Ectopic Production of Parathyroid Hormone in a Patient with Sporadic Medullary Thyroid Cancer

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Abstract. Elevation of serum parathyroid hormone (PTH) in patients with medullary thyroid cancer (MTC) is usually found in multiple endocrine neoplasia type 2A (Men2a). Ectopic production of PTH is rare and its molecular etiology remains largely uninvestigated. We report a case of ectopic production of PTH by a sporadic MTC. The etiology of ectopic PTH gene expression was examined, focusing on gCM2 which has a crucial role in developing parathyroid glands. We observed ectopic expression of the PTH and gCM2 genes in tissues from the tumor and metastatic lymph nodes. However, gCM2 gene expression was also detected in adjacent thyroid tissue and lymphoblasts, in which PTH gene expression was absent. Hypomethylation of the PTH promoter, which is reportedly associated with ectopic production of PTH, was not seen in either the tumor tissue or metastatic lymph nodes. Meanwhile, DNA hypomethylation was seen in a CpG island identified in the gCM2 promoter region, irrespective of the gCM2 gene expression status. We showed that transcriptional activity of the CpG island sequences cloned into a reporter plasmid was dependent upon DNA methylation. Finally, we present the first report of a PTH-producing MTC. There was no apparent association between ectopic PTH and gCM2 gene expression, despite co-expression of the two genes. Neither genomic rearrangement nor DNA hypomethylation in the PTH gene appeared responsible for ectopic production of PTH. Although DNA hypomethylation may be necessary for the gCM2 gene expression, ectopic expression of gCM2 won’t be possible by DNA hypomethylation alone.

Key words: PTH, gCM2, Medullary thyroid cancer, DNA methylation
femoral vein. These results suggested a combined MTC and parathyroid adenomas. The cytological diagnosis of MTC was confirmed after total thyroidectomy, with the lesion area suspected of being parathyroid adenomas diagnosed histologically as metastatic lymph nodes of the MTC (Fig. 1C). The tumor and metastatic lymph nodes histologically included no parathyroid gland, neither C-cell hyperplasia nor parathyroid adenoma was seen. no hyperplasia was observed in resected parathyroid tissues. she had no family history of MTC. genetic tests revealed a somatic mutation in the reT proto-oncogene occurring only in the tumor and metastatic lymph nodes, confirming a sporadic MTC with lymph node metastases (Figs. 2a and B).

A normal parathyroid gland at right upper pole of the thyroid was implanted in the forearm. The increased PTh concentration dropped down to a low level shortly after surgery, resulting in permanent hypocalcemia requiring long-term supplementation [<5-7.3 pg/mL (reference range, 12-60 pg/mL)].

Materials and Methods

Patient Profile
A 43-yr-old female patient was admitted to our department in May 2000 for investigation of a thyroid nodule in the left lobe associated with an increased serum calcitonin level [799.1 pg/mL (reference range, 17.1-58.7 pg/mL)]. Laboratory testing revealed an elevated parathyroid hormone level, indicating hyperparathyroidism. Ultrasound and computed tomography indicated no evidence of parathyroid enlargement, although Tc-99m-MIBI scintigraphy demonstrated foci of uptake just below the thyroid nodule, suspicious of a parathyroid adenoma (Table 1) (Fig. 1A). Moreover, selective venous sampling implied increased levels of PTH surrounding the MIBI foci (Fig. 1B). The selective sampling from cervical veins was performed after access through the right

<table>
<thead>
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<th>Table 1. Laboratory findings</th>
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<tr>
<td><strong>Calcitonin (pg/mL)</strong></td>
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</tr>
<tr>
<td>CEA (ng/mL)</td>
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<td>ProGRP (pg/mL)</td>
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<td>Mg (mg/dL)</td>
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<td>Intact PTH (pg/mL)</td>
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<td>C-PTHrP (pmol/L)</td>
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<td>Intact PTHrP (pmol/L)</td>
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<tr>
<td>1,25-dihydroxyvitamin D₃ (pg/mL)</td>
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<td>Adrenaline (ng/mL)</td>
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<td>Noradrenaline (ng/mL)</td>
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<td>Somatostatin (pg/mL)</td>
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CEA, carcinoembryonic antigen; proGRP, progastrin-releasing peptide; TRP, tubular reabsorption of phosphate; TmP/GFR, tubular maximal reabsorption rate of phosphate to GFR; C-PTHrP, C-terminal-region fragments of parathyroid-related peptide; PTHrP, parathyroid hormone-related peptide.
Immunohistochemistry of PTh and calcitonin was performed using standard techniques on formalin-fixed and paraffin-embedded (FFPE) sections of the thyroid specimen obtained at surgery. Anti-calcitonin and anti-PTh (1-34) (Dako, Kyoto, Japan) antibodies were used in these preparations.

