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Mutational analysis of GCMB, a parathyroid-specific transcription factor, in parathyroid adenoma of primary hyperparathyroidism

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Abstract

Sporadic primary hyperparathyroidism (PHPT), one of the most common endocrine disorders, is characterized by hypercalcemia and elevated PTH levels. The majority of cases are caused by a benign parathyroid adenoma, but somatic or *de novo* germ-line mutations that lead to adenoma formation have only been identified in few glands. GCMB is a parathyroid-specific transcription factor, which causes hypoparathyroidism when inactivated on both parental alleles or when a dominant-negative, heterozygous mutation is present. It is overexpressed in some parathyroid adenomas, and we therefore tested the hypothesis that GCMB mutations can be a cause of parathyroid adenomas. Nucleotide sequence analysis was performed on all coding exons and exon–intron borders of GCMB in 30 sporadic parathyroid adenomas and we identified several known polymorphisms that were either heterozygous or homozygous. In addition, one of the 30 investigated glands revealed a novel heterozygous missense mutation, c.1144G>A, which introduced methionine at position 382 for valine (V382M), a conserved amino acid residue. Western blot analysis using mutant GCMB (GCMB-V382M) from lysates of transiently transfected DF-1 fibroblasts, luciferase assays using extracts from these cells, and electrophoretic mobility assays failed to reveal differences between wild-type and mutant GCMB in expression level, transactivational capacity, and DNA-binding ability. Furthermore, pulse-chase experiments demonstrated no difference in half-life of wild-type and mutant protein. We conclude that mutations in the transcription factor GCMB do not seem to play a major role in the pathogenesis of PHPT.

Introduction

Primary hyperparathyroidism (PHPT) is a common endocrine disorder characterized by hypercalcemia and elevated PTH levels (Marx 2000). Many parathyroid adenomas are monoclonal in origin, indicating that they are derived from one single cell with a growth advantage (Arnold *et al*. 1988, 1995). However, in the majority of cases, the somatic or germ-line mutations leading to adenoma formation have not been identified. In fact, rearrangement and overexpression of the cyclin D1 gene (Motokura *et al*. 1991) and loss of the chromosomal region comprising the MEN1 tumor suppressor are the only known genetic

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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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defects leading to adenoma formation, which account for only a very small subset of parathyroid adenomas.

GCMB (one of the human orthologs of the *Drosophila* gene glial cells missing) is a transcription factor which is exclusively expressed during embryonic development in the parathyroid-specific domain that later gives rise to the parathyroid glands (Kim *et al.* 1998, Günther *et al.* 2000, Gordon *et al.* 2001). Inactivating or dominant-negative mutations have been found to cause familial forms of hypoparathyroidism that are autosomal recessive or autosomal dominant respectively (Ding *et al.* 2001, Baumber *et al.* 2005, Thomée *et al.* 2005, Mannstadt *et al.* 2008, Canaff *et al.* 2009, Bowl *et al.* 2010, Mirczuk *et al.* 2010). These findings provided insights into the importance of GCMB in parathyroid development and confirmed earlier observations in *Gcmb*-null mice, which lack parathyroid gland development (Günther *et al.* 2000). GCMB continues to be expressed in adult parathyroid glands where its postnatal function remains unknown. Overexpression of *Gcmb* mRNA has been reported in some parathyroid adenomas (Kebebew *et al.* 2004), making it plausible that *Gcmb* is a candidate gene, which contributes, if mutated, to the pathogenesis of parathyroid adenoma. We tested this hypothesis by sequencing its entire coding region in genomic DNA extracted from parathyroid adenoma of PHPT.

Materials and Methods

We studied 30 randomly selected surgically resected parathyroid adenomas from 30 patients with sporadic PHPT, who had undergone parathyroidectomy for the management of PHPT. Samples were obtained in accordance with protocols approved by the institutional review boards of the Massachusetts General Hospital, Boston. Samples were quickly frozen in liquid nitrogen and stored at −80 °C. Genomic DNA from ~30 mg tissue was extracted using proteinase K digestion followed by phenol–chloroform extraction and isopropanol precipitation as described earlier (Schipani *et al.* 1995). Intronic primers (Table 1) were used to amplify all coding exons and exon–intron borders of the gene encoding GCMB. PCRs were performed in 20 μl reaction volumes containing 50 ng genomic DNA, 20 pmol of each primer, 200 μM of each dNTP, 1·5 U Taq polymerase (Qiagen), and 2 mM $MgCl_2$. PCR conditions were as follows: 95 °C for 10 min; 35 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 10 min. PCR products were purified using ExoSap-It (Affymetrix, Santa Clara, CA, USA) and sequenced in forward and reverse directions using separate sequencing primers (Table 1). Nucleotide sequences were analyzed and compared to UCSC refseq sequence (ID: NM_004752) using the software Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA) and variants were compared to public databases.

