Identical Clonality of Sporadic Gastrinomas at Multiple Sites

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Abstract

Gastrinomas are neuroendocrine neoplasms that occur sporadically and in patients with multiple endocrine neoplasia type 1 (MEN1). In MEN1, multiple gastrinomas have been shown to arise by independent clonal events (Debelenko, et al., Cancer Res., 57: 2238–2243, 1997). The purpose of the present study was to analyze clonality in 20 sporadic gastrinomas from eight patients in whom the tumor was present in at least two separate sites. A combination of methods was used to assess clonality, including MEN1 gene mutation analysis, loss of heterozygosity analysis of the MEN1 locus, and analysis of X-chromosome inactivation at the human androgen receptor locus (human androgen receptor analysis). In three patients, a somatic MEN1 gene mutation was detected in the tumor. Identical mutations were found in other tumors at different sites within the same patients. Human androgen receptor analysis in three informative patients and loss of heterozygosity analysis in five patients revealed identical clonal patterns in the tumors from multiple sites in each patient. We conclude that sporadic gastrinomas at multiple sites are monoclonal and that MEN1 gene alterations in gastrinomas occur before the development of tumor metastases.

Introduction

Gastrinomas are the most common malignant functional enteropancreatic endocrine tumors, and between 60–90% of the sporadic gastrinomas are reported to be malignant (1). However, several observations have raised the possibility that sporadic gastrinomas at multiple sites in a single patient may not represent a primary tumor and its metastases. First, a significant proportion of patients with gastrinomas (20–25%) has the tumor as part of the MEN12 syndrome. Previous studies have shown that enteropancreatic endocrine tumors, including gastrinomas, in patients with MEN1 are frequently found simultaneously at multiple sites and arise through independent clonal events (2, 3). Secondly, clinical observations in patients with sporadic gastrinomas have cast doubt on the hypothesis that sporadic gastrinomas at multiple sites may not represent a primary tumor and accompanying metastases. In a recent large surgical series of gastrinomas (4), 10% of the patients had sporadic gastrinomas resected from a lymph node only and were disease-free 5 years after the operation. The possibility of primary lymph node gastrinomas has been previously raised by Arnold et al. (5). Furthermore, two reports identified isolated cells staining positive for gastrin in 15% of normal lymph nodes from the gastrinoma triangle (6) as well as rests of neuroendocrine cells in regional pancreatic lymph nodes from nongastrinoma patients (7). These observations raise the question of whether a gastrinoma in the duodenum or pancreas and, concomitantly, one or more lymph nodes in the same patient are multiple primary tumors rather than metastases originating from one primary tumor. Previous studies have also indicated that the tumor growth pattern in an individual patient may change (8). For example, a patient with no evidence of a gastrinoma by biochemical and imaging studies postresection may develop the clinical signs of an active gastrinoma in a different location years later. This raises the question of whether such a patient developed a second de novo gastrinoma or had metastatic growth of the tumor. Lastly, a recent study reported a mixture of polyclonal and monoclonal growth patterns in sporadic pancreatic endocrine tumors (9). This report included only one gastrinoma without reporting its clonal composition and did not distinguish precisely between individual tumors at multiple sites.

Analysis of clonality in sporadic gastrinomas at multiple sites provides an experimental measure to address the question of whether they represent multiple primary gastrinomas or primary tumors and metastases. Evidence of independent clonal events in these tumors would strongly suggest that tumors arise separately at different times and locations. In contrast, monoclonal growth would support the hypothesis of a single tumor clone and subsequent metastatic spread of the tumor.

In the present study, we analyzed the clonality of sporadic gastrinomas in multiple sites in a given patient with a variety of methods. Forty-four to 93% of sporadic gastrinomas have LOH at the MEN1 locus and 33% carry a mutation within the MEN1 gene (2, 10, 11). Therefore, first we analyzed the tumors for mutations of the MEN1 gene by sequencing. The clonal status of the tumors negative for MEN1 gene mutations was studied using a PCR-based analysis of X-chromosome inactivation in the androgen receptor (AR) gene. Because a subset of cases was not informative by this combined approach, we also analyzed patterns of LOH at the MEN1 locus in tumors at multiple sites.

