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Identification and characterization of C106R, a novel mutation in the DNA-binding domain of GCMB, in a family with autosomal-dominant hypoparathyroidism

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Summary

Overview—Glial cells missing B (GCMB) is a transcription factor that is expressed in the parathyroid hormone (PTH)-secreting cells of the parathyroid glands. Several mutations in *GCMB* have been reported to cause hypoparathyroidism (HP). We identified a family with two individuals in two generations (mother and son), who are affected by autosomal-dominant hypoparathyroidism (AD-HP). A novel heterozygous mutation in exon 2 of *GCMB* was identified in both affected individuals that changes cysteine at position 106 of the putative DNA-binding domain of GCMB to arginine (C106R).

Methods—We performed mutational analysis of the genes encoding GCMB, pre-pro PTH, GATA3 and CaSR using polymerase chain reaction (PCR)-amplified genomic DNA. The identified GCMB mutant was characterized by functional studies including nuclear localization, electrophoretic mobility shift assays (EMSA) and luciferase reporter assays, and homology modelling was performed to generate a three-dimensional structural model for the DNA-binding domain of GCMB to predict the structural consequences of the identified mutation.

Results—The C106R mutant of GCMB failed to interact with the DNA consensus recognition motif, as determined by EMSA. Furthermore, in comparison with wild-type GCMB, the C106R mutant demonstrated reduced transactivation in luciferase reporter assays; however, the mutant GCMB failed to reduce the activity of the wild-type protein. Consistent with the EMSA findings,

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homology modelling analysis suggested that replacement of cysteine 106 with arginine would interfere with DNA binding.

Conclusions—We have identified a novel GCMB mutation that may explain AD-HP in our family. However, the exact mechanism by which this heterozygous mutation leads to the disease in the described family remains to be elucidated.

Introduction

Calcium is one of the fundamental elements in the body; calcium ions are essential for processes as different as hormonal secretion, cardiac contraction, memory and neurotransmission. Regulation of the serum calcium concentration is tightly controlled by parathyroid hormone (PTH), which is secreted from parathyroid gland cells[1] and by activated vitamin D. Isolated hypoparathyroidism (HP) is a rare condition in which PTH levels are too low to maintain normal serum calcium concentration.[2] Affected individuals typically present with hypocalcaemia, hyperphosphatemia and low or normal 1,25-dihydroxyvitamin D levels.[2,3] Symptoms associated with HP, which are because of hypocalcaemia, include muscle cramps, tetany and seizures. Calcification of the basal ganglia, cataracts and dental abnormalites, including enamel hypoplasia, defects in dentin and dental caries, are frequently encountered in patients with chronic HP.[4]

Many cases of HP are sporadic; familial forms of HP show autosomal recessive (AR) or autosomal-dominant (AD) modes of inheritance, and HP can occur as part of a syndrome (e.g. DiGeorge syndrome), in conjunction with other abnormalities (e.g. renal abnormalities, deafness and hypoparathyroidism), or occurs as an isolated disorder.[2,5] Activating mutations in the calcium-sensing receptor (CaSR),[3,6,7] mutations in PTH that interfere with intracellular processing of the nascent protein[8] or mutations in the transactivation domain of glial cells missing B (*GCMB*)[9,10] show an autosomal-dominant pattern of inheritance. autosomal recessive hypoparathyroidism (AR-HP) can be caused by homozygous mutations in the genes encoding pre-pro PTH[11,12] or GCMB.[13–16]

Glial cells missing B is a transcription factor located on chromosome 6p24·2,[17] which is expressed exclusively in the PTH-secreting cells of the parathyroid glands.[18,19] Human *GCMB* consists of five exons, which encode for an 506 amino acid protein [19] that comprises a DNA-binding domain at the N-terminal region and two transactivation domains at the C-terminal region.[20] There are two human orthologs, *GCMA* and *GCMB*. The N-terminal DNA-binding domain is highly conserved in all vertebrates and some invertebrates and binds to a consensus motif (5′-ATGCGGGT-3′), while the C-terminal transactivation domains are not conserved.[20–22] Several homozygous, presumed loss-of-function mutations in *GCMB* have been described in families with AR-HP. To date, three different heterozygous *GCMB* mutations have been identified in families with AD-HP. Two heterozygous single-nucleotide deletions (c.1389delT and c.1399delC) and a missense mutation (N502H) are located in exon 5 that encodes the C-terminal tail of GCMB and contains the second transactivation domain.[9,10,23]

We report here a nonconsanguineous Korean family with two members presenting with HP. A novel heterozygous missense mutation in the second exon of *GCMB* was identified in both patients, but not in the healthy family members. We performed functional analysis and three-dimensional computer modelling of the *GCMB* DNA-binding domain complex to characterize the functional consequences of this mutation. We found that the novel mutation (C106R) impairs DNA-binding capability of GCMB. Homology modelling suggested that the mutation disrupts the structural integrity of the GCMB: DNA complex. Taken together, our results indicate that the C106R mutation could account for AD-HP in our family.