The mutation c.1144G>A leading to a novel amino acid change, V382M, was further tested *in vitro*. The mutation was introduced into the pcDNA3.1-based plasmid encoding wild-type human GCMB by QuikChange (Stratagene, La Jolla, CA, USA), which was confirmed by nucleotide sequence analysis of the entire insert. Fibroblast DF-1 cells were transiently transfected in 6-well plates with 1 μg plasmid per well using FuGENE 6 (Roche Diagnostics) and western blot analysis was performed as described earlier on cell lysates using the polyclonal GCMB antibody N-GCMB (Mannstadt *et al.* 2008). Reprobing the blot with anti-vinculin or anti-actin monoclonal antibody (Sigma Chemical Co.) was used for loading control. Luciferase assays were carried out as described (Mannstadt *et al.* 2008). Briefly, DF-1 cells were transiently transfected in 24-well plates with the plasmids encoding wild-type GCMB, GCMB-V382M, the previously described dominant-negative mutant c. 1389delT (GCMB-DN; Mannstadt *et al.* 2008) or empty vector pcDNA3.1, as well as plasmids encoding the firefly reporter 6xgbs luc (a gift from Drs Hashemolhosseini and Wegner, Erlangen, Germany (Schreiber *et al.* 1997)) and, to allow for normalization of the

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data, plasmids encoding *Renilla* luciferase (pRL-TK; Promega). After 48 h of transfection, cells were harvested and assayed for luciferase activity using the Dual Luciferase Reporter Assay (Promega). Three experiments were carried out in triplicates and data are presented as mean±S.D. of all experiments.

To assess the ability of wild-type and mutant GCMB to bind to DNA, electrophoretic mobility shift assays (EMSA) were performed as described earlier (Demay *et al.* 1990). Briefly, 0·5 ng ^{32}P-labeled double-stranded wild-type GCMB recognition element (5′-GATCCCGATGCGGGTGCA-3′; Schreiber *et al.* 1998) was incubated with 5 μg nuclear extracts from COS7 cells transiently transfected with plasmids encoding GCMB-WT or GCMB-V382M, or empty vector in 12 μl buffer containing 20 mM HEPES pH 7·9, 1 mM dithiothreitol, 2 mM $MgCl_2$, 10% glycerol, 150 mM KCl, and 1 μg dIdC for 20 min at room temperature. For competition experiments, extracts were preincubated for 5 min with 5× or 50× molar excess of unlabeled wild-type GCMB recognition element (specific competitor), or a mutant thereof (5′-GATCCCGAT<u>T</u>CGGGTGCA-3′; mutant competitor; Schreiber *et al.* 1998). For supershift assays, extracts were preincubated for 5 min with 1 μl C-GCMB antibody (Mannstadt *et al.* 2008) or pre-immune serum as control. Binding reactions were electrophoresed through a non-denaturing polyacrylamide gel, which was dried under vacuum with heat and exposed to a film at –80 °C using an intensifying screen.

To study the half-life of GCMB-V382M protein and compare it with that of wild type, metabolic labeling studies were performed as described earlier (Tuerk *et al.* 2000). In brief, COS7 cells were pulse labeled for 60 min using ^{35}S-labeled methionine and ^{35}S-labeled cysteine at a specific activity of 110 μCi/ml. After repeated washes, cells were incubated (chased) with regular medium at varying time intervals (10, 20, 40, 120, and 360 min). Labeled cells were used for immunoprecipitation using C-GCMB antibody, followed by SDS–PAGE and autoradiography. Specific bands were quantified using densitometric analysis and results of three independent experiments are shown.

Results

All five exons encoding GCMB were readily amplified by PCR from genomic DNA (Fig. 1, which also shows the scheme of our PCR strategy) from each of the 30 parathyroid adenomas making large homozygous deletions of entire exons unlikely. Sequence analysis did not reveal frameshift or nonsense mutations. Several heterozygous or homozygous single nucleotide polymorphisms were identified that had been previously reported (Maret *et al.* 2008) or can be found in public databases (Table 2). One adenoma revealed the known c. 844T>G variant in exon 5 that leads to the amino acid substitution Y282D, which had previously failed to show evidence for functional abnormalities *in vitro* (Maret *et al.* 2008).

In one of the 30 adenomas, a novel heterozygous missense mutation, c.1144G>A, was identified in exon 5 (Fig. 2), leading to the substitution of an evolutionarily conserved valine at position 382 to methionine (V382M). The valine from different vertebrate species, including mouse, dog, chicken and *Xenopus tropicalis*, is found at all residues equivalent to human 382, with the exception of the zebrafish ortholog, which has an isoleucine at this position (I391). The mutation, which was not found in public databases, was confirmed by endonuclease digestion using the enzyme *Cvi*AII (data not shown). To study its functional consequences, we introduced this mutation in the expression vector pcDNA3.1 carrying human wild-type GCMB and designated the resulting plasmid ‘GCMB-V382M’. First, we examined whether the c.1144G>A mutation changes the efficiency of protein expression. DF-1 fibroblast were transiently transfected with plasmid encoding wild-type GCMB or equal amount of GCMB-V382M; empty vector was used as control. Western blot analysis using one of our previously reported polyclonal GCMB antibodies and lysates from these

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cells demonstrated a protein band that was identical in size and similar in intensity to the wild-type protein (Fig. 3). Densitometric analysis of specific bands of six independent experiments demonstrates that the intensity of the band obtained using GCMB-V382M was 93±3% (mean±S.E.M.) of wild-type, which were not statistically different from each other. This indicates that the identified amino acid change does not significantly impair GCMB expression. In addition, the effect of the mutation on transactivational activity of the transcription factor in luciferase assays was analyzed. Transient transfection of DF-1 cells with plasmids encoding wild type and GCMB-V382M showed a similarly robust, 16-fold increase in luciferase activity (Fig. 4). Because the mutation was present in only one allele, we also studied the effect of cotransfecting equal amounts of wild-type and mutant GCMB; as control, we used the previously described dominant-negative mutant GCMB-DN (Mannstadt *et al.* 2008, Canaff *et al.* 2009). Cotransfection of wild-type GCMB and GCMB-V382M did not change luciferase activity induced by the native GCMB protein. The previously reported dominant-negative GCMB mutant, c.1389delT, showed the expected reduction in transactivation activity of wild-type GCMB, therefore confirming the dominant-negative effect of this previously described mutant. EMSAs were performed to determine whether GCMB-V382M binds differently to DNA. As shown in Fig. 5, a protein–DNA complex was identified when using nuclear extracts from COS7 cells transiently transfected with GCMB-WT (lane 2), but not when using extracts from COS7 cells transfected with empty vector (lane 1). The complex was specifically disrupted by excess amounts of unlabeled oligonucleotide (lanes 3 and 4), but only poorly, if at all, by an oligonucleotide carrying a point mutation (lanes 5 and 6). The polyclonal antibody C-GCMB, which is directed against carboxyl-terminal sequences of human GCMB, supershifted the DNA–protein complex (lane 7). This change in electrophoretic mobility was not observed when using pre-immune serum (lane 8). The GCMB-V382M mutant showed binding characteristics that were indistinguishable from those of GCMB-WT (lanes 9–16).

