

Identification of *MEN1* Mutations in Sporadic Enteropancreatic Neuroendocrine Tumors by Analysis of Paraffin-embedded Tissue

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Gastrinomas and other gastrointestinal neuroendocrine tumors may occur sporadically or as part of the inherited syndrome multiple endocrine neoplasia type 1 (*MEN1*). Mutations in the recently identified *MEN1* gene have been described in sporadic gastrinomas and insulinomas. This study describes techniques used to identify mutations in the *MEN1* gene in DNA extracted from paraffin-preserved tissue. Two novel mutations are identified in the *MEN1* gene from nine archived paraffin-embedded neuroendocrine tumors, demonstrating that retrospective genetic analysis can be used to identify mutations in the *MEN1* gene from preserved tissue. Conditions are provided by which paraffin-embedded tissue can be used as a source of genetic material for sequence information of sufficient quality for mutational studies of the *MEN1* gene. It should also be possible to apply this retrospective genetic analysis of paraffin-embedded tissue to other disease models.

Gastrinomas and other gastrointestinal neuroendocrine tumors may occur sporadically or as part of the autosomal dominant genetic syndrome multiple endocrine neoplasia type 1 (*MEN1*), characterized by parathyroid hyperplasia, pituitary adenomas, and islet cell tumors of the pancreas. The recently identified *MEN1* gene (1–3) encodes a putative tumor suppressor protein termed menin. Germ-line mutations were found in 94% of *MEN1* patients (4).

When a gene responsible for a hereditary cancer is identified, it is important to next determine if the same gene is also involved in sporadic forms of the cancer. A gene that affects both familial and sporadic forms of the

cancer is likely to be part of an important pathway and may provide insight about the mechanism of tumorigenesis. The breast cancer genes *BRCA1* and *BRCA2* are examples of genes that are often mutated in familial breast cancer but are not mutated in sporadic breast cancer cases. In the case of the *MEN1* gene, evidence is beginning to accumulate that suggests that its function is likely to be lost in some sporadic cancers of the same tissues affected in the hereditary *MEN1* syndrome. One study showed that ~21% of sporadic parathyroid tumors had *MEN1* mutations (5). Another study reported *MEN1* mutations in 5% of sporadic pituitary tumors (6).

Sporadic gastrinomas were first described by Zollinger and Ellison in 1955 (7). Relatively little is understood about the molecular mechanisms involved in gastrinoma tumorigenesis. A recent study performed using fresh-frozen tissue has demonstrated that *MEN1* mutations, detected by single-strand conformational polymorphism analysis, occur in 33% of sporadic gastrinomas and 17% of insulinomas (8). Other studies have found mutations in 6 of 23, 1 of 4, and 3 of 11 gastroenteropancreatic neuroendocrine tumors, respectively (9–11).

We were interested in confirming the role of *MEN1* mutations in sporadic gastrinomas and in determining if mutations in the *MEN1* gene could be identified in DNA extracted from paraffin-embedded gastrointestinal neuroendocrine tumors. A retrospective study was performed using nine archived paraffin-embedded gastrinomas and nonfunctioning neuroendocrine tumors from the tissue archives at the Ohio State University Medical Center. Heteroduplex analysis was used to screen 93% of the coding sequences for mutations of the *MEN1* gene. Using this strategy, we identified two novel frameshift mutations. These inactivating mutations, along with the finding of a loss of heterozygosity (LOH) in one tumor, are consistent with the *MEN1* gene product acting as a tumor suppressor. We have confirmed the role of *MEN1* mutations in sporadic enteropancreatic neuroendocrine

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tumors and have provided conditions for the use of archival paraffin-embedded tissue as a valuable source of genetic material for retrospective genetic studies.

Materials and Methods

TUMOR SPECIMENS

A total of six gastrinomas and three neuroendocrine tumors, one from each patient, were obtained at the time of surgical resection at the Ohio State Medical Center (Table 1). After resection, the tissues were paraffin-embedded according to routine protocol. The diagnosis of neuroendocrine tumors was made by histological analysis, and patients were considered to have gastrinomas if they had either increased fasting serum gastrin or paradoxical increases in serum gastrin in response to provocative testing with secretin. Patients were considered to have nonfunctioning pancreatic neuroendocrine tumors if they clinically demonstrated no evidence of hormonally active tumors in the presence of a pancreatic mass with neuroendocrine characteristics by histopathological analysis. All of these tumors were sporadic. All procedures performed for handling the subjects and tissues were in agreement with the ethics standards of the Ohio State University Human Subjects Committee.

