G\textsubscript{\alpha11} proteins resulted in a leftward shift of the concentration-response curves (Fig. 4C), with significantly lower half maximal (EC\textsubscript{50}) values, compared to wild-type Val340 G\textsubscript{\alpha11} and empty vector (Met340 = 2.44 mM [95% CI, 2.31 to 2.77 mM]; Val340 = 3.14 mM [95% CI, 3.03 to 3.26 mM]; empty vector = 3.12 mM [95% CI, 2.99 to 3.25 mM]; p < 0.0001) (Fig. 4D). There was no significant difference in the EC\textsubscript{50} values between Met340 and Leu341 (Leu341 = 2.60 mM; 95% CI, 2.49 to 2.72 mM; p > 0.05). Thus, the Val340Met missense substitution represents a novel gain-of-function G\textsubscript{\alpha11} mutation, similar to those reported in ADH2 patients.\textsuperscript{3,7}

## Discussion

The identification of a germline gain-of-function G\textsubscript{\alpha11} mutation, Val340Met, indicates ADH2 to be the likely cause of hypocalcemia in this family. To date, five other hypocalcemic patients and families have been reported to harbor germline G\textsubscript{\alpha11} mutations, consistent with a diagnosis of ADH2.\textsuperscript{3,6,7} Similar to the family characterized in this study, individuals with germline gain-of-function G\textsubscript{\alpha11} mutations have been reported to generally have mild-to-moderate hypocalcemia (serum adjusted-calcium concentration 2.44 to 2.77 mM; Val340 > 2.72 mM; Val340 > 2.72 mM; Met340 = 2.77 mM). The identifications of a novel gain-of-function G\textsubscript{\alpha11} mutation, similar to those reported in ADH2 patients.

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