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## A novel mutation in the *GCM2* gene causing severe congenital isolated hypoparathyroidism

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### Abstract

**Objective**—To investigate the *GCM2* gene in three siblings with congenital hypoparathyroidism and perform functional analysis.

**Materials and methods**—We sequenced the *GCM2* gene by PCR and analyzed the functional consequence of the mutation by transient transfection studies. Haplotype analysis was performed.

**Results**—We identified a nucleotide change, c.408C>A, in exon 3 that is predicted to truncate the Gcm2 protein (p.Tyr136Ter). All three affected siblings were homozygous and both parents were heterozygous for the mutation. Transfection studies revealed the mutant mRNA but not expression of the Gcm2 protein. Haplotype analysis revealed that the two mutant *GCM2* alleles shared genotypes on chromosome 6p24.2.

**Conclusions**—We describe the first *GCM2* mutation in exon 3 in patients with severe congenital hypoparathyroidism. Informative genetic markers could not exclude identity by descent for the mutant alleles. Gcm2 protein was not detected after transfection, suggesting that complete lack of Gcm2 action accounts for severe hypoparathyroidism.

### Keywords

GCMB; GCM2; isolated hypoparathyroidism; mutation

### Introduction

Early neonatal hypocalcemia is often mild, transient and resolves with minimal medical intervention. Severe neonatal hypocalcemia due to permanent hypoparathyroidism is rare and can cause permanent mental and physical deterioration when accompanied by seizures, laryngospasm and delayed treatment (1). Permanent hypoparathyroidism can be due to several genetic causes: mutation or hemizygosity of *TBX1* as part of the 22q11 deletion in

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#### Conflict of interest statement

The authors have nothing to disclose.

DiGeorge sequence (2); loss of function mutations in *TCBE* in Kenny-Caffey syndrome (3); loss of function mutation of *GATA3* in hypoparathyroidism, deafness, and renal dysplasia (HDR) syndrome (4); activating mutations of *CASR* in autosomal dominant hypercalciuric, hypocalcemia (5); and loss of function mutations of parathyroid hormone (*PTH*) in isolated hypoparathyroidism (6).

Recently, our group and others have demonstrated that either recessive amorphic mutations (7–11) or dominant inhibitor mutations (12, 13), in the Glial Cell Missing 2 (*GCM2*, formerly *GCMB*) gene located on chromosome 6p24.2 are the most common cause of isolated hypoparathyroidism (Figure 1). *GCM2* encodes a 506-amino acid transcription factor that is expressed principally in the developing and mature parathyroid glands. Genetic ablation of *Gcm2* in mice results in hypoparathyroidism because of aplasia of the parathyroid glands, which establishes *Gcm2* as critically important for parathyroid gland homeostasis. The human *GCM2* gene contains five coding exons, and all previous small mutations have been identified in exons 2 and 5 (8–13). These mutations interfere with DNA binding and nuclear localization or transactivation, respectively. We report the first mutation in exon 3 in a nonconsanguineous family with autosomal recessive isolated hypoparathyroidism (Table 1). Functional analysis showed that HEK293 cells transfected with the mutant cDNA did not contain *Gcm2* protein, suggesting that a complete lack of *Gcm2* led to severe hypoparathyroidism and absence of circulating PTH in the affected subjects.

## Patients and methods

The Institutional Review Board of the Alfred I. duPont Children's Hospital for Children approved the study protocol, and written informed consent was obtained from both parents on behalf of their younger children and assent obtained from the older brother (age 9 years).