DNA EXTRACTION

DNA extraction from paraffin-embedded tissue was modified from a previously described procedure (12). Several 50- μ m sections from a block of paraffin-embedded tumor tissue were placed in a 1.5-mL microcentrifuge tube. To dissolve the paraffin, the sections were immersed in 1 mL of xylene, vortex-mixed briefly, and incubated for 10 min at room temperature. The tube was then centrifuged for 5 min at 13 800 g. A pipette was used to remove the liquid. This procedure was repeated twice with 1-mL aliquots of xylene. The tissue was rehydrated gradually by repeating the above method with 1 mL of ethanol (twice), then with 800 mL/L ethanol, and then with 500 mL/L ethanol. One milliliter of H₂O was added, and the tube was refrigerated at 4 °C for 24 h to complete rehydration. To lyse the cells, the tissue was warmed to room temperature and then centrifuged for 5 min. The water was removed and replaced with 700 μ L of nuclei lysis buffer (10 mmol/L

Tris, 400 mmol/L NaCl, 2 mmol/L Na₂EDTA), and the sample was vortex-mixed. For solubilization of membranes and degradation of proteins, 70 μ L of proteinase K and 30 μ L of 200 g/L sodium dodecyl sulfate were added, and the sample was vortex-mixed and incubated at 55 °C for 24 h. If the tissue was not completely degraded, an additional 15 μ L of proteinase K was added and incubation was continued for another 24 h. Precipitation of degraded proteins was accomplished by adding 233 μ L of saturated NaCl solution. The tube was vortex-mixed for 15 s and spun for 5 min. The supernatant (containing the DNA) was transferred to a new tube, combined with 1 mL of ethanol, and inverted to precipitate the DNA.

PCR AMPLIFICATION

Tumor DNA was amplified in the presence of 150 ng of each oligonucleotide primer and 2.5 U of Taq polymerase (Cetus-Perkin-Elmer) in a final volume of 100 μ L of the following solution: 0.5 mmol/L deoxynucleotide triphosphates, 1 or 2 mmol/L MgCl₂, 67 mmol/L Tris (pH 8.8), 16.6 mmol/L ammonium sulfate, 6.7 μ mol/L EDTA, and 10 mmol/L 2-mercaptoethanol. The primer sequences (Table 2) were derived directly from the DNA sequence (Genbank accession no. U93237). Amplification proceeded by a denaturation step of 5 min at 95 °C, followed by 35 cycles (1 min at 95 °C, 2 min at 55 °C, 3 min at 72 °C), and ended with an extension step of 8 min at 72 °C (Ericomp Thermocycler). To achieve optimal amplification, it was sometimes necessary to increase the number of amplification cycles to 40. Ninety-three percent of the coding region was amplified; technical difficulties prevented us from amplifying the other 7% of the gene.

HETERODUPLEX ANALYSIS

The heteroduplex approach relies on the formation of heteroduplexes between the wild-type and mutant alleles, which are detected because they migrate differently than the corresponding homoduplexes in Hydrolink-MDE gels. It has been reported that there is a 93% rate of LOH in gastrinomas (8). Heteroduplexes would not form in the DNA of patients with a deleted allele; therefore, PCR products from different tumors were mixed, thus allowing for heteroduplex formation. These aliquots of PCR

Table 1. Gene mutations in gastrinomas and other neuroendocrine tumors.

Tumor no.	Tumor type	Location	<i>MEN1</i> mutation	Exon no.	
1	Gastrinoma	Metastasis	Liver	ND ^a	
2	NE	Primary	Pancreas	ND	
3	Gastrinoma	Primary	Stomach/duodenum	ND	
4	Gastrinoma	Metastasis	Lesser omental node	ND	
5	NE	Metastasis	Liver	320del7	2
6	NE	Primary	Pancreas	ND	
7	Gastrinoma	Metastasis	Liver	ND	
8	Gastrinoma	Metastasis	Liver	ND	
9	Gastrinoma	Metastasis	Peripancreatic node	634insC	3

^a ND, not detected; NE, neuroendocrine tumor.

