Endocrine tumors of the pancreas and gastrointestinal tract

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CHAPTER 7

DUCTULOINSULAR TUMORS OF THE PANCREAS:
ENDOCRINE TUMORS WITH ENTRAPPED
NONNEOPLASTIC DUCTULES


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LETTER TO THE EDITOR

Abstract
Rare pancreatic neoplasms have been reported that show both endocrine and exocrine differentiation in the neoplastic components. In addition, pancreatic endocrine tumors may contain small, cytologically bland ductules intimately admixed with the endocrine component. It was recently suggested that these ductules represent an intrinsic part of the tumor, i.e. that the ductules are neoplastic, and the term 'ductulo-insular tumors of the pancreas' was proposed. In the present study, the nature of the ductular component of 16 cases of ductule-containing pancreatic endocrine tumors was investigated at the molecular level. Molecular genetic changes often present in ductal pancreatic neoplasms were not found by immunohistochemistry for DPC4, p53 and ERBB2 and by sequence analysis of KRAS codon 12. An X-chromosome inactivation clonality assay of one such tumor from a female patient indicated that the neuroendocrine component was monoclonal, contrasting with the ductular component that was polyclonal. The lymph node and liver metastases from three patients only contained the neuroendocrine component and no ductules were observed. Although certain morphologic features of ductule-containing endocrine tumors are reminiscent of the embryonic development of the human pancreas, none of the tumors expressed PDX-1, a transcription factor essential in pancreatic organ development. Based on our results, it is suggested that the ductular component occasionally found in pancreatic endocrine tumors is the result of entrapment of preexisting nonneoplastic ductules, and that the tumors are otherwise not distinctive from conventional pancreatic endocrine tumors. Although the phenomenon is rare, it is important to recognize and to distinguish these tumors from true mixed ductal-endocrine neoplasms, which are generally more clinically aggressive. 'Pancreatic endocrine tumors with entrapped ductules' would be the preferred nomenclature, since it better reflects the nonneoplastic nature of the ductules.

Introduction
The human pancreas is an organ composed of exocrine (acinar and ductal) and endocrine cells. These cell types can appear in pancreatic neoplasms, and the corresponding tumors have distinctive histologic characteristics. Sometimes mixed tumors with exocrine as well as endocrine differentiation occur. These rare tumors are not well-characterized but have significant components of two cell lineages that are both considered to be neoplastic. Some pancreatic endocrine tumors (PETs) contain morphologically separate small ductules, suggestive of a mixed ductal-endocrine neoplasm. The main part of these tumors consists of endocrine cells, intermingled with small, bland-appearing ductules reminiscent of the nonneoplastic ductules found in pancreatic lobules, especially in regions of atrophy. These ductules are usually in close association with the endocrine cells, resembling so-called 'ductuloinsular complexes', but the two cell types are readily distinguishable by morphology and immunohistochem-
The origin of the ductular component is controversial and has never been investigated thoroughly. It has been suggested previously that the ductular component reflects entrapment of preexisting nonneoplastic ductules by an otherwise conventional endocrine tumor. However, a recent study suggested that the ductules should be considered as an intrinsic part of the tumor, i.e., they are neoplastic.

If this were true, one would expect to encounter some of the genetic changes frequently occurring in other ductular neoplasms of the pancreas, such as mutations in KRAS codon 12, inactivation of DPC4 and TP53 and amplification of ERBB2: at least there should be evidence of clonal origin of the ductules similar to the endocrine component. Also, because of the close relationship between the endocrine and ductular elements (resembling ductulosinsular complexes), another hypothesis is that ductule-containing endocrine tumors arise from multipotent stem cells and are mimicking embryonic pancreatic development. During embryonic pancreatic development multipotent stem cells give rise to the ducts. Some of the duct cells form committed endocrine cells, which grow into islets that are still connected to the ducts, but in a later stage separate and migrate into the exocrine pancreas. To determine whether ductulosinsular tumors have other than morphologic features related to embryonic pancreatic development, one could examine the protein expression of Pancreatic Duodenal Homeobox protein 1 (PDX1), an important transcription factor involved in the formation of primitive pancreatic ductal, endocrine and acinar cells.

When we recently encountered a case of a ductule-containing endocrine tumor, we investigated the nature of the ductular component at the molecular level. By reviewing the archives of three hospitals, 15 tumors showing the same microscopic features were found and similarly analyzed.

Case
A 27-year-old man presented with recurrent episodes of hyperinsulinemic hypoglycemia. Although the patient’s signs and symptoms were indicative of an insulinoma, a tumor could not be detected at clinical evaluation. A pancreatic tail resection was performed. Both grossly and by light microscopy the resected specimen showed no abnormalities. Postoperatively, the patient was treated with diazoxide to prevent the recurrence of hypoglycemia. He visited the hospital at irregular intervals. Fourteen years later, at the age of 41, the patient still had symptoms of hyperinsulinemic hypoglycemia. This time radiographic imaging showed a tumor in the remaining pancreas, which was enucleated. Postoperatively, the patient was normoglycemic.