The oldest affected sister was the product of an uncomplicated full-term pregnancy and spontaneous vaginal delivery to a 28-year-old mother who had recently immigrated to the United States from Guatemala. She was discharged home on a standard infant formula and presented at 7 days of age with a generalized tonic-clonic seizure and difficulty breathing. Her initial serum calcium was 5.6 mg/dL with a serum phosphorus of 10.3 mg/dL; serum intact PTH (iPTH) was undetectable. Fluorescence in situ hybridization (FISH) analysis for DiGeorge sequence showed two copies of the 22q11 locus. She was hospitalized and received intravenous (IV) calcium gluconate and was started on calcitriol by mouth twice daily. On the second day of hospitalization, she was transitioned to oral calcium supplements and was switched to a low-phosphorus infant formula (PM60:40, Mead Johnson). She maintained normal serum concentrations of calcium and phosphorus throughout childhood while treated with oral calcium and calcitriol, and developmental and cognitive milestones were all normal. Subsequent testing for a mutation in the *CaSR* gene was negative.

The second sister had her serum calcium evaluated at birth, and it was normal at 8.8 mg/dL. Subsequent testing showed asymptomatic hypocalcemia (6.3 mg/dL) on day-of-life 3 with undetectable iPTH. Molecular analysis of the 22q11 locus and *CaSR* gene was normal. A third sister also had asymptomatic hypocalcemia (7.3 mg/dL) when tested on day-of-life 2 with undetectable iPTH. Both sisters were treated with oral calcium and calcitriol and have done well.

The mother, father and an older brother of the affected children had normal serum levels of calcium and iPTH (Table 2). The parents had both immigrated to the United States from Guatemala and reported that while they were living in Guatemala, they had a 7-monthold

son who had died of intractable seizures. They did not know if the deceased child had ever had serum calcium measured. The parents denied consanguinity.

### DNA analysis

DNA was extracted from peripheral blood leukocytes using standard protocols (ArchivePure DNA Isolation Kit; 5Prime, Gaithersburg, MD, USA). Oligonucleotide primers were designed to amplify the five exons and adjacent intronic regions from the *GCM2* (OMIM 603716) gene. The polymerase chain reaction (PCR) was performed with five primer pairs (PCR conditions and primer sequences are available upon request), and amplicons were sequenced bidirectionally using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3130x1 Genetic Analyzer. Nucleotide sequences were compared with the control reference sequence (NG\_008970) by MacVector and Assembler Software (Cary, NC, USA).

### Biochemical studies

Site-directed mutagenesis was performed to introduce the single nucleotide replacement into the wild type *Gcm2* cDNA as previously described (7), and verified by sequence analysis. To analyze the mutant *Gcm2* protein, we performed transient transfection of HEK293T cells with mutant and wild type *Gcm2* cDNAs that were native or contained FLAG epitope tags. Briefly, HEK293T cells were seeded at 60%–80% confluence in six-well dishes, and 24 h later they were transfected with 1.0 µg of cDNA per well using Lipofectamine Plus. Cells were cultured in DMEM (Life Technologies, Rockville, MD, USA), supplemented with 10% fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 µg/mL) in a humidified (95%) atmosphere of 5% carbon dioxide at 37°C. Approximately 48 h after transfection, the culture medium was removed and RNA or total protein was extracted as previously described (7). Cell extracts were subjected to SDS-PAGE on 8% gels and immunoblot analysis was performed using an affinity-purified rabbit anti-human *GCM2* polyclonal antiserum (1:3000) as previously described (7) or M2 anti-FLAG monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA). Antibody binding was detected by use of peroxidase-labeled secondary antibody and was visualized using enhanced chemiluminescence. Reverse transcriptase-PCR for *Gcm2* was performed as previously described (7).

### Haplotype analysis

Primers were designed to amplify 11 loci on Chromosome 6 within and flanking the *GCM2* gene, including eight short tandem repeat (STR) loci (DS1640, D6S309, D6S470, rs6149425, D6S1721, D6S429, D6S1653, D6S260) and three intragenic single nucleotide polymorphisms (SNPs) (rs9461258, rs16870746, and rs2275387). The STR targets were amplified with unlabeled primers, run on an agarose gel to confirm their size, purified, and sequenced to confirm locus specificity. Genotyping of the family members was performed using 6-FAM labeled primers and an Applied Biosystems 3130xl Genetic Analyzer.

