Genetic Evidence for Early Divergence of Small Functioning and Nonfunctioning Endocrine Pancreatic Tumors: Gain of 9Q34 Is an Early Event in Insulinomas

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ABSTRACT

The malignant potential among endocrine pancreatic tumors (EPTs) varies greatly and can frequently not be predicted using histopathological parameters. Thus, molecular markers that can predict the biological behavior of EPTs are required. In a previous comparative genomic hybridization study, we observed marked genetic differences between the various EPT subtypes and a correlation between losses of 3p and 6q and gains of 14q and Xq and metastatic disease. To search for genetic alterations that play a role during early tumor development, we have studied 38 small (≤2 cm) EPTs, including 24 insulinomas and 10 nonfunctioning EPTs. Small EPTs are usually classified as clinically benign tumors in the absence of histological signs of malignancy. Using comparative genomic hybridization, we identified chromosomal aberrations in 27 EPTs (mean, 4.1). Interestingly, the number of gains differed strongly between nonfunctioning and functioning EPTs (3.4 versus 1.5, respectively; P = 0.0526), as did the number of aberrations in the benign (n = 30) and malignant (n = 8) tumors (3 versus 8.4, respectively; P = 0.0022). In the insulinomas, 9q gain (common region of involvement: 9q34) was most common (50%) and in nonfunctioning EPTs, gain of 4p was most common (40%). Most frequent losses in insulinomas involved 1p (20.8%), 1q, 4q, 11q, Xq, and Y (all 16.7%) and in nonfunctioning EPTs, 6q. Losses of 3p and 6q and gains of 17p and 20q proved to be strongly associated with malignant behavior in all of the small EPTs (P ≤ 0.0219). Our results demonstrate marked genetic differences between small functioning and nonfunctioning EPTs, indicating that these subtypes evolve along different genetic pathways. In addition, our study endorses the importance of chromosomes 3 and 6q losses to discriminate EPTs with a malignant behavior from benign ones.

INTRODUCTION

EPTs make up only 1–2% of all of the pancreatic tumors and can cause syndromes by uncontrolled expression of biologically active hormones. Such tumors are classified as functioning EPTs, whereas EPTs that do not release any hormone or produce hormones that do not lead to clinical symptoms are called nonfunctioning tumors (1). Because these clinical parameters do not provide information with respect to the biological behavior of EPTs, more indicative histopathological classification schemes to predict prognosis have been established (2, 3). Accordingly, well-differentiated EPTs are classified as benign if they are ≤2 cm in size, confined to the pancreas, nonangiogenic (with two or fewer mitoses per 10 high-power fields), and/or exhibit ≤2% Ki-67 positive cells. Most insulinomas fall into this category, as do nonfunctioning (micro)adenomas that are a rather common finding in carefully examined postmortem pancreases (4). EPTs that are ≥2 cm or show angiogenesis, higher numbers of mitoses, or percentages of Ki-67 positive cells are at risk of malignancy. They include many of the functioning tumors other than insulinomas.

Our understanding of the molecular mechanisms underlying the tumorigenesis of EPTs is still scarce. A small percentage of EPTs occur in association with the dominantly inherited MEN 1 and, to a lesser extent, VHL syndromes, the susceptibility genes of which have been cloned and mapped to chromosome bands 11q13 and 3p25.5, respectively (5, 6). The vast majority of EPTs, however, occur sporadically, and only a subset (15–20%) of these tumors harbor somatic MEN 1 mutations (7). In contrast, VHL mutations appear not to be relevant in the pathogenesis of sporadic EPTs (8), as are alterations of the K-ras, N-ras, and TP53 genes (9–11). The involvement of chromosomal regions 9p21 (p16 gene) and 17q12-q21 (c-erbB-2 gene) in EPT tumorigenesis is controversial (10–14).

Recently, we have applied CGH to identify chromosomal alterations that are important in the pathogenesis of EPTs (14). Various significant genetic changes could be identified, including losses of chromosomes 3, 6, 11, X, and Y and gains of chromosomes 5, 7, 9, 14, and 17. The total number of genetic changes per EPT appeared to be strongly correlated with both tumor size and disease stage. Moreover, genetic differences could be detected in the various hormonal subtypes, indicating that these groups may evolve along genetically different pathways. The objective of the present study was to characterize the genetic alterations of EPTs with a diameter ≤2 cm, because these lesions may provide information about the initiating genetic events in the pathogenesis of these tumors. We found marked genetic differences between small functioning and nonfunctioning EPTs, indicating that they evolve along different genetic pathways. In addition, by correlating genetic alterations with tumor subtype and malignant potential, additional evidence was obtained showing that losses of chromosomes 3 and 6q are related to malignancy in EPTs.

