Cellular Proliferative Fraction Measured With Topoisomerase IIα Predicts Malignancy in Endocrine Pancreatic Tumors

Jose Luis Diaz-Rubio, MD; Andres Duarte-Rojo, MD; Milena Saqui-Salces, Eng; Armando Gamboa-Dominguez, MD, MSci; Guillermo Robles-Diaz, MD

- Context.—Endocrine pancreatic tumors (EPTs) are rare lesions with varying biological behavior. Establishing malignancy is a challenge for clinicians and pathologists.

Objective.—To establish the role of proliferative, apoptotic, angiogenic, and hormonal markers as predictors of malignancy in EPTs.

Design.—Paraffin-embedded EPT samples were studied for prognostic markers.

Patients.—Twenty-one consecutive patients with a diagnosis of EPT.

Main Outcome Measures.—The proliferative fraction (topoisomerase IIα), microvascular density (CD34), vascular endothelial growth factor expression, and estrogen receptor-beta (ERβ) expression were studied by immunohistochemistry on all EPTs. Apoptosis was also assessed with terminal deoxynucleotidyl transferase nick-end labeling.

Results.—We identified 13 benign and 8 malignant tumors. Topoisomerase IIα was significantly increased in malignant tumors (P = .001), while there were no differences in apoptosis, microvascular density, or vascular endothelial growth factor expression in association with malignancy. No correlation could be identified between microvascular density and vascular endothelial growth factor expression, and ERβ was not detected. A receiver operating characteristic curve for topoisomerase IIα disclosed that above a labeling index of 13, the test had 88% sensitivity and 100% specificity for predicting malignancy.

Conclusion.—Cellular proliferation measured with topoisomerase IIα is a simple prognostic marker for malignancy in EPTs, unlike apoptosis, angiogenesis, or the presence of ERβ, which were not associated with malignant behavior. These findings designate a defined field for future research on endocrine pancreatic carcinogenesis and a possible target for chemotherapeutic agents.

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posed as a marker of good prognosis in some tumors, although its utility in EPTs has not been analyzed. The aim of the present study was to investigate the association between the biological behavior of EPTs and markers of proliferative activity, apoptosis, and angiogenesis, as well as estrogen receptor β (ERβ) expression.

**MATERIALS AND METHODS**

**Cases**

Twenty-one cases with a confirmed diagnosis of EPT were studied. Tumor samples were selected from the surgical pathology files when containing representative tissue for immunohistochemistry. Demographic and clinical data (laboratory data, imaging findings, surgical and histopathology reports, evolution, and outcome) for all 21 patients were obtained from the hospital records, when available.

Tumors were classified as functional when clinical symptoms related to hormone overproduction associated with serum elevation and immunohistochemical detection of the corresponding hormone were documented, and as nonfunctional when these conditions were absent, regardless of the presence of immunostaining for any hormone. Malignancy was established when invasion into neighboring organs or distant metastases was demonstrated by imaging techniques, surgery, and/or pathologic examination.

**Immunohistochemical Labeling**

Four-micrometer sections were cut from each fixed tumor sample and stored at −20°C until use. All staining procedures were performed according to the avidin-biotin complex method. Briefly, samples were deparaffinized in xylol and rehydrated in sequential alcohol baths. Antigen retrieval was performed with a microwave pressure cooker using 10 mM citrate buffer at pH 6.0 (CD34 and VEGF) and 10 mM EDTA buffer at pH 8.0 (ERβ and topo IIα). Slides were then allowed to cool at room temperature and were stained using the avidin-biotin-peroxidase complex detection method (DAB basic detection kit, Ventana Medical Systems Inc, Tucson, Ariz) in a Ventana NexES automated stainer.

The following primary antibodies were used for immunohistochemistry: mouse monoclonal antibody raised against human topo IIα (Dako, Glostrup, Denmark; dilution 1:50), CD34 (Dako; dilution 1:50), VEGF (R & D Systems, Minneapolis, Minn; dilution 1:100), and ERβ (Genetex, Inc, San Antonio, Tex; dilution 1:20). For topo IIα and ERβ, a labeling index was obtained according to the average number of tumor cells with evident nuclear staining in 10 high-power fields (×40). In the angiogenesis analysis, each section was examined under low power to identify intratumoral microvascular “hot spots”; sections were then observed at ×40 magnification. Structures with the morphologic features of microvessels that stained with the chromogen, irrespective of whether a lumen was present, were counted. The average from 10 microscopic fields was considered the microvessel-labeling index. The intensity of cytoplasmic VEGF staining within the tumor was scored semiquantitatively as absent (0), weak (1), moderate (2), or strong (3), and the proportion of cells staining was noted using a high-power field (×40). Positive controls for topo IIα, CD34, and VEGF staining were tonsillar tissue, while endometrium was used for ERβ. Cases stained without the primary antibodies were used as negative controls to rule out nonspecific staining. Immunohistochemical analysis was performed by an experienced pathologist (A.G.D.) who had no knowledge of the patients’ clinical data.