Materials and Methods

Patients

Eight patients who underwent exploratory laparotomy for ZES at the NIH between 1990 and 1999 and who had gastrinomas in more than one location were included in this study. The eight patients, six females and two males, were part of a prospective long-term study of ZES. The study protocol was approved by the Clinical Research Committee of the National Institute of Diabetes and Digestive and Kidney Diseases, and all patients gave informed consent. The diagnosis of ZES was established as previously reported (8). The absence of MEN1 was diagnosed by lack of a family history and lack of laboratory evidence of other endocrinopathies on a yearly evaluation. All patients initially underwent an exploratory laparotomy for attempted curative resection of the gastrinoma with an extensive intraoperative evaluation, and three patients had repeated operations performed as described previously (4). All patients had a duodenal or pancreatic gastrinoma and, additionally, a gastrinoma either in the lymph nodes or liver.

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2 The abbreviations used are: MEN1, multiple endocrine neoplasia type 1; LOH, loss of heterozygosity; ZES, Zollinger-Ellison syndrome; HUMARA, human androgen receptor analysis.
**Tumors**

**DNA Extraction.** Tumor samples were immediately snap frozen in liquid nitrogen during surgery and stored at –70°C or formalin-fixed and embedded in paraffin. Tumor DNA was extracted from 5-μm sections of the specimens using a commercial kit (DNA Mini Kit, Qiagen Inc., Santa Clarita, CA) after analyzing an adjacent slide with H&E staining to determine that the specimen contained at least 90% tumor cells. In those specimens with significant portions of normal tissue mixed in with the tumor, DNA was extracted after tumor microdissection as previously described (12).

**Mutational Analysis.** Primers for amplification of the MEN1 gene were obtained according to the published sequences (http://www.nhgri.nih.gov). PCR was carried out under previously published conditions (13). Direct sequencing of the exons was performed (AmpliCycle, Perkin-Elmer) in sense and antisense direction in duplicate. Leukocyte DNA from the same patients was simultaneously sequenced and an underlying germ-line mutation was ruled out.

**X-Chromosome Inactivation Analysis.** Extracted tumor DNA from female patients was digested with HpaII (Life Technologies, Inc., Gaithersburg, MD) and compared to the undigested DNA. The DNA was then PCR amplified with primers for the HUMARA using previously published conditions (14, 15). The case was considered informative if the undigested tumor DNA of the patient showed two different alleles of equal intensity (i.e., polyclonal). The absence or significant reduction of one allele in the digested tumor DNA was interpreted as evidence of monoclonality. Analysis was performed in duplicate and yielded the same results.

**LOH Analysis.** The four polymorphic DNA markers: D11S480, PYGM, D11S449, and INT-2 encompass the MEN1 locus on chromosome 11q13, and the method of LOH analysis has been previously published (2). The case was considered informative if the DNA from nontumor tissue of the patient showed two different alleles (i.e., heterozygosity). When comparing the two alleles in the tumor, the near complete (>90% decreased intensity) or complete absence of one allele was interpreted as LOH. Analysis was performed in duplicate and yielded the same results.

**Results**

A total of 20 tumors from eight patients with ZES were studied preoperatively. All patients had elevated fasting serum gastrin levels with hyperchlorhydria or an elevated secretin-stimulated calcium. At present, it is not known whether the events underlying tumor initiation and progression are the same in sporadic and inherited forms of gastrinomas. We have previously used LOH combined with X-chromosome inactivation analysis to demonstrate that multiple entero-pancreatic endocrine tumors, more specifically gastrinomas, in patients with familial MEN1 develop as independent clones (2, 3). This underscores the multifocal development of entero-pancreatic endocrine tumors in patients with MEN1 and indicates that the genetic events underlying tumor development or progression of gastrinomas may occur simultaneously at multiple sites.

In the present study, we provide several independent lines of evidence that in contrast to multiple gastrinomas in MEN1 patients, sporadic gastrinomas at different sites are of the same clonal composition. First, the mutational analysis of the MEN1 gene in different tumors of three patients revealed identical mutation in each tumor. The presence of the same mutation in all tested tumors from an individual patient indicates that all tumor cells are of monoclonal composition. The finding of identical MEN1 mutations in tumors at multiple sites strongly suggests a single mutational event that has been passed on to subsequent tumor cell generations rather than several independent mutational events that occurred in the course of the disease. This finding is significant especially in view of the fact that somatic and germ-line MEN1 gene mutations are scattered throughout the entire gene, and mutational hotspots have not been identified (10, 16). Second, the X-chromosome inactivation pattern was identical in all tumors from each of the three patients, suggesting that all tumors from different sites within a given patient were derived from the same clone. Third, all different tumors in each of the five informative