MATERIALS AND METHODS

Tumor Material and Patient Data. Thirty-eight EPTs with a maximum diameter of 2 cm (21 females, mean age 55.2 ± 21.3 years and 17 males, mean age 57.1 ± 15.1 years) were selected from the archives of the Departments of Pathology, Universities of Zurich and Basel, Switzerland and University of Turin, Turin, Italy (Table 1). The samples included 15 frozen and 23 formalin-fixed, paraffin-embedded EPTs, which were all sporadic tumors and not associated with the inherited MEN 1 or VHL syndromes. They were classified according to the most recent classification (15) and consisted of 10 nonfunctioning (6 benign and 4 malignant) and 28 functioning EPTs, including 24 insulinomas (22 benign and 2 malignant), 3 glucagonomas (2 benign and 1 malignant), and 1 malignant gastrinoma. Thirty-six patients had localized disease, as defined by the absence of extrapancreatic spread of the tumor, whereas only 2 patients had advanced disease (patients 23 and 24 in Table 1), with tumor spread into the lymph nodes (one case) or the liver (one case).

DNA Extraction. Genomic DNA from frozen samples was isolated by homogenizing ~5 mm³ of each sample prior to DNA extraction using the
Table 1  Patient characteristics and genetic findings in small EPTs

<table>
<thead>
<tr>
<th>Nr°</th>
<th>Sex</th>
<th>Age</th>
<th>Diagnosis</th>
<th>Ben/mal</th>
<th>Size (cm)</th>
<th>CGH results</th>
<th>Number of changes</th>
<th>FISH results&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>All Gains Losses</td>
<td>9c–9q34 6c–6q21 3c–4p16</td>
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<td></td>
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</tr>
<tr>
<td>1</td>
<td>Female</td>
<td>57</td>
<td>Ins</td>
<td>Ben</td>
<td>1.1</td>
<td>0</td>
<td>0 0 0</td>
<td>Pressed 0 0</td>
</tr>
</tbody>
</table>
| 2   | Female | 73 | Ins | Ben | 1.2 | 0 | 0 0 0 | D-5000 Puregene DNA isolation kit (Genta Systems, Minneapolis, MN). Genomic DNA from paraffin-embedded tumor material was isolated from 5 to 10 10-μm thick sections as described previously (16, 17). Briefly, unstained tissue sections were microdissected and scraped from the histological glass slides after identification of the appropriate tissue area using a stereomicroscope (Stemi DV4, Zeiss, Germany) at ×10–40. The paraffin material was collected in a 1.5-ml Eppendorf microcentrifuge tube containing 100% ethanol and centrifuged to the bottom of the tube (5 min, 14,000 rpm). After the ethanol was removed, 1 ml of xylol was added to the tissue material, and the tube was heated at 1 min at 55°C in a thermomixer. After centrifugation for 10 min at 14,000 rpm, the xylol was discarded, and the tissue material was treated two additional times with fresh xylol, as described above. Next, the specimens were treated subsequently with 100% and 70% ethanol with centrifugation steps in between (10 min, 14,000 rpm), after which the tissue material was dried in a speed-vac for 10 min at room temperature. Tissue was digested overnight with 1 mg/ml Proteinase K (Sigma Chemical Co., St. Louis, MO) in digestion buffer [50 mM Tris (pH 8.0), 1 mM EDTA, and 0.5% SDS] at 50°C. The DNA was purified by standard phenol-chloroform extraction and ethanol precipitation and quantified by spectrophotometry. CGH and Digital Image Analysis. CGH was performed essentially as described earlier (14). Briefly, 1 μg of tumor DNA was labeled with Spectrum Green-dUTPs (Vysis, Downsers Grove, IL) by nick translation (BioNick kit; Life Technologies, Inc., Basel, Switzerland). Spectrum Red-labeled normal reference DNA (Vysis) was used for cohybridization. The hybridization mix-

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<sup>a</sup> Nr, number; Ins, insulinoma; Ga, gastrinoma; Glu, glucagonoma; NF, nonfunctioning; Ben, benign; Mal, malignant; 9c,6c,3c, centromere 9,6,3.

<sup>b</sup> FISH results: Disomy, Trisomy: 2 and 3 copies per nucleus of both probes; 2–1 (0%); 2 copies of the first probe and 1 copy of the second probe in 1% of nuclei.

