Different Molecular Profiles Characterize Well-Differentiated Endocrine Tumors and Poorly Differentiated Endocrine Carcinomas of the Gastroenteropancreatic Tract

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ABSTRACT

Purpose: The molecular pathogenesis of gastroenteropancreatic endocrine tumors (ETs) is still largely unknown. The purpose of this work was a molecular characterization of 38 gastroenteropancreatic ETs with respect to the primary site and to the morphofunctional profile, pointing out useful diagnostic or prognostic molecular markers.

Experimental Design: Twenty-four well-differentiated ETs or carcinomas (WDET/Cs; 11 pancreatic, 3 gastric, and 10 intestinal) and 14 poorly differentiated endocrine carcinomas (1 pancreatic, 6 gastric, and 7 colorectal) were microallelotyped using 38 polymorphic microsatellite markers covering chromosomes 1, 3, 5, 6, 11, 17, and 18.

Results: Regardless of the primary site, a significantly higher percentage of allelic imbalances (AIs) was observed in poorly differentiated endocrine carcinomas than in WDET/Cs (P = 0.012), except for 3 of 8 nonfunctioning pancreatic endocrine tumors and 1 colorectal WDEC, exhibiting multiple AIs on chromosomes 1, 3, 6, and 11. A strong positive correlation between AI percentage and Ki-67 proliferation index was detected considering both the whole series of ETs (P = 0.004) and the group of WDET/Cs alone (P = 0.011). The survival analysis showed a positive correlation between low percentage of AI and longer survival (P = 0.01). No recurrent AIs at specific chromosomal regions were identifiable with respect to the primary site.

Conclusions: The malignant progression of endocrine tumors seems to be associated with complex allelotypes and chromosomal instability. Although no specific molecular markers of malignancy can be defined with certainty, the ploidy status and the degree of chromosomal derangements appear to be the most informative genetic factors with prognostic significance.

INTRODUCTION

Endocrine tumors (ETs) of the gut and pancreas are relatively rare neoplasms traditionally considered to originate from the cells of the dispersed endocrine system, which are scattered through the gastrointestinal mucosa or are present in the islets within the pancreatic parenchyma. They constitute a heterogeneous group of tumors, which includes different histopathological and prognostic classes. Regardless of the tumor site, they are classified into two major categories with significant differences in phenotype and clinical behavior, namely, well-differentiated ETs (WDETs) and poorly differentiated endocrine carcinomas (PDECs; Ref. 1). WDETs frequently show bland cytologic atypia, low proliferative rate, and a benign or low-grade malignant behavior. On the contrary, PDECs exhibit a highly atypical morphology with elevated proliferative index and behave in a highly malignant fashion presenting local invasion, early metastases, and a very poor prognosis. From both a morphological and a biological standpoint, these latter tumors may be considered as a gastroenteropancreatic (GEP) counterpart of the most common poorly differentiated endocrine carcinomas (small cell carcinoma; SCC) of the lung, although it is still unclear whether they share a common clinical course and a similar response to therapy. Currently, GEP ETs may represent a difficult task for the diagnosis and/or the clinical management, PDECs, because they may be misdiagnosed as a metastatic SCC of the lung and WDETs, because the histological evaluation alone is often not reliable in predicting the biological behavior and the prognosis of these tumors.

The past decade has seen the employment of cyto genetic and molecular approaches to improve our understanding of the biology of GEP ETs, and to define a panel of diagnostic and therapeutic targets for these neoplasms. However, no specific molecular markers of malignancy useful for the prognostic evaluation of these neoplasms have been found, and still very little is known about the pathogenesis of ETs. Molecular data for GEP PDECs are missing overall, with the exception of the high frequency of loss of heterozygosity at APC, DCC, and TP53 loci reported in 7 colorectal PDECs (2), and the evidence of “hot spots” for loss of heterozygosity on 8p, 15q, 17p, 11p, 12p, and 13q, and Xpq found in a small series of gastric PDECs (3–5).

