Oncogene Expression in Gastroenteropancreatic Neuroendocrine Tumors

Implications for Pathogenesis

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BACKGROUND. Neuroendocrine tumors of the gastroenteropancreatic system include pancreatic islet cell and carcinoid tumors. These tumors comprise a functionally and biologically heterogeneous group of neoplasms that rarely show reliable histopathologic signs of malignancy. No etiologic factors are proven to be associated with them, and their exact ontogeny and carcinogenesis remain unknown.
METHODS. Monoclonal antibodies were employed, along with microwave antigen retrieval and the avidin-biotin immunohistochemical method, to investigate the expression of c-*myc*, *bcl*-2, c-*erb* B-2, c-*erb* B-3, c-*jun*, and proliferating cell nuclear antigen (PCNA) in a retrospective series of 116 primary gastroenteropancreatic neuroendocrine tumors (GPNTs). The authors attempted to correlate this expression with the clinicopathologic outcome of the disease.
RESULTS. Immunoreactivities for c-*myc*, *bcl*-2, c-*erb* B-2, c-*erb* B-3, and c-*jun* were detected in 100%, 45%, 24%, 7%, and 24% of pancreatic neuroendocrine tumors (PNTs), respectively. In carcinoid tumors, immunoreactivities were detected for c-*myc* (63%), *bcl*-2 (28%), c-*erb* B-2 (31%), c-*erb* B-3 (6%), and c-*jun* (23%). There were significantly higher incidences of c-*myc*, *bcl*-2, and c-*erb* B-2 immunoreactivities in carcinoid tumors of the rectum than in those of the appendix, and significantly higher incidences of *bcl*-2 and c-*jun* immunoreactivities in carcinoid tumors of the bronchus than in those of the appendix. Incidence of PCNA immunoreactivity was significantly higher in malignant than in benign PNTs and also significantly higher in carcinoid tumors of the jejunum and ileum than in those of the appendix.
CONCLUSIONS. The oncogenes c-*myc*, *bcl*-2, c-*erb* B-2, and c-*jun* are frequently expressed in human GPNTs. The expression of these oncogenes may represent pathogenic events in the generation, malignant transformation, and progression of GPNTs. The immunohistochemical evaluation of cell kinetics in GPNTs by PCNA might be a useful adjunct to conventional diagnostic procedures. ***Cancer* 1997;80:668–75.** © *1997 American Cancer Society.*

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Neuroendocrine tumors of the gastroenteropancreatic system include two main groups, islet cell tumors of the pancreas and carcinoid tumors. These tumors secrete active hormones and may be associated with distinctive clinical syndromes. Pancreatic neuroendocrine tumors (PNTs) are rare, with an annual incidence of 0.2 in 100,000,[1] and are either inherited as part of the multiple endocrine neoplasia type 1 (MEN 1) syndrome or, more frequently, are sporadic. Since the first report by Nichols in 1902,[2] five main syndromes have been associated with PNTs: the hypoglycemic or insulinoma syndrome; the Zollinger–Ellison or gastrinoma syndrome; the Verner–Morrison, watery diarrhea hypokalemia achlorhydria (WDHA), or va-

soactive intestinal polypeptide (VIP)oma syndrome; the glucagonoma syndrome; and the somatostatinoma syndrome. About one-third of PNTs fail to produce any hormone-related syndrome and are therefore described as nonfunctioning.[1] Carcinoid tumors are the most common of the gastrointestinal tract (GI) neuroendocrine neoplasms and account for 55–86% of all such tumors.[3,4] According to the site of origin, carcinoid tumors have been classified as tumors of the foregut (bronchus, pancreas, stomach, and proximal duodenum), midgut (distal duodenum, jejunum, ileum, appendix, proximal colon, and ovary), and hindgut (distal colon and rectum).[5] The histochemical characteristics, biochemical properties, and clinical manifestations of carcinoid tumors vary according to their embryonic origin. These tumors comprise a functionally and biologically heterogeneous group of neoplasms that rarely show reliable histopathologic signs of malignancy. Despite recent advances in the diagnosis, localization, and treatment of these tumors, no etiologic factors are proven to be associated with them, and their exact ontogeny and carcinogenesis remain unknown.

