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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)

PRIMARY HYPERPARATHYROIDISM (HPT) is frequently encountered as a nonfamilial disorder. Familial HPT, which comprises 10% of total HPT, encompasses a clinically and genetically heterogeneous group of disorders including multiple endocrine neoplasia (MEN) type 1 [MEN1, Online Mendelian Inheritance in Man (OMIM) 131100], MEN type 2A (MEN2A, OMIM 171400), familial hypocalciuric hypercalcemia (OMIM 145980, 145981, 600740), HPT-jaw tumor syndrome (HPT-JT, OMIM 145001), and familial isolated HPT (FIHP, OMIM 145000).

The prevalence of FIHP is 1% of total HPT and is inherited in an autosomal dominant pattern (1). The affected family members have an age of onset of 20–25 yr old, which is 30 yr earlier than sporadic cases (2). The parathyroid tumors in these patients are frequently described as a multiglandular asymmetric hyperplasia, although they may involve the development of polyclonal and clonal tumors (3, 4). A subset of these patients has been suggested to be an allelic variant of *MEN1*, because germline mutations in the *MEN1* gene have been found in about 20% of reported affected kindreds (1, 2).

MEN1 is an autosomal dominant disease characterized by the combined occurrence of tumors of the parathyroid glands, the pancreatic islet cells, and the anterior pituitary. The mutational hypothesis in this gene proposes that the first hit or mutation is inherited as a germline mutation and the second hit occurs as a somatic mutation in the predisposed endocrine cell. This second mutation may occur by chromosome loss (*e.g.* lost of heterozygosity), chromosome loss with duplication, mitotic recombination, or another localized event such as a point mutation (5). As a consequence of these two mutations, both alleles of the MEN1 gene are inactivated, allowing the tumor growth. To date, at least 20 kindreds with FIHP have been associated with germline mutations in the *MEN1* gene, but there is no evidence that a particular type of *MEN1* gene mutation can cause FIHP (1, 6–23). Because HPT is the most frequent and earliest expression in MEN1, FIHP may be a prelude to typical MEN1, an atypical expression of *MEN1*, or a phenocopy caused by a mutation in other genes (24–27).

The *MEN1* gene is located in chromosome 11q13 and consists of 10 exons that encode a 610-amino-acid protein, named menin (28). The coding region of this gene is organized into nine exons (exons 2–10) and eight introns. The 2.8-kb *MEN1* transcript is expressed in a wide variety of tissues including lymphocytes, spleen, testes, ovaries, thymus, pancreas, and thyroid (5). Menin is predominantly a nuclear protein that binds to the JunD subunit of the activator protein-1 transcriptional factor and represses JunD-mediated transcriptional activation *in vitro* (29). The exact biological function of JunD is not well characterized, and some studies reveal that the absence of JunD can increase or suppress the cell proliferation (30). There are five known functional domains of menin: three JunD interacting domains (residues 1–40, 139–242, and 323–428) and two nuclear localization signals (NLS) at the C terminus (residues 479–497 and 588–608) (31).

Abbreviations: CT, Computed tomography; FIHP, familial isolated HPT; HC, healthy control; HPT, hyperparathyroidism; HPT-JT, HPT-jaw tumor; MEN, multiple endocrine neoplasia; NLS, nuclear localization signal; OMIM, Online Mendelian Inheritance in Man.

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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 osteitis 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, thyroidectomy, 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, thyroidectomy, and thymectomy, with findings of multiple parathyroid hyperplasias (32). Extensive investigation has been performed in the proband (II-4) to identify the presence of other neoplastic lesions suggestive of MEN1 or HPT-JT syndrome. However, the prolactin and gastrin blood levels, as well as an abdominal and selar computed tomography (CT) scan, did not show evidence of other MEN1-related endocrinopathies. Orthopantography of the jaw and abdominal ultrasound were carried out, but no case of jaw tumor or renal lesions (bilateral renal cysts, renal hamartoma, and Wilms' tumor) was found. Moreover, 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 and abdominal CT scan, normal gastrin and prolactin values). In three affected relatives (II-5, II-6, III-6), the gastrin and prolactin levels were normal. In one relative (II-2), we detected a mild elevation of the gastrin level (290 pg/ml; normal value, <200 pg/ml) in the presence of an atrophic gastritis (diagnosed by gastroscopy, biopsy, and gastric 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 dideoxy 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 *Hph*I (New England Biolabs, Beverly, MA) recognizes the normal sequence GGTGA $(N)_8$/N and cut the normal 284-bp PCR prod-