Regarding the GEP WDETs, more information is available, especially for pancreatic ETs (PETs), but only small or heterogeneous series have been analyzed, without a consistent tumor phenotype analysis among different studies. The application of comprehensive genome-wide approaches such as comparative genomic hybridization and high-resolution allelotyping have led to the identification of a wide spectrum of genetic aberrations in PETs, including chromosomal losses of 1p, 3pq, 6q, 11q, Xq,
Allelotypes of GEP Endocrine Tumors

prognostic molecular markers. A comprehensive approach was undertaken to define specific patterns of allelic imbalance (AI) with microsatellite analysis, including 17p LOH, 18q21 LOH, 11q LOH, 11q13–q14 LOH, 1q LOH, TP53 mutation, p53 abnormalities, MEN1 gene mutation, p16 and p14 promoter hypermethylation, and dpc4 abnormalities. The review of the status of these findings, reported in Table 1, reveals a very low frequency of alterations for almost all of the TSGs analyzed in GEP sporadic WDETs including MEN1, VHL, DPC4, PTEN, TP53, and p16 genes. The only exception was a relatively high frequency of MEN1 gene mutation and of p16 inactivation either by promoter hypermethylation or by homozygous deletions of the gene. On the contrary, loss of heterozygosity studies of PETs and, in a few reports, of gastrointestinal WDETs, have identified variable frequencies of allelic losses at several arms and chromosomal regions (Table 1), but these findings alone are not compelling for a specific role in the endocrine tumorigenesis.

In the present study we investigated the genetic profiles of a well-characterized series of GEP WDETs and PDECs evaluating multiple chromosomal regions by a microsatellite typing analysis. The aim of this comprehensive approach was to define specific patterns of allelic imbalance (AI) with respect to the primary site and to the morphofunctional profile of each tumor, pointing out useful diagnostic or prognostic molecular markers.

**Materials and Methods**

**Patients and Samples.** Thirty-eight ETs of the gut and pancreas (9 gastric, 5 ileal, 1 appendiceal, 11 colorectal, and 12 pancreatic tumors) were obtained from 38 patients who had undergone surgical or endoscopic resection. For the morphological evaluation, tumor samples were fixed in buffered formalin and embedded in paraffin wax. Five μm-thick sections were stained with H&E and Grimelius’ silver stain. The size of the primary tumors, the occurrence and the location of the metasteses, the presence of necrosis and vascular invasion, and the mitotic index (number of mitotic figures in 10 high power field ×400) were recorded for each case. The tumors were classified according to the WHO Classification of ETs (1), and the clinicopathological features of the 38 ETs studied are reported in Table 2. The staging of malignant tumors was performed, at first diagnosis, as follows: (a) stage I, tumor confined to the site of origin; (b) stage II, tumor with loco-regional extension (direct invasion of contiguous organs and/or regional lymph node metastases); and (c) stage III, widespread disease, with distant ematogenous metastases (Table 2).

Follow-up data were collected by contacting clinicians and/or by consulting the Tumor Registry of Lombardia Region (Italy).

**Immunohistochemistry.** Immunohistochemistry for the detection of general endocrine markers and of Ki67 antigen was performed on 3 μm-thick sections obtained from paraffin blocks and collected on poly-L-lysine-coated slides. Immunostainings were performed using the ABC-peroxidase technique (46), with the following primary antibodies: monoclonal antinsulin (clone SY88; BioGenex, San Ramon, CA), monoclonal anti-N-CAM (clone 123C3; Monosan, Uden, the Netherlands), monoclonal anti-NSE (clone MIG-N3; Monosan), polyclonal anti-PGP9.5 (Biogenesis, New Field, United Kingdom), mono-