## Results

Sequence analysis of the *GCM2* gene revealed a novel nucleotide change, c.408C>A, in exon 3 (Figure 2). The proband and her affected sisters were all homozygous for this mutation, while her parents were heterozygous carriers. The proband's older brother was homozygous for wild type *GCM2* alleles. The single base transversion replaces tyrosine with a premature stop at codon 136 (p.Tyr136Ter). We assessed the functional consequence(s) of this nonsense mutation by transient transfection of HEK293T cells. Cells that had been transfected with wild type *Gcm2* cDNA expressed a recombinant *Gcm2* protein of expected 55-kDa molecular weight. By contrast, no *Gcm2* protein was detected

using either the FLAG antibody or anti-Gcm2 antibody in cells that had been transfected with the Gcm2 c.408C>A cDNA. Finally, cells that had been transfected with equal amounts of mutant Gcm2 cDNA plus wild type Gcm2 containing a FLAG epitope tag expressed only the wild type Gcm2 protein and in expected quantities. Reverse transcription (RT)-PCR demonstrated equivalent expression of transcripts corresponding to wild type and c.408C>A forms of Gcm2 in transfected cells.

Haplotype analysis is summarized on the pedigree shown in Figure 3. Seven markers were informative, and spanned a 9.7 Mb region on 6p24.2 that included the *GCM2* locus. All three affected siblings shared the same genotype. The informative intragenic SNP markers found in both mutated parental chromosomes that were passed on to the affected siblings were identical, and thus we cannot exclude that the two parents shared a common ancestor and that these two mutant alleles were identical by descent.

## Discussion

Gcm2 is a transcription factor that is crucial for parathyroid gland homeostasis (16). In transgenic mice, loss of Gcm2 results in absence of the parathyroids, and in humans spontaneous mutations of *GCM2* are associated with congenital hypoparathyroidism (17). We report a novel point mutation in exon 3 of the *GCM2* gene that is associated with a null-protein defect and severe early onset hypoparathyroidism. The proband presented with tetany and a hypocalcemic seizure at 7 days of age. She has, thus far, had normal developmental and cognitive milestones throughout early childhood on calcium and calcitriol supplementation. Her two affected siblings were also ascertained within the first few days of life, and have similarly done well with calcitriol therapy. The three affected subjects had undetectable levels of serum intact PTH, which differs from reports of low serum levels of PTH in previously reported patients who carried small mutations that lead to Gcm2 variants with reduced function or that mislocalize within the cell (8–13). Thus, it is conceivable that residual Gcm2 action allows development of small amounts of parathyroid tissue that can secrete detectable but physiologically insignificant amounts of PTH into the circulation.

The c.408C>A null-protein mutation follows a predictable autosomal recessive mode of inheritance with three siblings who each have a homozygous *GCM2* mutation and two heterozygous, non-consanguineous parents. The parents are both of Guatemalan ethnicity, which is similar to the first reported human cases of isolated hypoparathyroidism caused by a different, large intragenic deletion of *GCM2* (18). In both the previous family and the family reported here, molecular analysis demonstrated that the mutant alleles shared similar haplotypes and thus likely arose within common founders. By contrast, two other *GCM2* mutations – the R47L missense (9, 11, 12) and the I298fsX307 frameshift (11) – have each been identified in three unrelated kindreds, and thus may represent potential mutational ‘hotspots’.

Mutational hotspots are often associated with repetitive sequences, such as homonucleotide runs, direct and inverted repeats and microsatellite repeats (18). For these mutation hotspots, the exact DNA sequence is not critical but only the fact that a sequence motif is repeated. Alternatively, mutation hotspots can depend on nucleotide sequence context (mutable motifs, subsequences) nucleotide substitutions or deletions within homonucleotide repeats, or during the repair of mismatches caused by heteroduplex formation between imperfect direct repeats (18). Although our haplotype analysis suggests identity by descent for the c.408C>A mutation that we have identified, this substitution occurs within a four cytosine homonucleotide repeat, and it is conceivable that the C to A transversion could have arisen independently.