Using metabolic labeling to analyze degradation of newly synthesized protein (Tuerk *et al.* 2000, Maret *et al.* 2008), we determined the half-life of GCMB-V382M, which was ~50 min and indistinguishable from the half-life of wild-type GCMB (Fig. 6).

Discussion

PHPT is one of the most common endocrine disorders with an incidence of ~1 in 1000. Its hallmark is hypercalcemia due to excessive secretion of PTH. In about 80% of the patients, it is caused by a benign, single parathyroid adenoma. Monoclonality has been documented for some investigated tissue samples from patients with PHPT making it likely that each adenoma is derived from one single cell that acquired a growth advantage. However, for most adenomas, the molecular mechanism allowing this monoclonal expansion remains to be determined. Two genetic alterations have been shown to play a role: overexpression of the cyclin D1 oncogene or inactivation of the menin tumor suppressor gene. Rearrangement that brings the cyclin D1 gene under the control of the PTH promoter leading to cyclin D1 overexpression has been found in three parathyroid adenomas (Motokura *et al.* 1991). Consistent with an important pathogenetic role of cyclin D1 in these rare cases, transgenic mice overexpressing cyclin D1 under the control of the PTH gene promoter developed abnormal parathyroid cell proliferation and chronic hyperparathyroidism (Imanishi *et al.* 2001). Likewise, somatic mutations in *Menin*, a tumor suppressor gene causing multiple endocrine neoplasia type 1 when inactivated, have been detected in parathyroid tumors (Heppner *et al.* 1997). However, these two genes seem to have a disease-causing effect in only a very small percentage of cases. Other genes have been studied for their role in the pathogenesis of parathyroid adenoma, including the 3′-UTR of the *PTH* gene (Costa-Guda *et al.* 2006), β-catenin (Costa-Guda & Arnold 2007), the p27 cyclin-dependent kinase

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inhibitor CDKN1B (Lauter & Arnold 2008), and the 25-hydroxyvitamin D-1α-hydroxylase (Lauter & Arnold 2009), but no causative mutations have been found.

We studied the transcription factor GCMB as a plausible candidate gene for its role in parathyroid adenoma development. There is a growing list of transcription factors that lead to tumor formation when mutated. Examples include CEBPA associated with acute myeloid leukemia (Smith *et al.* 2004), FOXL2 with granulosa-cell tumors of the ovary (Shah *et al.* 2009), and CCCTC-binding factor (CTCF) with a variety of tumors (Filippova *et al.* 2002). GCMB is unique in its expression pattern, because it is exclusively expressed in the parathyroid glands. It belongs to a small family of transcription factors with homology to the *Drosophila* gene glial cells missing. Human GCMB comprises 503 amino acids and contains an amino-terminal DNA-binding domain and two carboxy-terminal transactivation domains. In the mouse embryo, it is expressed as early as embryonic day 9·5 in the region of the third pharyngeal pouch that subsequently develops into the parathyroid glands (Gordon *et al.* 2001, Liu *et al.* 2007). GCMB is a master regulator of parathyroid gland development because homozygous *GCMB*-null mice lack parathyroid glands (heterozygous mice are phenotypically normal) and thus develop hypoparathyroidism, which results in considerable mortality shortly after birth (Günther *et al.* 2000). Patients with homozygous inactivating mutations or heterozygous dominant-negative GCMB mutations develop hypoparathyroidism, presumably from failure to develop parathyroid glands. Interestingly, GCMB continues to be expressed in the adult parathyroid glands, yet its physiological role after embryonic development is completely unclear. Likewise, target genes of GCMB have not been identified, with the exception of the calcium-sensing receptor (CaSR), which was reported to be downregulated after ‘knock-down’ of GCMB in primary human parathyroid cell cultures (Mizobuchi *et al.* 2009). Both promoters of the CaSR can be transactivated by GCMB (Canaff *et al.* 2009).

GCMB has been reported to be downregulated, unchanged, or upregulated in human parathyroid adenoma (Correa *et al.* 2002). Interestingly, the parathyroid adenoma of a recently described patient with PHPT revealed over 200-fold upregulation of GCMB expression, which together with PTH, belonged to the six most highly expressed genes in microarray analysis (Au *et al.* 2008). Besides the normal allele, a heterozygous mutation in the *PTH* gene leading to a truncated PTH molecule was identified in the patient's peripheral blood cells, while the parathyroid adenoma revealed only the presence of the mutant allele with evidence for deletion of the wild-type allele. It is plausible that continuous overexpression of GCMB contributed to the particularly large size (5 g) of the adenoma.