<p>| Table 1 Review of molecular findings reported in well-differentiated endocrine tumors |
|-----------------------|---------------------|----------------|------------------|---------------------|-------------------|---------------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>Pancreas</th>
<th>Stomach</th>
<th>Duodenum</th>
<th>Ileum</th>
<th>Appendix</th>
<th>Colonrectum</th>
</tr>
</thead>
<tbody>
<tr>
<td>VHL gene mutation</td>
<td>1/60 (1%)</td>
<td></td>
<td>1/164 (54%)</td>
<td>1/6 (16%)</td>
<td>10/25 (40%)</td>
<td>(14–16)</td>
</tr>
<tr>
<td>3p25–q21 LOH</td>
<td>10/4202 (51%)</td>
<td></td>
<td>0/5 (0%)</td>
<td>0/1 (0%)</td>
<td>0/2 (0%)</td>
<td>(14, 15, 17, 18)</td>
</tr>
<tr>
<td>3p26–3q29 LOH</td>
<td>13/21 (62%)</td>
<td></td>
<td>0/5 (0%)</td>
<td>0/1 (0%)</td>
<td>0/2 (0%)</td>
<td>(19)</td>
</tr>
<tr>
<td>PTEN gene mutation</td>
<td>1/33 (3%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(20)</td>
</tr>
<tr>
<td>pten abnormalities (IHC)</td>
<td>1/24 (4%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEN1 gene mutation</td>
<td>42/182 (23%)</td>
<td></td>
<td>1/6 (16%)</td>
<td>10/25 (40%)</td>
<td>0/5 (0%)</td>
<td>(21–26)</td>
</tr>
<tr>
<td>11q LOH</td>
<td>12/23 (52%)</td>
<td>3/6 (50%)</td>
<td>4/24 (17%)</td>
<td>3/11 (27%)</td>
<td>4/8 (50%)</td>
<td>(29–31)</td>
</tr>
<tr>
<td>TP53 mutation</td>
<td>1/20 (5%)</td>
<td>0/4 (0%)</td>
<td>0/6 (0%)</td>
<td>1/9 (11%)</td>
<td>(32, 33)</td>
<td></td>
</tr>
<tr>
<td>p53 abnormalities (IHC)</td>
<td>9/30 (30%)</td>
<td>8/16 (50%)</td>
<td>0/10 (0%)</td>
<td>0/9 (0%)</td>
<td>0/3 (0%)</td>
<td>(34–36)</td>
</tr>
<tr>
<td>17p LOH</td>
<td>7/20 (35%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(32)</td>
</tr>
<tr>
<td>DPC4 gene mutation</td>
<td>4/34 (11%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(17, 37)</td>
</tr>
<tr>
<td>18q21 LOH</td>
<td>10/47 (21%)</td>
<td>2/7 (28%)</td>
<td></td>
<td></td>
<td></td>
<td>(17, 37)</td>
</tr>
<tr>
<td>dpc4 abnormalities (IHC)</td>
<td>1/17 (5%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(38)</td>
</tr>
<tr>
<td>p16 promoter hypermethylation</td>
<td>10/23 (43%)</td>
<td>1/2 (50%)</td>
<td>9/22 (40%)</td>
<td>2/7 (28%)</td>
<td>2/4 (50%)</td>
<td>(39–41)</td>
</tr>
<tr>
<td>p14 promoter hypermethylation</td>
<td>1/11 (9%)</td>
<td>2/2 (100%)</td>
<td>0/2 (0%)</td>
<td>4/7 (57%)</td>
<td>1/4 (25%)</td>
<td>(39)</td>
</tr>
<tr>
<td>p16, p15, p14 abnormal expression</td>
<td>9/26 (35%)</td>
<td>4/9 (44%)</td>
<td></td>
<td></td>
<td></td>
<td>(42)</td>
</tr>
<tr>
<td>Xp–Xq LOH</td>
<td>7/17 (41%)</td>
<td>5/30 (17%)</td>
<td>2/9 (22%)</td>
<td>1/6 (16%)</td>
<td>0/2 (0%)</td>
<td>(5, 43)</td>
</tr>
<tr>
<td>lp–1q LOH</td>
<td>10/23 (43%)</td>
<td>6/19 (31%)</td>
<td></td>
<td></td>
<td></td>
<td>(26, 44)</td>
</tr>
<tr>
<td>6q13–q27 LOH</td>
<td>58/93 (62.2%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(45)</td>
</tr>
</tbody>
</table>
Table 2  Clinicopathological data of the 38 gastroenteropancreatic endocrine tumors analyzed

