11q13 Allelic Loss in Pituitary Tumors in Patients with Multiple Endocrine Neoplasia Syndrome Type 1

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INTRODUCTION

MEN 1 is characterized by tumors of the pancreas, parathyroid, and pituitary glands (1). It is an autosomal, dominantly inherited disorder with 94% penetrance by age 50 years (1, 2). The gene, which is localized to chromosome 11q13, has recently been cloned and found to contain 10 exons (3). It encodes a protein termed menin whose function remains undetermined (3). The number of different inactivating mutations in the MEN 1 gene and evidence for loss of the wild-type allele in different tumor types in MEN 1 patients suggest that the MEN 1 gene functions as a tumor suppressor gene, consistent with Knudson’s two-hit model (3, 4).

Parathyroid adenomas, the most common endocrine tumor in MEN 1, occur in 90–97% of cases (1, 2). Pancreatic neuroendocrine tumors have been observed in 30–82% of cases, and duodenal tumors have been observed in 25–60% of cases (1, 5). Tumors of the anterior pituitary occur in 35–60% of patients (1, 2, 5). Pro-lactinomas are by far the most common pituitary tumor in MEN 1, accounting for 40–80% of pituitary tumors in most series; other tumors commonly found include GH-secreting, nonfunctioning, and ACTH-secreting tumors (6, 7).

Two principal theories of pituitary tumorigenesis exist: (a) a hypothalamic hormonal influence; and (b) an intrinsic pituitary defect (8). In favor of the former are normal responses of adenomas to hypothalamic hormones, development of tumors in mice transgenic for GHRH, and overexpression of the GHRH gene in some GH-secreting pituitary tumors (9, 10). On the other hand, although ectopic GHRH-producing tumors such as small cell lung carcinoma induce somatotroph hyperplasia, they are not known to be associated with GH-secreting adenomas; similarly, ectopic corticotropin releasing hormone production has not been shown to stimulate the development of a corticotroph adenoma (8).

The evidence for an intrinsic pituitary defect in sporadic and hereditary pituitary tumors is more compelling. X chromosome inactivation analysis has been used in PRL-, GH-, and ACTH-secreting and nonsecreting adenomas to demonstrate the monoclonal origin of pituitary adenomas (11–13). In contrast, hyperplastic and normal pituitary tissue is polyclonal (14).

G proteins, which are involved in transmembranous cell signal transduction, are heterotrimeres of three distinct subunits. In up to 40–50% of sporadic human GH-secreting adenomas, somatic heterozygous missense activating point mutations of the Gs α subunit are found, which convert the G protein into a constitutively active protein, the oncogene gsp (15).

Loss of tumor suppressor gene function by mutations or

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3 The abbreviations used are: MEN 1, multiple endocrine neoplasia type 1; LOH, loss of heterozygosity; GH, growth hormone; GHRH, GH-releasing hormone; ACTH, adrenocorticotropic hormone; PRL, prolactin; SSCP, single-strand conformational polymorphism.
deletions in the p53, retinoblastoma, p16, and nm23 genes, however, has not been identified as a common feature in pituitary adenomas (16–19). Similarly, growth factors such as basic fibroblast growth factor, fibroblast growth factor 2, and transforming growth factor family members have been only tangentially implicated in pituitary tumorigenesis. In addition, the tumors were screened for protein activation.