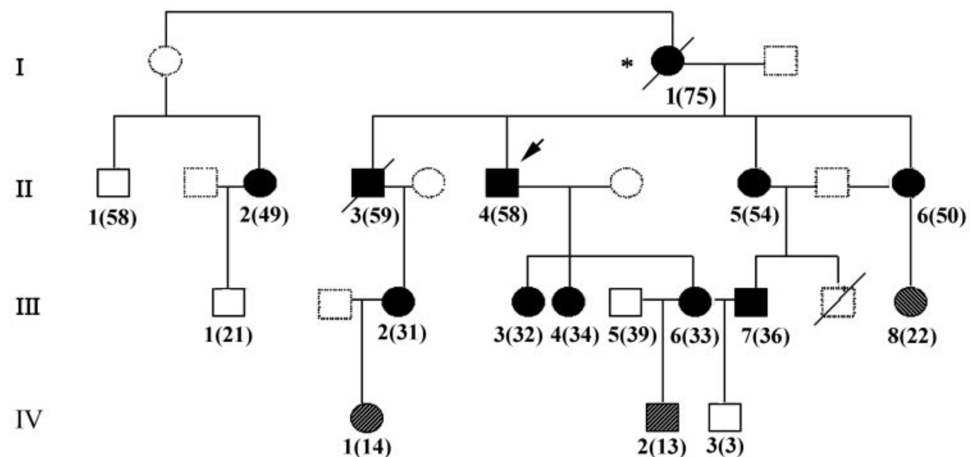

FIG. 1. Family pedigree. *Filled black symbols* indicate members affected with HPT and heterozygote for the mutation. *Open symbols* are family members unaffected with normal MEN1 gene, and *filled gray symbols* show unaffected family members who are heterozygous. The family member who died with parathyroid 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

Exon	Name	Forward	Size	Reverse	Name
2	2F	GGAACCTTAGCGGACCCTGGGAG	780	AGAGGTGAGGTTGATGATTTGGAG	2R
3–4	3F	AGTGTGGCCCATCACTACCT	685	TCCCACAGCAAGTAAGTCTGG	4R
5–6	5F	CCCTGTTCCGTGGCTC	285	CCCCTGCCTCAGCCACTGTTA	6R
7	7F	CCTCAGCCAGCAGTCCTGTAG	384	GGACGAGGGTGGTTGGAAACTG	7R
8	8F	TGGTGAGACCCCTTCAGACCCTAC	278	CCATCCCTAATCCCGTACATGC	8R
9	9F	CTGCTAAGGGGTGAGTAAGAGAC	284	AGTCTGACAAGCCCGTGGCTGCTG	9R
10	10F	CAGCCACTGGCCGGCAACCTT	684	TTTGATACAGACTGTCTCGGG	10R
9–10	9F-B	GCGATTCTACGACGGCATCT	629	GTGGGGACCCAGGCTC	10R-B
9–10	9F-B	GCGATTCTACGACGGCATCT	174	AGTCTGACAAGCCCGTGGCTGCTG	9R

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 *Hph*I 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 *Hph*I 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 *Eco*RI and *Hin*dIII (New England Biolabs), and the insert of each clone was purified using Wizard PCR preps (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% CO_2. 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 $MgCl_2$[. 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 intronic 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 *Hph*I 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.