Patients and methods

Patients

The 41-year-old proband is the daughter of Korean, nonconsanguineous parents, who are both normocalcaemic (Fig. 1a, individual II-5). She presented with a hypocalcaemic seizure during infancy, was diagnosed with idiopathic HP and treated with calcitriol and calcium. She gave birth to a boy (Fig. 1a, individual III-1) at 27 years of age who himself had tetany because of hypocalcaemia (total calcium, 1·65 mM; normal range, 2·05–2·7 mM) when he was 14 months old. The proband and her son were otherwise healthy and had no evidence of an immunodeficiency disease, hearing or renal abnormalities. Physical examination of both patients was unremarkable, and there were no dysmorphic features suggestive of DiGeorge syndrome. PTH level was lower than 1·0 pg/ml in both patients (normal range, 10–65 pg/ml), and phosphorus level was 1·45 mM (normal range, 0·81–1·52 mM) and 2·71 mM (normal range, 0·94–1·74 mM), respectively, in the proband and her son. Familial idiopathic hypoparathyroidism was diagnosed, and treatment with calcium and calcitriol was initiated for the son. PTH and phosphorus levels of the proband were checked during the treatment, those of her son were determined before the treatment was initiated.

Mutational analyses of genes encoding PTH, CaSR, GATA3 and GCMB

Venous blood samples were collected from the two affected individuals (Fig. 1a, II-5 and III-1), the parents of the proband (Fig. 1a, I-3 and I-4) and the siblings of the proband (Fig. 1a, II-6 and II-7); control DNA from nonrelated Korean healthy volunteers was collected after obtaining informed consent. The study was approved by the Institutional Review Board of Gachon University Gil Hospital in Incheon, Korea. Genomic DNA was extracted from 10 ml of peripheral blood using a standard method. All coding exons and intron–exon junctions for the *PTH*, *CaSR*, *GATA3* and *GCMB* genes were amplified and sequenced. The sequences of primers are available on request. The PCR products were electrophoresed through polyacrylamide gels and read in both directions using an ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA). DNA sequence abnormalities were also confirmed by digesting DNA with a restriction endonuclease. The T-to-C substitution at nucleotide 316 in exon 2 (c.316T>C) introduces a restriction site for the *Bst*UI enzyme. For PCR-RFLP analysis, a DNA fragment of exon 2, including the C106R mutation, was amplified using forward (5′-GGGCGAGTCGATTAACCTC-3′) and reverse (5′-TGAAGGAAGAAGGGGAAAATTAG-3′) primers with 35 cycles of PCR at 55 °C for 1 min, then digested with five units of *Bst*UI enzyme at 60 °C for 16 h. After digestion, the DNA fragments were separated on 1·5% agarose gel and visualized with ethidium bromide.

GCMB expression constructs

Using the plasmid encoding wild-type human GCMB as a template, the c.316T>C cysteine-to-arginine (C106R) mutation was introduced using site-directed mutagenesis, according to the manufacturer's protocol (Quikchange; Stratagene, Santa Clara, CA, USA). The resulting plasmid (designated C106R), as well as plasmids harbouring four previously reported mutations (R47L, G63S, c.1389delT and c.1399delC), was used for transient transfections. R47L and G63S were previously reported to lead to AR-HP[13,15], and c.1389delT and c.1399delC were identified in AD-HP and shown to possess dominant-negative properties.[9] All GCMB constructs were verified by direct nucleotide sequence analysis.

Western blot analysis of GCMB

HEK293T cells, cultured in Dulbecco's modified Eagle's Medium supplemented with 10% foetal bovine serum, were transiently transfected with plasmids encoding wild-type or mutant GCMB using LipofectAMINE according to the manufacturer's protocol (Invitrogen,

Carlsbad, CA, USA). After 48 h, cells were lysed and Western blot analysis of total cell extracts was performed using polyclonal antibodies detecting amino- and carboxy-terminal epitopes of GCMB (N-GCMB and C-GCMB, respectively[9]) following standard procedures.

Nuclear localization studies

HEK293 cells were seeded into 4-well plates and transiently transfected with GCMB expression constructs using LipofectAMINE. The cells were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature and then permeabilized with 0·5% Triton X-100 in PBS. Next, the cells were stained with GCMB-specific antibody and subsequently with an Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody (Invitrogen). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). After staining, fluorescence images were acquired using an LSM700 confocal microscope (Carl Zeiss, Thornwood, NY, USA).