The unique expression of GCMB in the parathyroid gland, its continued expression in the adult parathyroids with an unclear role, as well as reports demonstrating expression and possible upregulation of this protein in parathyroid adenoma, make this transcription factor an excellent candidate for contribution to the adenoma formation in PHPT. Remarkably, GCMB contains a unique inhibitory domain located between amino acids 258 and 347, which significantly reduces the activity of the adjacent transactivation domains. Removal of the inhibitory domain resulted in a tenfold increase in transactivation activity *in vitro* (Tuerk *et al.* 2000). Mutations in the inhibitory domain could, therefore, directly fulfill our hypothesis that mutations leading to an activation of GCMB could play a causative role in parathyroid adenoma.

Our sequence analysis of GCMB in 30 parathyroid adenomas from patients with PHPT revealed several known polymorphisms in the gene encoding GCMB. We also identified one novel missense mutation in the last exon of GCMB, which was present in the heterozygous state in one parathyroid adenoma. However, western blot analysis, luciferase reporter assays to assess transactivational activity, metabolic labeling to determine protein half-life, and

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EMSA revealed properties of the GCMB-V382M mutant that were indistinguishable from those of the wild-type protein. Although these assays may not detect all relevant biological properties with sufficient sensitivity and/or specificity, we conclude that functionally important mutations in GCMB were absent in our samples. This suggests that mutations in this transcription factor contribute infrequently, if at all, to the pathogenesis of PHPT.

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References

Arnold A, Staunton CE, Kim HG, Gaz RD, Kronenberg HM. Monoclonality and abnormal parathyroid hormone genes in parathyroid adenomas. New England Journal of Medicine. 1988; 318:658–662. (doi:10.1056/NEJM198803173181102). [PubMed: 3344017]

Arnold A, Brown MF, Urena P, Gaz RD, Sarfati E, Drueke TB. Monoclonality of parathyroid tumors in chronic renal failure and in primary parathyroid hyperplasia. Journal of Clinical Investigation. 1995; 95:2047–2053. (doi:10.1172/JCI117890). [PubMed: 7738171]

Au AY, McDonald K, Gill A, Sywak M, Diamond T, Conigrave AD, Clifton-Bligh RJ. PTH mutation with primary hyperparathyroidism and undetectable intact PTH. New England Journal of Medicine. 2008; 359:1184–1186. (doi:10.1056/NEJMc0802570). [PubMed: 18784115]

Baumber L, Tufarelli C, Patel S, King P, Johnson CA, Maher ER, Trembath RC. Identification of a novel mutation disrupting the DNA binding activity of GCM2 in autosomal recessive familial isolated hypoparathyroidism. Journal of Medical Genetics. 2005; 42:443–448. (doi:10.1136/jmg.2004.026898). [PubMed: 15863676]

Bowl MR, Mirczuk SM, Grigorieva IV, Piret SE, Cranston T, Southam L, Allgrove J, Bahl S, Brain C, Loughlin J, et al. Identification and characterization of novel parathyroid-specific transcription factor glial cells missing homolog B (GCMB) mutations in eight families with autosomal recessive hypoparathyroidism. Human Molecular Genetics. 2010; 19:2028–2038. (doi:10.1093/hmg/ddq084). [PubMed: 20190276]

Canaff L, Zhou X, Mosesova I, Cole DE, Hendy GN. Glial cells missing-2 (GCM2) transactivates the calcium-sensing receptor gene: effect of a dominant-negative GCM2 mutant associated with autosomal dominant hypoparathyroidism. Human Mutation. 2009; 30:85–92. (doi:10.1002/humu.20827). [PubMed: 18712808]

Correa P, Akerström G, Westin G. Underexpression of Gcm2, a master regulatory gene of parathyroid gland development, in adenomas of primary hyperparathyroidism. Clinical Endocrinology. 2002; 57:501–505. (doi:10.1046/j.1365-2265.2002.01627.x). [PubMed: 12354132]

Costa-Guda J, Arnold A. Absence of stabilizing mutations of beta-catenin encoded by CTNNB1 exon 3 in a large series of sporadic parathyroid adenomas. Journal of Clinical Endocrinology and Metabolism. 2007; 92:1564–1566. (doi:10.1210/jc.2006-2554). [PubMed: 17284619]

Costa-Guda J, Lauter K, Naveh-Many T, Silver J, Arnold A. Mutational analysis of the PTH 3′-untranslated region in parathyroid disorders. Clinical Endocrinology. 2006; 65:806–809. (doi:10.1111/j.1365-2265.2006.02670.x). [PubMed: 17121534]

Demay MB, Gerardi JM, DeLuca HF, Kronenberg HM. DNA sequences in the rat osteocalcin gene that bind the 1,25-dihydroxyvitamin D_3 receptor and confer responsiveness to 1,25-dihydroxyvitamin D_3. PNAS. 1990; 87:369–373. (doi:10.1073/pnas.87.1.369). [PubMed: 2153298]

Ding C, Buckingham B, Levine MA. Familial isolated hypoparathyroidism caused by a mutation in the gene for the transcription factor GCMB. Journal of Clinical Investigation. 2001; 108:1215–1220. (doi:10.1172/JCI13180). [PubMed: 11602629]

Filippova GN, Qi CF, Ulmer JE, Moore JM, Ward MD, Hu YJ, Loukinov DI, Pugacheva EM, Klenova EM, Grundy PE, et al. Tumor-associated zinc finger mutations in the CTCF transcription factor

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selectively alter tts DNA-binding specificity. Cancer Research. 2002; 62:48–52. [PubMed: 11782357]

