Novel Intronic Mutation of MEN1 Gene Causing Familial Isolated Primary Hyperparathyroidism

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Primary hyperparathyroidism may occur as part of hereditary syndromes, including multiple endocrine neoplasia types 1 and 2A (MEN1 and MEN2A), hyperparathyroidism-jaw tumor syndrome, and the familial isolated hyperparathyroidism (FIHP). It is unclear whether FIHP corresponds to a different genetic entity or a variant of MEN1 (or hyperparathyroidism-jaw tumor syndrome). We report a patient and 11 family members with FIHP in whom we identified a heterozygous G-to-A mutation at nucleotide 7361 of tumor suppressor MEN1 gene. This mutation is located in the first base of intron 9 (IVS9 + 1 G>A). All the family members with hyperparathyroidism were heterozygous for the intronic mutation. In vitro studies were performed in COS cells transfected with minigenes carrying the coding regions spanning exon-intron 9 and 10 with the mutant and the wild-type sequences. RT-PCR analyses showed an abnormal mRNA of greater size (829 bp) in the mutated MEN1 gene than the normal transcript (629 bp). The longer PCR product includes the exon 9, the unspliced intron 9, and part of exon 10. RT-PCR of MEN1 mRNA from patient’s blood confirmed the existence of unspliced intron 9 in mature mRNA. In summary, we report a case of FIHP associated with a new intronic heterozygous germline mutation (IVS9 + 1 G>A) of MEN1 gene. This mutation produces an aberrant splicing of mRNA that could lead to a truncated protein, without activity, explaining the clinical picture of this patient and his family. (J Clin Endocrinol Metab 89: 4124–4129, 2004)
This communication reports a Chilean family with FIHP with a heterozygous germline mutation in the MEN1 gene, which has not been described previously either in full expression of MEN1 disease or in FIHP.

Subjects and Methods

Subjects

The index case (Fig. 1, II-4) is a 45-yr-old Chilean male who was diagnosed with HPT [serum calcium, 2.69 mmol/liter (normal range, 2.12–2.61 mmol/liter); midfraction (44–68) PTH, >12,000 ng/liter (normal value, <1600 ng/liter)] during a study for metatarsal bone pain that revealed an ostetis fibrosa cystica. A single enlarged parathyroid gland was removed, and then he abandoned the medical controls. He came back 10 yr later with a recurrence of hyperparathyroidism and three enlarged parathyroid glands. Total parathyroidectomy, thymectomy, and parathyroid autograft were performed.

The familiar history revealed that his mother (I-1), one brother (II-3), two sisters (II-5, II-6), one cousin (II-2), and three daughters (III-3, III-4, III-6) presented with HPT and kidney stones. Two daughters (III-3, III-4) and a cousin were also treated with total parathyroidectomy, and parathyroid autograft were performed. The mother’s proband (I-1) died by the age of 75 yr with parathyroid carcinoma, without evidence of other MEN1-related endocrinopathies or clinical jaw tumor (normal selar pH determination). In this patient, the prolactin levels were normal, as was the abdominal CT scan.

Informed consent was obtained from all participants in this study according to the guidelines of the Declaration of Helsinki, and the protocol was approved by the Research Committee of the School of Medicine at Pontificia Universidad Católica de Chile.

DNA amplification and sequence analysis of the MEN1 gene

Genomic DNA was isolated from leukocytes of the index case (II-4), 16 family members (Fig. 1), and 100 healthy controls (HCs) using a commercially available DNAzol reagent (Invitrogen, Carlsbad, CA). DNA sequence analysis of the entire 1830-bp coding region including exon/intron boundaries of the MEN1 gene in the index case was amplified and sequenced. Oligonucleotide primers used in each reaction are described in Table 1. PCR amplification was carried out with the following cycling conditions: 4 min at 94 C, 10 cycles of 1 min at 94 C, 1 min at 65 C (step down −0.5 C/cycle), and 2 min at 72 C, adding 30 cycles of 1 min at 94 C, 1 min at 60 C, and 1 min at 72 C. Amplified gene products were purified by QIAquick gel extraction purification kit (QIAGEN, Valencia, CA). Sequence analyses of MEN1 gene was performed using specific primers (Table 1) by the fluorescent dyeoxy chain terminator method in the ABI Prism 377 DNA genetic analyzer (Applied Biosystems, Foster City, CA). Sequences were matched with the published MEN1 gene (GenBank accession no. 1945388) with BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