Electrophoretic mobility shift assays

To assess the DNA-binding ability of the C106R mutant, electrophoretic mobility shift assays (EMSAs) was performed using a ^{32}P-labelled double-stranded probe containing GCMB recognition sites (5′-ATGCGGGT-3′). HEK293T cells were transiently transfected with either wild-type or mutant GCMB constructs. Forty-eight hours after transfection, nuclear protein was isolated, as previously described[22] and incubated with DNA probes for 30 min at room temperature. The mixture was loaded onto 6% native polyacrylamide gels and electrophoresed in 0·5× TBE buffer at 80 V for 75 min. The gels were dried and developed by autoradiography. The amounts of nuclear proteins were normalized with the level of NF1 protein. Competition assays using a 50-fold molar excess of unlabelled wild-type DNA probe or mutant DNA probe (5′-ATTCGGGT-3′) confirmed the specificity of the band. The super shift of the DNA-GCMB protein complex with anti-GCMB antibody, but not with IgG, also confirmed the specificity of the shifted band detected by EMSAs.

Luciferase reporter assay

To analyse the transcriptional activity of the GCMB mutants, luciferase assays were performed as described.[9] In brief, DF-1 fibroblasts were transiently transfected in triplicates in 24-well plates with the reporter construct pTATAluc-6xgbs[21,24] and plasmids encoding wild-type and mutant GCMB. The amount of DNA transfected is indicated in the figure. The data shown here represent the mean ± SEM for three separate experiments.

Homology modelling of GCMB DNA-binding domain structure

The three-dimensional crystal structure of the DNA-binding complex with DNA of the murine GCM1 has been previously reported.[25] The DNA-binding domains of murine GCM1 and human GCMB are 68% identical (82% similar). A structural model for the GCMB DNA-binding domain mutant C106R was generated using SWISS-MODEL.[26] The resulting model was superimposed on the GCM domain from the murine GCM1: DNA complex (PDB ID: 1ODH) to yield structural models for the GCMB C106R: DNA complex and the GCMB R110W: DNA complex. The final structural model was visualized by PyMOL program (Schrödinger, Portland, OR, USA).

Results

GCMB mutation in a family with AD-HP

The mode of inheritance in our family, that is mother and son being affected by HP, is consistent with an autosomal-dominant mode of inheritance (Fig. 1a). No variants in the DNA sequence of the genes encoding *PTH, GATA3* or *CaSR* were found in the proband

(data not shown). However, DNA sequence analysis of the entire 1521-bp coding region and associated splice sites of the *GCMB* gene revealed the presence of a heterozygous missense mutation (c.316T>C) because of a T → C substitution at nucleotide 316 in both patients (Fig. 1b). The novel *GCMB* mutation was confirmed by restriction site analysis with *Bst*UI (Fig. 1c). Amplified genomic DNA from four healthy family members, including the parents of the proband (Fig. 1a, individual I-3 and I-4), did not show the restriction digest pattern indicative of the mutation; instead only the pattern of wild-type DNA was observed, which was also confirmed by direct sequencing. This mutation was absent in 100 unrelated normal chromosomes from subjects of similar ethnicity, and this mutation was not found in public databases. These results indicate that the c.316T>C variant is unlikely to be a polymorphism.

This mutation leads to a change in amino acid at codon 106: a change of cysteine residue at codon 106, located in the DNA-binding domain, to arginine (C106R). This cysteine residue is conserved across species, for example, human, mouse, chicken, zebrafish, frog and drosophila (Fig. 1d). We hypothesized that the C106R mutation is able to disrupt formation of the GCMB DNA-binding domain: DNA complex and therefore performed *in vitro* and *in silico* experiments to prove this hypothesis.

Expression of GCMB in vitro

Western blot analysis of lysate of cells transiently transfected with plasmid encoding wild-type GCMB using the polyclonal N-GCMB antibody revealed a protein band of expected size of about 60 kDa (Fig. 2a); no immunoreactivity was observed in lysates from cells transfected with empty vector (v). Lysates from cells transfected with plasmids encoding the previously described mutants c.1389delT and c.1399delC showed a protein band that was slightly larger as expected from the addition of unrelated amino acids.[9] Lysates from cells expressing R47L, G63S and C106R showed a protein band that was similar in size to wild-type protein (Fig. 2a). A protein band of the expected size was also observed in lysates from cells expressing wild-type GCMB, R47L, G63S and C106R when the blot was reprobed with the C-GCMB antibody, which had been raised against a portion of the protein sequence that is replaced in c.1389delT and c.1399delC. Consistent with the specificity of this antibody, no protein band was detected in lysates from cells transfected with c.1389delT and c.1399delC (Fig. 2a).

Nuclear localization and DNA-binding studies

Nuclear localization of wild-type or mutant GCMB proteins was confirmed by immunofluorescence confocal microscopy. HEK293 cells were transfected with expression constructs of each GCMB proteins, fixed and stained with antibody against GCMB (red). Cell nuclei were stained with DAPI (blue). Nuclear localization (pink) was revealed by merging the GCMB and DAPI images (Fig. 2b).