We expressed the c.408C>A Gcm2 cDNA in mammalian cells to assess the biochemical consequences of the mutation. The mutation introduces a premature stop codon (p.Try136Ter) within the DNA-binding domain or GCM domain that is proximal to the nuclear localization signal and transactivation domain 1 (Figure 1) (9).

GCM2 is a member of a small family of GCM transcription factors that are present in vertebrates and some invertebrates (11, 19), and which share a conserved zinc-coordinating DNA-binding domain (20) that binds to the consensus motif 5-ATGCGGGT-3 (21, 22). Mutations in the GCM domain are predicted to cause structurally significant effects that have been shown to interfere with DNA binding or transactivational activity (10, 11).

To date, previously described small mutations in *GCM2* have been described in exons 2 or 5 and have disrupted or eliminated sequences required for DNA binding, nuclear localization or transactivation. Accordingly, mutations in exon 2 have been associated with hypomorphic alleles and recessive hypoparathyroidism, while mutations in exon 5 have been associated with inhibitory function and dominant hypoparathyroidism (Figure 1).

Theoretically, the p.Tyr136Ter mutation could lead to nonsense-mediated decay of transcripts, a truncated protein that lacks the nuclear localization signal as well as the transcriptional activation domains, or an unstable protein that is rapidly degraded. Biochemical studies performed in our laboratory showed that Gcm2 transcripts but not protein were expressed in HEK293T cells that had been transfected with the Gcm2 p.Try136Ter cDNA.

Hence, we suggest that severe hypoparathyroidism occurred in the three affected subjects we studied because the premature termination reduced stability of the Gcm2 protein, further enhancing the rapid rate of degradation (23) and leading to a null-protein phenotype.

In conclusion, this is the first report of a mutation in exon 3 of the *GCM2* gene and a mutation that leads to a null-protein phenotype. All three affected children presented with profound hypocalcemia and hyperphosphatemia during the neonatal period, and intact PTH was undetectable in the circulation. These results increase our understanding of the spectrum of *GCM2* mutations that cause autosomal recessive isolated hypoparathyroidism and suggest that minimal GCM2 activity may allow development of sufficient parathyroid tissue to account for small amounts of circulating PTH.