Restriction analysis of MEN1 gene

A restriction analysis was performed to determine the haplotype of the patient and his family. Likewise, a restriction analysis of 100 HCs was carried out to identify the normal restriction pattern. The restriction endonuclease HphI (New England Biolabs, Beverly, MA) recognizes the normal sequence GGTGA (N)8/N and cut the normal 284-bp PCR product.

FIG. 1. Family pedigree. Filled black symbols indicate members affected with HPT and heterozygote for the mutation. Open symbols are family members unaffected with normal MEN1 gene, and filled gray symbols show unaffected family members who are heterozygous. The family member who died with parathyroid carcinoma is marked with an asterisk; and the proband is indicated with the arrow. The age of each family member is shown in parentheses.

TABLE 1. Oligonucleotide primers (5′→3′) used for PCR amplification of MEN1 gene and mRNA

<table>
<thead>
<tr>
<th>Exon</th>
<th>Name</th>
<th>Forward</th>
<th>Size</th>
<th>Reverse</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2F</td>
<td>GGAACTTACGCCGACCTGGAG</td>
<td>780</td>
<td>AGAGGGTGGGTATGATGTTGGAG</td>
<td>2R</td>
</tr>
<tr>
<td>3−4</td>
<td>3F</td>
<td>AGGTGTGCCCCAATCTACTACTT</td>
<td>685</td>
<td>TCCCGAGCAGAAGTCTGG</td>
<td>4R</td>
</tr>
<tr>
<td>5−6</td>
<td>5F</td>
<td>CCTCTGTTCTGCTGGCTC</td>
<td>285</td>
<td>CCCCTGCTCAAGCCACTGTA</td>
<td>6R</td>
</tr>
<tr>
<td>7</td>
<td>7F</td>
<td>CCTGACGGCCAGCTCTCGTAG</td>
<td>384</td>
<td>GGACAGGGGTGCTGGAATC</td>
<td>7R</td>
</tr>
<tr>
<td>8</td>
<td>8F</td>
<td>TGGTGAACCCCTCGACGCTC</td>
<td>278</td>
<td>CCCATCCCTAATCCCGTACATGC</td>
<td>8R</td>
</tr>
<tr>
<td>9</td>
<td>9F</td>
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<td>284</td>
<td>AGTCGGACAGACCCGCTGGTGGCG</td>
<td>9R</td>
</tr>
<tr>
<td>10</td>
<td>10F</td>
<td>CACCGCTCTGGCGCCACACCT</td>
<td>684</td>
<td>TTTGATACAGACTGTCTCGGG</td>
<td>10R</td>
</tr>
<tr>
<td>9−10</td>
<td>9F-B</td>
<td>GCCATTTCTCGACAGCCTAT</td>
<td>629</td>
<td>GGGCGCCAGCGACT</td>
<td>10R-B</td>
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<tr>
<td>9−10</td>
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<td>GCCGATTTCTCGACAGCCTAT</td>
<td>174</td>
<td>AGTCGGACAGAAGCCTGGTGGCG</td>
<td>9R</td>
</tr>
</tbody>
</table>

Sequences are referred to the MEN1 gene sequence (accession no. 1945388) published in the GenBank database. F, Forward, R, reverse.
uct in four fragments, 208, 40, 22, and 14 bp. The mutation G to A in intron 9 (IVS9 + 1G>A) reported here changes the recognition site for the restriction endonuclease HphI from G/GTGA to G/ATGA. Restriction analyses with primers 9F and 9R (Table 1) amplified the normal or mutated 284-bp product. These fragments were digested with 0.5 U HphI for 16 h at 37 °C. The mutated product (flanking the exon 9-intron 9 region) is protected from cleavage, and the resulting restriction fragments are 248, 22, and 15 bp. Products of restriction assays were electrophoresed in 6% polyacrylamide gel and visualized by staining with ethidium bromide.