We performed EMSAs using nuclear extracts from HEK293T cells transfected with constructs encoding wild-type or mutant GCMB to investigate the DNA-binding activities of the GCMB proteins. The C106R mutation involves a highly conserved residue within the GCMB DNA-binding domain (residues 19–174). EMSA assays demonstrated that C106R did not bind to the DNA consensus recognition motif, which is similar to R47L, whereas c. 1389delT did bind to the DNA consensus recognition motif as expected likely due to intact DNA-binding domain (Fig. 2c). The DNA-GCMB protein complex was blocked by adding a 50-fold molar excess of unlabelled wild-type DNA probe, but not when mutant DNA probe was added (Fig. 2c). The super shift of the DNA-GCMB protein complex was checked by adding GCMB antibody (positive control, GCMB antibody), but there was no shift when we added unrelated antibody (negative control, normal IgG) to DNA probe (Fig. 2c).

Luciferase reporter assays

DF-1 cells transiently transfected with plasmids encoding wild-type GCMB showed an approximately 35-fold higher luciferase activity than cells transfected with empty vector. Cells transfected either with the previously reported mutant c.1399delC or with the new mutation C106R showed a much reduced luciferase activity that was about 5-fold and 6-fold, respectively. DF-1 cells co-transfected with both wild-type and c.1399delC showed the previously reported dominant-negative property of that mutant. However, co-transfection of cells with plasmids encoding wild-type and C106R did not show a decrease in wild-type activity (Fig. 3).

Homology modellings of the GCMB DNA-binding domain mutant C106R: DNA complex and R110W: DNA complex

To model the effect of the C106R mutation, a homology-based structure of the mutant protein was generated (Fig. 4). The presence of the C106R mutation is predicted to cause a local conformational change in the GCMB-DNA complex, thus preventing complex formation. When the most commonly found rotamer was modelled for Arg-106, the side chain was in too close proximity to the target DNA in the GCMB: DNA complex, resulting in impairment of the DNA-binding ability of the GCMB protein. The presence of Arg-106 is likely to sterically hinder duplex formation, as Cys-106 (wild-type) has a smaller side chain than Arg-106 and does not appear to cause a less distorting or destabilizing effect than Arg-106. The presence of the R110W mutation is predicted to cause a local conformational change in the loop consisting of residues 103–107 (‘103–107 loop’) and the following helix containing R110W (‘110 helix’). In the current homology model, the side chain of Trp-110 would sterically clash with the 10–107 loop that is involved in DNA recognition, which in turn may promote a conformational change in the 110 helix. Such conformational changes in both the 103–107 loop and the 110 helix would impair the DNA-binding capability of the 103–107 loop, resulting in the loss of DNA binding by the GCMB protein.

Discussion

The pathogenesis of many forms of familial HP is not completely understood. Mutations in the *preproPTH* and *CaSR* genes influence PTH secretion, while mutations in GCMB might lead to parathyroid dysgenesis.[6,12,27] GCMB knockout mice have HP and parathyroid agenesis revealing a key role of the GCMB protein in foetal parathyroid development.[27] Moreover, *GCMB* gene is also abundantly expressed in adult human parathyroid gland, and GCMB is considered to be an important transcriptional factor, which transactivates *CaSR* gene.[23] Recently, it was reported that GCMB also regulates the expression of PTH in cooperation with MafB.[28]

Several *GCMB* gene mutations have been shown to cause familial isolated hypoparathyroidism (Fig. 5). Each of these mutations revealed different molecular and clinical characteristics (Table 1).[9,10,13,15,16,29,30] For example, the *GCMB* mutations that cause AR-HP (R39X, R47L, G63S, R110W and Y136X) all occur in the DNA-binding domain or result in a large deletion or no protein at all.[13–16,30] In contrast, the mutations leading to AD-HP (c.1389delC, c.1399delT, and N502H) are located in the transactivation domain 2.[9,10,23] However, the R110W mutation is associated with AD-HP as Tomar *et al.*[29] found heterozygosity for this amino acid change in 11 of 101 patients with isolated HP, but not in 655 controls. Heterozygous R110W did not affect DNA binding *in vitro* and the same mutation was identified in asymptomatic family members indicating either the R110W is very rare polymorphism or that additional genetic (e.g. the T370M change) or nongenetic factors contribute to this disease.[29]

In the present study, a novel missense *GCMB* mutation was identified in a Korean family with two individuals affected by AD-HP. This mutation was neither found in unaffected family members, nor in control DNA from 50 unrelated healthy Koreans. The identified heterozygous T-to-C substitution in exon 2 (c.316T>C) leads to a change in codon 106 from cysteine to arginine (C106R) located in the DNA-binding domain. The nucleotide substitution leading to this amino acid change seems to be *de novo*, because the parents of the proband do not harbour this substitution.