**Table I** Patient characteristics

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (yr), sex</th>
<th>fMEN1</th>
<th>Parathyroid disease</th>
<th>Pancreatic disease</th>
<th>Other conditions</th>
<th>Pituitary</th>
<th>LOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>65, M</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>M: PRL</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>31, F</td>
<td>Yes</td>
<td>Yes</td>
<td>Islet cell</td>
<td>M: PRL</td>
<td>NF*</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5, M</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>M: PRL</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>31, F</td>
<td>Yes</td>
<td>Yes</td>
<td>Insulinoma</td>
<td>m: ACTH</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>46, F</td>
<td>No</td>
<td>Yes</td>
<td>NET*</td>
<td>m: ACTH</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>42, M</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Ocular melanoma</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>53, F</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>M: GH</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>52, F</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>m: GH</td>
<td>Yes</td>
<td></td>
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<tr>
<td>9</td>
<td>35, M</td>
<td>Yes</td>
<td>Yes</td>
<td>Insulinoma</td>
<td>M: NS</td>
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<td></td>
</tr>
<tr>
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<td>Yes</td>
<td>Insulinoma</td>
<td>M: PRL</td>
<td>Yes</td>
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<tr>
<td>11</td>
<td>38, F</td>
<td>No</td>
<td>Yes</td>
<td>NET</td>
<td>M: NS</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>

* M: macroadenoma; m: microadenoma; NS, nonsecretory tumor.
* fMEN1, familial MEN 1 patient.
* NF, noninformative for the polymorphic markers studied.
* NET, neuroendocrine tumor of the pancreas that stained for multiple polypeptides but did not secrete.

pituitary tumors were evaluated with magnetic resonance imaging; eight were macroadenomas (greatest dimension, >10 mm), and three were microadenomas. One patient (case 5) had two separate synchronous tumors, each producing a single hormone; immunohistochemical staining revealed staining for only one hormone (ACTH or PRL) in each tumor, both of which were removed at the same surgery. However, only material from the PRL-secreting adenoma was available for genetic study. Three tumors were reported in a previous study, before the MEN 1 gene had been identified (25). Nine of 11 were familial cases identified as having at least two typical endocrine neoplasms as well as one or more features in at least one first-degree relative with MEN 1-related endocrinopathies. Two sporadic cases (cases 5 and 11) were identified on the basis of having at least two typical endocrine neoplasms and the presence of germ-line mutations identified by dideoxy fingerprinting sequencing and analysis of blood DNA. All tumor specimens were obtained from surgery performed (by E. H. O.) at the NIH as part of an institutional review board-approved protocol for which informed consent was obtained. Both freshly frozen and paraffin-embedded tissues were used in this study.

**Materials and Methods**

**Study Population.** Eleven patients with MEN 1 syndrome who underwent trans-sphenoidal microsurgery for symptomatic pituitary adenomas were studied. The biochemical, clinical, and pathological characteristics of the 11 patients are presented in Table 1. There were five males and six females. The average age at surgery was 38 years (range, 5–65 years); the average age at initial clinical diagnosis of MEN 1 disease was 29.5 years (range, 5–48 years). In all cases, immunohistochemical staining of the surgical specimens confirmed the biochemical findings. Ten of 11 patients had parathyroid disease; one 5-year-old patient (case 3) had elevated serum total and ionized calcium levels, but normal parathyroid hormone levels. Six patients had neuroendocrine tumors of the pancreas. All pituitary tumors were evaluated with magnetic resonance imaging; eight were macroadenomas (greatest dimension, >10 mm), and three were microadenomas. One patient (case 5) had two separate synchronous tumors, each producing a single hormone; immunohistochemical staining revealed staining for only one hormone (ACTH or PRL) in each tumor, both of which were removed at the same surgery. However, only material from the PRL-secreting adenoma was available for genetic study. Three tumors were reported in a previous study, before the MEN 1 gene had been identified (25). Nine of 11 were familial cases identified as having at least two typical endocrine neoplasms as well as one or more features in at least one first-degree relative with MEN 1-related endocrinopathies. Two sporadic cases (cases 5 and 11) were identified on the basis of having at least two typical endocrine neoplasms and the presence of germ-line mutations identified by dideoxy fingerprinting sequencing and analysis of blood DNA. All tumor specimens were obtained from surgery performed (by E. H. O.) at the NIH as part of an institutional review board-approved protocol for which informed consent was obtained. Both freshly frozen and paraffin-embedded tissues were used in this study.