<sup>c</sup> Incidentally discovered at autopsy, strongly positive for glucagon by immunohistochemistry.

<sup>d</sup> Incidentally discovered at autopsy, strongly positive for glucagon by immunohistochemistry. No information available on exact (small) tumor size.
ture consisted of 200 ng of Spectrum Green-labeled tumor DNA, 200 ng of Spectrum-Red labeled normal reference DNA, and 10–20 μg of human Cot-1 DNA (Life Technologies, Inc.) dissolved in 10 μl of hybridization buffer [50% formamide and 2× SSC (pH 7.0)]. Hybridization was carried out for 3 days at 37°C to denatured [5 min at 75°C in 70% formamide and 2× SSC (pH 7.0)] normal male human metaphase spreads (Vysis). Slides were washed at 45°C three times for 10 min in 50% formamide/2× SSC followed by two times for 5 min in 2× SSC. The chromosomes were counterstained with 4,6-diamidino-2-phenylindole for identification.

Digital images were collected from six to seven metaphases using a Photometrics cooled CCD camera (Microimager 1400; Xillix Technologies, Vancouver, Canada) attached to a Zeiss Axiostep microscope and a Power G3 Macintosh computer. The software program QUIPS (Vysis) was used to calculate average green:red ratio profiles for each chromosome. At least four observations per autosome and two observations per sex chromosome were included in each analysis. In a subset of cases, the fluorochrome labels for tumor and reference DNA were reversed in the CGH to confirm the reproducibility of the detected chromosomal abnormalities.

Positive, negative, and sex-mismatched controls were applied as described by Richter et al. (18). Gains and losses of DNA sequences were defined as chromosomal regions where both the mean green:red fluorescence ratio and its SD were >1.20 and <0.80, respectively. Over-representations were considered amplifications when the fluorescence ratio values in a subregion of a chromosomal arm exceeded 1.5. In negative control hybridizations, the mean green:red ratio occasionally exceeded the fixed 1.2 cutoff level at the following chromosomal regions: 1p32-pter, 16p, 19, and 22. Gains of these known G-C-rich regions were therefore excluded from all of the analyses.

Contingency table analysis was used to analyze the relationship between genomic alterations and malignancy, and hormonal subtype. Student’s t test and ANOVA were applied to compare the number of genomic alterations between the different EPT hormonal subtypes.

**Confirmation of CGH Data by FISH.** To validate CGH data independently, touch preparations of six EPTs were subjected to FISH using a combination of a chromosome 9-specific centromere probe (pMR9α) and a cosmid probe (cos-ABL-8) containing the abl gene at 9q34 (19, 20), as well as a chromosome 6-specific centromere probe (p308; Ref. 21) and a PAC probe (66H14) mapping to the 6q21 region (kindly provided by Dr. P. Sinclair, Royal Free and University College School of Medicine, London, United Kingdom). Cell processing, probe labeling, in situ hybridization, and detection and evaluation of the observed nuclear FISH signals were performed as described recently (22, 23). In addition, paraffin-embedded tissue sections (5 μm) of four EPTs, each showing different alterations for chromosomes 3 and 4 (Table 1), were used for independent FISH analysis with a combination of a chromosome 3-specific centromere probe and a chromosome band 4p16-specific cosmid probe (c5.5; Ref. 24, 25), as described previously (26).

**RESULTS**

**CGH Findings in Association with Tumor Subtype and Malignant Outgrowth.** Table 1 and Fig. 1 summarize all of the DNA sequence copy number changes detected by CGH in the 38 EPTs ≤2 cm, and representative CGH data are shown in Fig. 2A. Genetic aberrations were found in 27 of 38 EPTs (71%), and the average number of chromosome arm aberrations per tumor was 4.1 ± 4.6 (range, 0–13). Chromosomal losses (mean, 2.1; range, 0–9) were as frequent as gains (mean, 2.0; range, 0–10), and no evident amplifications could be detected (Table 1 and 2). The average number of aberrations was approximately two times higher in nonfunctioning EPTs than in functioning EPTs (6.1 versus 3.4, respectively), and of particular note, the difference between the average number of gains in both groups (3.4 versus 1.5, respectively) almost reached statistical significance (P = 0.0526; Table 2). In addition, the mean number of chromosomal changes, gains, and losses all were strongly associated with malignancy (P ≤ 0.0242; Table 2).