<table>
<thead>
<tr>
<th>Site</th>
<th>N°</th>
<th>Sex</th>
<th>Age</th>
<th>Diagnosis</th>
<th>Size (cm)</th>
<th>Benign/ malignant</th>
<th>local invasion</th>
<th>Necrosis</th>
<th>Stage</th>
<th>Angiовascularization</th>
<th>Mitosis (%)</th>
<th>Ki67 (%)</th>
<th>Follow-up Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>1</td>
<td>F</td>
<td>76</td>
<td>WDET (Type I)</td>
<td>2</td>
<td>UB</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>2.8</td>
<td>DOC</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>F</td>
<td>68</td>
<td>WDET (Type I)</td>
<td>1</td>
<td>B</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>0.2</td>
<td>ANED</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>F</td>
<td>56</td>
<td>WDEC (Type III)</td>
<td>5.5</td>
<td>M</td>
<td>LN</td>
<td>II</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>0.4</td>
<td>ANED</td>
</tr>
<tr>
<td>Ileum</td>
<td>4</td>
<td>M</td>
<td>67</td>
<td>PDEC</td>
<td>3</td>
<td>M</td>
<td>LN/liver</td>
<td>III</td>
<td>+</td>
<td>+</td>
<td>39</td>
<td>63.7</td>
<td>DOD</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>M</td>
<td>59</td>
<td>PDEC</td>
<td>5</td>
<td>M</td>
<td>LN</td>
<td>II</td>
<td>+</td>
<td>+</td>
<td>44</td>
<td>50.9</td>
<td>DOD</td>
</tr>
<tr>
<td>Colon-rectum</td>
<td>11</td>
<td>F</td>
<td>68</td>
<td>WDEC</td>
<td>0.9</td>
<td>M</td>
<td>-</td>
<td>-</td>
<td>I</td>
<td>+</td>
<td>0</td>
<td>0.1</td>
<td>ANED</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>F</td>
<td>41</td>
<td>WDEC</td>
<td>2.1</td>
<td>M</td>
<td>LN</td>
<td>-</td>
<td>II</td>
<td>-</td>
<td>0</td>
<td>0.2</td>
<td>ANED</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>F</td>
<td>64</td>
<td>PDEC</td>
<td>2.2</td>
<td>M</td>
<td>LN</td>
<td>II</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0.4</td>
<td>ANED</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>M</td>
<td>48</td>
<td>PDEC</td>
<td>2.2</td>
<td>M</td>
<td>LN</td>
<td>II</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0.6</td>
<td>ANED</td>
</tr>
<tr>
<td>Appendix</td>
<td>21</td>
<td>M</td>
<td>13</td>
<td>WDET</td>
<td>2.5</td>
<td>UB</td>
<td>-</td>
<td>-</td>
<td>UB</td>
<td>-</td>
<td>0</td>
<td>0.8</td>
<td>ANED</td>
</tr>
<tr>
<td>Colon</td>
<td>16</td>
<td>M</td>
<td>29</td>
<td>WDET</td>
<td>1.2</td>
<td>B</td>
<td>UK</td>
<td>-</td>
<td>B</td>
<td>NE</td>
<td>0</td>
<td>0.2</td>
<td>ANED</td>
</tr>
<tr>
<td>Pancreas</td>
<td>27</td>
<td>F</td>
<td>64</td>
<td>WDET (Insulinoma)</td>
<td>1.6</td>
<td>B</td>
<td>-</td>
<td>-</td>
<td>B</td>
<td>-</td>
<td>1</td>
<td>0.8</td>
<td>ANED</td>
</tr>
</tbody>
</table>

*WDET, well-differentiated endocrine tumor; WDEC, well-differentiated endocrine carcinoma; PDEC, poorly differentiated endocrine carcinoma; Sst, somatostatinoma; NF, nonfunctioning; B, benign; M, malignant; UB, uncertain behavior; NE, not evaluable; DOC, dead of other causes; ANED, alive with not evidence of disease; DOD, dead of disease; AWD, alive with disease; LN, lymph node; UK, unknown.*

Antichromogranin A (clone Phc5; Enzo Diagnostics, New York, NY), monoclonal anti-insulin (clone AE9D6; BioGenex), monoclonal antigucaon/glycentin (Milab, Malmö, Sweden), monoclonal antipancreatic polypeptide (Cambridge Research Biochemicals, Cambridge, United Kingdom), monoclonal antigastrin-CCK-Cerulein COOH-terminal peptide (clone B4; Farmitalia, Milan, Italy), and monoclonal anti-Ki67 (clone MIB-1; Immunotech, Marseilles, France).
additional microsatellite analysis was performed using at least 17p13.2 TP53 17q D17S787- 18p D18S63- 18q21 (DCC, DPC4) D18S457

% of AI 18 38 8 57 60 27 44 38 76 0 0 13 18 25 0 15 31 54 8 21 43 25 62 20 45

* A*, appendix; I, allelic imbalance; R, allelic retention; --, not informative locus; blank, not available result.

The Ki67 proliferative index was determined in percentage by counting the number of positive nuclei in 2000 tumor cells, according to La Rosa et al. (35).

**DNA Extraction.** Normal and tumor DNA of 12 patients (cases 27–38 in Table 2) was isolated from frozen tissues by homogenizing ~5 mm³ of each sample before the extraction using DNeasy Tissue kit (Qiagen, Milan, Italy). For the remaining 26 cases normal and tumor DNA was obtained from archival paraffin-embedded specimens after microdissection as described previously (47).

**Allelotyping PCR.** Normal and tumor DNA samples were allelotyped using a panel of 38 polymorphic markers covering a total of 17 different chromosomal regions (Table 3, A and B). Thirty of these markers are physically mapped near known TSGs or loss of heterozygosity regions reported as critical in endocrine tumorigenesis (1ppq, 3p, 5q, 6q, 11p, 17p, and 18q). For tumors exhibiting AIs in any of these regions, an additional microsatellite analysis was performed using at least one microsatellite marker on the opposite chromosomal arm (3q, 6p, 17q, and 18p) to verify the extension of the AI region to the entire chromosome. The 38 markers included in this study, were selected on the basis of their chromosomal location and heterozygosity using the Unified Data Base for Human Genome Mapping (4) and the eGenome Catalogue of Human genome. The primer sequences of 29 dinucleotide loci were obtained from the Genome database, whereas for the remaining 9 markers, the primer sequences were redefined by using the Primer Express software (Applied Biosystems; version 1.0) to reduce the length of the primer product and to improve the PCR efficiency using DNA from paraffin-embedded tissues. The sequences were as