Grossly, the resected specimen contained a reddish, circumscribed but not encapsulated lesion with a diameter of 1 cm, surrounded by a small rim of pancreatic tissue. Microscopically, the main part of the lesion showed the histological characteristics of an endocrine tumor. It was formed by cells arranged in nests and trabecula. The cells had centrally located nuclei with finely dispersed chromatin.
Figure 7.1 A: One of the tumors (index case) consisted of closely intermingled ductular and endocrine components (hematoxylin and eosin). B: Positivity for synaptophysin confirms the endocrine nature of the main part of the tumor. The ductules are negative for endocrine markers. C: The ductular component is cytokeratin 7 positive, whereas the endocrine cells are cytokeratin 7 negative. D: The immunohistochemical stain for PDX-1 is negative in both components. The insert shows fetal pancreas, used as a positive control.

and inconspicuous nucleoli. In the central part of the lesion another component was observed. This component consisted of tubular structures, reminiscent of non-neoplastic pancreatic ductules, intermingled with the endocrine cells. The cells forming the ductules showed no nuclear atypia. Mitotic activity was absent in both the endocrine and in the ductular elements. There was no infiltrative, perineural or vasoinvasive growth. In the surrounding rim of pancreatic tissue outside the tumor no abnormal islets or periductal endocrine cells were found. A diagnosis of 'pancreatic endocrine tumor with entrapped ductules' was made (Fig. 7.1A). Positive immunohistochemical staining for chromogranin, synaptophysin and neuron-specific enolase confirmed the endocrine nature of the main part of the lesion (Fig. 7.1B). The ductular component was negative for endocrine markers, but expressed pankeratin, cytokeratin 8, cytokeratin 7 and carcinoembryonic antigen, markers for which the endocrine part of the tumor was negative (Fig 7.1C). The endocrine cells, but not the ductules, showed strong positivity for
insulin, consistent with the symptoms of the patient. Stains for glucagon, somatostatin, pancreatic polypeptide, adrenocorticotropic hormone, gastrin, calcitonin, serotonin and S100 were negative in both components. The proliferative activity of the tumor, assessed by staining with Ki67, was variable, but generally low (less than 1%), especially in the ductular component. Both components were negative for PDX-1 (Fig 7.1D).

By ultrastructural examination of the paraffin-embedded tissue, separate cells with either an endocrine or a ductular phenotype could be identified in the tumor. The ductules were generally separated from the endocrine cells by a continuous basement membrane. However, some endocrine cells were present within the ductular structures, interspersed between the basement membrane and the basal surface of the ductular cells. No transitional cell types were found. Acinar cells were present adjacent to, but not in, the tumor.

The expression of a number of gene products known to be involved in neoplastic transformation of pancreatic ductal cells was examined immunohistochemically. Stains for p53 and ERBB2 were negative in both parts of the tumor. Normal expression of DPC4 was found in the endocrine and in the ductular component. A mutation in codon 12 of the KRAS gene could not be detected by sequence-analysis of microdissected tumor tissue.

Review of the slides from the pancreatic tail resection performed 14 years earlier did not reveal any histologic abnormalities in either the endocrine or exocrine compartment.

Material and methods

Patients

The study was extended with 15 additional cases retrieved from the archives of the Departments of Pathology of the Academic Medical Center, Amsterdam The Netherlands (6 cases), of Memorial Sloan-Kettering Cancer Center, New York, NY (5 cases) and of the University Hospitals, Kiel, Germany (4 cases). Tumors were included if the main portion consisted of endocrine cells, if the tumor contained a ductular component, which was not only present at the peripheral rim of the tumor, but also in more centrally located areas amidst tumor fields and if there was close association of the ductules with the endocrine cells (resembling ductuloinsular complexes).

Immunohistochemistry

Immunohistochemical stains were performed on 5-μm sections of paraffin-embedded tumor tissue as described previously. The tumors from the Academic Medical Center, Amsterdam and from the Memorial Sloan-Kettering Cancer Center, New York were formalin-fixed. The tumors from the University Hospital, Kiel were fixed in Bouin’s-fixative.

The index case was stained with all antibodies shown in table 7.1.
## Table 7.1 Antibodies and pretreatment used for the immunohistochemical stains.