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Gender/age</th>
<th>Tumor sites</th>
<th>MEN1 mutation</th>
<th>HUMARA</th>
<th>LOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F/44</td>
<td>Duodenum (1)</td>
<td>None</td>
<td>NI</td>
<td>LOH</td>
</tr>
<tr>
<td>2</td>
<td>F/42</td>
<td>Duodenum wall (2)</td>
<td>None</td>
<td>NI</td>
<td>LOH</td>
</tr>
<tr>
<td>3</td>
<td>F/40</td>
<td>Duodenum</td>
<td>730delG</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>F/28</td>
<td>LN</td>
<td>730delG</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>F/59</td>
<td>Duodenum</td>
<td>None</td>
<td>NI</td>
<td>LOH</td>
</tr>
<tr>
<td>6</td>
<td>F/44</td>
<td>PH LN</td>
<td>None</td>
<td>Mono</td>
<td>No LOH</td>
</tr>
<tr>
<td>7</td>
<td>M/62</td>
<td>Duodenum (1)</td>
<td>1212del7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>M/58</td>
<td>LN (2)</td>
<td>1212del7</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Patients in whom tumors were removed during different operations. In all other patients, the tumors were removed during the same surgery. 

* The number in brackets represents the number of the operation. 

**Discussion**

Gastrinomas occur sporadically (75%) and as a manifestation of MEN1 (25%) and can occur at multiple sites, suggesting either multifocal tumor origin or metastatic spread from a primary tumor (1). Interestingly, the sporadic gastrinoma and the MEN1-associated variant are indistinguishable by morphological criteria and both may show heterogeneous immunohistochemistry staining for various peptides. At present, it is not known whether the events underlying tumor initiation and progression are the same in sporadic and inherited forms of gastrinomas. We have previously used LOH combined with X-chromosome inactivation analysis to demonstrate that multiple enteropancreatic endocrine tumors, more specifically gastrinomas, in patients with familial MEN1 develop as independent clones (2, 3). This underscores the multifocal development of enteropancreatic endocrine tumors in patients with MEN1 and indicates that the genetic events underlying tumor development or progression of gastrinomas may occur simultaneously at multiple sites.
patients tested for LOH with four polymorphic markers at the MEN1 locus had identical patterns of loss or retention of heterozygosity. This suggests that the inactivation of the second allele of the MEN1 gene by LOH occurred in a single cell and was propagated to all daughter cells.

Our data support the conclusion that sporadic gastrinomas in multiple sites are of clonal origin. Therefore, the common clinical presentation of a sporadic duodenal or pancreatic gastrinoma simultaneously with a lymph node or liver tumor represents a primary tumor metastases as opposed to independent tumors. A recent study by Perren et al. (9) reported that other sporadic pancreatic endocrine tumors were as likely to be polyclonal as monoclonal. However, the authors observed a predominantly polyclonal growth pattern among tumors referred to as “benign” (5/7; 71%) and oligo- or monoclonal growth pattern in those tumors defined as “malignant” (8/13; 62%). The authors proposed that a growth pattern of sporadic pancreatic endocrine tumors may initially involve several tumor clones and then, once a single clone has a distinct growth advantage, a monoclonal growth pattern may evolve, i.e., clonal evolution (9). The results of the present study could be interpreted as evidence that molecular pathogenesis of sporadic gastrinomas in multiple sites differs from the pathogenesis of other pancreatic endocrine tumors (nongastrinoma) or as evidence for the clonal evolution of sporadic gastrinomas. The former interpretation would be supported by the fact that we only found evidence for a monoclonal growth pattern in sporadic gastrinomas and we did not observe any polyclonal growth patterns in the gastrinomas using multiple different techniques. The latter interpretation should also be considered in view of the fact that our goal was to study gastrinomas at multiple sites in individual patients. Therefore, we selected for patients with possibly aggressive disease. Such patients represent the largest group of gastrinomas patients encountered clinically because 60–90% of all gastrinomas are reported to be malignant (1).

MEN1 gene mutations are found in about 33% of sporadic gastrinomas (10, 11), but the functional relevance of such a mutation in the tumor development has not been elucidated. Identical mutations and deletions in the primary tumor and its metastases within the same patient not only indicate that multiple tumors are derived from one founding cell with these genetic alterations but also provide insight into the timing of such an event. Identical mutation and deletion patterns of the MEN1 gene were detected in the primary gastrinoma as well as in all metastases; therefore, the genetic changes must have occurred before the spread of the tumor.

In summary, we provide molecular evidence that sporadic gastrinomas in multiple sites are monoclonal in growth and in fact represent a primary tumor and subsequent metastases as opposed to independent tumors. Furthermore, the finding of identical MEN1 gene mutations and LOH patterns in primary tumors and its metastases within an individual patient is indicative of mutational/deletional events that have occurred before the development of metastases.

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References


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