We have provided insight into the structural-functional relationships of the GCMB mutant protein through several approaches. Functional studies using nuclear localization and EMSA indicated that the C106R mutant GCMB did not have the ability to bind the consensus GCM recognition motif. Luciferase assays showed a significant decrease in luciferase activity compared with the wild-type protein. Therefore, the mutation could explain HP in this family; however, we could not demonstrate dominant-negative effects of C106R by co-transfection with wild-type GCMB in our *in vitro* system. Three possible explanations are considered as reasons for the absence of a dominant-negative effect by C106R: (i) the construct containing 6xgbs is artificial; using the natural GCMB promoter for luciferase reporter assays, if sufficiently strong, may provide evidence for a dominant-negative effect; (ii) the heterozygous C106R mutation may need to be combined additional, as-of-yet undefined mutations in the same gene (compound) or in other genes (digenic or polygenic), which may be similar to the requirements of the heterozygous R110W mutation to cause AD-HP.[29] In fact, C106R and R110W are predicted to cause conformational changes leading to the loss of DNA binding, and it is intriguing to note that patients who are heterozygous for either the R110W allele,[29] or, as described here, for the C106R allele develop AD-HP when *in vitro*, they act like recessive mutations. Wecan only speculate whether secondary stressors, either genetic (modifier genes) or nongenetic (environmental factors including diet), cause the HP to develop in these patients; and (iii) although theoretically possible, parent-specific genetic imprinting of GCMB appears very unlikely, as a similar mechanism would have to occur also in other AD-HP families, in which the disease is caused by heterozygous GCMB mutations. However, this form of the disease can be caused by maternal or paternal inheritance of c.1399delT, the mutation that eliminates transactivation domain 2, thus making allele-specific silencing highly unlikely.[9,10]

We also performed computer-based homology modelling analysis of the DNA-binding domain of the C106R (and R110W) mutant to reveal the possible underlying reason for the disruption of the DNA binding. The rotamer, which is a side chain of arginine, appears too close (spatially) to the DNA in the GCMB-DNA complex, thereby resulting in impairment in the DNA-binding ability of the GCMB protein.

Thus far, only three GCMB mutants have been reported causing AD-HP. The two single-nucleotide deletion mutants, leading to a shift in reading frame (c.1389delC and c. 1399delT), and the missense mutant (N502H) are located within the C-terminal transactivation domain 2 (amino acids 428–506); all three were shown to have a dominant-negative effect in luciferase reporter assays[9,10,23]. These results suggest that mutations in the transactivation domain 2 are important for the pathogenesis of AD-HP. However, unlike previous mutations, the C106R mutation is located in the N-terminal DNA-binding domain and is found in a family with autosomal-dominant HP. It is formally possible that mutations in a different gene cause HP in our family. However, the absence of this mutation in healthy family members and in normal controls of the same ethnic background, together with our demonstration of a loss of DNA-binding ability of the mutant protein by EMSAs and a striking reduction in transactivation capacity in luciferase reporter assays, makes an involvement of this mutation in the pathogenesis of HP in our family likely. A dominant-negative effect of the mutant protein could not be demonstrated *in vitro*. Further studies are

therefore necessary to reveal the exact mechanism by which C106R leads to HP; these may provide novel insights into the mechanisms through which GCMB regulates parathyroid function.

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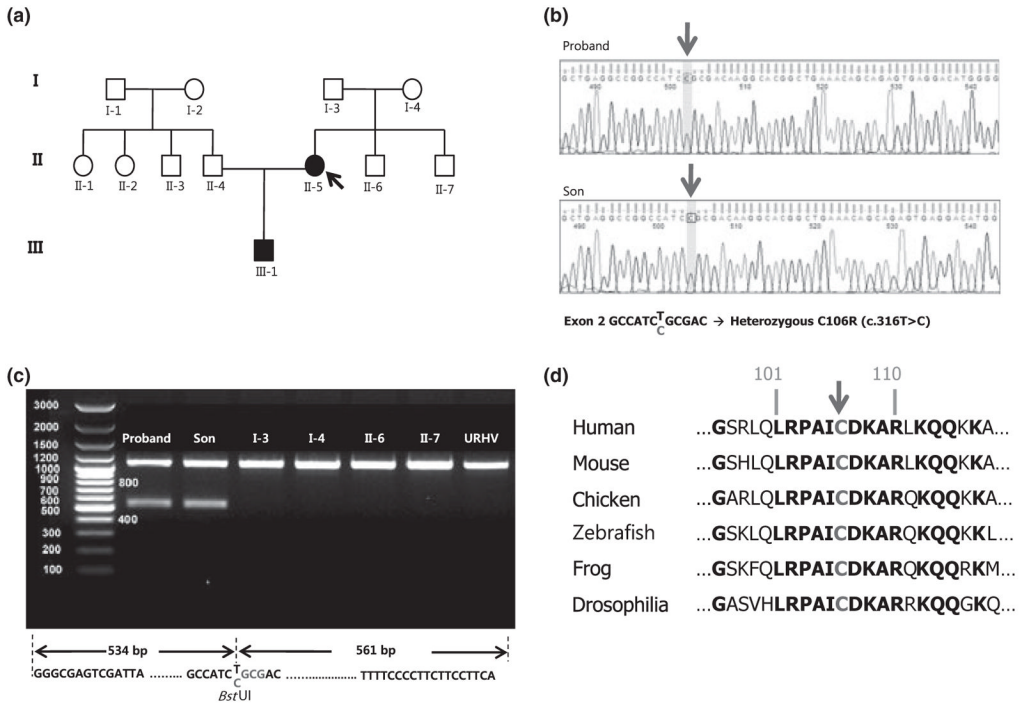