Apoptotic cells were identified by in situ detection of DNA fragmentation, using the terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) method. Staining was performed on deparaffinized slides following incubation with protease for 15 minutes at 37°C, washing with Tris, and quenching of endogenous peroxidase by incubation in 3.0% (vol/vol) hydrogen peroxide in phosphate-buffered saline for 5 minutes. Single-stranded DNA was identified using a commercially available kit (ApopTag Plus; Intergen Co, New York, NY), according to the manufacturer’s protocol, and the tissue was counterstained with hematoxylin for 1 minute. Sections were dehydrated in ethanol, cleared in xylene, and mounted with glass coverslips. The apoptotic labeling index was defined as the average number of apoptotic cells in 10 random fields (×40).

**Statistical Analysis**

Data are expressed as medians and ranges. Statistical comparisons were made using the Mann-Whitney U test for continuous variables and the Fisher exact test or χ² analysis for categorical variables. For significant associations identified in the univariate analysis, receiver operating characteristic (ROC) curves were performed to determine the ideal cutoff value and operational characteristics of the test. Correlation coefficient and Spearman rank correlation tests were applied, as appropriate, to test dependence between variables. Differences were considered significant at P < .05. The program SPSS 10.0 for Windows (SPSS, Inc, Chicago, Ill) was used for statistical analysis.

**RESULTS**

Demography and Biological Behavior

Eight tumors occurred in men and 13 were diagnosed in women, with a median age at diagnosis of 45 years (range, 17–70 years). Thirteen of the EPTs were benign and 8 were malignant, with no difference regarding age or sex. Twelve of the tumors were functional (9 insulinomas and 3 gastrinomas), and 9 were nonfunctional. Malignancy occurred in 1 of the functional tumors (insulinoma) and in 7 of the nonfunctional tumors (P = .002). Two benign insulinomas were classified as multiple endocrine neoplasia type I (MEN-1). Four patients with malignant EPTs died (2 during hospitalization, and 2 more 3 and 8 months after discharge); the remaining 4 patients abandoned outpatient follow-up after 5 to 25 months. Eleven cases with benign EPTs were followed for at least 2 years without evidence of tumor recurrence (the remaining patients were followed for 19 months and 1 month).

**Immunohistochemistry**

Results of topo IIα, CD34, and apoptosis (TUNEL) studies are shown in Table 1. No immunostaining was observed for ERβ. The only marker associated with malignancy was topo IIα (Figure). Semiquantitative expression of VEGF is demonstrated in Table 2. There was no association for VEGF, even when neoplasias with strong immunostaining were compared with the remaining tumors (P = .13).

<table>
<thead>
<tr>
<th>Table 1. Labeling Indices for Proliferation, Apoptosis, and Microvessel Density in Endocrine Pancreatic Tumors*</th>
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<tbody>
<tr>
<td>Proliferation (topo IIα)</td>
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<tr>
<td>Benign, Median (Range)</td>
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<tr>
<td>P</td>
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<td>0.4 (0–1.2)</td>
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* topo IIα indicates topoisomerase IIα; TUNEL, terminal deoxynucleotidyl transferase nick-end labeling.
functioning by controlling DNA conformation, replication, recombination, and/or other transcriptional events. The α isoform of topoisomerase II predominates mainly in proliferating cells, making it a marker of cell proliferation that correlates with aggressive clinical behavior in other neoplasms. In this study, the topo IIα-labeling index was associated with malignant behavior in EPTs, which is in agreement with other studies in which the tumoral growth fraction was assessed using proliferating cell nuclear antigen and/or Ki-67. Similar findings were also reported in a previous study using antibodies to topo IIα; however, only nonfunctional tumors were studied, excluding the functional counterparts that are actually more common in clinical practice. The current work found that topo IIα was also able to distinguish malignancy in functional EPTs. Furthermore, this marker showed high sensitivity and specificity at a labeling index greater than 13, which allows us to propose it as a useful marker for evaluating the tumoral growth fraction and to predict malignancy in EPTs.

Suppression of apoptosis is considered essential for tumor development and/or progression. Experimental studies have demonstrated the crucial role of antia apoptotic (Bcl-2, Bcl-xL) and proliferative/apoptotic (c-Myc) signals in islet cell tumorigenesis. Also, expression of Bcl-2 has been reported in 45% of EPTs, and induction of apoptosis has been observed in tumor biopsies of patients treated with somatostatin analogs. However, the decreased TUNEL-labeling index in malignant EPTs did not reach statistical significance, nor was any correlation found between apoptosis and cellular proliferation. Our results do not support apoptosis as a key factor for malignancy in EPTs, as it has been shown in other neoplasias, and an independent alteration on cellular proliferation seems to trigger the malignant behavior of these tumors.

The role of angiogenesis and angiogenic factors has been widely studied in models of pancreatic β-cell carcinogenesis. Transformation of premalignant lesions into solid and invasive tumors requires an angiogenic switch in which VEGF plays a pivotal role, although the precise molecular mechanisms involved in the process are poorly understood.