The medullary tumors have an ominous histological pattern, with solid masses of cells with large vesicular nuclei.

Fig. 1. (A) Parathyroid scintigraphy using 99m-Tc-methoxyisobutylisonitrile (99m-Tc-MIBI). Two positive spots were detected just below the left thyroid lobe (double arrows). (B) Venous sampling of parathyroid hormone (PTH) and calcitonin. The patient’s serum PTH concentration was increased at 3, 8, and 9 positions close to the foci localized by MIBI accumulation. (C) Histological features of the nodular lesion in the left thyroid (i, x100; ii, x400) and metastatic lymph nodes (iii, x40; iv, x100). The medullary tumors have an ominous histological pattern, with solid masses of cells with large vesicular nuclei.

Treatment didn’t completely correct the elevation of serum calcitonin (160-290 pg/mL). An increased level of carcinoembryonic antigen was postoperatively normalized (Table 1). An increased progastrin-releasing peptide level have not yet been completely normalized [29.8-65.0 pg/mL (reference range, <46.0 pg/mL)] (Table 1). Repetitive imaging tests have not yet disclosed any recurrence following surgery.

All samples were acquired with informed consent in accordance with protocols approved by the human subject protection committees. Experimental protocols were approved by the institutional review boards of Kanazawa University.

**Immunohistochemistry**

Immunohistochemistry of PTH and calcitonin was performed using standard techniques on formalin-fixed and paraffin-embedded (FFPE) sections of the thyroid specimen obtained at surgery. Anti-calcitonin and anti-PTH (1-34) (Dako, Kyoto, Japan) antibodies were used in these preparations.
Mutational Analysis of the RET gene

We analyzed the DNA from the patient’s tumor tissue, metastatic lymph nodes, adjacent thyroid and blood leukocytes. The DNA from the paraffin-embedded tumor and lymph node samples was isolated, using a PS isolation kit (Wako, Osaka, Japan). DNA sequencing was performed for exons 10, 11, 13, 14, 16 and the flanking intron sequences of the RET gene using the ABI PRISM BigDye Terminator cycle sequencing kit (Applied Biosystems Japan, Tokyo, Japan). The primers used in this study were as follows: exon 10 F; 5’-ACA CTG CCC TGG AAA TAT GG-3’ and 10 R; 5’-TGC TGT TGA GAC CCT TGT GG-3’, exon 11 F; 5’-ATA CGC AGC CTG TAC CCA GT-3’ and 11 R; 5’-GGA GGG CAG GGG ATG TTC-3’, exon 13 F; 5’-GAT CGTTTG CAA CCT GCT CT-3’ and 13 R; 5’-GGA GAA CAG GGC TGT ATG GA-3’, exon 14 F; 5’-AAG ACC CAA GCT GCC TGA C-3’ and 14 R; 5’-GCT GGG TGC AGA GCC ATA-3’, and exon 16 F; 5’-CTG AAA GCT CAG GGA TAG GG-3’ and 16 R; 5’-TGT AAC CTC CAC CCC AAG AG-3’. The sequencing reactions were performed according to the manufacturer’s instructions and were analyzed on an ABI310 DNA Sequencer (Applied Biosystems Japan). Digestion of the PCR products with FokI restriction enzyme (New England Biolabs, Beverly, MA, USA) confirmed the mutation.

Analysis of gene expression

Total RNA (1 µg) was reverse-transcribed using Superscript II (Invitrogen) and random hexamer primers. Reverse-transcribed products were amplified by 40 cycles of PCR using various sets of primers: F; 5’- AAA ATC GGA TGG GAA ATC TG-3’ and R; 5’-GCA GCA TGT ATT GTT GCC CTA-3’ for the PTH gene; F; 5’-AAC TCC CGC ATC CTC AAG AAG TCC-3’ and R; 5’-CAT GGC TCT TCT TGC CTC AGC TTC-3’ for the GCM1 gene; F; 5’-AGT TCA TTC CTT GTC GAG GGC ACA-3’ and R; 5’-TGT TGC TGA AAT GAC CAB TGC TGT C-3’ for the GCM2 gene; and F; 5’-CAA GAG ATG GCC ACG GCT GCT-3’ and R; 5’-TCC TTC TGC ATG ATC CTC TCC GCA-3’ for the β-actin gene. The RT-PCR products were separated on 1% agarose gels and stained with ethidium bromide. MTC tissue from a 64-yr-old male patient with familial MEN2A and lymphoblasts from a 28-yr-old male control were included in the gene expression analyses. We also used normal parathyroid tissue from a female patient, resected during a thyroidectomy because of Basedow’s disease.

