Somatic Mutations of the MEN1 Tumor Suppressor Gene in Sporadic Gastrinomas and Insulinomas


Abstract

Gastrinomas and insulinomas are frequent in multiple endocrine neoplasia type 1 (MEN1). The MEN1 tumor suppressor gene was recently identified. To elucidate the etiological role of the MEN1 gene in sporadic enteropancreatic endocrine tumorigenesis, we analyzed tumors (28 gastrinomas and 12 insulinomas) from 40 patients for MEN1 gene mutations and allelic deletions. One copy of the MEN1 gene was found to be deleted in 25 of 27 (93%) sporadic gastrinomas and in 6 of 12 (50%) sporadic insulinomas. MEN1 gene mutations were identified in 9 of 27 (33%) sporadic gastrinomas and 2 of 12 (17%) insulinomas and were not seen in corresponding germ-line DNA sequence. A specific MEN1 mutation was detected in one gastrinoma and in the corresponding germ-line DNA of a patient who had no family history of MEN1. Somatic MEN1 gene mutations and deletions play a critical role in the tumorigenesis of sporadic gastrinomas and may also contribute to the development of a subgroup of insulinomas.

Introduction

Gastrinomas and insulinomas occur sporadically (1) and as a part of an autosomal dominant syndrome, MEN12 (2). Insulinomas usually follow a benign clinical course and are cured by resection of the tumor. In contrast, gastrinomas have a high malignant potential, with regional lymph node or liver metastases developing in up to 90% of the cases, and are curable in the long term in only 30% of the cases. The role of the tumor suppressor genes oncogenes in enteropancreatic endocrine tumorigenesis remains uncertain (3, 4). The Knudson tumor suppressor gene hypothesis states that hereditary cancers develop due to the inheritance of a mutated tumor suppressor gene and that a somatic mutational event involving the wild-type allele of the gene leads to a neoplasm (5). The model predicts that sporadic tumors of the same cell type would arise after somatic inactivation of both copies of the gene responsible for the familial tumor (5). The MEN1 gene was linked to chromosome 11q13 in 1988 (6). Previous studies demonstrated LOH with polymorphic markers on 11q13 in sporadic gastrinomas and insulinomas, suggesting that the MEN1 gene may play a role in sporadic enteropancreatic endocrine tumorigenesis (3, 7).

The MEN1 tumor suppressor gene was recently identified (8), and germ-line mutations in the gene were detected in 47 of 50 kindreds with familial MEN1 (9). To evaluate whether sporadic gastrinomas and insulinomas are associated with functional inactivation of the MEN1 gene, the gene was analyzed for deletions and mutations in 40 sporadic enteropancreatic tumors.

Patients and Methods

Subjects. Tumors from 40 patients who underwent exploratory laparotomy for pancreatic or duodenal endocrine tumors at the NIH were included in the study. All patients gave informed consent in a protocol approved by the NIDDK Institutional Review Board. Clinical and family history were reviewed in each case, and all 40 patients were thought to have a sporadic neoplasm at the time of surgery. The diagnosis of insulinoma required a fasting blood sugar of less than 45 mg/dl, hypoglycemia symptoms, and a simultaneous elevated plasma insulin level of >5 microunits/ml (10). The diagnosis of ZES (gastrinoma) was established as described previously (11) and required meeting at least two of the following criteria: an elevated fasting serum gastrin level; a basal acid output of more than 15 mEq/h if the patient had not had previous gastric surgery or of more than 5 mEq/h if the patient had had previous gastric surgery; and an increase in the serum gastrin level of 200 pg/ml after iv. secretin, an increase in serum gastrin level of 395 pg/ml after an i.v. calcium infusion, or a positive histological diagnosis of gastrinoma.

A diagnosis of sporadic insulinoma was made by an absence of a family history of endocrinopathies associated with MEN1 (renal stones, hyperparathyroidism, pituitary disease, or pancreatic endocrine tumors) and normal serum calcium and albumin measurements. If results were unclear, serum prolactin and gastrin measurements and family screening were done in some cases. A diagnosis of sporadic gastrinoma required a negative family history (as described above) and normal values for total serum calcium, ionized calcium, albumin, plasma PTH level (mid-PTH portion), plasma PTH (intact PTH), serum prolactin, urinary cortisol, and normal computed tomography or magnetic resonance imaging of the sella turcica (12).