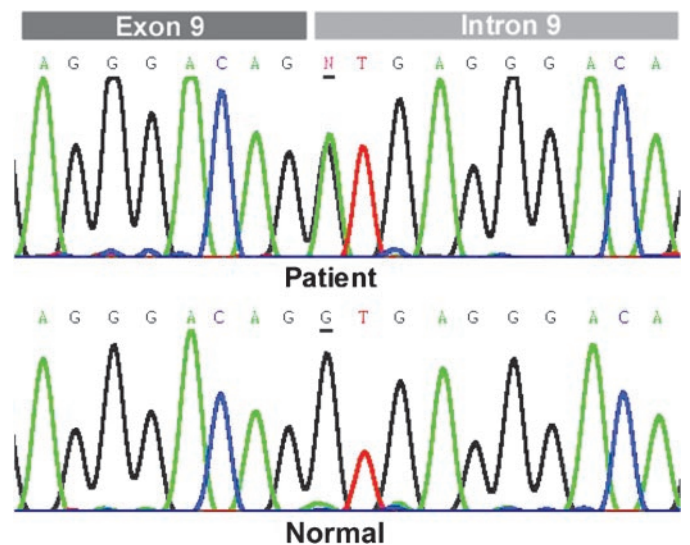

FIG. 2. *MEN1* gene sequencing analysis in the patient and control. *Underlined* base shows the heterozygous mutation (IVS9 + 1G>A), one nucleotide from splice donor site of exon 9 (t*op sequence*), and the normal consensus sequence G/GT (*bottom*).

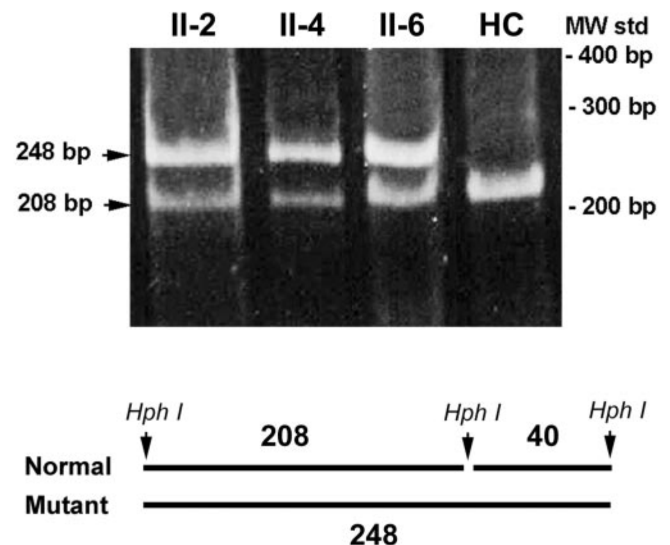

FIG. 3. Restriction analysis of *MEN1* with *Hph*I. A 6% polyacrylamide gel electrophoresis shows the PCR products digested with the restriction endonuclease *Hph*I in the patient, cousin, sister, and a HC. Healthy volunteers (n = 100) have only one fragment at 208 bp, whereas heterozygotes have two fragments, one at 208 bp (normal allele) and another at 248 bp (mutant allele). Only one subject is shown. MW std, Molecular weight marker.

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 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/<u>G</u>T to G/<u>A</u>T 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 selar 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

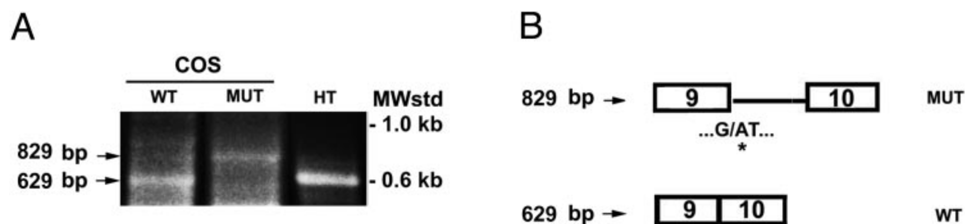

FIG. 4. *In vitro* expression of MEN1 minigene in COS cells. A, A 1% agarose gel showing the electrophoretical pattern of RT-PCR products amplified by RNA obtained from COS cells transfected with either the wild-type (WT; 629 bp) or the mutant (MUT) minigene (829 bp). Human testis was used as control to identify the normal RT-PCR product. MWstd, Molecular weight marker. B, Consensus splice sequence G/GT is disrupted by the G to A mutation (∗) causing retention of intron 9.