CEA = carcinoembryonic antigen, NSE = neuron-specific enolase, PP = pancreatic polypeptide, ACTH = adrenocorticotropic hormone.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clone</th>
<th>Dilution</th>
<th>Source</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pankeratin</td>
<td>Polyclonal</td>
<td>1:10 000</td>
<td>DAKO</td>
<td>Pepsin</td>
</tr>
<tr>
<td>CK8</td>
<td>CAM5.2</td>
<td>1:100</td>
<td>Becton &amp; Dickinson</td>
<td>Pepsin</td>
</tr>
<tr>
<td>CK7</td>
<td>OV-TL</td>
<td>1:500</td>
<td>Biogenex</td>
<td>Pepsin</td>
</tr>
<tr>
<td>CK19</td>
<td>RCK 108</td>
<td>1:1600</td>
<td>Biogenex</td>
<td>Pepsin</td>
</tr>
<tr>
<td>CEA</td>
<td>Polyclonal</td>
<td>1:2000</td>
<td>DAKO</td>
<td>-</td>
</tr>
<tr>
<td>Chromogranin A</td>
<td>Polyclonal</td>
<td>1:1000</td>
<td>DAKO</td>
<td>-</td>
</tr>
<tr>
<td>Synaptophysin</td>
<td>Polyclonal</td>
<td>1:200</td>
<td>DAKO</td>
<td>Citrate</td>
</tr>
<tr>
<td>NSE</td>
<td>Polyclonal</td>
<td>1:10 000</td>
<td>Seralab</td>
<td>-</td>
</tr>
<tr>
<td>Insulin</td>
<td>Polyclonal</td>
<td>1:200</td>
<td>DAKO</td>
<td>-</td>
</tr>
<tr>
<td>Glucagon</td>
<td>Polyclonal</td>
<td>1:1600</td>
<td>DAKO</td>
<td>-</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>Polyclonal</td>
<td>1:3200</td>
<td>DAKO</td>
<td>-</td>
</tr>
<tr>
<td>PP</td>
<td>Polyclonal</td>
<td>1:1000</td>
<td>DAKO</td>
<td>-</td>
</tr>
<tr>
<td>ACTH</td>
<td>Polyclonal</td>
<td>1:400</td>
<td>Organon</td>
<td>-</td>
</tr>
<tr>
<td>Gastrin</td>
<td>Polyclonal</td>
<td>1:2000</td>
<td>DAKO</td>
<td>-</td>
</tr>
<tr>
<td>Calcitonin</td>
<td>Polyclonal</td>
<td>1:6000</td>
<td>DAKO</td>
<td>-</td>
</tr>
<tr>
<td>Serotonin</td>
<td>Polyclonal</td>
<td>1:100</td>
<td>Biogenex</td>
<td>-</td>
</tr>
<tr>
<td>α&lt;sub&gt;1&lt;/sub&gt;-antichymotrypsin</td>
<td>Polyclonal</td>
<td>1:10 000</td>
<td>DAKO</td>
<td>-</td>
</tr>
<tr>
<td>S100</td>
<td>Polyclonal</td>
<td>1:3200</td>
<td>DAKO</td>
<td>-</td>
</tr>
<tr>
<td>Ki67</td>
<td>MIB-1</td>
<td>1:100</td>
<td>DAKO</td>
<td>Citrate</td>
</tr>
<tr>
<td>P53</td>
<td>DO-7+BP53-12</td>
<td>1:200</td>
<td>Neomarkers</td>
<td>Citrate</td>
</tr>
<tr>
<td>ERBB2</td>
<td>E2-4001</td>
<td>1:200</td>
<td>Neomarkers</td>
<td>Citrate</td>
</tr>
<tr>
<td>DPC4/Smad 4</td>
<td>B8</td>
<td>1:100</td>
<td>Santa Cruz</td>
<td>Citrate</td>
</tr>
<tr>
<td>PDX-1</td>
<td>Polyclonal</td>
<td>1:5000</td>
<td>Palle Serup</td>
<td>Citrate</td>
</tr>
</tbody>
</table>
In the other tumors stains for chromogranin, synaptophysin, cytokeratin 7 and cytokeratin 19 were performed to confirm the endocrine nature of the main part of the tumor and to confirm the nonendocrine nature of the ductules. All tumors were stained for PDX-1, p53, ERBB2, DPC4 and Ki67 to examine the relationship with pancreatic embryonic development and to assess the ductules for abnormalities commonly detected in pancreatic ductal neoplasms. The PDX-1 antibody was a gift from Palle Serup from the Hagedorn Research Institute, Gentofte, Denmark. Human fetal pancreas at 17 weeks of gestation served as a positive control for the PDX-1 staining. The primary antibody was replaced by phosphate-buffered saline as the negative control.

**KRAS codon 12 analysis**

Sequence-analysis of KRAS codon 12 was performed on all tumors after microdissection of paraffin-embedded tissue that contained both the ductular and the endocrine components. If the results of the paraffin-embedded material were inconclusive, fresh frozen tumor tissue was also analyzed. Cell suspensions consisting of the colon carcinoma cell line SW 480 (homozygous for a GGT-GTT KRAS codon 12 mutation) and the colon carcinoma cell line HT 29 (wild type KRAS) were used as controls. Placental DNA was used as a control for nonspecific hybridization.