Chromosomal regions that were most often lost (>15% of EPTs) included 1p, 6q, 11p, and Xq, with the highest frequency of losses on chromosome 11q (21.1%; Fig. 1 and Table 3). In addition, Xq losses were more frequent in female than in male patients (28.6% versus 5.8%, respectively), whereas chromosome Y loss was detected in 5 of 17 (29.4%) males. Increased DNA sequence copy number (>15% of EPTs) most often involved chromosomes 3q, 9q, and 14q, with the highest frequency of gains on chromosome 9q (36.8%; Fig. 1 and Table 3). A comparison between nonfunctioning and functioning EPTs revealed marked differences in genetic alterations between these two groups (Fig. 1 and Table 3). In particular, chromosome 6q losses (CRI, 6q12–22) and chromosome 4 gains were mainly detected in the nonfunctioning EPTs (P = 0.0027; Table 3), whereas 9q gains (CRI, 9q34) were strongly associated with functioning EPTs (P = 0.0404). The latter genetic aberration was detected in 12 of 24 (50%) insulinomas (P = 0.0288) and was the sole alteration in 4 of these tumors. Chromosomal losses of 3pq and 6q (P ≤ 0.0005) as well as gains of 17q and 20q (P ≤ 0.0219) proved to be significantly correlated with malignant outgrowth of EPTs. Our data demonstrate that small EPTs harbor fewer but similar genetic alterations as have been found in larger EPTs by CGH (14) and indicate that already in small tumors an evident genetic divergence can be detected between nonfunctioning and functioning EPTs as well as between benign and malignant tumors.

**Confirmation of CGH Data by FISH.** FISH analysis on touch preparations of six EPTs confirmed the CGH results of chromosome arms 9q and 6q (Table 1). Two benign insulinomas without chromosome imbalances (patients 2 and 3) presented two copies of the
centromere and locus-specific probe per nucleus for both chromosome arms in the FISH analysis, indicating that these tumors have a diploid DNA content. Of two additional insulinomas showing 9q34 gain as the sole alteration by CGH, one tumor (patient 10) displayed three copies of the centromere 9 probe and six copies of the 9q34 probe per nucleus in the major cell population, confirming the CGH finding by FISH. Also, a trisomy for both chromosome 6 probes was observed in this EPT, and this tumor appeared to be triploid. The other insulinoma (patient 11) turned out to harbor two genetically heterogeneous cell populations exhibiting three and four copies of the chromosome 9 probes, respectively. Because of the blocking of repeated sequences by Cot-1 DNA, the centromeric region cannot be analyzed by CGH, which probably explains the detection of only the 9q34 gain. Discrimination of the two different cell populations was not possible with the chromosome 6 probes that demonstrated three copies per nucleus in most cells. In the EPT of patient 15, the gain of 9q33–34 identified by CGH could be detected in 51% of the nuclei by FISH, whereas no alterations for chromosome 6 were found by both methods. In the malignant insulinoma of patient 24, FISH again detected different cell populations with the chromosome 9 probes. Next to a major population with four copies of both centromere 9 and 9q34, 25% of nuclei demonstrated a clear duplication of the cABL target at 9q34. In addition, a number of very large nuclei was observed in between these nuclei containing two sometimes very large centromere spots together with up to twelve 9q34 signals, suggesting that amplification of this target had occurred (Fig. 2B). The frequency of these cells (5%) was, however, much too low to identify this amplification as such by CGH. The loss of 6q found by CGH in this tumor was reflected by the loss of one copy of 6q21, whereas the centromere 6 target was still present as two copies per nucleus in the major cell population. Paraffin sections of four EPTs were subjected to FISH with a combination of a centromere-3 probe and a 4p16-specific probe.