Fig. 1.

(a) Pedigree structure of the family with autosomal-dominant hypoparathyroidism (AD-HP). White symbols, unaffected; arrow, proband; black symbols, affected individuals. (b) Sequence chromatogram of parts of exon 2 of the *GCMB* gene from the proband (II-5) and her son (III-1) with HP showing presence of a heterozygous T-to-C transition (c.316T>C). The arrows in the chromatogram indicate the presence of heterozygous missense mutation because of T → C substitution at nucleotide 316 in both affected individuals. (c) PCR-RFLP analysis of the C106R mutation. PCR was used to amplify a 1095-bp fragment containing the c.316T>C mutant, which creates a new restriction site for BstUI, yielding 534- and 561-bp fragments when the mutation is present. Both patients show the presence of two bands, the 1095 bp wild-type band and a band between 500 and 600 bp corresponding to the mutant allele. Parents and siblings of the proband (I-3, I-4, II-6, and II-7) and 50 unrelated healthy individuals only showed the wild-type band (1095 bp) (49 of 50 unrelated healthy individuals' data not shown). Unrelated healthy volunteers. (d) Sequence alignment of human, mouse, chicken, zebrafish, frog and drosophila protein in DNA-binding domain. Conserved amino acids are expressed in bold; numbers indicate amino acid positions of human Glial cells missing B protein. The amino acid cysteine with the arrow was substituted with arginine in C106R mutation.

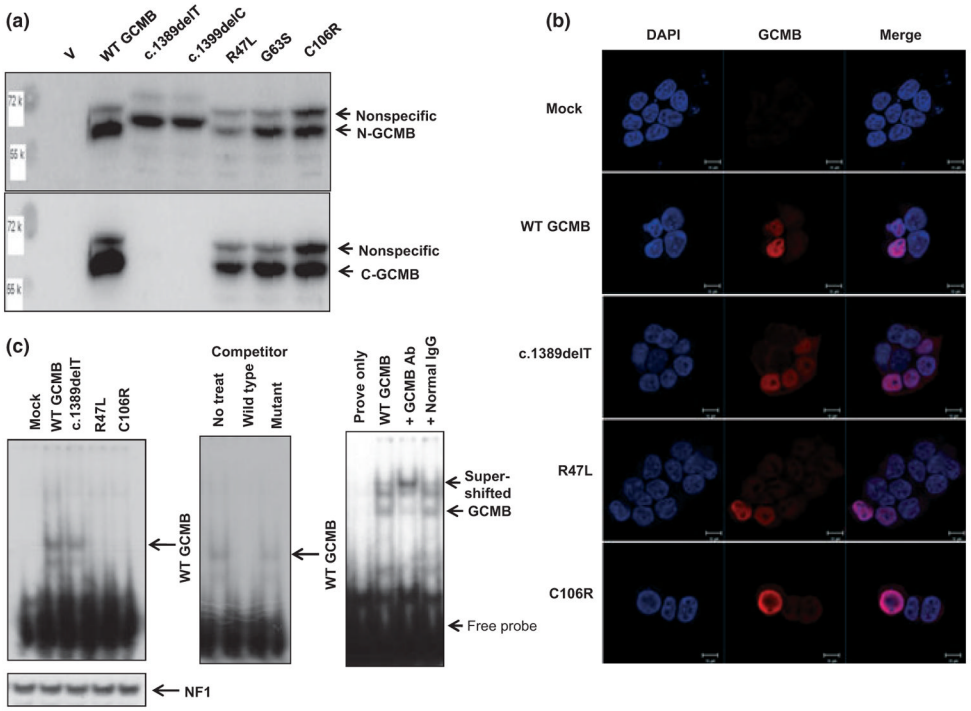

Fig. 2.