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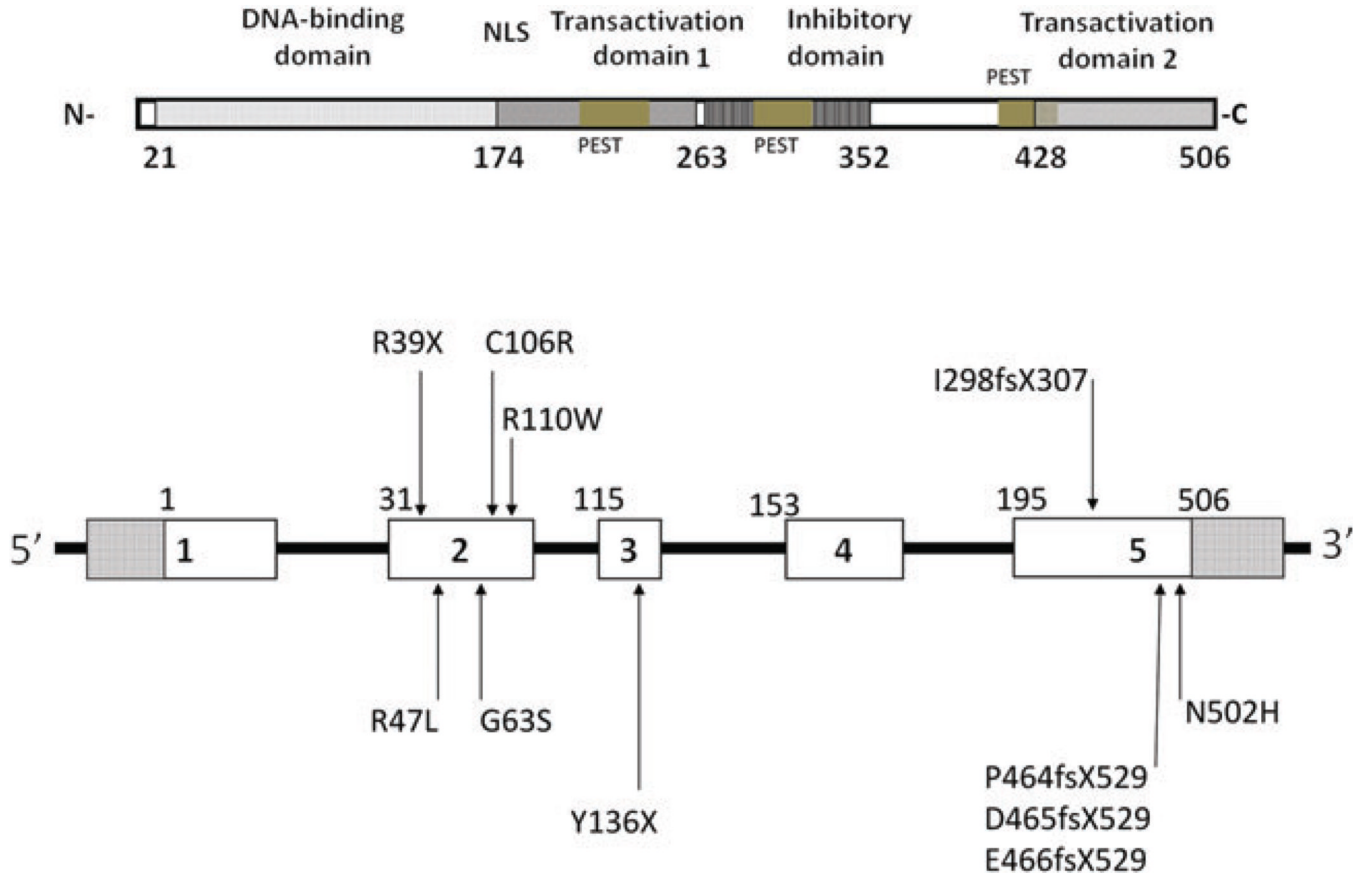
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