Tumor tissue from 10 slides of 5 μm was microdissected. DNA was purified with the Puregene DNA Purification system (Gentra systems Minneapolis, MN) according to the manufacturer’s instructions and dissolved in a final volume of 15 μl. A first PCR was performed as described previously with 1 μl DNA, 25 pmol primer forward (5'-CTGAATATAAAGTGGTGAGTTTGACCT-3'), 25 pmol primer reverse (5'-CATGAAAAATGGTCAGAGAAACC-3'), 2.0 mM MgCl₂, 100 μM dNTP and 0.35 U Platinum Taq (Invitrogen, Carlsbad, CA). Fifteen cycles of 30 seconds at 94°C, 30 seconds at 55°C and 1 minute at 72°C were run, starting with a denaturation step of 3 minutes at 94°C and finishing with an annealing step of 5 minutes at 72°C. The PCR-product was purified with the Qiaquick PCR purification kit (Westburg, Leusden, The Netherlands) and incubated during 1 hour with a mixture 10 U restriction enzyme BSTN-1 (Bio-labs, Bend, OR in the buffer provided by the manufacturer and 0.2 μg BSA (purified BSA 100x, Bio-labs) in a total volume of 10 μl. Then a second PCR was performed using 1 μl digested DNA, 25 pmol primer forward-2 (5'-CTCGCAACT-GAATATAAAGTGGTGAGTTTGACCT-3'), 25 pmol primer reverse-2 (5'-TCAAAGAATGGTCCTGACCC-3'), 3.5 mM MgCl₂, 100 μM dNTP and 1 U Platinum Taq (Invitrogen). Thirty-five cycles were run using the same PCR program. The PCR-product was purified with the Qiaquick purification kit. The sequence reaction was performed with the DNA sequencing kit, BigDye-Terminator Cycle sequencing Ready Reaction (Applied Biosystems, Warrington, UK) according to the manufacturer’s instructions, using primer forward-3 (5'-CTCGCAACT-GAATATAAAGTGGTGAGTTTGACCT-3') and primer reverse-3 (5'-TCAAAGAATGGTCCTGACCC-3').
GAATGGTCCTGGACC-3'). The sequence reaction products were run on an ABI Prism 3100 Automatic Sequencer (Perkin Elmer biosystems, Foster City, CA) and analyzed subsequently.

**X-chromosome inactivation clonality assay**

A clonality assay based on X-chromosome inactivation by methylation in females and amplification of the Human Androgen Receptor (HUMARA) gene on the X-chromosome was performed on case 7. The endocrine and ductular components were analyzed separately after microdissection of both components by laser capsule microdissection using the PALM® Laser Microbeam Microdissection System (Microlaser Technologies, Bernried, Germany) on 10µm unfixed fresh-frozen sections counterstained with hematoxylin. DNA was purified with the Puregene DNA purification kit (Gentra systems) according to the manufacturer’s instructions. The DNA concentration was measured with the PicoGreen kit (Turner designs, Sunnyvale, CA).

DNA of both the endocrine and ductular components was incubated with the restriction enzyme Hpa II (Roche, Basel, Switzerland), which is methylation sensitive. As a control for the digestion DNA of both the endocrine and the ductular components was incubated with the restriction enzyme Msp I (Roche), which recognizes the same restriction site, unmethylated and hemimethylated. As an internal control DNA of a tumor from a male patient was added to all samples. All samples (17.5 µl) contained 70 U enzyme and buffer provided by the manufacturer, 50 ng purified DNA of either the endocrine or ductular component and 5 ng DNA of the internal control. Samples to which no enzymes were added served as a negative control. After incubation for 5 h at 37°C an identical amount of enzyme and buffer was added in a final volume of 35 µl. The samples were incubated overnight at 37°C.

After digestion a nested PCR was performed to amplify the HUMARA gene. The first PCR was done with the complete digested sample, 175 ng primer forward (5'-GCTGTGAAGGTTGCTGTTCCTCAT-3'), 175 ng primer reverse (5'-CGTC-CAAGACCTACCGAGGAGCTT-3') and a total concentration of 3.6 mM MgCl₂. Twenty cycles of 1 minute at 96°C, 1 minute at 66°C and 1 minute at 72°C were run, starting with a denaturation step at 96°C for 1 minute and finishing with an annealing step at 72°C for 7 minutes. The PCR products were purified with the Qiaquick PCR purification kit (Westburg) and dissolved in an end-volume of 30 µl. The second PCR was performed with 2 µl purified DNA, 100 ng primer forward-2 (5'-TCCAGAATCTG TTCCAGAGCGTGC-3'), 100 ng primer reverse-2 (5'-ATGGGCTTGGGGAGAACCATCCTC-3') and a total concentration of 1.8 mM MgCl₂. The same PCR program was run as for the first PCR. Finally, 1.5 µl of the PCR product was analyzed by an automatic ABI3100 sequencer and Genescan 2.1 software (Perkin Elmer Biosystems).
CHAPTER 7

Results

Patients

In order to strengthen the evidence for the nonneoplastic nature of the ductular component in the pancreatic endocrine tumor described above, 15 pancreatic tumors from 10 male and 5 female patients with a mean age of 59 years (range 19-79 years) were analyzed additionally. Eight of the 15 tumors behaved clinically as an insulinoma, one of the tumors produced the glucagonoma syndrome and the remaining 6 tumors were nonfunctioning. Three of the 15 patients had lymph node metastases. In 1 of these patients the tumor had also metastasized to the liver. The clinical data are shown in table 7.2.