(a) Western blot analysis of cell lysates obtained from 293HEK cells transiently transfected with equal amounts of plasmids encoding Glial cells missing B (GCMB) wild-type or mutant proteins. Polyclonal antibodies raised against an amino-terminal peptide of GCMB were used in *upper panel* (N-GCMB), and antibodies raised against an carboxyl-terminal peptide of GCMB were used in *lower panel* (C-GCMB). GCMB has an apparent molecular size of about 60 kDa. The bands obtained with the previously described mutants c.1389delT and c.1399delC show slightly larger size (see text). C106R is detected by both N-GCMB and C-GCMB antibodies. The mutant proteins encoded by c.1389delT and c.1399delC are not detected by C-GCMB antibody, as expected. (b) Subcellular localization of wild-type or mutant GCMB proteins was confirmed by immunofluorescence confocal microscopy. HEK293 ells were transfected with expression constructs of each GCMB proteins, fixed and stained with antibody against GCMB (red). Cell nuclei were stained with DAPI (blue). Nuclear localization (pink) was revealed by merging the GCMB and DAPI images. Scale bars: 10 μm. (c) *left panel*, EMSAs showed the DNA-binding ability of wild-type GCMB protein (lane 2); the protein containing c.1389delT bound to the GCM-binding site of double-stranded DNA oligonucleotides (lane 3); however, the proteins containing R47L and C106R did not bind (lane 4 and 5), *middle panel*, the DNA-GCMB protein complex was blocked after addition of a 50-fold molar excess of unlabelled wild-type competitor to the binding reactions (lane 2); therefore, the result indicates that GCMB subunits are responsible for the observed band (lane 1). However, the DNA-GCMB protein complex was intact when we added mutant competitor (lane 3), *right panel*, DNA-protein super shift occurred after the addition of GCMB subunit-specific antibodies to the binding reactions (lane 3); therefore, the result indicates that GCMB subunits are responsible for the observed shifted bands. However, there was no shift when we added unrelated antibody (negative control, normal IgG) to DNA probes (lane 4). V, empty vector; WT, wild-type.

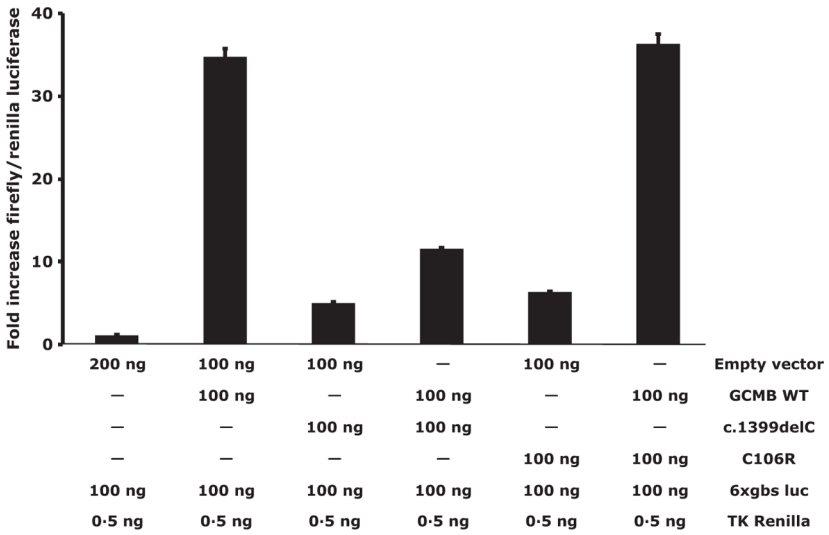

Fig. 3.

Luciferase reporter assay using wild-type and mutant Glial cells missing B (GCMB). DF-1 fibroblasts were transiently transfected with plasmids encoding empty vector, wild-type GCMB, the previously identified dominant-negative mutant c.1399delC or C106R. In addition, cells were co-transfected with plasmids encoding wild-type GCMB and different mutants, as indicated, to test for dominant-negative activity. Each well was co-transfected with plasmids encoding 6xgbs luc reporter and *Renilla*, those were used for normalization. Cells were harvested 48 h after transfection and assayed for luciferase activity. Results are shown as the means from three experiments, each performed with triplicate wells. Bars denote SEM. Luciferase activity obtained with empty plasmid was defined as 1.