Tumors. Twenty-eight frozen gastrinomas (4 pancreatic, 8 duodenal, and 1 common bile duct) and metastases (14 lymph node and 1 liver) and 12 benign pancreatic insulinomas were obtained from the file of the Laboratory of Pathology, National Cancer Institute, NIH (Bethesda, MD). All tumors were frozen in liquid nitrogen at the time of surgery and stored at −70°C. Paired normal DNA was obtained from frozen normal exocrine pancreas, duodenum, lymph node, or blood of each patient. Histological evaluation of tumors revealed characteristic neuroendocrine features and positive staining for chromogranin A (Boehringer Mannheim, Indianapolis, IN) and/or synaptophysin (Zymed, San Diego, CA) by immunohistochemistry. The clinical diagnoses of gastrinoma and insulinoma were confirmed by positive immunostain for gastrin (DAKO Corp., Carpenteria, CA) and insulin (BioGenex, San Ramon, CA), respectively.

Tumor and normal cells were selected from 5-μm-thick H&E-stained histological slides, microdissected under direct light microscope visualization as...
described previously (13), and used for PCR-SSCP analysis (14). For this study, dissections were not performed to obtain pure populations of tumor cells but to enrich tumor samples sufficiently to perform mutation analysis by SSCP. Procured cells were resuspended in 30 μl solution containing Tris-HCl (pH 8.0), 0.1 M EDTA (pH 8.0), 1% Tween 20, and 0.1 mg/ml proteinase K and incubated overnight at 37°C. Following thermal inactivation of proteinase K (95°C for 5 min), 1–1.5 μl of the DNA extract were used for PCR amplification.

**MEN1 Allelic Deletion Analysis.** FISH was performed using cosmid clone c10B11 (size, 40 kb) containing the MENJ gene as a probe (15). Touch preparations of frozen tumor specimens were made on glass slides and used for FISH analysis as described previously (16, 17). Hybridization signals were scored using a Zeiss Axioshot epifluorescence microscope, and two-color images were captured on a Photometrics charge-coupled device camera (Photometrics, Ltd., Tucson, AZ) using IP Lab image software (Signal Analytics Corporation, Vienna, VA). At least 100 interphases with strong hybridization signals were scored for each tumor. The presence of cells with one MENJ signal at a frequency of more than 30% was interpreted as an allelic deletion (loss of one copy of the MENJ gene). Seventy % of the cases with allelic deletion detected by FISH showed, upon touch preparation, cells with one MENJ signal at a frequency of more than 70%, and 30% of the cases showed cells with one MENJ signal at a frequency of 35–70%. The percentage of tumor and somatic cells in each frozen tissue imprint correlated with percentage of tumor and somatic cells in the H&E-stained frozen tissue sections from which the touch preparations had been prepared. Normal frozen pancreatic (exocrine and endocrine) tissue controls showed cells with one MENJ signal at a frequency of 3%.

In cases with no detectable MENJ allelic deletion by FISH, DNA from precisely microdissected tumor and normal samples (13) was screened for LOH using three polymorphic markers on 11q13 as described previously (3, 7). The markers included intragenic marker D11S4946, located in the MENJ 5′-flanking region, and the flanking markers PYGM and D11S449 (data not shown). No mutation in the MENJ gene was detected by SSCP analysis in any exons from 17 of 27 sporadic gastrinomas and 5 of 12 insulomas that showed allelic deletion. Observed MENJ gene mutations were limited to tumor DNA and were not detected in germ-line DNA in any case, except for one patient who showed an exon 8 mutation in both tumor (G-1229) and germ-line DNA (Table 2). This patient had no family history of MENI. Preoperatively, this patient’s MENJ status was unclear because his serum calcium, prolactin, and (mid-PTH portion) PTH levels were normal; however, he had a slightly elevated intact PTH level and a history of neck surgery for an unknown reason 15 years prior to admission.