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1 Internet address: http://bioinformatics.weizmann.ac.il/udb.
2 Internet address: http://egenome.chop.edu.
3 Internet address: www.gdb.org.
follows: 5’-TCACCTGACGTCCTTAAATA-3’/5’-CAACA-
AACATGCTCCTTGAGTGTGCT-3’ (D1S2842); 5’-CATTTTCTCCT-GCTATGTGCTTGTAATCCC-3’ (D6S470); 5’-CCAGAAAGCAGAGACAAC-3’/5’-TAT-TCTACGAAAGCGCATTTGGT-3’ (D6S415); 5’-AGAAAAC-
AAAACGGATGGATAT-3’/5’-CCTTACAATAGGATCT-GTGTT-3’ (D6S193); 5’-GCTTCTAAGAGATAGCTCA-
CCC-3’/5’-TGCACTGTATTTGTTTGTC-3’ (D6S297); 5’-GCTTGA-
GTTGACAAGCTGCAC-3’/5’-GGAATAGCTGATT-
TCCAGGT-3’ (PYGM); 5’-CGAGGAGAGCTCTTT-3’/ 5’-TCTGGGTGTCTGAAATG-3’ (INT2); 5’-GCGTGG-
GCAATAGTGAC-3’/5’-GAAGAGGTCTCGTGTCG-
CAGA-3’ (D11S1347); 5’-CCCCCATCCTACCTCCATTCCATC-3’/5’-
TGGGATGTTTCCCTG-3’ (D18S452). Forward prim-
eres were synthesized with a fluorescent tag (FAM, HEX, or TET) on the 5’ end and purified using standard high-
performance liquid chromatography. Fifty ng of DNA was am-
plified in a 15-μl reaction solution containing 1.5 μl of 10×
buffer (Roche, Mannheim, Germany), 1.25–2.25 mM MgCl₂, 0.3
μM primer pairs, 200 μM deoxynucleoside triphosphate, and 2
units of DNA polymerase (Roche, Mannheim, Germany). Am-
plications were performed using a 5-min initial denaturation at
95°C, followed by 10 cycles of 50 s at 94°C, 50 s at 55°C, and
50 s at 72°C, and by 25 cycles of 30 s at 85°C, 30 s at 55°C,
and 30 s at 72°C. The fluorescently labeled PCR products were
electrophoresed on an Applied Biosystems 310 automated DNA
sequencer (Applied Biosystems, Milan, Italy), and the fluores-
cent signals from the different sized alleles were recorded and
analyzed using Genescan software (version 2.1; Applied Bio-
systems).

**Definition of Al.** An AI, suggestive either of an allelic loss or of an allelic gain, was scored by the ratio of relative allelic peak height in the tumor DNA to relative allelic peak height in the corresponding normal DNA. The formula used for the calculation was T2: T1/N2: N1 where T1 and N1 were the height values for the smaller allele and T2 and N2 were the height values for the larger allele of the tumor and normal samples, respectively. For informative markers AI was scored
when signal reduction for one allele was of 50% or more (AI factor $\geq 0.5$ or $\leq 2$).

Random preferential allele amplification resulting in false AIs may be sometimes observed when DNA from archival sections is used. In all of the DNA from paraffin-embedded tissues, AI was assigned only when at least three repeated experiments consistently showed an AI.

For each tumor allelotype the percentage of AI was calculated as the number of the chromosomal regions with AIs divided by the total number of the informative regions among the 17 analyzed (Table 3, A and B).

Statistical Analysis. Statistical analysis was performed using Fisher’s exact test, the $\chi^2$ test with Yates’ correction, the independent sample $t$ test, and the correlation analysis (SPSS 7.5 software).

The survival analysis was performed by using the Kaplan-Meier product limit estimate of probability of survival against time, producing a product limit survival curve for each of the following variables: size of the primary tumor, metastases and/or local invasion, diagnosis (WDET versus WDEC versus PDEC), tumor necrosis, angioinvasion, Ki-67 index, mitotic index, and percentage of AI. For each survival curve, the relative risk of failure associated with being in the poor prognostic group and the results of the standard Mantel Haenzel log-rank test were calculated. For all of the survival analysis, the program survan XL, version 1.14 (copyright 1995–1997) was used.