Histology

By definition, the histological features of the tumors were similar to the features of the index case. The main part of the tumors consisted of endocrine cells. All

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age (in years)</th>
<th>Hormone-production</th>
<th>Tumor size (cm)</th>
<th>Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (index case)</td>
<td>M</td>
<td>41</td>
<td>Insulinoma</td>
<td>1</td>
<td>?</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>68</td>
<td>Glucagonoma</td>
<td>7</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>37</td>
<td>Nonfunctioning</td>
<td>3</td>
<td>?</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>66</td>
<td>Insulinoma</td>
<td>1.5</td>
<td>?</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>56</td>
<td>Nonfunctioning</td>
<td>1.7</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>69</td>
<td>Insulinoma</td>
<td>1.5</td>
<td>?</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>74</td>
<td>Nonfunctioning</td>
<td>3</td>
<td>5 lymph nodes</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>75</td>
<td>Insulinoma</td>
<td>1</td>
<td>?</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>70</td>
<td>Nonfunctioning</td>
<td>2.5</td>
<td>3 lymph nodes</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>40</td>
<td>Nonfunctioning</td>
<td>4</td>
<td>2 lymph nodes, liver</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>52</td>
<td>Insulinoma</td>
<td>4</td>
<td>No</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>79</td>
<td>Nonfunctioning</td>
<td>2.8</td>
<td>No</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>46</td>
<td>Insulinoma</td>
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<td>No</td>
</tr>
<tr>
<td>14</td>
<td>M</td>
<td>19</td>
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<td>No</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>77</td>
<td>Insulinoma</td>
<td>2</td>
<td>No</td>
</tr>
<tr>
<td>16</td>
<td>M</td>
<td>52</td>
<td>Insulinoma</td>
<td>2.3</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 7.2 Clinical data of the patients included in the study.
M = male, F = female
Ductuloinsular tumors of the pancreas

Figure 7.2 A: One of the tumors not only contained ductules, but also non-neoplastic islets (case no. 10, hematoxylin and eosin). B-D: The normal arrangement of hormone-producing cells shown by immunohistochemical stains for insulin, glucagon and somatostatin, respectively, confirms the presence of a normal islet in the nonfunctioning tumor, which is negative for these hormones.

primary tumors contained a variable number of ductules, also in the central part of the tumor. There was close association of the endocrine elements with the ductules. The ductules were lined by bland cuboidal epithelium without mitotic activity or evident cytoplasmic mucin. Importantly, in the 10 lymph node metastases from 3 patients, only the endocrine component but no ductules were found. Also the liver metastasis from one of the patients consisted only of endocrine cells.

In 15 of the 16 tumors including the index case, at least a small rim of surrounding pancreas was available for histologic examination. In 4 cases this surrounding pancreatic tissue showed severe chronic pancreatitis with ductular transformation of the acinar cells and extensive fibrosis, which extended into the tumor. In a few cases not only ductules, but also normal islets were found in the fibrotic tissue, which was intermingled with the tumor (Fig. 7.2).

In 7 of the 16 tumors the surrounding pancreas showed focal signs of chronic pancreatitis. In the other patients pancreatitis was not a striking feature.
Figure 7.3 A: The ductular component before digestion with Hpa II: two alleles of the HUMARA gene from the ductules and one allele of the (unmethylated) HUMARA gene from the internal control are present. B: The ductular component after digestion with Hpa II: still two alleles of the HUMARA gene from the ductules are present. Compared with A there is no important shift in the ratio between the two alleles (from 1:0.99 to 1:1.56), indicating that the ductules are polyclonal. The allele from the internal control has been digested completely. C: The endocrine component before digestion with Hpa II: two alleles of the HUMARA gene from the endocrine cells and one allele of the HUMARA gene from the internal control are present. The alleles from the endocrine cells show a difference in amplification (ratio 1:5.88). D: The endocrine component after digestion with Hpa II: still two alleles of the HUMARA gene from the endocrine cells are present, but they show a large shift in the ratio between the two alleles (from 1:5.88 to 1:1.69), which indicates that the same allele in the endocrine cells has been digested by Hpa II, although not completely, and that the same allele is methylated. These results suggest that the endocrine cells are monoclonal. The allele from the internal control has been digested completely.
Ductuloinsular tumors of the pancreas

**Immunohistochemistry**

In all tumors the endocrine component was positive for the endocrine markers chromogranin and synaptophysin and negative for cytokeratin 7. Sometimes, the endocrine component also expressed cytokeratin 19, but always more weakly than the ductules. In all cases, the ductular component was strongly positive for cytokeratin 7 and cytokeratin 19, but negative for chromogranin and synaptophysin. In cases in which the surrounding pancreas showed severe chronic pancreatitis, the expression of cytokeratin 7 and cytokeratin 19 had increased in the areas with ductular transformation, compared with normal exocrine pancreatic tissue. In all tumors the Ki67 stain showed a low proliferative rate, both in the endocrine and in the ductular component. None of the ductules expressed PDX-1. Also the stains for p53 and ERBB2 were negative. There was a normal expression of DPC4 in all cases.

