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 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 Gmb-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 Gmb mRNA has been reported in some parathyroid adenomas (Kebebew et al. 2004), making it plausible that Gmb 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 MgCl2. 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 universal 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). Reproducing the blot with anti-vinulbin 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 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, 5′-32P-labeled double-stranded wild-type GCMB recognition element (5′-GATCCCCGATGCG-GGTGCA-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 MgCl2, 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′-GATCCCCGATGCGGTGCA-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.

**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.

**Table 1** Primers used for PCR amplification and for nucleotide sequence analysis, and expected size of the PCR products

<table>
<thead>
<tr>
<th>PCR primers</th>
<th>Product</th>
<th>Sequencing primers</th>
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<tbody>
<tr>
<td>Exon 1</td>
<td>295</td>
<td>F 5'-GCCAGTTTCAAAATGCTGAG-3′</td>
</tr>
<tr>
<td>Exon 2</td>
<td>1095</td>
<td>R 5'-TGGTGGTGGAGTCGTGAG-3′</td>
</tr>
<tr>
<td>Exon 3</td>
<td>302</td>
<td>F 5'-GATCCCCGATGCG-GGTGCA-3′</td>
</tr>
<tr>
<td>Exon 4</td>
<td>305</td>
<td>R 5'-GATCCCCGATGCG-GGTGCA-3′</td>
</tr>
<tr>
<td>Exon 5 1</td>
<td>716</td>
<td>F 5'-GCCAGTTTCAAAATGCTGAG-3′</td>
</tr>
<tr>
<td>Exon 5 2</td>
<td>753</td>
<td>R 5'-GCCAGTTTCAAAATGCTGAG-3′</td>
</tr>
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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 (Tuirk 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 CvaII (data not shown). To study its functional consequences, we introduced this mutation in the expression vector pCNA3.1 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 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 reported dominant-negative GCMB mutant (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

### Table 2

<table>
<thead>
<tr>
<th>Location</th>
<th>Variant</th>
<th>Number of glands with nucleotide change</th>
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<tbody>
<tr>
<td>5' of exon 1</td>
<td>c.−74C&gt;T</td>
<td>30</td>
</tr>
<tr>
<td>5'-UTR</td>
<td>c.−44T&gt;C</td>
<td>1</td>
</tr>
<tr>
<td>Intron 1</td>
<td>IVS1−271A&gt;G</td>
<td>5</td>
</tr>
<tr>
<td>Intron 1</td>
<td>IVS1−242G&gt;A</td>
<td>9</td>
</tr>
<tr>
<td>Intron 2</td>
<td>IVS2+163G&gt;A</td>
<td>8</td>
</tr>
<tr>
<td>Exon 5</td>
<td>c.844T&gt;G</td>
<td>1</td>
</tr>
<tr>
<td>Exon 5</td>
<td>c.1144G&gt;A</td>
<td>1</td>
</tr>
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</table>
Due to excessive secretion of PTH, 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 (Motoyama 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 (Hepner 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 inhibitor CDKN1B (Lauter & Arnold 2008), and cyclin D1 overexpression has been found in three parathyroid adenomas (Motokura et al. 2001).

Using metaphase labeling to analyze degradation of newly synthesized protein (Tuork 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).
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 transactivation activity, metabolic labeling to determine protein half-life, and EMSA 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.

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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