RESULTS

PDEC and WDET/C Allelotypes. Thirty-eight microsatellite markers covering a total of 7 chromosomes including 1, 3, 5q, 6, 11, 17, and 18 were investigated in a series of 38 GEP ETs (Table 2) using standardized fluorescence-based methodology and apparatus. The detailed results of the allelotyping analysis together with the percentage of AI calculated for each tumor, are shown in Table 3, A and B, for gastrointestinal and pancreatic tumors, respectively. Representative electropherograms of the microsatellite analysis are reported in Fig. 1.

Regardless of the primary site of the tumor, very different allelotypes were observed when comparing the 14 cases of PDECs with the remaining 24 cases of WDET/Cs. In particular, the percentage of AI was significantly higher in PDECs (mean percentage of AI = 43.3%) than in WDET/Cs (mean percentage of AI = 21.7%; $P = 0.012$). Considering the...
WDET/C group, 8 cases (33.3%) showed no AI in any of the chromosome regions analyzed, and 12 cases (50%) exhibited only scattered AIs that were mainly localized at 3p21-p14 and 11p15 markers (5 of 11 cases and 7 of 12, respectively for the two chromosomal regions). The remaining 4 WDET/Cs showed percentage of AI >50%, similar to PDECs. Three of these tumors were nonfunctioning pancreatic tumors (2 malignant and 1 with uncertain behavior; cases 32, 33, and 36 in Table 3B) and 1 case was a colorectal WDEC (case 19 in Table 2 and Table 3A). Interestingly, 3 of these 4 tumors (cases 19, 33, and 36), although correctly classified as WDEC (cases 19 and 36) or WDET with uncertain behavior (case 33), showed, at the histological review, a degree of cytological atypia higher than usually observed in well-differentiated endocrine neoplasia. In detail, these tumors presented enlarged nuclei, prominent and sometimes multiple nucleoli, and a condensed chromatin pattern. These atypical nuclear features, however, were accompanied by the presence of moderately abundant and granular cytoplasm, which was strongly reactive with Grimelius’ silver stain and chromogranin A and, in case 19, showed the strongly eosinophilic granules typical of EC-cell tumors (Fig. 1B). The fourth tumor (case 32) did not show significant nuclear atypia; however, it presented a higher proliferative index (9.5%) than the other WDECs, and it is of note that the patient harboring this WDEC died of disease after 36 months of follow-up, whereas all of the other patients with WDECs with a similar or longer follow-up are alive (Table 2).

The high percentage of AI observed in PDECs was explained by the number of chromosome arms entirely involved in AIs (Table 3, A and B). These large AI regions often subtended AIs of the whole chromosomes as the additional microsatellite analysis on 3q, 6p, 17q, and 18p showed in 13 of 42 regions analyzed in PDECs. On the contrary, large AI regions were very rare among WDET/Cs with the only exception for the 4 pancreatic and colorectal tumors exhibiting the high percentage of AI reported above. In these cases most of the chromosomal regions showing AI were extensive to the whole chromosomes and, namely, these alterations involved chromosomes 1, 3, 6, and 11.

The second noteworthy difference between PDECs and WDET/Cs, regardless of the primary site, was the significantly higher percentage of TP53 AI observed in PDECs (12 of 13 informative cases) compared with WDET/Cs (1 of 21 informative cases; P < 0.01). Intriguingly, the only WDEC exhibiting TP53 AI was case 19 whose uncommon cytological atypia and unusually high percentage of AI have been reported above.

Considering the allelotype of each tumor (Table 3, A and B) in relationship to the primary site, it seems clear that the lowest values of AI percentage among WDET/Cs were observed in functioning PETs and WDET/Cs of the ileum and appendix (mean percentage of AI = 9.8%) compared with gastric, colorectal, and nonfunctioning pancreatic tumors (mean percentage of AI = 30%). Analogously, among PDECs the mean percentage of AI was lower in colorectal (32%) than in gastric and pancreatic (54.4%) neoplasms. Due to the few cases in each subgroup, no other observations as well as no recurrent AIs at specific chromosomal regions have been identifiable with respect to the site of origin of the tumors.

AI Percentage and Clinicopathological Parameters.

On the basis of the observation of a positive correlation between high degrees of AI and malignant behavior of the tumor, AI percentage for each neoplasm was correlated with all of the clinicopathological parameters known to be of prognostic value, namely, local invasion, metastasis, angioinvasion, size, and proliferation rate of the tumor (Table 2).

A strong positive correlation between AI percentage and Ki-67 proliferation index was detected considering both the whole series of ETs (P = 0.004; Fig. 2A) and the group of WDET/Cs alone (P = 0.011; Fig. 2B). Similarly, considering all of the 38 ETs, AI percentage was positively correlated with the number of mitoses observed in the tumors (P = 0.043). By contrast, no significant relationship was observed between AI and tumor size and stage.