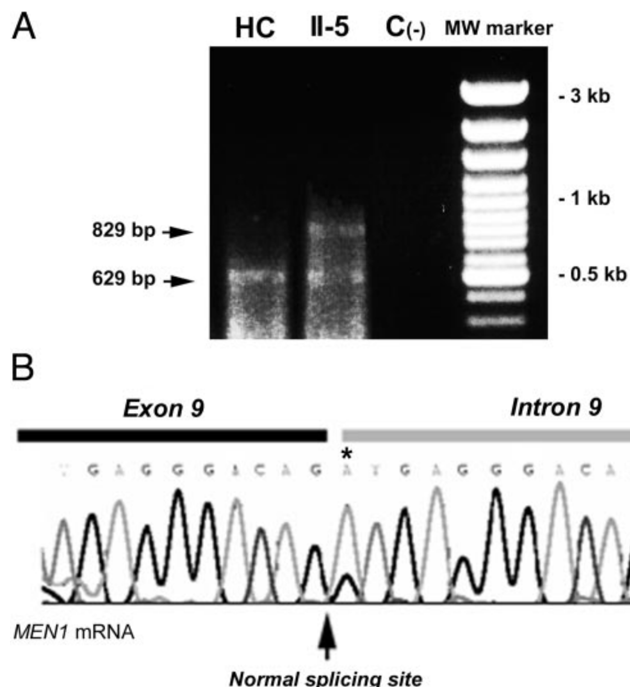

FIG. 5. A, RT-PCR of *MEN1*. Total RNA was isolated from patient's peripheral blood cells (II-2, II-4, II-5). RT-PCR showed an 829-bp PCR product that is not present in the HC and negative control [C(−)]. RT-PCR was performed with primers 9F-B and 10R-B. All experiments were normalized with glyceraldehyde phosphate dehydrogenase (not shown). MW, Molecular weight. B, Sequencing analysis of cDNA from patient leukocytes. Electropherogram shows the G>A germline mutation that originates the retention of intron 9 in the *MEN1* mRNA.

(I-1) died by the age of 75 yr without biochemical and radiological evidence of selar 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/G̲T that changed to G/A̲T in the mutant gene), producing the retention of the intron (*in vitro* experiments). The COS cells transfected with the mutant minigene did not remove the intron 9 of the *MEN1* gene, generating an mRNA of greater size (Fig. 4), which would result in a protein with a new 30-residues-longer C-terminal region. Identical results were demonstrated in RNA isolated from the patient's leukocytes (II-5; Fig. 5), in which two RT-PCR products were identified, one in a 629-bp fragment and another one in a 829-bp fragment, representing a normal and a mutated amplified allele, respectively. This result demonstrates the retention of intron 9, which adds 200 bp to the amplified product of the mutant allele.

The intron 9 retention in tumor tissues would result in the truncation of menin and, secondarily, the loss of the two NLSs (NLS-1 and NLS-2) encoded by exon 10, impairing the adequate translocation of menin to the nucleus. Previous studies in lymphocytes of a patient without a NLS-2 region resulted in a substantial portion of menin in the cytoplasm and a faster degradation than wild-type menin. These experiments indicate that NLS-2 may be critical for the efficient transfer of menin to the nucleus and might also contribute to the stability of the protein (37). On the other hand, if the skipping of exon 9 of MEN1 gene occurs, the mutation could induce a partial loss of the downstream JunD binding domain (residues 323–428) and, secondarily, the JunD transcriptional activity repression mediated by menin, modifying the functional synergy in the tumor suppressor activity.

In summary, we describe a family with FIHP with a new heterozygous intronic mutation (IVS9 + 1G>A) in the *MEN1* gene not described previously. The mutation causes an aberrant splicing of primary RNA of *MEN1* that leads to a truncated protein without activity, explaining the clinical picture of this patient and his family.

Acknowledgments

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