Table 2. Primer sequences and conditions for amplification.

Exon	Primer sequence	bp ^a amplified	MgCl ₂ , mmol/L	Anneal temp, °C
2A-F	5'-GAGCAGAGGCTGAAGAGG-3'	358	1	55 (Hot start)
2A-R	5'-CGGGGCTGGGCTGGAAGGT-3'			
2B-F	5'-GGCTTCGTGGAGCATTTTC-3'	369	2	55
2B-R	5'-TTGAAGAAGTGGGTCATGGAT-3'			
3-F	5'-GGTCACAGGCTTGAAAGG-3'	393	2	55
3-R	5'-AGAAAATGGAGTCCCTTGG-3'			
4-F	5'-GAAGCAGGCACAGGGTGG-3'	239	2	55 (Hot start)
4-R	5'-CACAGCAGTCAAGTCTGG-3'			
5-6-F	5'-GGACCCGTTCTCCTCCCT-3'	319	2	55
5-6-R	5'-CCCTGCCTCAGCCACTGTTAGG-3'			
7-F	5'-GGTGGGAGTGGAGATGGAGA-3'	334	2	55
7-R	5'-TGGTTGGAAACTGATGGAGG-3'			
8-F	5'-GACCCACCTACTCCCCAG-3'	350	2	55
8-R	5'-TCCTGCCATCCCTAATCCCG-3'			
9-F	5'-TATGCTTACCTTTTCTGGAG-3'	379	2	55
9-R	5'-GGGGATGGGCAGATGCTG-3'			
10B-F	5'-GCCCAGGAAGCCAGCACTG-3'	385	2	55
10B-R	5'-GGTCCCACAAGCGGTCC-3'			
10C-F	5'-CACTGTCTTTCCTCAAGC-3'	250	2	55
10C-R	5'-AGACCTATATTCTAACGACTG-3'			
10D-F	5'-AGTGTGATCAAACCCACGAT-3'	322	2	55
10D-R	5'-GTGCGGAAATATACTCCTA-3'			
10E-F	5'-CAAAGGGAGCACAGGTC-3'	244	2	55
10E-R	5'-AGTTTCGTCAGGAAGAGGG-3'			

^a bp, base pair; temp, temperature.

product were heated to 95 °C for 5 min, and then slowly ramped down to 37 °C for 30 min (13). A 20- μ L aliquot of each mixture was then combined with 4 μ L of 6 \times Triple Dye Loading Buffer (FMC BioProducts) and was electrophoresed through a 50-cm Hydrolink-MDE gel (AT Biochem) containing 150 g/L urea in Tris-borate-EDTA buffer (80.3 mmol/L Tris base, 48.5 mmol/L boric acid, 1.53 mmol/L Na₂EDTA) for 15 h at 1000 V. The gel was stained in a solution of Tris-borate-EDTA buffer containing 1 mg/L of ethidium bromide, and the DNA was photographed under ultraviolet light. This screening method would detect mutations in exons of the gene as well as in splice donor and acceptor sites. Because none of the primer pairs were designed to amplify promoter or intron sequences, we would not have been able to detect intronic cryptic splice site mutations, mutations in the promoter, or mutations in exon 1.

SEQUENCE ANALYSIS

Sequencing was performed on samples that produced heteroduplexes on the MDE gel. The Wizard PCR Preps DNA Purification System (Promega) was used to isolate longer double-stranded DNA strands away from the degraded DNA, which is abundant in preserved tissue and decreases the quality of the sequence. Double-stranded sequencing of the purified PCR product was performed using the double-stranded DNA cycle sequencing system (Life Technologies). Primers were end-labeled in a volume of 5 μ L containing 1 μ L of 5 \times kinase

buffer (Life Technologies), 2.5 μ L (3 ng/ μ L) of primer, 1 μ L of [γ -³²P]-ATP, 0.5 μ L of T4 polynucleotide kinase (Life Technologies); and were incubated at 37 °C for 20 min, 55 °C for 10 min; and then placed on ice. The sequencing reaction proceeded in a volume of 37 μ L: 5 μ L of end-labeled primer, 26 μ L of distilled H₂O, 4.5 μ L of 10 \times Taq sequencing buffer (Life Technologies), 1 μ L of template DNA (from PCR amplification), and 0.5 μ L (5 U/ μ L) of Taq DNA polymerase. The cycle sequencing program consisted of the following: 6 min at 95 °C, 20 cycles (30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C), followed by 10 cycles (30 s at 95 °C, 1 min at 68 °C), and 8 min at 72 °C. The product from the sequencing reaction was analyzed using a 50-cm 5% denaturing polyacrylamide gel. Tumor DNA was compared with that of a control patient without MEN1. Mutations were confirmed by repetition and by separately labeling both forward and reverse primers.