Gordon J, Bennett AR, Blackburn CC, Manley NR. Gcm2 and Foxn1 mark early parathyroid- and thymus-specific domains in the developing third pharyngeal pouch. Mechanisms of Development. 2001; 103:141–143. (doi:10.1016/S0925-4773(01)00333-1). [PubMed: 11335122]

Günther T, Chen ZF, Kim J, Priemel M, Rueger JM, Amling M, Moseley JM, Martin TJ, Anderson DJ, Karsenty G. Genetic ablation of parathyroid glands reveals another source of parathyroid hormone. Nature. 2000; 406:199–203. (doi:10.1038/35018111). [PubMed: 10910362]

Heppner C, Kester MB, Agarwal SK, Debelenko LV, Emmert-Buck MR, Guru SC, Manickam P, Olufemi SE, Skarulis MC, Doppman JL, et al. Somatic mutation of the MEN1 gene in parathyroid tumours. Nature Genetics. 1997; 16:375–378. (doi:10.1038/ng0897-375). [PubMed: 9241276]

Imanishi Y, Hosokawa Y, Yoshimoto K, Schipani E, Mallya S, Papanikolaou A, Kifor O, Tokura T, Sablosky M, Ledgard F, et al. Primary hyper-parathyroidism caused by parathyroid-targeted overexpression of cyclin D1 in transgenic mice. Journal of Clinical Investigation. 2001; 107:1093–1102. (doi:10.1172/JCI10523). [PubMed: 11342573]

Kebebew E, Peng M, Wong MG, Ginzinger D, Duh QY, Clark OH. GCMB gene, a master regulator of parathyroid gland development, expression, and regulation in hyperparathyroidism. Surgery. 2004; 136:1261–1266. (doi:10.1016/j.surg.2004.06.056). [PubMed: 15657585]

Kim J, Jones BW, Zock C, Chen Z, Wang H, Goodman CS, Anderson DJ. Isolation and characterization of mammalian homologs of the Drosophila gene glial cells missing. PNAS. 1998; 95:12364–12369. (doi:10.1073/pnas.95.21.12364). [PubMed: 9770492]

Lauter KB, Arnold A. Mutational analysis of CDKN1B, a candidate tumor-suppressor gene, in refractory secondary/tertiary hyperparathyroidism. Kidney International. 2008; 73:1137–1140. (doi:10.1038/ki.2008.28). [PubMed: 18288099]

Lauter K, Arnold A. Analysis of CYP27B1, encoding 25-hydroxyvitamin D-1alpha-hydroxylase, as a candidate tumor suppressor gene in primary and severe secondary/tertiary hyperparathyroidism. Journal of Bone and Mineral Research. 2009; 24:102–104. (doi:10.1359/jbmr.080903). [PubMed: 18767934]

Liu Z, Yu S, Manley NR. Gcm2 is required for the differentiation and survival of parathyroid precursor cells in the parathyroid/thymus primordia. Developmental Biology. 2007; 305:333–346. (doi:10.1016/j.ydbio.2007.02.014). [PubMed: 17382312]

Mannstadt M, Bertrand G, Muresan M, Weryha G, Leheup B, Pulusani SR, Grandchamp B, Jüppner H, Silve C. Dominant-negative GCMB mutations cause an autosomal dominant form of hypoparathyroidism. Journal of Clinical Endocrinology and Metabolism. 2008; 93:3568–3576. (doi:10.1210/jc.2007-2167). [PubMed: 18583467]

Maret A, Ding C, Kornfield SL, Levine MA. Analysis of the GCM2 gene in isolated hypoparathyroidism: a molecular and biochemical study. Journal of Clinical Endocrinology and Metabolism. 2008; 93:1426–1432. (doi:10.1210/jc.2007-1783). [PubMed: 18182452]

Marx SJ. Hyperparathyroid and hypoparathyroid disorders. New England Journal of Medicine. 2000; 343:1863–1875. (doi:10.1056/NEJM200012213432508). [PubMed: 11117980]

Mirczuk SM, Bowl MR, Nesbit MA, Cranston T, Fratter C, Allgrove J, Brain C, Thakker RV. A missense glial cells missing homolog B (GCMB) mutation, Asn502His, causes autosomal dominant hypoparathyroidism. Journal of Clinical Endocrinology and Metabolism. 2010; 95:3512–3516. (doi:10.1210/jc.2009-2532). [PubMed: 20463099]

Mizobuchi M, Ritter CS, Krits I, Slatopolsky E, Sicard G, Brown AJ. Calcium-sensing receptor expression is regulated by glial cells missing-2 in human parathyroid cells. Journal of Bone and Mineral Research. 2009; 24:1173–1179. (doi:10.1359/jbmr.090211). [PubMed: 19257819]

Motokura T, Bloom T, Kim HG, Jüppner H, Ruderman JV, Kronenberg HM, Arnold A. A novel cyclin encoded by a bcl1-linked candidate oncogene. Nature. 1991; 350:512–515. (doi: 10.1038/350512a0). [PubMed: 1826542]

Schipani E, Weinstein LS, Bergwitz C, Iida-Klein A, Kong XF, Stuhrmann M, Kruse K, Whyte MP, Murray T, Schmidtke J, et al. Pseudohypoparathyroidism type Ib is not caused by mutations in the coding exons of the human parathyroid hormone (PTH)/PTH-related peptide receptor gene.