**KRAS codon 12 analysis**

In none of the tumors was a mutation in KRAS codon 12 detected.

**X-chromosome inactivation analysis**

A clonality assay based on at random X-chromosome inactivation by methylation in females and amplification of the HUMARA gene on the X-chromosome was performed on the ductular and endocrine component of the tumor from a female patient (case no. 7). Both components were microdissected and analyzed separately. Figure 7.3 panel A shows that two different alleles of the HUMARA gene were present in the ductules before digestion with the methylation-sensitive restriction enzyme Hpa II.

If the ductules were monoclonal, in all ductular cells the same allele of the HUMARA gene would be methylated and the same unmethylated allele would be digested by the Hpa II enzyme: after digestion only one allele would remain. However, after digestion with Hpa II there were still two peaks with only a small shift in the ratio between the two alleles (from 1:0.99 to 1:1.56, Fig. 7.3B), suggesting that the alleles of the HUMARA gene in the ductular cells were methylated at random: thus the ductular cells were polyclonal.

Before digestion the endocrine component also showed two different alleles of the HUMARA gene. The ratio of 1:5.88 indicates that there was a difference in the amplification of the two alleles (Fig. 7.3C). After digestion with Hpa II these two peaks remained (Fig. 7.3D). However, a large shift in the ratio (from 1:5.88 to 1:1.69) between the two alleles occurred, indicating that the same allele in the endocrine cells had been digested by Hpa II, although not completely, and that the same allele in these cells was methylated. These results indicate that, in contrast to the ductular component, the endocrine cells were monoclonal.
Discussion

Pancreatic neoplasms with both ductal and endocrine elements are rare. Various terms have been used in the literature for these entities, including ‘ductuloinsular tumor’, ‘duct-acinar-islet cell tumor’, and ‘mucin-producing islet cell adenoma’. Whereas some of these reported cases contain neoplastic elements with both endocrine and ductal differentiation, we think that others represent conventional pancreatic endocrine tumors with entrapped, nonneoplastic ductules. The characteristics of this second group of tumors have not been clearly elucidated and the present study is the first in which the nature of the ductular component has been investigated at the molecular level.

To establish whether the ductules are neoplastic or not, the expression of a number of gene products known to be involved in neoplastic transformation of the ductal tumors in the pancreas was investigated. Amplification of ERBB2 and point mutations in codon 12 of the KRAS oncogene are early genetic changes in the stepwise development of pancreatic ductal adenocarcinoma and are also commonly observed in other ductal neoplasms (including intraductal papillary-mucinous neoplasms and mucinous cystic neoplasms). Inactivation of the TP53 and DPC4 tumor suppressor genes appears to occur late in this multistage sequence. In the tumors included in this study no evidence for genetic changes were found by immunohistochemistry for ERBB2, p53 and DPC4 and by sequence analysis for a KRAS codon 12 mutation. Therefore these data do not lend support to the hypothesis that the ductular component is neoplastic. The results of the X-chromosome inactivation analysis, performed on both components of the tumor from a female patient, even more strongly suggest that the ductules are nonneoplastic. In interpreting this analysis one has to be aware of difficulties that can arise: because X-chromosome inactivation in females shows a patchy distribution, nonneoplastic cells from the same area can have inactivation of the same X-chromosome, incorrectly suggesting monoclonality. To prevent this in our case, tissue was sampled in different areas of the tumor. Results apparently suggesting polyclonality can be found due to contamination with inflammatory or stromal cells. In our case, contamination was avoided as much as possible by using laser capsule microdissection for obtaining tissue from the ductules, which is a very accurate method for isolating small cell groups or even single cells for further analysis. Because the X-chromosome inactivation analysis is based on comparing amplification of two alleles of a gene before and after digestion with a restriction enzyme, the results are dependent of the completeness of digestion and equal amplification of the alleles on both X-chromosomes. Therefore, we used an internal control. Taking these considerations into account, the results of the X-chromosome analysis in the present study indicate that the ductules are polyclonal while the endocrine component is monoclonal.