In vitro expression of MEN1 minigene

We performed an in vitro study using MEN1 minigenes that contain the MEN1 gene sequence spanning exon 9, intron 9, and a partial region of exon 10, carrying either the mutant or the wild-type sequence. Minigenes were synthesized using primers 9F and 10R (Table 1). Briefly, after PCR amplification, the PCR products from index case and a HC were cloned into pCR 2.1 TOPO TA cloning kit (Invitrogen). Plasmid DNA from positive clones was digested with EcoRI and HindIII (New England Biolabs), and the insert of each clone was purified using Wizard PCR prep (Promega, Madison, WI) and subcloned in pCR3 expression vector (Invitrogen). The pCR3 clones containing the mutated or the normal minigene were confirmed by restriction and sequencing analysis. Plasmidial DNA for transfection experiments was purified by CONCERT-nucleic acid purification system (Invitrogen), and 4 μg was transfected into COS cells using LipofectAMINE 2000 reagent (Invitrogen). COS cells were grown on DMEM supplemented with 10% (vol/vol) fetal bovine serum and antibiotics (100 IU/ml penicillin, 100 mg/ml streptomycin) at 37 °C and 5% CO2. After 48 h, total RNA was isolated with TRIZOL LS reagent (Invitrogen). Single-stranded cDNA was synthesized from total RNA by RT-PCR using the SuperScript II kit (Invitrogen). An aliquot of the reaction mixture was heated at 95 °C for 5 min and then added to a PCR. The PCR conditions to amplify cDNA were the same as those described above, but with primers 9F-B and 10R-B (Table 1).

RNA isolation and RT-PCR of MEN1 from peripheral blood cells

Total RNA from freshly sampled peripheral blood cells of three patients (II-2, II-4, II-5) and five HCs was extracted using TriZol LS reagent according to the manufacturer’s protocol. RT was performed with 3 μg of total RNA using SuperScript II in 20 μl of reaction buffer [5 mM Tris-HCl buffer (pH 8.3), 75 mM KCl, 0.1 mM dithiothreitol, 3 mM MgCl2], RT-PCR of MEN1 mRNA was performed with primers 9F-B and 10R-B. To perform sequencing analysis, the mutant fragment was re-amplified with primers 9F-B and 9R. The reaction was normalized with the amplification of a constitutive expression gene, glyceraldehyde phosphate dehydrogenase. RT-PCR products were visualized using 1% agarose gel electrophoresis, purified by Qiaquick gel extraction kit, and subjected to automated sequencing.

**Results**

**Molecular analysis of the MEN1 gene**

The entire coding regions of the MEN1 gene from the index case (Fig 1, II-4), including the exon-intron boundaries, were sequenced. The sequencing analysis revealed a heterozygous G to A mutation in the nucleotide position +1 of intron 9 (IVS9 +1G>A) (Fig. 2). No other mutations were found in the coding regions and flanking introns sequences of exons 2–10 of the MEN1 gene.

**Restriction analysis**

To confirm the IVS9 +1G>A germline mutation, we performed a restriction analysis using the HphI restriction enzyme. As shown in Fig. 3, the patient (II-4) and two relatives (II-2, II-6) with HPT had the noncleaved 248-bp fragment and cleaved 208-, 40-, 22-, and 14-bp fragments indicating that they are heterozygous (Fig. 3 only shows the 248- and 208-bp fragments). All the family members with HPT were heterozygous for the intronic mutation (data not shown). The same pattern was observed in three healthy family members who were 22, 14, and 13 yr old (III-8, IV-1, IV-2). We did not identify the mutation in the remaining unaffected relatives and 100 healthy volunteers.
Expression of the MEN1 minigene in COS cells

The functional consequences of this splice donor mutation were analyzed in COS cells transfected with mutant and normal minigenes. The RT-PCR product containing the mutant minigene had 829 bp and the normal minigene had 629 bp. The 629-bp product is the size predicted for the normally spliced region (exons 9–10) (Fig. 4). The longer PCR product (829 bp) includes the amplification of exon 9, the unspliced intron 9, and part of exon 10. The unspliced intron 9 adds 200 bp to the mutant RT-PCR product, explaining its abnormal size. As shown in Fig. 4, the amplification control was obtained from human testis tissue, which amplified the normally 629-bp fragment.