**Microdissection of Tumor Samples.** In nine cases, tumor cells were procured from 6–μm sections taken from paraffin-embedded blocks using tissue microdissection (28), as demonstrated in Fig. 1, A and B. In ambiguous cases, reticulin staining was performed in adjacent sections to differentiate tumor tissue with complete loss of a reticulin network from adjacent normal pituitary tissue (Fig. 1, C and D). Microdissection of pure tumor tissues was performed in all nine cases, as demonstrated in Fig. 1, E and F. Procured cells were then suspended in 30 μl of DNA extraction solution containing 50 mM Tris-HCl, 1 mM EDTA, 0.5% Tween 20, and 0.2–0.4 mg/ml proteinase K (pH 8.0). This mixture was incubated overnight at 37°C; proteinase K was inactivated by heating at 95°C for 5 min.

In two patients (cases 3 and 11), phenol-chloroform extraction was performed on freshly frozen tumor. DNA was extracted in a 0.7-ml solution of 50 mM Tris (pH 8.0), 100 mM EDTA (pH 8.0), 100 mM NaCl, 1% SDS, and 0.5 mg/ml
proteinase K. Samples were extracted four times in phenol-chloroform, precipitated with ethanol, and resuspended in 1× TE (pH 7.4).

Constitutional DNA was derived from peripheral blood or normal tissues obtained at the time of surgery. Peripheral blood DNA was extracted using the Qiagen Blood and Cell DNA kit (Chatsworth, CA).

**LOH Analysis.** DNA from tumor and normal samples was screened for LOH using two polymorphic markers on 11q13 as described previously (29). These were two microsatellite markers flanking the MEN 1 gene, *PYGM* and *D11S449* (30, 31).

**SSCP Analysis of gsp Mutations.** Missense activating point mutations in Gs α were screened using the two primer sets described in Landis et al. (15): (a) exon 8, 5'-CGCCCGC- CGCCGCCGCCTCCGCCCAGGAACATGATCTCTGTATTATA-3' and 5'-TCGTTGGCTTTTTGTAGATCCA-3'; and (b) exon 9, 5'-CGCCCGC- CCGGCCGCTCCGCGCGCGCCCGCCAGTCTGGAATAAACCAG-3' and 5'-AACTGCAAGCCAGTCCCT- CTGGAATAAACCAG-3'. All 11 cases were screened by SSCP analysis of each exon for the presence of aberrant bands in tumor DNA as compared with normal DNA, obtained as described above. Tissue from a GH-secreting tumor was used as a positive control. The following PCR conditions were used: 92°C for 60 s; 58°C for 60 s; and 72°C for 60 s for a total of 35 cycles.

**PCR Amplification of Genomic DNA.** Genomic DNA was isolated from patients 3 and 11 as described above. Primers used for the amplification of the 323-bp fragment in exon 2 were as follows: (a) 5' primer, GGCTTCGCTTGGAGGCATTTCT; and (b) 3' primer, CTGATGAAAGCTGAAGGGACT. PCR conditions were as described in the section above.
RESULTS

Table 1 illustrates the principal clinical and pathological characteristics of the patients. Tumor cells were selectively microdissected from paraffin-embedded formalin-fixed specimens and freshly frozen tissue, carefully avoiding potentially contaminating cells (including WBCs, fibrous cells, and normal tissues) as illustrated in Fig. 1, A–F. The microdissection technique increases the purity of tumor and normal cell populations, permitting precise analysis of the two distinct populations (28).

In a previous study, genetic analysis of blood DNA from the 11 patients had demonstrated characteristic germ-line MEN 1 gene mutations in all patients (32).