Mutations observed among enteropancreatic endocrine tumors in the study included four deletions, two insertions, and five missense mutations (Table 2 and Fig. 2). No tumor was found to have more than one MENJ gene mutation. Mutations were identified in exons 2, 3, 4, 8, and 10 (Table 2). Five of nine (56%) somatic mutations in sporadic gastrinomas occurred in exon 2. Two insulomas showed mutations, one in exon 2 and one in exon 10. Deletions or insertions resulting in a frameshift of the protein coding sequence accounted for 55% (6 of 11) of the mutations. These mutations produce altered transcripts, with 11% (1 of 9) localized (duodenal and pancreatic) gastrinomas examined (Table 2).

Table 1 PCR primer sets used for MENJ gene mutation analysis

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer sequence</th>
<th>Size (bp)</th>
<th>Annealing temperature (°C)</th>
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<td>60</td>
</tr>
<tr>
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<td>2C*</td>
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<td>297</td>
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</tr>
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<td>5'-tctacccacccctcctcag-3'</td>
<td>249</td>
<td>55</td>
</tr>
<tr>
<td>4</td>
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<td>171</td>
<td>58</td>
</tr>
<tr>
<td>5</td>
<td>5'-atccttctcctcctcctcaca-3'</td>
<td>58</td>
<td>98</td>
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<td>6</td>
<td>5'-cccctccttcctccctcctc-3'</td>
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<td>5'-tctctctctctctctctctctc-3'</td>
<td>219</td>
<td>60</td>
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</table>

* Several primer sets were used to amplify exons 2 and 10 due to their large size.

**Results**

Gastrinomas from 28 patients (15 males and 13 females; mean age = 50 years, range = 15–71 years) and insulinas from 12 patients (7 males and 5 females; mean age = 47 years, range = 17–81 years) were examined for allelic deletions and mutations of the MENJ gene.

One copy of the MENJ gene was found to be deleted in 25 of 27 (93%) sporadic gastrinomas and in 6 of 12 (50%) sporadic insulinas as determined by FISH using a probe containing the MENJ gene (Fig. 1). MENJ gene somatic mutations were identified in 33% (9 of 27) of sporadic gastrinomas and 17% (2 of 12) of sporadic insulinas analyzed (Table 2 and Fig. 2). All but two of the tumors with mutations also showed MENJ gene deletion by FISH, indicating that both copies of the gene were inactivated (Table 2). The absence of allelic deletion in tumors G-1236 and In-1257 was confirmed by LOH analysis using an intragenic microsatellite marker (D11S446) at the MENJ 5′ region and two flanking 11q13 markers, PYGM and D11S449 (data not shown). No mutation in the MENJ gene was detected by SSCP analysis in any exons from 17 of 27 sporadic gastrinomas and 5 of 12 insulinas that showed allelic deletion. Observed MENJ gene mutations were limited to tumor DNA and were not detected in germ-line DNA in any case, except for one patient who showed an exon 8 mutation in both tumor (G-1229) and germ-line DNA (Table 2). This patient had no family history of MENI. Preoperatively, this patient’s MENJ status was unclear because his serum calcium, prolactin, and (mid-PTH portion) PTH levels were normal; however, he had a slightly elevated intact PTH level and a history of neck surgery for an unknown reason 15 years prior to admission.

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We identified three benign polymorphisms R171Q (CGG/CAG), D418D (GAC/GAT), and A541T (GCA/ACA) in exons 3, 9 and 10, respectively, in three sporadic insulinoma cases. These polymorphisms were identical to the three most common polymorphisms observed previously (8, 9).