RT-PCR of MEN1 from total RNA extracted from peripheral blood cells

The RT-PCR of MEN1 (with primers 9F-B and 10R-B) in a HC amplified a 629-bp product. In one affected subject (II-5), we demonstrated the presence of the normal fragment (629 bp) and an additional fragment carrying the unspliced intron 9 (829 bp) (Fig. 5A). Sequencing analysis of the mutant fragment revealed the presence of the intron 9 and the nucleotide change G/GT to G/AT in the exon-intron boundary region (Fig. 5B).

Discussion

In this study we identified a novel intronic heterozygous germline mutation (IVS9 + 1 G>A) of MEN1 gene in a Chilean family affected with FIHP. The diagnosis of FIHP was supported by an extensive investigation to exclude MEN1 syndrome. We found normal prolactin and gastrin values in five affected relatives (I-1, II-4, II-5, II-6, III-6), excluding strongly the possibility of these endocrine tumors. The mild elevation of gastrin level in relative II-2 was attributed to atrophic gastritis (33). Moreover, the proband and three relatives (I-1, II-2, II-4, II-6) had abdominal and sellar CT scans, which did not show pancreatic or pituitary tumors. The absence of biochemical (gastrin and prolactin) or radiological evidence of MEN1 is not definitive proof of FIHP, because we cannot predict whether any member of this family will express MEN1 phenotype in the future. However, all but one of the family members tested are older than 40 yr of age, the age at which at least one nonparathyroid tumor should be expressed (34). Moreover, the proband’s mother...
(1-1) died by the age of 75 yr without biochemical and radiological evidence of solar or pancreatic tumor, which strongly supports the FIHP diagnosis.

The presence of parathyroid carcinoma in the proband’s mother raises the diagnostic possibility of HPT-JT syndrome. However, the jaw orthopantography in the proband and in one daughter did not identify jaw lesions. Moreover, the abdominal ultrasound did not find the typical renal lesions (bilateral renal cysts, Wilms’ tumor, or renal hamartomas) described in this syndrome. The presence of parathyroid carcinoma has been described in other cases of FIHP, suggesting that long-term hyperactivity of the parathyroid glands may result in carcinoma (35, 36).

The role of this germline mutation in the origin of FIHP is supported by its presence in family members affected by HPT and its absence in a 58-yr-old cousin without HPT (II-1). The absence of this intronic mutation in 100 HCs argues against the presence of a polymorphic change and supports the presence of a novel mutation. The three unaffected family members carrying the mutation corresponded to the younger members (II-8, III-1, III-2), so we cannot predict whether they will express the disease later in life.

In our index case (I-4) we found a G to A mutation at the first base of intron 9, which disrupts the consensus sequences, critical for the splicing reaction (original donor sequence is G/GT that changed to G/AT in the mutant gene), producing the retention of the intron (in vitro experiments). The COS cells transfected with the mutant minigene did not produce the retention of the intron (in vitro experiments), suggesting that long-term hyperactivity of the parathyroid glands may result in carcinoma (35, 36).

In summary, we describe a family with FIHP with a new mutation in the MEN1 gene. The absence of this intronic mutation in 100 HCs argues against its presence in a polymorphic change and supports the presence of a novel mutation. The three unaffected family members carrying the mutation correspond to the younger members (II-8, III-1, III-2), so we cannot predict whether they will express the disease later in life.

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Carrasco et al. • Novel Mutation in MEN1 Gene