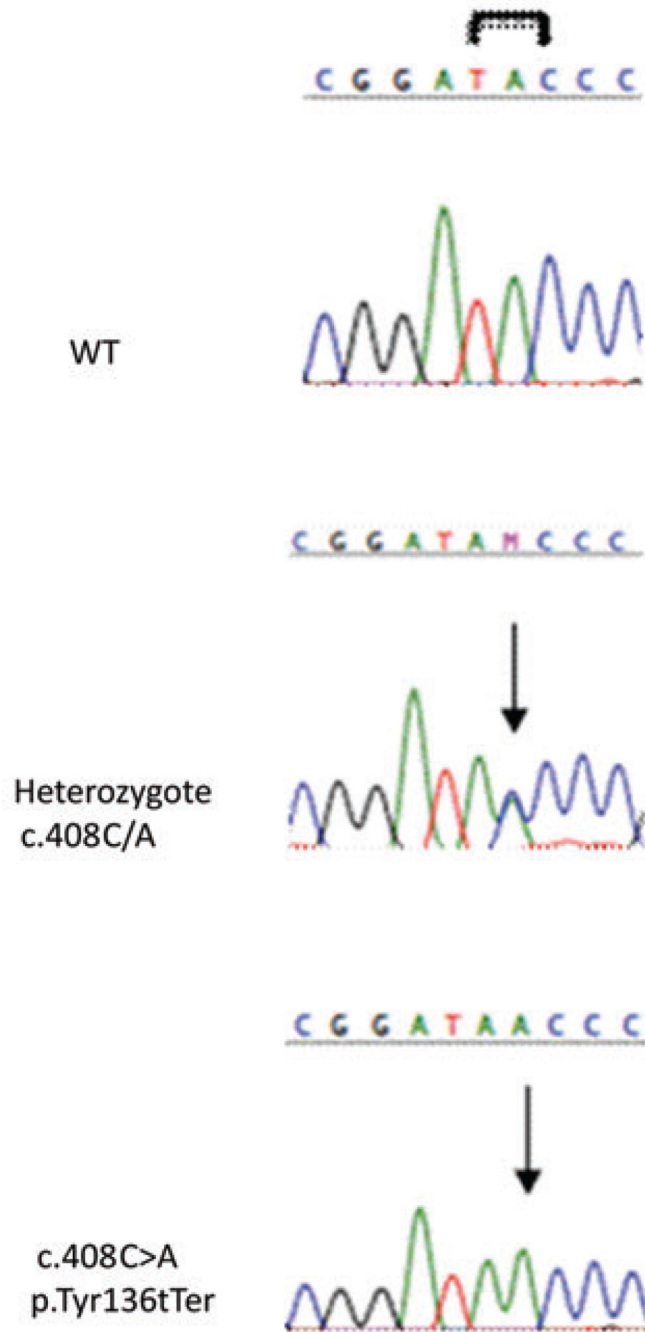
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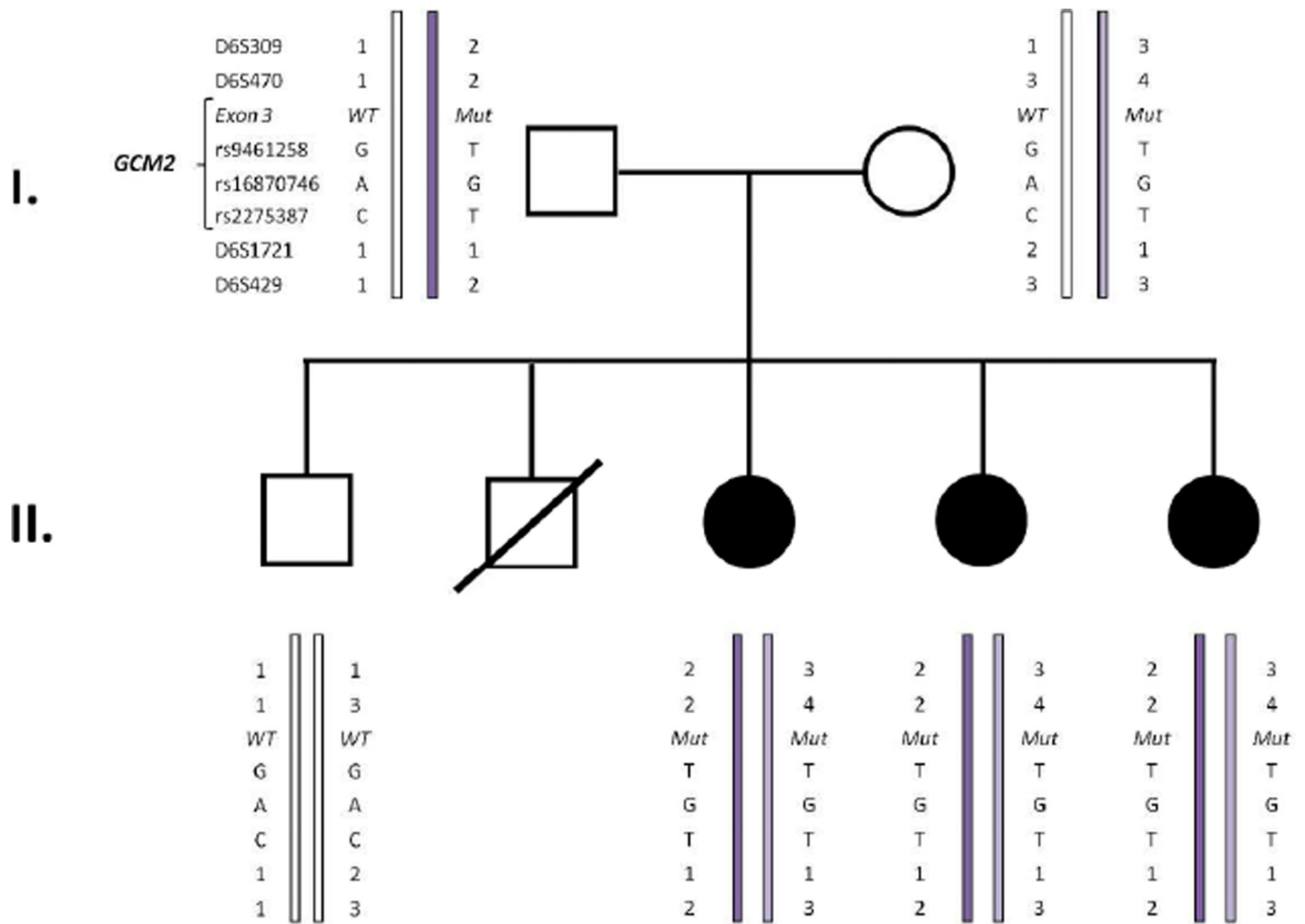