Southern blot analysis for the PTH promoter

A 10 µg aliquot of genomic DNA was digested with PstI (New England Biolabs), fractionated on a 1% agarose gel and then blotted onto nylon membranes. Prehybridization, hybridization and probe labeling were performed using AlkPhos Direct (GE Healthcare UK Ltd., Buckinghamshire, England), as reported previously [8]. The probes comprised the PCR products, using the following forward and reverse primers for the PTH promoter region (2986 bp): 5’-AAG CAG TTC ACA CTC AAA TGA CCA ACA-3’ and 5’-TCC AAA AGC TTC TCA TGA AAA CCA ACC-3’.

DNA methylation analysis for the PTH and GCM2 genes

Genomic DNA from the FFPE resection specimens was collected, treated with bisulfite and amplified using primers specific for the PTH gene (60 bp) (F; 5’-
TGG GAT TAG AGT TGA GAG AAT TGA-3' and R; 5'-TTA CCT TCC CAC CAA AAA TCC-3'), and the GCM2 gene (84 bp) (F; 5'-GAG YGA GTT GGG TAG ATG-3' and R; 5'-AAT ATC CCA ACT AAA CTA CAT CC-3').

The PCR products of the PTH promoter region were digested with Hpy188I, which cuts only methylated DNA after bisulfite conversion, and then subjected to electrophoresis on 5% agarose gels, followed by staining with ethidium bromide.

For the GCM2 gene, at least ten PCR product clones generated using bisulfite-converted DNA from each FFPE specimen were chosen in order to analyze DNA methylation status, as described previously [9-10].

GCM2 promoter constructs

The flanking region of the GCM2 gene promoter was amplified from human genomic DNA, using a method we have reported previously [10-11]. After digestion with KpnI and NheI, the PCR-amplified fragments were subcloned into the pGL4.10 luciferase reporter plasmid. The inserts were sequenced to ensure fidelity of the amplified sequences. The oligonucleotides used were -45 KpnI F (5'-ATG GGG TAC CCC TTC ACA CAC CCC ACT TCT-3'), -190 KpnI F (5'-ATG GGG TAC CAG CTG CCA GAG GTC GAT G-3'), and +250 NheI R (5'-ATC GGC TAG CTC CGC AGA CTC TTC AAG AAC-3').

Luciferase assay

The effect of DNA methylation on promoter activity was assessed, using a method we have reported previously [10]. Briefly, Sss I methylase was used for in vitro methylation of the GCM2 promoter luciferase constructs in a pGL4.10 vector. The constructs contained 2 different lengths of the GCM2 promoter luciferase constructs in a pGL4.10 vector. The constructs contained 2 different lengths of the GCM2 promoter. In each case, half of the DNA sample was methylated using Sss I and the other half was incubated with Sss I methylase in the absence of S-adenosylmethionine (mock-methylation). The efficiency of methylation or mock-methylation was determined with the methylation-sensitive restriction enzyme, NaeI. The human adrenocortical H295R cells were transfected with the following plasmids using FuGene HD (Roche Laboratories, Basel, Switzerland): (1) 300 ng of modified pGL4.10 firefly luciferase reporter plasmid containing methylated or unmethylated GCM2 promoter and (2) 15 ng of pRL-TK Renilla luciferase control reporter vector that contained cDNA encoding Renilla luciferase (Promega, Madison, WI, USA) as an internal control of transfection efficiency. The day before transfection, the H295R cells were seeded onto a 96-well plate and transfected at 80% confluency. The cells were incubated for 24 h following transfection. Firefly and Renilla luciferase assays were performed on 10 µL of cell lysates using a dual-luciferase reporter assay system kit (Promega). The results were calculated as the mean of triplicate assays and expressed as the ratio to the internal standard Renilla luciferase.