No other clinicopathological parameter was correlated with...
the degree of AIs, and no significant association was observed between AI at specific chromosome regions and any of the clinicopathological parameters reported in Table 2.

Univariate Analysis. Follow-up data were available for 35 of the 38 patients. One patient (case 1) died for a cause unrelated to the ET. Twenty-three patients (77%) were living after a median follow-up time of 48 months (range, 9–120), whereas 11 patients (23%) died of disease after a median time of 5 months (range, 1–36). Among the tested variables, the presence of metastases and/or local invasion, the presence of tumor necrosis, the presence of angioinvasion, a diameter >2 cm, and a Ki-67 index >2% were significantly correlated with a poorer prognosis (P = 0.026, P < 0.0001, P < 0.0001, P = 0.0007, P < 0.05, and P = 0.0005, respectively). In addition, survival was significantly related to tumor stage, with stage I showing the best, stage III the worst, and stage II intermediate figures (P = 0.001). The diagnosis of PDEC carried a strongly significant poorer prognosis, compared with a diagnosis of WDET/C (P < 0.0001), whereas the survival curves of WDETs and WDECs were not significantly different. The percentage of AIs at the chromosomal regions analyzed was related to patient outcome, because very low percentage of AI was significantly associated with a longer survival. In particular, we found significantly different survival curves when comparing patients with tumors bearing an AI percentage higher or lower than 15% (P = 0.03). This statistical significance was still high if the AI percentage cutoff was raised to 20% (P = 0.01; Fig. 3).

DISCUSSION

For the first time with this report, a series of both well-differentiated ETs and poorly differentiated endocrine carcinomas of the GEP tract was allelotyped at the main chromosomal regions involved frequently in the endocrine tumorigenesis (Table 1). Because of the rarity and the clinicopathological heterogeneity of these tumors, the series of ETs was large and well characterized, as the morphofunctional profile of each tumor was carefully reviewed according the WHO criteria (Ref. 1; Table 2). First of all, the goal of this study was to define specific and recurrent molecular profiles of ETs in relationship to their histological grade and biological behavior. Moreover, the comprehensive analysis of three different sites of tumor origin (stomach, intestine, and pancreas) was performed to identify distinct genotypic differences that may reflect the primary site of the tumor and thereby assist in the diagnosis and classification of these neoplasms. Microsatellite analysis is a useful approach to identify AIs attributable both to deletion of sequences harboring TSGs and to gain or amplification of specific chromosome regions. For this reason, this method allows an accurate analysis of minimal deleted regions looking for candidate TSGs, but it may be also used to detect genomic alterations at chromosomal level particularly for tumor genotypes exhibiting chromosomal instability (CIN).

The findings of the microsatellite analysis reported in this work demonstrate very different allelotypes when comparing PDECs with WDET/Cs. As shown in Table 3, A and B, the molecular profile of the highly malignant gastrointestinal (13 cases) and pancreatic (1 case) PDECs was characterized by very frequent AIs often involving extensive chromosome regions or entire chromosomes. These allelotypes are strongly suggestive for frequent losses or gains of whole chromosomes and/or large chromosome portions as we could verify with interphasic fluorescent in situ hybridization assays indicating that aneuploidy is the main feature of these karyotypes (data not shown). Although the molecular findings about GEP PDECs are very scanty and fragmentary (2–5), these data are in agreement with a wealth of cytogenetic and molecular results about lung PDECs showing a high degree of CIN and very complex karyotypes with nonrandom numerical and structural chromosome aberrations (48, 49).

On the contrary, the subgroup of WDET/Cs was mainly characterized by the absence of AI, or by very low percentage of AI with minimal values among functioning PETs and WDET/C of the ileum and appendix compared with gastric, colorectal, and nonfunctioning pancreatic WDET/Cs. The AIs were mostly scattered even if two chromosomal regions, namely 3p21-p14 and 11p15, appear more frequently involved. These findings are in agreement with previous studies that suggested an early involvement of 3p21-p14 region in the tumorigenesis of PETs (19), whereas frequent losses of the short arm of chromosome 11 has been reported in a series of PETs by several comparative genomic hybridization studies (6–10). Among the 24 WDET/Cs analyzed, 4 tumors exhibited unusual allelotypes characterized by high percentage of AI often extensive to the whole chromosomes appearing more similar to those observed in PDECs rather than in WDET/Cs (cases 32, 33, 36, and 19). Three of these tumors were nonfunctioning PETs and exhibited allelotypes very close to those described by Rigaud et al. (11). In this high-resolution allelotyping of 16 nonfunctioning PETs, the authors found that 44% of cases showed frequent and large allelic deletions, whereas the remaining 56% of cases were characterized by a few random losses, demonstrating also that the average fractional allelic loss was positively correlated with the ploidy status and the malignant progression of the tumor. In our study we confirm these results, identifying with similar frequency a subgroup of nonfunctioning PETs (3 of 8 nonfunctioning PETs; 42%) characterized by frequent AIs, malignant morphological features (a higher degree of cytological atypia and/or a higher proliferative index), and a poorer prognosis (1 of 3 versus 0 of 4 patients dead of disease) than observed in the remaining nonfunctioning PETs analyzed. Similarly, in the
colon-rectum, we found a fourth case of WDEC with very similar allelotype and clinicopathological characteristics (case 19 in Table 3A).