Microvascular density did not differ between benign and malignant tumors. Results for VEGF were similar, al-

The DNA topoisomerases are necessary for normal cell

COMMENT

Establishment of malignancy in EPTs is a difficult endeavor when tumor infiltration or metastases are not found, because apparently benign tumors can show malignant behavior after months or even years of observation. A marker capable of distinguishing malignancy would anticipate the need for close observation and early adjuvant chemotherapy, improving survival. Tumor development is considered a multistep process, involving multiple molecular abnormalities and leading to the transition from a normal to a malignant cellular state. Some of these abnormalities include proliferative, apoptotic, and angiogenic pathways, as well as alterations in sex-hormone signaling. In the present study, we tested 5 molecular markers involved in tumorigenesis, as predictors of malignancy, in a group of 21 patients with EPTs. All but 1 of the benign cases were followed for at least 19 months without evidence of recurrence; thus, the possibility of being malignant and misclassified is remote.

The DNA topoisomerases are necessary for normal cell

Table 2. Immunostaining Score for Vascular Endothelial Growth Factor*

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<td>Benign EPTs</td>
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<td>5</td>
<td>3</td>
<td>0</td>
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<tr>
<td>Malign EPTs</td>
<td>2</td>
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* $\chi^2$: $P = .28$. EPTs indicates endocrine pancreatic tumors.

No correlation between microvascular labeling index (CD34) and VEGF expression was observed ($r = 0.25; P = .28$). However, when only benign EPTs were analyzed, a moderate correlation was found ($r = 0.53; P = .05$). On the other hand, topo IIα and apoptosis were not related ($r = 0.05; P = .82$), even when comparing benign and malignant tumors separately.

The ROC curve analysis of the topo IIα labeling index demonstrated an area under the curve of 0.947 (95% confidence interval, 0.754–0.992). When a labeling index of 13 was established as the ideal cutoff value, a sensitivity value of 88% and specificity of 100% were found, with positive and negative predictive values of 100% and 93%, respectively. The only functional tumor found to be malignant had a topo IIα labeling index of 22.

Patterns of immunohistochemical staining of topoisomerase IIα (topo IIα) in endocrine pancreatic tumor. A, Positive topo IIα expression in benign endocrine pancreatic tumor (streptavidin-biotin peroxidase immunostaining, original magnification ×40). B, Strongly nuclear-positive topo IIα expression in malignant endocrine pancreatic tumor (streptavidin-biotin peroxidase immunostaining, original magnification ×40).

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though the semiquantitative analysis found strong immunostaining only in malignant tumors. To our knowledge, microvascular density had not been assessed in EPTs on clinical grounds, and the lack of association between this parameter and tumor behavior contrasts with what has been demonstrated in other neoplasms (ie, breast and prostate).29,30 Terris et al13 found VEGF expression in 16 of 20 EPTs, without correlation with tumor stage.13 Taken together, these findings suggest that angiogenesis and VEGF expression are not as crucial for tumor invasion in EPTs, and other factors of malignancy may be more important. Also, this is in agreement with the recent finding of the role of VEGF on a well-characterized model of β-cell carcinogenesis, in which it was responsible for early angiogenesis, progression from adenoma to carcinoma, and accelerated tumor growth, but did not increase tumor invasion or metastasis. These authors concluded that VEGF-mediated angiogenesis is required, but not sufficient, for the progression to tumor malignancy.31 These findings are especially noteworthy in EPTs, in which the main definition of malignancy derives from invasion and metastasis, thus precluding the role of vascular morphology and VEGF as early markers of malignancy. Other factors of malignancy, such as lymphangiogenesis and VEGF-C, cannot be discarded.32

Correlation between microvascular density and VEGF was found only in benign tumors. This association can be due to the dependence of early angiogenesis on VEGF (the angiogenic switch) that may be lost as carcinogenesis progresses, reflecting the less critical role of VEGF on an already initiated process,33 the need for other angiogenic factors (ie, matrix metalloproteinases), or the loss of antiangiogenic regulators.34,35

Finally, we found no ERβ immunostaining in any EPT, excluding its utility as a marker of benign behavior, as suggested in breast, ovarian, and colon cancer,16,17,18 or its possible role as a regulator of apoptosis and angiogenesis, according to some proapoptotic and antiangiogenic properties previously attributed to this receptor.35,36

In conclusion, topo IIα is a potentially useful marker of malignancy that can help the clinician to formulate a more specific approach for the search for metastasis and that can facilitate the early administration of adjuvant chemotherapy and/or somatostatin analogs. Furthermore, the cellular content of this enzyme can be used to identify the sensitivity to topo II-targeted chemotherapeutic agents.19

The identification of cellular proliferation as the only factor associated with malignancy designates a specific field for more in-depth studies on endocrine pancreatic carcinogenesis.

References