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Journal of Clinical Endocrinology and Metabolism. 1995; 80:1611–1621. (doi:10.1210/jc.80.5.1611). [PubMed: 7745008]

Schreiber J, Sock E, Wegner M. The regulator of early gliogenesis glial cells missing is a transcription factor with a novel type of DNA-binding domain. PNAS. 1997; 94:4739–4744. (doi:10.1073/pnas.94.9.4739). [PubMed: 9114061]

Schreiber J, Enderich J, Wegner M. Structural requirements for DNA binding of GCM proteins. Nucleic Acids Research. 1998; 26:2337–2343. (doi:10.1093/nar/26.10.2337). [PubMed: 9580683]

Shah SP, Kobel M, Senz J, Morin RD, Clarke BA, Wiegand KC, Leung G, Zayed A, Mehl E, Kalloger SE, et al. Mutation of FOXL2 in granulosa-cell tumors of the ovary. New England Journal of Medicine. 2009; 360:2719–2729. (doi:10.1056/NEJMoa0902542). [PubMed: 19516027]

Smith ML, Cavenagh JD, Lister TA, Fitzgibbon J. Mutation of CEBPA in familial acute myeloid leukemia. New England Journal of Medicine. 2004; 351:2403–2407. (doi:10.1056/NEJMoa041331). [PubMed: 15575056]

Thomée C, Schubert SW, Parma J, Le PQ, Hashemolhosseini S, Wegner M, Abramowicz MJ. GCMB mutation in familial isolated hypoparathyroidism with residual secretion of parathyroid hormone. Journal of Clinical Endocrinology and Metabolism. 2005; 90:2487–2492. (doi:10.1210/jc.2004-2450). [PubMed: 15728199]

Tuerk EE, Schreiber J, Wegner M. Protein stability and domain topology determine the transcriptional activity of the mammalian glial cells missing homolog, GCMb. Journal of Biological Chemistry. 2000; 275:4774–4782. (doi:10.1074/jbc.275.7.4774). [PubMed: 10671510]

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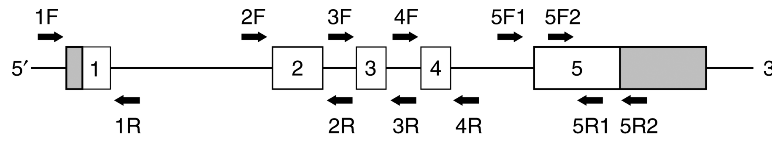

Figure 1.

GCMB gene structure and sequencing strategy. Genomic structure of human GCMB (not to scale) with exons boxed and untranslated regions in gray. Amplification primers are marked with arrows. See Table 1 for sequences of amplification and sequencing primers and PCR product sizes.

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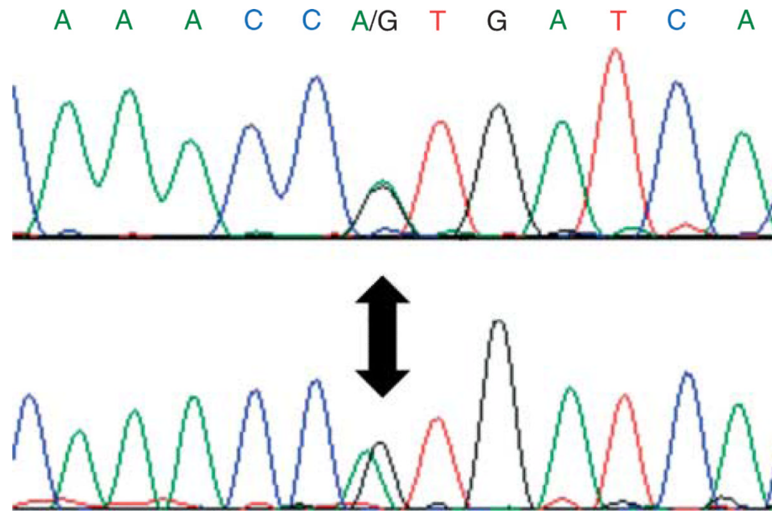

Figure 2.
Nucleotide sequence analysis of GCMB using genomic DNA from one of the investigated parathyroid adenoma. A portion of the nucleotide sequence of exon 5 is shown. The arrow indicates the heterozygous transition G>A at position 1144 in exon 5 of the cDNA (c. 1144G>A), which causes a substitution of an evolutionary conserved valine at position 382 to methionine (V382M). Corresponding chromatograms of the 5′ to 3′ (upper panel) and 3′ to 5′ sequences (lower panel) are shown.

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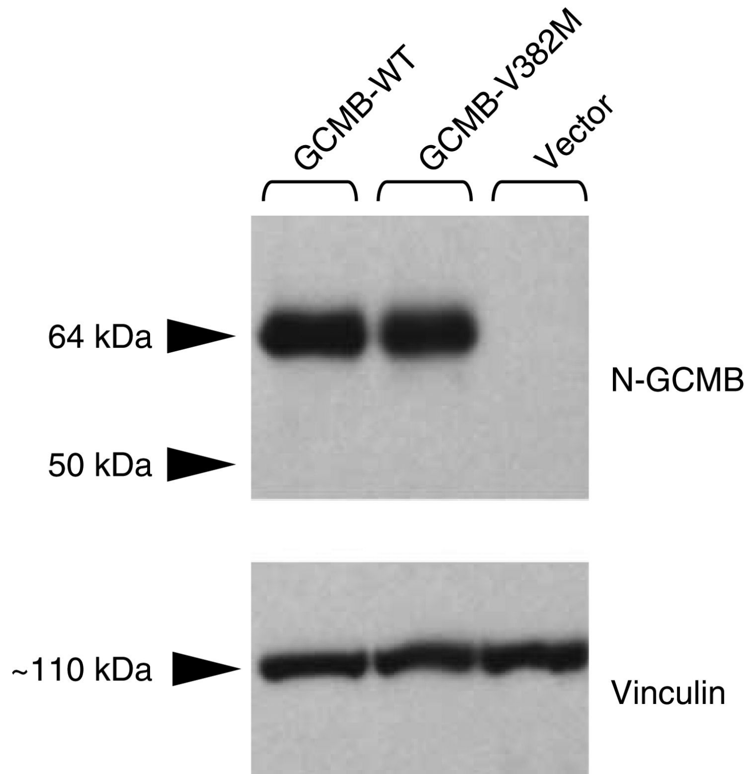

Figure 3.