The past decade has seen major advances in the understanding of the molecular genetics of cancer, and many of these advances have stemmed from studies of cellular oncogenes and tumor-suppressor genes. Several oncogenes have been well studied over the years and characterized with regard to their roles in the development of specific human tumors. For example, in 1980, a translocation involving the c-*myc* oncogene was found to be associated with Burkitt's lymphoma, and this oncogene was subsequently implicated in the development of many other types of human tumors. The proto-oncogene c-*erb* B-2 is a member of the type 1 growth factor family, which also includes c-*erb* B-3 and epidermal growth factor receptor (EGFR).[6] The c-*jun* proto-oncogene is a member of the early response gene family. c-*jun* appears to occupy a central role in the regulation of proliferation and is involved in signal transduction processes.[7] Amplification and overexpression of these oncogenes have been implicated in experimental cellular transformation and tumorigenesis in a wide range of human cancers, including neuroendocrine tumors and neuroendocrine-differentiated cells.[8–17] *bcl*-2 appears to constitute a member of a new category of oncogenes, namely, regulators of programmed cell death.[18] Recently, *bcl*-2 has been described in neuroblastoma and other human tumor cell lines of neural origin[19,20] and implicated in the tumorigenesis of GI tumors.[21,22]

However, the expression and roles of oncogenes in the pathogenesis and progression of gastroenteropancreatic neuroendocrine tumors (GPNTs) have not been intensively studied. Only a few studies, with limited numbers of cases, have been reported.[8,23] In this study, we have analyzed, by immunohistochemistry using monoclonal antibodies, the expression of five dominant oncogenes—c-*myc*, *bcl*-2, c-*erb* B-2, c-*erb* B-3, and c-*jun*—in a retrospective series of 116 primary human GPNTs, including islet cell tumors of the pancreas and carcinoids of foregut, midgut, and hindgut origin, and we have attempted to correlate this expression with the clinicopathologic outcome of the disease. Additionally, as measurement of cell proliferation may provide useful information concerning tumor prognosis and may aid in diagnosis, we examined the correlation between the fraction of proliferating cell nuclear antigen (PCNA) positive nuclei in the tumor and the outcome in this series of GPNT patients.

MATERIALS AND METHODS

Patients

All cases of primary GPNTs from the period 1986–1995 in the archival files of the Northern Ireland Neuroendocrine Tumor Register, including PNTs (n = 29) and carcinoid tumors (n = 87), were reviewed retrospectively. This tumor register was established in 1978 by the Department of Medicine, the Queen's University of Belfast.[1] Detailed clinicopathologic data on patients (including age, gender, symptoms, tumor location, tumor size, and occurrence of metastases) were available, and each patient's clinical progress had been followed and recorded since initial diagnosis. The tumor identity was confirmed in each case by the diagnostic pathology service of the Royal Group of Hospitals, Belfast, Northern Ireland. The confirmations were made by histologic methods, electron microscopy, and immunohistochemistry for general neuroendocrine markers and specific regulatory peptides.

Tumor Samples

Samples of GPNTs were collected, and tumor tissue had been fixed in modified Susa and processed to paraffin wax according to a constant protocol.[24] In addition, samples of two normal pancreata were obtained and processed identically.

Immunohistochemistry

Oncoprotein expression was detected with commercially prepared monoclonal antibodies specific for each protein. These were anti-c-*myc*, clone 9E11, at a dilution of 1:150; anti-c-*erb* B-2, clone CB11, 1:50; anti-c-*erb* B-3, clone RTJ1, 1:50; anti-c-*jun*, clone DK4, 1:50; anti-PCNA, clone PC10, 1:100 (all from Novocastra, Newcastle upon Tyne, UK); and anti-*bcl*-2, clone 124, 1:50 (Dakopatts, Glostrup, Denmark).

Immunohistochemical studies were performed

by the avidin-biotin complex (ABC) method as described previously.[25] Briefly, sections 4 μm thick were dewaxed and subjected to microwave antigen retrieval by immersion in citrate buffer pH 6.0. Endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide. The slides were treated with 20% normal rabbit serum for 30 minutes at room temperature, then incubated with the primary antibodies (c-*myc*, *bcl*-2, c-*erb* B-2, c-*erb* B-3, and PCNA) for 1 hour at room temperature and with c-*jun* for 18 hours at 4°C, in a moist chamber according to the manufacture's instructions. The sections were then washed and incubated with a biotinylated secondary antibody (Dakopatts, Glostrup, Denmark). After 35 minutes of incubation in ABC (Dakopatts, Glostrup, Denmark), the reaction product was visualized using 3,3-diaminobenzidine tetrahydrochloride (Sigma, Poole, UK).

Controls utilized throughout the staining procedure included staining of sections that were previously determined as either positive or negative for each oncoprotein as well as incubation and development of sections using normal blocking serum without incubation in primary antibody.

The immunohistochemical results were assessed without knowledge of the clinical diagnosis and evaluated semiquantitatively as described previously.[25]

Statistical Analysis

The chi-square test was used with Yate's correction for the statistical analysis of the data in which specimens containing more than 10% positive tumor cells were included. Statistical significance was set at $P < 0.05$.

RESULTS

Oncoproteins and PCNA immunoreactivity in PNTs and carcinoid tumors are given in Tables 1 and 2, in which only specimens with more than 10% of tumor cells stained are included.