LOH

A semiquantitative technique was used in an attempt to determine the number of *MEN1* alleles present in comparison to the number of alleles of the *CFTR* gene. Primers M1-7 (see Table 2) and CF621 (forward: 5'-AGTCACCAAAGCAGTACAGC-3'; reverse: 5'-GGGCCTGTGCAAGGAAGTGTTA-3') were end-labeled in the same reaction mixture: 1.2 μ L of doubly distilled H₂O, 0.5 μ L of forward primers (15 ng/ μ L each primer), 0.6 μ L of kinase buffer [350 mmol/L (pH 7.6), 500 mmol/L KCl, 5 mmol/L

2-mercaptoethanol, 50 mmol/L $MgCl_2$, 0.1 μ L of [γ - ^{32}P]-ATP, 0.1 μ L of T4 polynucleotide kinase (10 U/ μ L), and 0.2 μ L of *Pfu* DNA polymerase (2.5 U/ μ L; Stratagene). The end-labeling reaction mixture was incubated at 37 °C for 20 min and 55 °C for 10 min, and then transferred to ice. The PCR amplification protocol was a variation of that described by McAndrew et al. (14). The PCR reaction mixture consisted of 14.9 μ L of distilled H_2O , 0.25 μ L of dimethyl sulfoxide, 2.5 μ L of 10 \times PE Buffer (Cetus-Perkin-Elmer), 0.75 μ L of 100 mmol/L $MgCl_2$, 0.5 μ L of reverse primers (15 ng/ μ L each primer), 0.5 μ L of 25 mmol/L deoxynucleotide triphosphates, and 0.1 μ L of Taq polymerase (5 U/ μ L). This was added to the end-labeled primers and to 2 μ L of DNA (1 g/L) and amplified as follows: 6 min at 95 °C, 16 cycles (1 min at 95 °C, 2 min at 55 °C, 3 min at 72 °C), and 8 min at 72 °C. The amplified products were analyzed on a 50-cm 5% denaturing polyacrylamide gel and were quantified by autoradiography. Densitometry of the bands was performed on a Shimadzu CS-9000. The genomic *CFTR* and *MEN1* ratio was determined for all samples. Tumor 5 was used as a positive paraffin control because its LOH status was determined by sequence analysis.

Results

MUTATION ANALYSIS

Nine tumor specimens, including six gastrinomas and three nonfunctioning neuroendocrine tumors, were evaluated for *MEN1* mutations, using the technique of heteroduplex analysis. Aberrantly migrating bands were identified in two of nine tumors at the time of electrophoresis, indicating the presence of mutations (Fig. 1). Direct sequencing of tumor 9 revealed a novel one-base pair insertion at nucleotide 643 (643insC) in exon 3 of the *MEN1* gene (Fig. 2), which produced a shift of the reading frame. The resulting reading frame was terminated by a new stop codon in the subsequent triplet codon. Tumor 5 was shown to have a novel seven-base pair deletion in

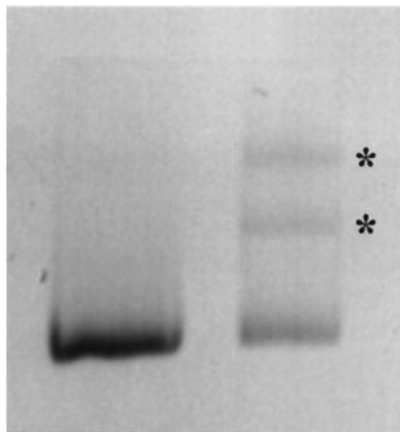


Fig. 1. Heteroduplex analysis of exon 3 of tumor 9 (right) and a healthy control (left).

* indicates heteroduplex.