**Figure 1.** Schematic illustration of the genomic structure of the *GCM2* gene. The upper portion illustrates the location of the various domains of the gene and the approximate number of amino acids encoded in each domain, as well as the cumulative number of 506 amino acids encoded by the *GCM2* gene. The protein contains a GCM DNA-binding domain, two transactivation domains, an inhibitory domain and a predicted nuclear localization signal (NLS) at residues 176–191. The lower portion illustrates the known mutations for isolated hypoparathyroidism and the location of each with respect to the five codons in the gene. Mutations in exon 2 cause recessive hypoparathyroidism while many of the mutations in exon 5 are associated with dominant hypoparathyroidism. Putative mutational hotspots would include the R47L missense and the I298FS307 frameshift mutation; both of which arose non-consanguineously [after Ref. (11)].





**Figure 2.** Sequence chromatograms of exon 3 region showing the *GCM2* Y136X mutations. Affected individuals show the presence of a C>A transition leading to a stop codon within exon 3. Each unaffected heterozygote parent carries the change within one copy of their *GCM2* gene.



**Figure 3.** Haplotype analysis using chromosome 6p23-24 loci and the *GCM2* mutation in the reported AR-HPT family. The genotypes at the chromosome 6p23-24 microsatellite polymorphic loci and SNPs were ascertained. The paternal haplotypes are on the left and the maternal haplotypes are on the right. The two parental haplotypes share genotypes at 5 loci, thereby indicating that the c.408C>A mutation may have arisen in a shared ancestor of the father and mother.

**Table 1**

Currently recognized GCMB mutations that result in reduced expression or function of protein.

<b>Mutation</b>	<b>Inheritance</b>	<b>Location</b>	<b>Study</b>
R39X	AR	Exon 2	Bowl et al. (11)
R47L	AR	Exon 2	Baumber et al. (9)
G63S	AR	Exon 2	Thomee et al. (10)
C106R	AD	Exon 2	Yi et al. (14)
R110W	AR	Exon 2	Bowl et al. (11)
Y136X	AR	Exon 3	Doyle et al. (current report)
1298fsX307	AR	Exon 5	Bowl et al. (11)
P464fsX529	AD	Exon 5	Mannstadt et al. (13)
D465fsX529	AD	Exon 5	Mannstadt et al. (13)
E466fsX529	AD	Exon 5	Canaff et al. (12)
N502H	AD	Exon 5	Mirczuk et al. (15)

AR, autosomal recessive; AD, autosomal dominant.

**Table 2**

Laboratory values for the three affected siblings at the time of diagnosis as neonates and of their parents.

Family member	iPTH, pg/mL	Ca, mg/dL	Phos., mg/dL	AlkP	250HD, ng/mL	1,250HD, pg/mL
Mother	36	9.8	-	-	29	61
Father	40.7	9.7	-	-	-	-
Sibling 1	<3	5.1	10.4	270	23	78
Sibling 2	<3	7.2	9.8	151	24	80
Sibling 3	<3	7.0	10.9	303	41	104

-, not done. HD, hydroxyl; iPTH, intact parathyroid hormone.