Western blot analysis using polyclonal antibody against human GCMB and lysates from transiently transfected fibroblasts. Chicken DF-1 cells were transiently transfected with wild-type GCMB (lane 1), GCMB-V382M (lane 2), or empty vector (lane 3). Cells were lysed with SDS–PAGE sample buffer and cellular proteins were separated on an 8% SDS–PAGE under reducing conditions; size marker: SeeBlue Plus2 (Invitrogen). After transfer onto a PDVF membrane and blocking with 5% non-fat milk, the GCMB antibody N-GCMB (upper panel) was added at a final dilution of 1:5000 for 1 h at room temperature. The second antibody was HRP-conjugated goat anti-rabbit IgG and the blot was visualized by chemiluminescence. After stripping, the blot was reprobed with an antibody against vinculin to assess whether similar amounts of protein had been loaded (lower panel). Using antibody N-GCMB, protein bands of the expected sizes were detected in lysates from cells expressing wild-type GCMB and the mutant, but not in lysates from cells transfected with empty vector.

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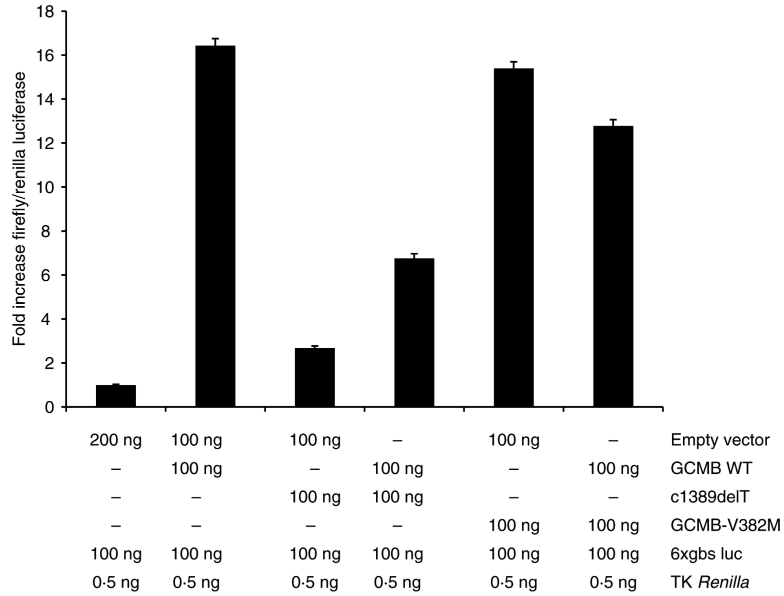

Figure 4.

Luciferase reporter assay using chicken fibroblast DF-1 cells. Cells were co-transfected with plasmids encoding empty vector, wild-type human GCMB (GCMB WT), the dominant-negative c.1389delT mutant (Mannstadt *et al.* 2008), the identified GCMB-V382M mutant, or a combination as indicated; amount of the different plasmids transfected per well is indicated below each graph; each well was furthermore co-transfected with 100 ng/well of 6xgbs luc reporter plasmid and with 0·5 ng/well of plasmid encoding *Renilla* that was used for normalization. Results are shown as the means from three experiments, each performed in triplicate wells; bars denote s.d. Luciferase activity obtained with empty plasmid was defined as 1.

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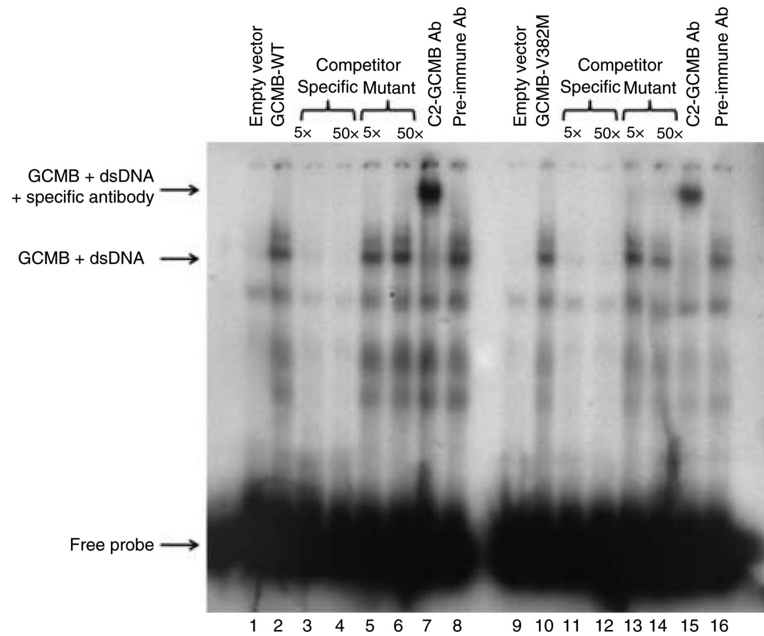

Figure 5.