**Discussion**

The role of the MENJ gene in sporadic enteropancreatic endocrine tumorigenesis was suggested previously by LOH studies on 11q13 (3, 7). Our previous work using 10 polymorphic markers flanking the arm of the putative MENJ gene on chromosome 11q13 demonstrated that 44% of sporadic gastrinomas showed LOH (3, 7). The closest flanking markers used were PYGM (centromeric) and D11S449 and PPP1CA (telomeric), each located more than 100 kb from the MENJ gene. Here, we tested for allelic deletions at the precise location of the...
gene. The significant increase in deletion rate, 93% versus 44%, indicates that a substantial subset of gastrinomas may contain limited interstitial deletions of a 300 kb or less in size that would not be evident at flanking markers PYGM, D11S449, and PPP1CA. Interestingly, the LOH profile observed in gastrinomas differs from that seen in MEN1-associated parathyroid tumors, which exclusively show large deletions of several megabases or more (13). The significance, if any, of the different deletion profiles between the two tumor types is currently not clear.

The high frequency of MEN1 mutations (33%) and allelic deletions (93%) in sporadic gastrinomas indicates that, analogous to MEN1-associated tumors, MEN1 gene alterations are critical events in sporadic gastrinoma tumorigenesis. Mutational inactivation of one MEN1 allele coupled with deletion of the second allele strongly implicates the MEN1 gene in the pathogenesis of these tumors, as defined in the two-hit recessive (loss-of-function) mechanism (5). MEN1 mutations were detected in localized duodenal and pancreatic gastrinomas and...

### Table 2. MEN1 gene mutations and allelic deletions in gastrinomas and insulinomas

<table>
<thead>
<tr>
<th>Exon</th>
<th>Tumor code</th>
<th>Tumor location</th>
<th>Mutation</th>
<th>Allelic deletion</th>
</tr>
</thead>
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<tr>
<td>2</td>
<td>G-344</td>
<td>Duodenum</td>
<td>358del46</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>G-58</td>
<td>Lymph node</td>
<td>358del25</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>G-187</td>
<td>Duodenum</td>
<td>186F (ATC — TTC)</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>G-1351</td>
<td>Pancreas</td>
<td>483del11</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>G-1236</td>
<td>Pancreas</td>
<td>W126G (TGG — GGG)</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>In-1147</td>
<td>Pancreas</td>
<td>545insT</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>G-40</td>
<td>Lymph node</td>
<td>F159C (TTC)</td>
<td>TGT)</td>
</tr>
<tr>
<td>8</td>
<td>G-520</td>
<td>Lymph node</td>
<td>875insA</td>
<td>+</td>
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<td>8</td>
<td>G-1491</td>
<td>Lymph node</td>
<td>1212delTT</td>
<td>+</td>
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<tr>
<td>8</td>
<td>G-1229</td>
<td>Lymph node</td>
<td>E363del (term-line)</td>
<td>+</td>
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<tr>
<td>10</td>
<td>In-1257</td>
<td>Pancreas</td>
<td>A535V (GCT — GTT)</td>
<td>—</td>
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<tr>
<td>10</td>
<td>G-555</td>
<td>Lymph node</td>
<td>554L (TCA — TTA)</td>
<td>—</td>
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</tbody>
</table>

* G, gastrinoma; In, insulinoma.
* Mutation abbreviations follow standard nomenclature (27).
* Presence (+) or absence (−) of MEN1 allelic deletion as detected by FISH.
* Deleted nucleotides are CTGT.
* Metastasis.
* Deleted nucleotides are TATCTA TCATGCCGC CCTCTATGC.
* Deleted nucleotides are GCCATAG.

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Fig. 1. Representative results of FISH in lymphoblast cell line and sporadic enteropancreatic endocrine tumors. Green signal, α-satellite centromeric marker; red signal, chromosome 11q13 probe containing the MEN1 gene (cosmid c10B11). A, the MEN1 gene localized to 11q13 on metaphase chromosome preparation from a normal lymphoblast cell line by FISH. B–D, interphase touch preparations of sporadic enteropancreatic endocrine tumors. B, sporadic insulinoma (In-756) showing no deletion of the MEN1 gene by FISH. Two red signals, two alleles of the MEN1 gene. C, allelic deletion of the MEN1 gene detected in sporadic insulinoma cells (In-1147; one red signal) as compared to a normal somatic cell with two red signals (left). D, allelic deletion of the MEN1 gene in sporadic gastrinoma cells (G-1544; one red signal).
metastatic (lymph node) gastrinomas examined (Table 2), suggesting that they occur at an early stage of sporadic gastrinoma tumorigenesis.