It is interesting to note that in other tumors with both exocrine and endocrine components, microallelotyping has been used to demonstrate that both compo-
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Components were clonal and closely related. These tumors from the stomach and colon demonstrated clearly neoplastic features in both exocrine and endocrine components, the latter largely representing high grade neuroendocrine carcinomas. Thus, it is possible to prove the relationship of morphologically diverse components within a ‘mixed’ tumor.

In contrast to the results presented here other authors consider the ductular component to be neoplastic. In a recent study, a ductular component in otherwise classical pancreatic endocrine tumors was a rather common finding, present in 15 of 92 cases (16.3%). It was suggested that the ductular component consists of neoductules, which arise from transformed endocrine tumor cells and thus are neoplastic. However, no convincing evidence supporting this hypothesis was provided. Some of the tumors described by others as ‘ductuloinsular’ are worthy of comment here. Some cases showed neoplastic epithelium, invasion of surrounding tissue, perineural growth and sometimes metastasis, which all suggests a neoplastic nature and tumor biology. It is felt that these tumors are either true mixed ductal-endocrine carcinomas or pancreatic endocrine tumors with reactive atypia of entrapped ductules. Another reported case had a liver metastasis that contained ductules while the primary tumor consisted of an endocrine component only, suggesting a neoplastic nature to the ductules. However, these ductules might represent nonneoplastic bile ducts entrapped by a conventional endocrine tumor. In our series the 3 cases with metastases only showed the endocrine component in the lymph nodes and liver. Furthermore, in all our cases the nuclear morphology was bland without atypia and without proliferative activity.

Even if the ductular component is not neoplastic, it could still be an intrinsic part of the tumor. The pathogenesis of pancreatic endocrine tumors might be related to (or recapitulate) embryonic pancreatic development, during which islet cells arise from primitive ducts. Islet formation from ducts, mimicking this process, occurs, for example, in nesidioblastosis, but this is a more generalized phenomenon, that does not produce a circumscribed mass. If embryonic ducts may give rise to the endocrine neoplasm, expression of PDX-1 could be found in the ductular component of the tumors, because PDX-1 is considered to be an important regulator of early pancreatic development. However, immunohistochemical stains for the PDX-1 protein were negative, and we have no evidence to support this hypothesis. Because the results of this study support neither the hypothesis of a neoplastic origin of the ductules nor the hypothesis of mimicry of embryonic development, the most obvious explanation for the presence of a ductular component in endocrine tumors of the pancreas, is entrapment of pre-existing pancreatic ductules. The chronic pancreatitis observed in the surrounding pancreas in 11 of the 16 cases is compatible with entrapment: in chronic pancreatitis the number of ductules increases by ductular transformation of acinar cells and in the fibrotic tissue that results from the inflammation, ductules and islets may remain as the only preexisting structures. Also, in chronic pancre-
atatis there may be close association of nonneoplastic ductules and islets ('ductuloin- 
sector complexes'), closely resembling the juxtaposition of endocrine and 
ductular elements observed in the tumors we studied.

In summary, although some authors consider ductules found within pancreatic 
endocrine tumors to be neoplastic and part of the original tumor, and although 
the histological features of these tumors are reminiscent of pancreatic organ 
development, the results presented here favor another pathogenesis. Most likely, 
the lesion reflects the simple entrapment of preexisting pancreatic ductules by a 
'conventional' pancreatic endocrine tumor, possibly enhanced by accompanying 
chronic pancreatitis in the surrounding pancreas. It is important to recognize 
this lesion and to appreciate the nonneoplastic nature of the ductules, which 
may be less rare than previously thought. It is suggested to call these tumors 
'pancreatic endocrine tumors with entrapped ductules' to describe their nature 
more precisely. The biology is similar to the biology of a conventional pancreatic 
endocrine tumor.

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To the editor

We would like to compliment van Eeden and colleagues on their meticulous study, which confirms the nonneoplastic nature of ductules encountered in pancreatic endocrine tumors.\(^3\)

They have concluded from clonality studies and immunohistochemistry that the ductular component is entrapped rather than a neoplastic process. We have commented previously on the presence of ductules in pancreatic endocrine tumors,\(^1\) and we wish to present some additional observations.

Four cases of so-called ductuloinsular pancreatic endocrine tumors were retrieved from the files of the Departments of Pathology, University Health Network, Toronto, and Wayne State University, Harper University Hospital, Detroit. All 4 cases were clinically symptomatic insulin-producing tumors. It is worth noting that 9 of 16 cases reported by van Eeden et al\(^3\) and 6 of 15 cases in the series by Deshpande et al\(^2\) were insulin-producing endocrine tumors.

Microscopically, all 4 cases showed tubules intimately associated with the endocrine elements. Many of the cytologically benign tubules were centrally located within these tumors. Focal chronic pancreatitis, remote to the tumor mass, was present. In addition, all the tumors were characterized by dense stromal sclerosis in which the ductules were embedded. This latter feature was also commented on by Deshpande et al.\(^2\)

We agree with van Eeden et al\(^3\) that the ductules are nonneoplastic; we would like to reiterate that the prevalence of insulin production and stromal fibrosis raises the question whether these two features are involved in the process. It would appear that both insulin and stromal sclerosis are recurring and common threads in this characteristic pancreatic endocrine tumor.