LOH analysis was performed using microsatellite markers D11S449 and PYGM, flanking the MEN 1 gene locus. One case (patient 2) with familial MEN 1 and a known germ-line mutation was not informative with the markers studied. Confirmation of wild-type allelic deletion was obtained in 8 of 10 informative cases by a combination of family pedigree analysis (33), which provided information about the mutated allele retained in the tumor in 6 cases, and restriction enzyme digestion (2 cases) directed to the mutated allele (Fig. 3). In these eight cases, the combination of family pedigree analysis and restriction digestion demonstrates that LOH is directed exclusively at the wild-type allele.

SSCP analysis for gsp gene mutations was carried out in all 11 patients using primers for the activating mutations in either exon 8 or exon 9 as described by Landis et al. (15). No patient, including the four patients with GH-secreting tumors, showed a gsp-activating mutation.

DISCUSSION

We tested for allelic deletions at the MEN 1 gene locus on chromosome 11q13 in 11 MEN 1-associated pituitary tumors. All 11 patients had a germ-line mutation of the MEN 1 gene. LOH analysis was informative in 10 tumors, all of which had 11q13 allelic deletions. Previous studies suggest that LOH in MEN 1 tumors reflects the deletion of the wild-type allele (33, 34). To confirm this hypothesis, we show that allelic deletion occurs on the wild-type allele in 8 of our 10 informative cases through a combination of family pedigree analysis (33) and germ-line restriction analysis of the mutated allele. This is the first study to demonstrate both mutation of one allele and deletion of the second allele in pituitary tumors associated with MEN 1 syndrome. Our findings implicate the inactivation of both the mutated and wild-type alleles of the MEN 1 gene in the pathogenesis of these pituitary tumors, as defined by Knudson’s two-hit tumor suppressor gene hypothesis (4).

The results of this systematic study of pituitary tumors in MEN 1 patients suggest that mutation of one copy of the MEN
I gene, coupled with the deletion of the second, wild-type allele, plays a causative role in pituitary tumor formation. MEN 1 gene deletion was observed in tumors irrespective of their functional or hormonal status. Furthermore, whereas gsp-activating mutations are generally found in up to 50% of GH-producing tumors (15), none of the four GH-secreting tumors in our MEN 1 patients showed evidence of a gsp-activating mutation by SSCP screening, nor were gsp gene mutations found in the seven tumors examined that did not secrete GH.

These results suggest that the mechanism of MEN 1-associated pituitary tumor development parallels that of MEN 1-associated parathyroid adenomas and neuroendocrine tumors of the pancreas and duodenum. Therefore, the MEN 1 gene seems to represent a critical pathway for MEN 1-associated pituitary tumorigenesis. Furthermore, both mutation and deletion, the two genetic abnormalities prescribed by Knudson's tumor suppressor theory (4), seem essential for the development of these tumors. These findings differ from studies of sporadic pituitary adenomas, which showed MEN 1 gene alterations in only a small number of cases (10% or less). In one large series, only 4 of 39 sporadic tumors showed a deletion of one copy of the MEN 1 gene; furthermore, a specific MEN 1 gene mutation in the remaining allele was detected in only two of these tumors (27). Similar results have been identified in sporadic parathyroid tumors and insulinomas. (34, 35).

Current evidence is inconclusive with respect to the exact pathogenesis of sporadic pituitary adenomas. A systematic approach beginning with cell transformation, most likely the result of somatic mutations, and progressing along a continuum of pituitary tumor promotion or growth fostered by the proliferative actions of hypothalamic hormones, growth factors, and abnormal signaling pathway molecules may provide a unitary, inclusive hypothesis for pituitary adenoma formation. At present, the absence of gsp mutations in these MEN 1 pituitary tumors suggests that G protein activations are not involved or are only involved at a late stage in pituitary tumorigenesis; however, mutations and deletions of the MEN 1 tumor suppressor gene seem to play a prominent, early role in tumor formation in these patients. This situation seems different from that in sporadic pituitary tumors (27). Our results indicate that divergent origins of pituitary adenoma tumorigenesis exist in patients with MEN 1 and those with sporadic tumors.

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