In the present study, we observed an apparent clustering of mutations in exon 2 of the MEN1 gene in sporadic gastrinomas. Exon 2 is the first coding exon in the MEN1 gene, and four of the five exon 2 mutations observed in the gastrinomas produce frameshifts resulting in a protein that, even if biologically stable, contains little of the wild-type amino acid sequence. Thus, it is possible that tumors which share essentially complete knockout of menin may also share phenotypic characteristics. Clearly, the number of cases examined is small and needs to be expanded before firm genotypic/phenotypic conclusions can be drawn.

Here, a MEN1 gene mutation was found in exon 8 and tumor DNA in a patient who had no family history of MEN1. The patient had ZES with normal serum calcium and prolactin levels, conflicting levels of plasma PTH values, and an unclear past history of neck surgery. Interestingly, the E363del mutation in this patient was identical to a germ-line mutation previously described in two unrelated MEN1 families (8, 9). Patients with MEN1 can initially present with enteropancreatic endocrine tumors as the sole manifestation of their disease (12, 21) and can, therefore, be difficult to distinguish from patients with sporadic tumors without MEN1. In one study, one-third of the 28 patients with ZES with MEN1 initially presented with ZES and only developed hyperparathyroidism or pituitary disease years later (12). Because 20–25% of patients with ZES have MEN1 and because their clinical management differs significantly from that of patients with the sporadic form of the disease, the ability to screen patients with ZES for MEN1 germ-line mutations will be a valuable tool for evaluation of ZES patients in the future.

Somatic MEN1 mutations were detected in 2 of 12 (17%) sporadic insulinomas. One of the two insulinomas also showed a loss of the second copy of the MEN1 gene. Five insulinoma cases had a loss of one copy of the MEN1 gene without a detectable MEN1 gene mutation in the other copy. In 50% (6 of 12) of insulinomas, neither MEN1 mutation nor deletion could be detected. Thus, the MEN1 gene appears to be altered in a subset of sporadic insulinomas. Other, as yet unidentified tumor suppressor genes/oncogenes may contribute to the pathogenesis of the majority of insulinomas (22). The data reported here for insulinomas are similar to recent data obtained in a group of sporadic parathyroid tumors analyzed for MEN1 gene mutations (23). MEN1 mutations were identified in 7 of 33 (21%) sporadic parathyroid tumors, and LOH on 11q13 was detected in 13 of 33 (39%) parathyroid tumor samples.

No mutation in the MEN1 gene was detected by SSCP analysis in any exons from 17 of 27 sporadic gastrinomas and 5 of 12 insulinomas that showed allelic deletion. This work may, in fact, underestimate the prevalence of MEN1 somatic mutations in sporadic enteropancreatic endocrine tumors, as the sensitivity rate of SSCP analysis for detection of single base substitutions is estimated at 80% (24). Moreover, some of these tumors may contain a mutation in regions of the MEN1 gene that were not screened, such as the promoter. In the absence of a mutation or deletion, an alternative mechanism, such as hypermethylation of a CpG island as described in other tumor suppressor genes (25, 26), may inhibit transcription of the second copy of the MEN1 gene. Lastly, another as yet unidentified tumor suppressor gene that is important for pathogenesis of some enteropancreatic endocrine tumors may exist near the MEN1 locus. Further studies are necessary to investigate such alternative mechanisms in enteropancreatic endocrine tumorigenesis.

In summary, a high frequency of MEN1 mutations and allelic deletions was observed in localized and metastatic sporadic gastrinomas. The finding that the MEN1 tumor suppressor gene has an etiological role in sporadic gastrinoma should lead to a better understanding of the molecular pathogenesis of this malignant tumor and should aid in methods of diagnosis and treatment of patients with gastrinoma. The study suggests that MEN1 mutations and allelic
deletions may also contribute to the development of a subgroup of benign insulinomas.

Acknowledgments

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References

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