Electrophoretic mobility shift assay using nuclear extracts prepared from transiently transfected COS7 cells and ^{32}P-labeled double-stranded (ds) oligonucleotides. Lanes 1 and 9, extracts from COS7 cells transfected with empty vector as control; lanes 2–8 and lanes 10–16, extracts from COS7 cells expressing GCMB-WT and GCMB-V382M respectively. Lanes 3, 4, 11, and 12, unlabeled oligonucleotide, which corresponds to the DNA recognition site of GCMB, was used in molar excess as indicated as a specific competitor; lanes 5, 6, 13, and 14, unlabeled oligonucleotide corresponding to a mutated DNA recognition site of GCMB was used as a nonspecific (mutant) competitor; lanes 7 and 15, supershift assay using polyclonal C-GCMB antibody; lanes 8 and 16, supershift assay using pre-immune serum as negative control. Free probe, complex of GCMB and ^{32}P-labeled oligonucleotide, and complex of GCMB, oligonucleotide, and specific antibody are indicated (arrow).

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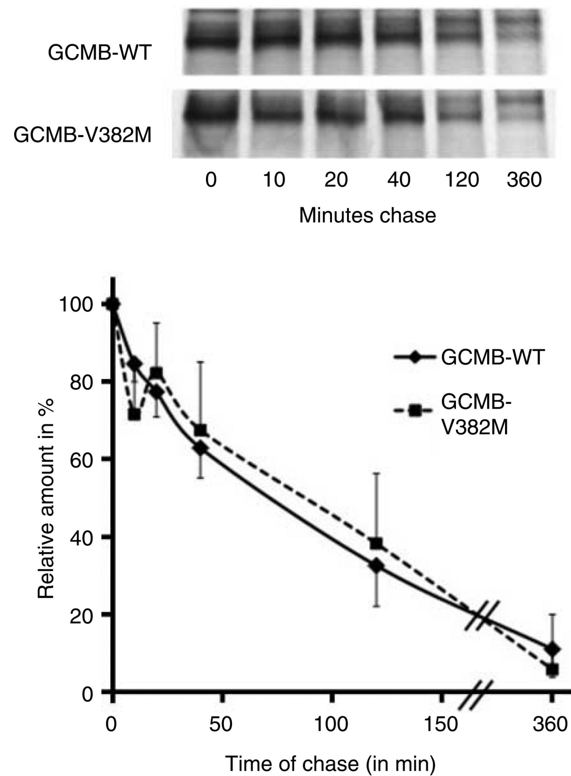

Figure 6.

Protein half-life studies using metabolic labeling. COS7 cells transiently transfected with plasmids encoding GCMB-WT or GCMB-V382M were pulse-labeled for 1 h with ^{35}S-labeled methionine and ^{35}S-labeled cysteine and incubated (chased) for 10, 20, 40, 120, and 350 min. GCMB proteins were immunoprecipitated using C-GCMB antibody and the precipitates were separated on an SDS polyacrylamide gel followed by autoradiography. Upper panel: representative autoradiogram. Lower panel: densitometrical analyses derived from three different, independent experiments (mean±S.D.); data are expressed relative to the amount at time 0. Solid line, GCMB-WT; dashed line, GCMB-V382M.

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Table 1

Primers used for PCR amplification and for nucleotide sequence analysis, and expected size of the PCR products

PCR primers		Product	Sequencing primers
Exon 1	F 5′-CCTTCACACACCCCACTTTC-3′	295	F 5′-CACCTGGCGCACCTGTC-3′
	R 5′-TCCGCAGACTCTTCAAGAAC-3′		R 5′-AATGCCATTCTCCCTCCTTC-3′
Exon 2	F 5′-GGGCGAGTCGATTAACCTC-3′	1095	F 5′-ATGTGTGCAGTTTGGACTGG-3′
	R 5′-TGAAGGAAGAAGGGGAAAATTAG-3′		R 5′-AAAACTCATGACTCCAAGGTCAC-3′
Exon 3	F 5′-TTCTTTGTTCCCAGCTAATTTTC-3′	302	F 5′-CTTCAGAAGGGTCTGGGGTC-3′
	R 5′-TGTATTTTGTTTGGCCCAGG-3′		R 5′-GTTTGGCCTTTGTGGTCTG-3′
Exon 4	F 5′-CTTGAGATGTTGGGAAAGGC-3′	305	F 5′-TTGGGCAACATTGTCAGC-3′
	R 5′-TGACCTTCATATTTTGCATAACG-3′		R 5′-AACGATCAGCGTATCTTGGG-3′
Exon 5 1	F 5′-AAGCTTAGCAACCCCTGGAC-3′	716	F 5′-TTGTTACAGGTGAGGGTGTCC-3′
	R 5′-CTGGTGGTGGAGTCGTGAG-3′		R 5′-GTACCTGCAGGGAAGCTCTG-3′
Exon 5 2	F 5′-TTCACCAACAAACAGCATGG-3′	753	F 5′-GGAAACCAGCTCTTGGAAAAC-3′
	R 5′-GCCAGTTTCAAAATGCTGTG-3′		R 5′-TCCAACTGATTATTTTCTCAGTTACTC-3′

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Table 2

Variants identified in 30 parathyroid adenomas. All variants were heterozygous except c. – 74C>T, which was homozygous in all samples

Location	Variant	Number of glands with nucleotide change
5′ of exon 1	c. – 74C>T	30
5′-UTR	c. – 44T>C	1
Intron 1	IVS1 – 271A>G	5
Intron 1	IVS1 – 242G>A	9
Intron 2	IVS2 + 163G>A	8
Exon 5	c.844T>G	1
Exon 5	c.1144G>A	1