A second important result of our work regards the positive correlation between AI percentage and Ki-67 proliferation index of each tumor, considering both the whole series of ETs ($P = 0.004$; Fig. 2A) and the group of WDET/Cs alone ($P = 0.011$; Fig. 2B). As expected, this finding clearly demonstrates that actively proliferating cells are more prone to accumulate genetic anomalies and that the resultant genomic instability is likely to accelerate the malignant progression of the tumor. These results support and extend to gastrointestinal sites the findings reported by Rigaud et al. (11) for nonfunctioning PETs, concerning the strong positive association between ploidy status and Ki-67 index. Intriguingly, the authors indicated these variables as independent prognostic factors at a multivariate survival analysis highlighting the potential value of the combination of ploidy status and Ki-67 index in predicting the malignant potential of PETs. Our results are in agreement with these findings and additionally underline the importance of including the two latter parameters in the evaluation of the prognosis of ETs in addition to classical clinicopathological features, such as tumor stage and size. As an example, case 3, although being a large (5.5 cm in size) sporic ET of the stomach, metastatic to lymph nodes and, as such, bearing a poor prognosis, showed a low proliferative index (1 mitosis/10 high power fields and Ki67-index 0.4%), a low AI percentage (7.7%), and is alive and free of disease after 8 years. In addition, our univariate analysis of survival both confirms the relationship between the proliferative index and the probability of survival, and suggests a significant prognostic value for the rate of AI in tumor cells.

In this scenario, it is intriguing to find that AI at locus TP53 was observed in our series as an almost distinctive feature of PDECs rather than WDET/Cs (12 of 13 informative PDECs versus 1 of 21 informative WDET/Cs; $P < 0.01$). Noteworthy, the only WDEC exhibiting TP53 AI was a colorectal tumor (case 19 in Fig. 1B) of which the uncommon cytological atypia and unusual high percentage of AI appeared close to those usually observed in PDECs. These findings are consistent with many studies indicating the concurrence of p53 loss and aneuploidy during the tumorigenesis, leading to the hypothesis that p53 may function to actively repress CIN because of its direct involvement in cellular responses against double-strand breaks of DNA (50, 51). Very recently, Bunz et al. (52) demonstrated that inactivation of p53 alone does not lead to CIN in diploid human cell lines, but it is expected that the absence of functional p53 could exacerbate a pre-existing tendency toward CIN likely caused by other genes involved in the regulation of anaphase initiation (53).

In conclusion, the data presented in this work clearly suggest that the two distinct molecular profiles exhibited by PDECs and WDET/Cs are consistent with the markedly different biological and clinical features of these tumors, and support the hypothesis that two different genetic pathways may underlie the malignant progression of these neoplasms. In particular, it is conceivable that the typical CIN exhibited by PDECs may originate from early alterations in a cell cycle checkpoint that monitors the integrity of the spindle apparatus, a structure critical for proper bipolar segregation of duplicated sister chromatids at mitosis. Additional cytogenetic studies of large series of both lung and extrapulmonary PDECs will be useful to define specific anomalies in relationship to the primary site of the tumor, as well as a molecular analysis of larger series of atypical WDET/Cs, will be conclusive to detect which gene defects may cause the malignant conversion of these tumors. On the other hand, the less complex allelotypes usually exhibited by typical WDET/Cs evoke an instability at molecular level attributable to specific genetic and/or epigenetic defects. In agreement with this hypothesis, some authors demonstrated very recently that a subgroup of GEP WDET/Cs showed a CpG island methylator phenotype studying the promoter methylation status and/or the expression of a panel of genes methylated frequently in other gastrointestinal malignancies (39–42).

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Different Molecular Profiles Characterize Well-Differentiated Endocrine Tumors and Poorly Differentiated Endocrine Carcinomas of the Gastroenteropancreatic Tract

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