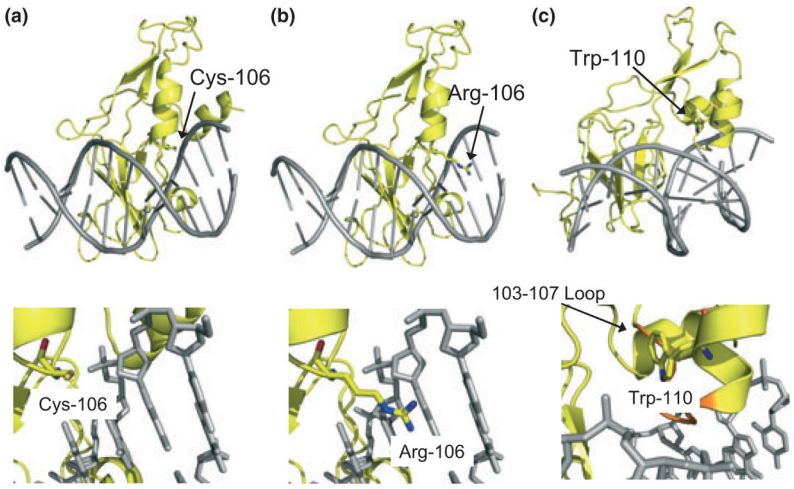

Fig. 4.

Three-dimensional models of the DNA-binding domain of wild-type GCMB (a), mutant Glial cells missing B (GCMB) C106R in complex with DNA (b) and mutant GCMB R110W in complex with DNA (c). The homology model shows the structural effect of the C106R GCMB mutant associated with autosomal-dominant hypoparathyroidism (AD-HP) in our family. The DNA-binding domain of GCMB is shown as ribbon representations with the side chain of Arg-106 in stick models. The target DNA is presented in stick models. The presence of Arg-106 is likely to disrupt the stacking interaction between bases in the DNA, leading to distortion or destabilization of the duplex structure. Cys-106, in contrast, has a smaller side chain than Arg-106 and is predicted to provide an ample space accommodating DNA. The presence of the R110W mutation is predicted to cause a local conformational change in the loop consisting of residues 103–107 (‘103–107 loop’) and the following helix containing R110W (‘110 helix’). The side chain of Trp-110 would sterically clash with the 103–107 loop that is involved in DNA recognition, which in turn may promote a conformational change in the 110 helix. Such conformational changes in both the 103–107 loop and the 110 helix would impair the DNA-binding capability of the 103–107 loop, resulting in the loss of DNA binding by the GCMB protein.

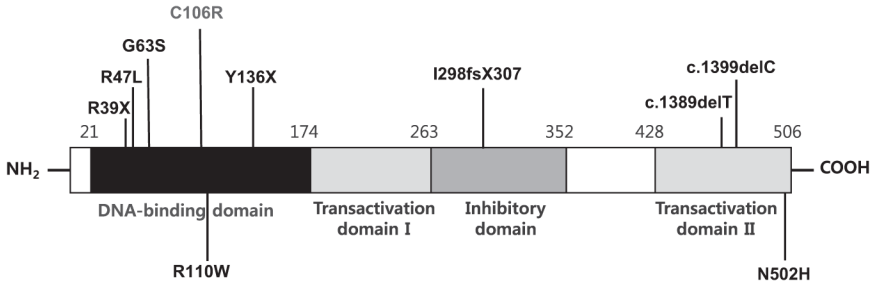

Fig. 5.

Schematic structure of Glial cells missing B (GCMB) protein. Mutations reported in the literature that were associated with familial hypoparathyroidism are indicated in addition to the C106R mutation identified in this study.

Table 1

Molecular characteristics of glial cells missing B (GCMB) mutants identified in patients with familial hypoparathyroidism and reported in the literature

Mutation	Nuclear localization	DNAbinding ability	Mode of inheritance	Luciferase reporter assay
R39X	–	–	AR	–
R47L	Localized	Decreased	AR	Decreased[1]
G63S	Localized	Unaltered	AR	Decreased[1]
C106R	Localized	Decreased	AD	Decreased[*]
R110W	Localized	Decreased	AR/AD	Decreased[1]
Y136X	–	–	AR	–
I298fsX307	Localized	Unaltered	AR	Decreased[1]
c.1389delT	Localized	Unaltered	AD	Decreased[2]
c.1399delC	–	–	AD	Decreased[2]
N502H	Localized	Unaltered	AD	Decreased[2]

AR, autosomal recessive; AD, autosomal dominant; HP, hypoparathyroidism; WT, wild-type.

Nuclear localization suggests unaltered protein expression after transfection with cDNA encoding mutant GCMB and can be confirmed by compact and intense nuclear staining by immunofluorescence.

The AR-HP causing mutations showed ‘Decreased[1]’ transactivation potential when transfected alone, that is mimicking an AR/homozygous state.

The AD-HP causing mutations showed ‘Decreased[2]’ transactivation potential when transfected with WT, that is mimicking an AD/heterozygous state. However, the C106R mutant only showed ‘Decreased[*]’ transactivation potential when transfected alone, that is mimicking an AR/homozygous state. It did not show decreased transactivation potential when transfected with WT, that is mimicking an AD/heterozygous state.