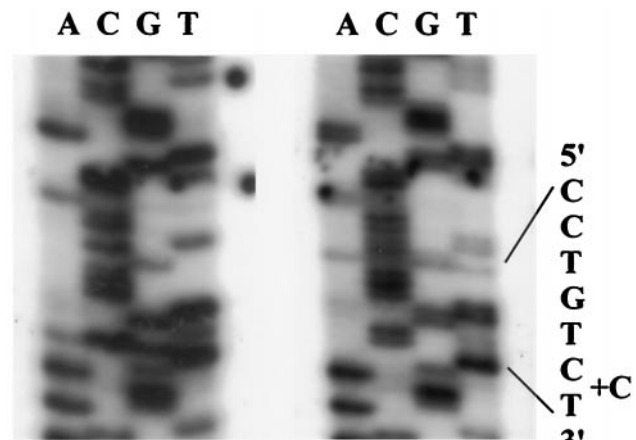


Fig. 2. Portions of the sequencing gel of DNA from a healthy control (left) and a patient (right).

Sequencing from primer M1-3R showed the patient to have an insertion of a single C at nucleotide 643 in exon 3.

exon 2 of the *MEN1* gene (320del7; Fig. 3). This mutation also caused a frameshift, which produced a severe truncation of the message 44 codons downfield from codon 71.

We observed mutations in one of six (17%) gastrinomas and one of three (33%) neuroendocrine tumors for an overall frequency of 22%. Although this frequency is lower than the 33% (9 of 27) reported previously (8), it is not inconsistent given that we looked at fewer tumors. Zhuang et al. (8) used the method of single-strand conformational polymorphism to scan for mutations, whereas we used heteroduplex analysis, which has been shown to have comparable sensitivity (15). More than 60% of the *MEN1* gene mutations that were previously identified in sporadic gastrinomas were located in exons 2 and 3 (8). Both of the mutations found in this study were also found in exons 2 and 3.

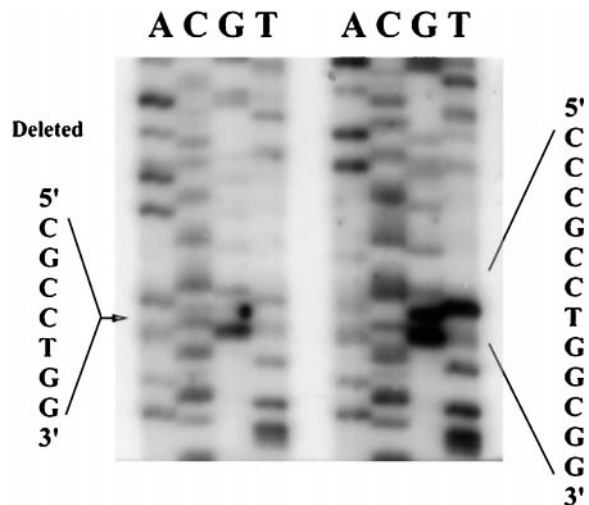


Fig. 3. Portions of the sequencing gels of DNA from a patient (left) and a healthy control (right).

Sequencing is from primer M1-2BR. The patient was found to have a deletion of seven nucleotides starting at nucleotide 320 in exon 2.

LOH

LOH is common in pancreatic endocrine tumors (16, 17) and was detected in 93% of sporadic gastrinomas and 50% of sporadic insulinomas investigated by Zhuang et al (8). LOH was confirmed in one of the two tumors that we analyzed by sequence analysis. Tumor 5 was shown to have only a single sequence ladder despite a seven-base pair shift in the sequence of the homologous allele (Fig. 3). If both the wild-type allele and the mutant allele were present, overlapping sequence ladders would be apparent. The fact that no wild-type sequence was observed indicates that the wild-type allele had been deleted. In contrast, tumor 9 had a one-base pair insertion that did demonstrate two overlapping sequence ladders (Fig. 2). Because we were dealing with old preserved tumors, we did not have access to blood samples from the corresponding patients and were, therefore, not able to perform standard LOH analysis. We attempted to identify LOH in our tumor samples by semiquantitative coamplification of exon 7 of the *MEN1* gene and exon 10 of the *CFTR* gene, which served as an endogenous internal control known to have a disomic copy number. Any variation between samples or between the conditions of the PCR reaction would, therefore, not affect the ratio for each sample, because both amplified products would be affected equally (18). Amplification proceeded for 16 cycles so that polymerase enzyme, nucleotides, and primer concentrations were in excess over the template DNA concentration. This ensured that amplification was exponential and quantitative. The ratios, however, were not markedly different for any of the samples including the positive control, tumor 5. The results are most consistent with amplification of contaminating stromal tissue from the archival blocks, which prevented discrimination between the presence of one or two alleles of the gene.