It is well recognized that factors, such as insulin-like growth factor and transforming growth factor alpha and beta, are capable of inducing fibrosis. Are the ductules merely resident entrapped ductules or are they proliferating in reaction to the extensive stromal fibrosis and growth factors? In our cases, the ductules were clustered and multiple, and their number, size and distribution suggest a nonneoplastic, proliferative process. It is possible that these ductules are proliferating in response to the "trophic" or "proxicrine" effect of the endocrine cells.

If this is the case, then this phenomenon may introduce a new perspective to endocrine-exocrine interaction in the pancreas, and to the age-old question of whether local endocrine activity has any role in the initiation and progression of ductal neoplasia, and indeed, in influencing the aggressiveness of ductal adenocarcinoma by exerting a "proxicrine" effect, a term coined by Dr. Murray Korc (personal communication, Utah, September 1999). This term has been used to describe the trophic effect of endocrine cells that are in close proximity to ductal cells, as opposed to either autocrine or paracrine activity. These are issues that warrant separate consideration and further scrutiny.

The nature of the ductules may present an intriguing intellectual exercise, but
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from a practical point of view, it is important to recognize this particular type of endocrine tumor at frozen section as the ductules embedded within a sclerotic stroma can be mistaken for ductal carcinoma of the pancreas.

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Authors' reply
The comments by Chetty and colleagues are very much appreciated, and we agree that it is critical to appreciate the nonneoplastic nature of the ductules for proper diagnosis. As far as the mechanisms and the pathogenesis are concerned, a number of questions remain.

Whether or not the phenomenon of entrapped ductules needs to be regarded as quite typical for insulinomas depends on the prevalence of insulinomas in the group of pancreatic endocrine tumors (PETs) with entrapped nonneoplastic ductules compared to the number of insulinomas in the group of conventional PETs. Up to 60% of the conventional PETs have been reported to be insulinomas.\(^4\) In this respect, the findings of 6 insulinomas in the group of 15 PETs with entrapped nonneoplastic ductules (40%) reported by Deshpande et al.\(^2\) and 9 in 16 cases (56%) in our own study\(^5\) are not unexpected. That ductules were found in the majority of 40 PETs reviewed at the Toronto institution as reported in a previous letter\(^1\) also suggests that this phenomenon is not specific for insulinomas. Based on the observation that the ductules are multiple and clustered, Chetty and colleagues propose that they result from a nonneoplastic proliferative process. However, in our experience the ductules do not contain mitotic figures and
The metastasis contains both entrapped hepatocytes (A), as confirmed in the CD10 immunostaining for bile canaliculi (B), and ductules (C), as confirmed in the cytokeratin 7 immunostaining (D).

show a very low proliferative rate based on Ki67 immunohistochemical staining. Therefore, if proliferation plays a role, it has to be an extremely slow process. In our opinion, ductular transformation is another possible pathogenetic mechanism. Our results are not consistent with ductular transformation of the neoplastic endocrine cells, as suggested by Deshpande et al., but ductular transformation of entrapped acinar cells from the surrounding pancreas, a phenomenon frequently observed in chronic pancreatitis, is possible. Alternatively, the ductules could represent residual entrapped preexisting small intralobular ductules that remain within the growing endocrine tumor following atrophy of the acinar elements. A similar juxtaposition of ductules and islets is seen in areas of chronic pancreatitis where there is extensive acinar atrophy ("ductuloinsular complexes"). Indeed, chronic pancreatitis was observed in 11 of our 16 cases. Moreover, as we have shown, ductules are not the only structures entrapped by PETs. In some of our cases, normal pancreatic islets were also found within the tumors. Interestingly, after submission of our manuscript, one of the patients included in our study (case no. 5), underwent resection of liver metastases. He also had developed symptoms of hypergastrinemia. Histologically, the tumor
nodules in the liver showed the features of a metastatic PET, and in one of these nodules not only ductules, but also clusters of hepatocytes were found (Fig. 7.4). While the ductules within the metastatic tumor may be either entrapped bile ducts or transformed hepatocytes, the presence of pancreatic islets in primary PETs and hepatocytes in a liver metastasis indicates that entrapment of nonneoplastic elements certainly occurs, perhaps accompanied by transformation or proliferation.

These observations do not exclude, as suggested by Chetty et al., that PETs produce trophic factors that exert their influence on the surrounding tissue and induce the fibrosis observed in the tumors. But taking into account that not all PETs with entrapped structures are insulinomas, it is most likely that any such trophic factors can be produced by different types of PETs.

We agree with Chetty and colleagues that this is an intriguing issue and that more research is needed before the pathogenesis of PETs with entrapped nonneoplastic ductules will be fully understood.


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