**Table 2** Genomic changes per case in small EPTs

<table>
<thead>
<tr>
<th>Tumor</th>
<th>n</th>
<th>All</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Gains</th>
<th>P&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Losses</th>
<th>P&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>All EPTs</td>
<td>38</td>
<td>4.1 ± 4.6</td>
<td>2.0 ± 2.7</td>
<td>2.1 ± 2.6</td>
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<tr>
<td>Nonfunctioning EPTs</td>
<td>10</td>
<td>6.1 ± 5.1</td>
<td>3.4 ± 4.1</td>
<td>2.6 ± 2.9</td>
<td></td>
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<tr>
<td>All functioning EPTs</td>
<td>28</td>
<td>3.4 ± 4.3</td>
<td>NS&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.5 ± 1.9</td>
<td>0.0526</td>
<td>1.9 ± 2.6</td>
<td>NS</td>
</tr>
<tr>
<td>Insulinomas</td>
<td>24</td>
<td>3.5 ± 4.5</td>
<td>NS&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.5 ± 1.9</td>
<td>0.0725</td>
<td>1.9 ± 2.7</td>
<td>NS</td>
</tr>
<tr>
<td>Benign EPTs</td>
<td>30</td>
<td>3 ± 4</td>
<td>1.5 ± 2.5</td>
<td>1.5 ± 2.2</td>
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<tr>
<td>Malignant EPTs</td>
<td>8</td>
<td>8.4 ± 4.4</td>
<td>0.0022</td>
<td>3.9 ± 2.7</td>
<td>0.0242</td>
<td>4.3 ± 3</td>
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<sup>a</sup> ANOVA analysis.
<sup>b</sup> NS = not significant.
<sup>c</sup> P for functioning versus nonfunctioning EPTs.
<sup>d</sup> P for insulinomas versus nonfunctioning EPTs.
Table 3 Frequent genomic changes (%) in small EPTs and association with malignancy

<table>
<thead>
<tr>
<th>Locus</th>
<th>All EPTs n = 38</th>
<th>Nonfunca n = 10</th>
<th>Functa n = 28</th>
<th>Pb</th>
<th>Ins n = 24</th>
<th>P</th>
<th>Ben n = 30</th>
<th>Mal n = 8</th>
<th>P</th>
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<tr>
<td>1p</td>
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<td>37.5</td>
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<td>16.7</td>
<td>NS</td>
<td>10</td>
<td>12.5</td>
<td>NS</td>
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<td>10</td>
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<td>20</td>
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<td>6.7</td>
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a Nonfunc, nonfunctioning EPTs; Funct, functioning EPTs; Ins, insulinomas; Ben, benign; Mal, malignant; NS, not significant.

b Contingency table analysis.

shown in Table 1 and Fig. 2B, FISH confirmed the chromosomal imbalances found for chromosomes 3 and 4p by CGH in all of the four cases.

DISCUSSION

With the exception of insulinomas, EPTs ≤2 cm in diameter are seldom, because most EPTs have a larger size at the time of diagnosis, present with local invasion or have already developed metastases in regional lymph nodes or the liver in ≤50% of patients (1). Nevertheless, small EPTs can provide us with important information on genetic changes underlying early tumor development, because they mainly consist of benign tumors, including most insulinomas, which can cause hyperinsulinemic hypoglycemia already at early tumor stages, as well as nonfunctioning (micro)adenomas that are incidentally detected at autopsy. It was the goal of this study to identify genetic alterations in small EPTs by CGH, to correlate the overall number of genetic alterations with clinical and histopathological parameters, and to evaluate if the identified chromosomal alterations are similar to those described previously in larger EPTs of different hormonal subtypes (14). CGH detected chromosomal aberrations in 71% of EPTs, most commonly involving losses on chromosomes 1p, 6q, 11p, Xq, and Y and gains on chromosomes 7q, 9q, and 14q. Hence, these chromosomal regions may harbor genes critically playing a role in the pathogenesis of small EPTs. In addition, marked genetic differences were observed between the functioning and nonfunctioning EPTs, and a strong correlation was found between the number of genomic changes per tumor and malignant behavior.

The observed average number of genetic changes per tumor in this study was in good agreement with the results described earlier for a limited number of EPTs ≤2 cm [Ref. 14; 4.1 versus 3.8, respectively] and proves that genetic instability already begins to emerge in small EPTs. Interestingly, we noticed a strong tendency toward a difference between the mean number of genetic alterations in nonfunctioning and functioning tumors being most evident comparing the number of gains in both tumor groups (3.4 versus 1.5, respectively; P = 0.0526). This difference has shown to be highly significant with increasing tumor size (14). Furthermore, a number of genomic changes could be characterized that were strongly correlated to either small functioning or nonfunctioning EPTs. One of the most striking findings in this study was the gain of chromosome 9q in 46.4% of functioning tumors and particularly in 50% of insulinomas, with the CRI being 9q34.