Results

Immunohistochemistry for PTH and calcitonin

Immunodetection of PTH was not obvious in MTC tissue, while calcitonin-immunoreactivity was detected.

Identification of an activating mutation in the RET proto-oncogene

Sequencing analysis of the RET gene in genomic DNA from MTC tissue revealed a heterozygous missense mutation that changed codon 918 from ATG to ACG (Met to Thr) in exon 16. This mutation is present in almost 50% patients with a sporadic MTC [12-13] (Fig. 2A). FokI digestion also confirmed the mutation in metastatic lymph nodes, but neither adjacent thyroid nor leukocytes had evidence of a sporadic MTC (Fig. 2B).

Gene expression of the PTH, GCM1 and 2 genes

RT-PCR confirmed ectopic gene expression of PTH by the tumor tissue and metastatic lymph nodes (Fig. 3). To assess the molecular mechanisms underlying this ectopic expression of PTH, we analyzed gene expression of the GCM1 and 2 genes, which have been shown to play a major role in PTH secretion in gcm2-null mice [14]. GCM2 was expressed strongly in normal parathyroid tissue, consistent with a previous report [15]. GCM2 expression was also observed in both the tumor and metastatic lymph nodes, whereas GCM1 expression was seen only in the tumor, but not in the metastatic lymph nodes (Fig. 3). Despite co-expression of PTH and GCM2 in the patient’s MTC, we also detected GCM2 expression in the adjacent normal thyroid and lymphoblasts from a healthy control, in which PTH expression was absent. These results indicated GCM2 expression was not absolutely necessary for PTH gene expression.
and defined as follows; relaxed showed gray shading on the map (200 bp minimum length, 50% or higher G + C content, 0.60 or higher observed Cpg / expected Cpg, post-processing: merge islands ≤ 100 bp apart), while strict showed black shading on the map (500 bp minimum length, 50% or higher gC content, 0.60 or higher observed Cp g / expected Cp g) (Fig. 5 a).

Bisulfite sequencing revealed CpG dinucleotides with -
in first exon of the GCM2 gene were largely unmeth-
- in all the tissues investigated (Fig. 5C).  since the gCM2 Cpg island was hypomethylated with -out gCM2 gene expression in a MTC tissue from a Men2a patient (Fig. 3), DNA hypomethylation didn’t appear to determine gCM2 gene expression.

Southern analysis for the PTH promoter

As amplification and rearrangement of the PTH promoter has been reported as a cause of ectopic PTH expression in ovarian cancer [4], we performed Southern blotting which revealing no evidence of similar abnormalities (data not shown).

DNA methylation analysis for the PTH and GCM2 genes

Hypomethylation of the PTH promoter has also been reported in a case of pancreatic cancer [7]. We analyzed DNA methylation status at the CpG site that is reportedly associated with PTH gene expression in parathyroid glands [16]. Combined bisulfite and restriction assays did not demonstrate hypomethylation in either the tumor or metastatic lymph nodes. The methylation status of the MTC was similar to that of the adjacent normal thyroid, whereas hypomethylation was seen in normal parathyroid, as reported previously [16] (Fig. 4). As PTH expression was present in the MTC tissue and absent in the adjacent normal thyroid, this indicated DNA hypomethylation at the CpG site was not necessary for ectopic expression of PTH.

CpG islands of the GCM2 gene were identified in MapViewer, showing regions of high G + C content in the assembled genome sequence. Two sets of criteria, “strict” and “relaxed” were used for CpG islands and defined as follows; relaxed showed gray shading on the map (200 bp minimum length, 50% or higher G + C content, 0.60 or higher observed CpG / expected CpG, post-processing: merge islands ≤ 100 bp apart), while strict showed black shading on the map (500 bp minimum length, 50% or higher GC content, 0.60 or higher observed CpG / expected CpG) (Fig. 5A). Bisulfite sequencing revealed CpG dinucleotides within first exon of the GCM2 gene were largely unmethylated in all the tissues investigated (Fig. 5C). Since the GCM2 CpG island was hypomethylated without GCM2 gene expression in a MTC tissue from a MEN2A patient (Fig. 3), DNA hypomethylation didn’t appear to determine GCM2 gene expression.
PTh-Producing MTC

rum PTh concentration caused by ectopic production of PTh by a sporadic MTC. Ectopic production of PTh is very rare [3-7, 17-23]. In our patient, the serum level of PTh was elevated, a change which is not generally observed in cases of sporadic MTC. Histological examination confirmed a diagnosis of MTC with metastatic lymph nodes. After removal of the tumor and metastatic lymph nodes, the patient's serum PTh level decreased to within the normal range. Although immunohistochemistry was negative for PTh, ectopic PTh expression was confirmed by selective venous sampling and RT-PCR.