Discussion

During this study, mutations in the *MEN1* gene were identified in two paraffin-preserved sporadic enteropancreatic neuroendocrine tumors. These mutations in tumors 9 and 5 were frameshifting mutations in the 5' region of the gene, which would cause a severe truncation of the menin protein. Tumor 9 was from a 52-year-old woman who presented with multiple gastric ulcers and diarrhea. Her serum gastrin response to provocation with secretin was consistent with gastrinoma. The patient's tumor was preoperatively localized by computer tomography and octreotide scintigraphy. At the time of surgery, she was found to have a single metastatic lymph node, which was histologically confirmed to be a gastrinoma. No primary tumor was identified. Tumor 5 was from a 73-year-old man who was noted to have multiple liver nodules at the time of a distal pancreatectomy and splenectomy. These were resected and appeared histologically consistent with metastatic neuroendocrine tumor. The primary tumor was presumably in the tail of the pancreas. Because the patient did not exhibit biochemical evidence

of a hormonally active tumor, he was subsequently diagnosed as having a metastatic nonfunctioning pancreatic neuroendocrine tumor. Furthermore, the identification of both an allelic deletion and a one-base pair insertion in tumor 5 is consistent with the two-hit hypothesis of a tumor suppressor gene (19).

Tissue preserved in paraffin is an important resource for genetic studies. There is more background and the sequence data are often of lesser quality than are found from sequenced fresh tissue DNA, because much of the DNA from paraffin-preserved tissue is degraded, but it is still quite feasible to find mutations. We established conditions under which we were able to identify two mutations in DNA extracted from paraffin in nine embedded blocks. We found that amplification of relatively small products was effective for DNA extracted from paraffin-preserved tissue. Because of the highly degraded nature of the DNA, there is likely to be more short-length DNA that would provide an intact template. In our study, the ratio of intact to degraded DNA was increased by using a DNA purification system (see *Materials and Methods*). This procedure was also found to increase the quality of the sequence data.

LOH analysis using microsatellite markers from the *MEN1* locus was not feasible because we did not have access to blood samples from the patients. However, we attempted to apply a semiquantitative dosage assay by comparing PCR-amplified product from *MEN1* exon 7 to amplified *CFTR* exon 10, which served as our endogenous internal control. The dosage ratios were not consistent, which was most likely because of the masking of possible allelic loss by contaminating stromal cells. Extraction from paraffin-embedded tissue as described here is sufficient for heteroduplex analysis and for DNA sequencing; however, gene dosage assays are complicated by the presence of small amounts of contaminating stromal tissue in preserved surgical sections. Recently, techniques have been described that might make a dosage assay on DNA from preserved tissue feasible. Immunostaining was used by Lubensky et al. (20) to determine that the endothelial component in *MEN1* parathyroid tissue accounted for the majority of the stromal component of the excised parathyroid tumors. They used a 30-gauge needle under a light microscope to select tumor cells and to minimize the amount of contamination by stromal tissue. They were quite successful at identifying quantitative differences in *MEN1* copy number between DNA from tumor tissue and from blood. A similar technique may be used for a successful semiquantitative dosage assay on DNA from paraffin-embedded tissue.

We have performed a retrospective mutation analysis of the *MEN1* gene, using archival paraffin-embedded tissue. This is important, because enteropancreatic neuroendocrine tumors are rare, and the collection of tissue for prospective genetic studies may require long periods of time. Analyzing archival tissue will expedite the process of understanding the role of the *MEN1* gene in

tumorigenesis and would be an aid in understanding the role of other disease genes. Molecular diagnosis of familial *MEN1* mutations from preserved tissue of deceased relatives would provide an opportunity for accurate family studies and counseling for at-risk relatives. Finally, by identifying mutations in both alleles of the gene of one patient, we have presented supporting evidence that the *MEN1* gene is a tumor suppressor gene involved in the tumorigenesis of some enteropancreatic neuroendocrine tumors.

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