Four insulinomas exhibited 9q gain as the only detectable aberration, and, thus, one might consider this aberration as an important genetic event early in tumorigenesis. FISH analysis verified the 9q gains in four different tumors using a centromere-9 and 9q34-specific probe, and moreover, showed the amplification of the cABL target at 9q34 in one malignant insulinoma. Gains and amplification of 9q34 have been reported to occur in several other human neoplasms including squamous cell carcinomas of the head and neck, adrenocortical carcinomas, pheochromocytomas, and the chronic myeloid leukemia-derived cell line K562 (23, 27–29). The oncogenic significance of the cABL gene, however, has only been established in hematopoietic cells thus far, so that additional studies are needed to clarify its possible role in the development of EPTs. Another candidate gene is the oncogene VAV2 (30) located near the TSCI gene at 9q34. Strangely enough, insulinomas are occasionally detected in TSC patients (31), and these tumors are rather expected to harbor a mutation in the TSCI TSG together with a loss of the other allele. We did not observe loss of heterozygosity using microsatellite marker D9S66 in ten of our sporadic insulinoma cases. However, a TSC-related insulinoma may also develop via an alternative genetic pathway including inactivation of the TSCI gene at 16p13.3. Gains of chromosome 4p and 4q were exclusively found in the nonfunctioning EPTs (in 40% and 30% of cases, respectively), a finding that is in accordance with the results of Terris et al. (32) who found among other alterations gains of chromosome 4 in 7 of 11 nonfunctioning EPTs. Nevertheless, this genomic change cannot be considered a potential initiating genetic event, because chromosome 4 gains were only identified in neoplasms exhibiting ≤10 chromosome arm aberrations per tumor. This also accounts for the most frequently detected genetic alteration in nonfunctioning EPTs, i.e., loss of 6q in 50% of cases. This alteration proved to be significantly more present in nonfunctioning than in functioning EPTs (P = 0.0027), but caution should be taken into account by interpreting these data, because three of five neoplasms were malignant, and a strong association between 6q losses and malignant outgrowth have been observed by us in this (see below) as well as in previous studies accounting for EPTs in general, thus including functioning EPTs. Thus, an early genetic event in nonfunctioning EPTs is difficult to assign on the basis of our CGH results. The difference in genetic makeup compared with functioning EPTs, however, is evident, and the identification of a high number of aberrations already in rather small tumors strengthens our hypothesis that one or
more genes playing a role in the guarding of genetic stability may be inactivated in nonfunctioning EPTs.

Genomic changes that were commonly encountered in both functioning and nonfunctioning tumors involved losses of 1p, 1pq, Xq, and Y, as well as gains of 7q and 14q, alterations that have been observed previously in EPTs (14, 32). Chromosomal losses and loss of heterozygosity at 11q13 are well-known findings in EPTs, because this locus harbors the MEN 1 gene (6) as well as an additional putative TSG more telomeric of the MEN 1 gene (33, 34). The significance of copy number decreases of the sex chromosomes (Xq in female and Y in male patients) is unknown, and such losses are seen in many malignancies, as well as in bone marrow cells of healthy elderly people (35–37). Nevertheless, in our patient series, a number of EPTs of relatively young patients proved to harbor these sex chromosome losses, suggesting that chromosome X may contain one or more TSGs, most likely at the pseudautosomal region on Xp or at Xq22–23. Gains of chromosome 7 have been reported to be present in 43–68% of EPTs by three independent CGH studies (14, 32, 38), and hepatocyte growth factor, epidermal growth factor receptor, and MET have been suggested as potential genes involved in the tumorigenesis of EPTs. So far, however, none of these genes has been proven to be relevant.

Losses of 1p and gains of 14q have been associated with metastatic disease in EPTs (14, 39), and these changes were, indeed, more frequently seen in the malignant than in the benign tumors in this study. A statistically significant correlation, however, was observed between the average number of genomic changes per tumor ($P = 0.0022$), in particular losses of 3pq and 6q and gains of 17pq and 20q ($P \leq 0.0219$), and malignant outgrowth. This implicates that these chromosomal regions may contain genes playing a role in malignant outgrowth, and we and others have previously found allelic losses, as well as genomic deletions at chromosomes 3 and 6q to be associated with clinically malignant EPTs (8, 13, 14, 40–42). The CRLs include 3p25, 3q22–26, and 6q21–22, but the respective genes of interest still need to be identified.

In summary, our data show that EPTs $\leq 2$ cm harbor chromosomal imbalances similar to larger tumors but at a lower frequency. Furthermore, small functioning EPTs already clearly differ in their pattern of genetic aberrations from small nonfunctioning tumors, indicating that these EPT subgroups follow different genetic routes during tumorigenesis. In particular, a copy number increase of chromosome 9q34 represents a hitherto unrecognized but probably important early genetic event in insulomas. In addition, our study provides additional evidence for the association of chromosomes 3 and 6q losses and malignant outgrowth of EPTs.

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