Effect of DNA methylation on GCM2 promoter activity

To assess the transcriptional activity of methylated and unmethylated GCM2 promoter, luciferase reporter constructs containing varying lengths of the GCM2 promoter and its 5'-flanking sequence were transfected into H295R cells (Fig. 6). Basal luciferase expression levels of unmethylated constructs were consistently higher than in the methylated constructs. These results suggested that DNA methylation within the GCM2 promoter region decreased GCM2 promoter transactivational activity.

Discussion

In this paper, we report a case with elevation of serum PTH concentration caused by ectopic production of PTh by a sporadic MTC. Ectopic production of PTh is very rare [3-7, 17-23]. In our patient, the serum level of PTh was elevated, a change which is not generally observed in cases of sporadic MTC. Histological examination confirmed a diagnosis of MTC with metastatic lymph nodes. After removal of the tumor and metastatic lymph nodes, the patient's serum PTh level decreased to within the normal range. Although immunohistochemistry was negative for PTh, ectopic PTh expression was confirmed by selective venous sampling and RT-PCR.

Elevation of serum PTH and calcitonin was observed in our patient. PTh elevates blood Ca++ level by dissolving the salts in bone and preventing their re-
nal excretion. Calcitonin causes reduction in serum calcium, an effect opposite to that of PTH. The elevated calcitonin level appeared to interfere with the action of PTH, leaving serum calcium concentration unchanged in the patient.

Mitochondria-rich oxyphil cells presumably account for MIBI uptake in parathyroid lesions [24]. MIBI foci in metastatic lymph nodes led to the diagnosis of PTH-producing MTC in our patient. However, no increased uptake was observed in the MTC tumor itself. Blood flow and capillary permeability, plasma and mitochondrion membrane potentials, and cellular mitochondrial contents play important roles in its uptake by tumor cells [25-26]. These factors may explain a difference in MIBI uptake between the MTC tumor and metastatic lymph nodes.

The specific absence of parathyroid glands has been reported in glial cells missing two (Gcm2)-null mice. These mice had normal parathyroid hormone levels. Expression and ablation studies identified the thymus, where Gcm1 is expressed, as an additional, downregulatable source of PTH [14]. Moreover, mutations in GCM2 transcription factors have been implicated in syndromes of hypoparathyroidism [27-28]. GCM1 is primarily expressed in the placenta whereas GCM2 expression is restricted to parathyroid cells in humans. In our case, we therefore paid attention to the GCM2 gene in order to investigate the cause of ectopic PTH expression. However, this transcription factor was expressed in thyroid and lymphoblasts lacking PTH expression. We confirmed that whereas GCM2 might be necessary for parathyroid development, it was not absolutely necessary for PTH expression.

Genomic rearrangement or hypomethylation of the PTH promoter region have been shown to be associated with ectopic PTH expression. However, we did not find these abnormalities, indicating that the cause of PTH transactivation was heterogeneous. As we identified no genetic and epigenetic changes within the PTH promoter, this indicated that some other trans-acting elements, except for GCM2, were involved in ectopic PTH activation in our case.

Epigenetic regulation by methylation of 5'-cytosine of Cpg dinucleotides regulates gene expression. Cpg methylation of the promoter down-regulates transcription by preventing binding of positive transcription factors to their recognition sequences and by recruiting repressor molecules, such as methyl-Cpg-binding domain proteins and DNA methyltransferases [10, 29]. A CpG island containing the promoter and exon 1 of GCM2 was largely hypomethylated in all tissues we investigated (Fig. 5), regardless of whether or not the GCM2 gene was expressed. We couldn’t draw the conclusion that ectopic expression of GCM2 was dependent upon DNA methylation. DNA methylation, however, repressed the transcriptional activity of reporter constructs containing the GCM2 promoter (Fig. 6). DNA hypomethylation may be a prerequisite, but isn’t a sufficient condition for the GCM2 gene expression.

This is the first reported case of sporadic MTC producing PTH in which there was no apparent association between PTH and GCM2 expression. The causes of ectopic expression of PTH in this case appeared to be attributable to several diverse characteristics.

**Disclosure statement**

The authors have nothing to declare.

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