Oncoprotein Immunoreactivity in PNTs

Strong immunoreactivity for c-*myc* was widespread, generally cytoplasmic, and with some nuclear staining identified in all 29 tumor samples (Fig. 1A). Cytoplasmic *bcl*-2 immunoreactivity, heterogeneous in intensity, was present in 13 samples (Fig. 1B). In the normal pancreata, moderately intense c-*myc* immunoreactivity was observed in the acinar cell cytoplasm, and weak immunoreactivity was detected in the cytoplasm of cells of the islets of Langerhans. Only ductular epithelial cells showed *bcl*-2 immunoreactivity, and endocrine tissue was negative.

Immunoreactivity for c-*erb* B-2 occurred in the cytoplasm and occasionally in the cell membranes in 7 of 29 PNTs in which more than 10% of tumor cells reacted positively (Fig. 1C). Immunoreactivity for c-*erb* B-3 occurred in only one insulinoma in which less than 10% of cells were positive and in one glucagonoma in which 10–50% of cells were positive, and this was localized exclusively to the cytoplasm. In the normal pancreas, c-*erb* B-2 immunoreactivity occurred in the cytoplasm of some acinar cells and in the cell membrane of ductal cells, whereas weak cytoplasmic c-*erb* B-3 was only detected in cells of the islets of Langerhans.

Cytoplasmic and nuclear c-*jun* immunoreactivity was observed in 7 of 29 tumor samples (Fig. 1D). No c-*jun* immunoreactivity was detected in normal pancreatic tissues.

Oncoprotein Immunoreactivity in Carcinoid Tumors

Of the 87 carcinoid tumors from different sites examined, c-*myc* immunoreactivity was found in 55 tumors (63%), and 24 tumor samples (28%) showed immunoreactivity for *bcl*-2 (Table 2). The majority of positive cases displayed a cytoplasmic staining pattern of variable intensity for both oncoproteins, and occasional nuclear staining was observed for c-*myc* (Fig. 2A).

Immunoreactivity for c-*erb* B-2 was found in 27 carcinoid tumors (31%). Staining was cytoplasmic, with no distinct cell membrane staining observed (Fig. 2B). Only one ileal carcinoid tumor and one appendiceal carcinoid tumor displayed immunoreactivity for c-erb B-3.

c-*jun* immunoreactivity was detected in 20 of 87 carcinoid tumors. In most positive cases, the staining was cytoplasmic and occasionally nuclear, with the intensity of staining varying from case to case (Fig. 2C, 2D).

PCNA Immunoreactivity in GPNTs

Nuclear immunoreactivity to PCNA was found in 17 of 29 PNTs with more than 10% positive cells (Fig.3A). In the normal pancreas, PCNA immunoreactivity was detected in occasional acinar cells alone (Fig. 3B).

PCNA immunoreactivity was found in carcinoid tumors from different sites, including the bronchus (86%), stomach (80%), jejunum and ileum (90%), cecum (100%), appendix (60%) and rectum (71%). Unequivocal nuclear staining was present in all positive cases.

Association of Oncoprotein Expression

Consecutive sections of all tumor samples were used in immunostaining for oncoproteins and PCNA. A marked association was observed between the presence of oncoprotein immunoreactivity, particularly

TABLE 1
Oncoprotein and PCNA Immunoreactivities in Pancreatic Neuroendocrine Tumors

Tumor type	No. of tumors	No. metastatic	c-*myc*	*bcl*-2	c-*erb* B-2	c-*erb* B-3	c-*jun*	PCNA
Insulinoma	11	0	11 (100%)	4 (36%)	2 (18%)	0 (0%)	1 (9%)	3 (27%)
Gastrinoma	6	3	6 (100%)	3 (50%)	1 (17%)	0 (0%)	2 (33%)	5 (83%)
Glucagonoma	4	2	4 (100%)	3 (75%)	3 (75%)	1 (25%)	1 (25%)	2 (50%)
VIPoma	3	2	3 (100%)	2 (67%)	1 (33%)	0 (0%)	2 (67%)	3 (100%)
Nonfunctioning	5	4	5 (100%)	1 (20%)	0 (0%)	0 (0%)	1 (20%)	4 (80%)
Total	29	11	29 (100%)	13 (45%)	7 (24%)	2 (7%)	7 (24%)	17 (59%)
Total benign	18	0	18 (100%)	9 (50%)	2 (11%)	1 (6%)	3 (17%)	7 (39%)
Total malignant	11	11	11 (100%)	4 (36%)	5 (45%)	0 (0%)	4 (36%)	10 (91%)[a]
Normal pancreas	2	0	2 (100%)	0 (0%)	0 (0%)	2 (100%)	0 (0%)	2 (100%)

PCNA: proliferating cell nuclear antigen.
[a] $P = 0.018$ versus benign pancreatic neuroendocrine tumors.

TABLE 2
Oncoprotein and PCNA Immunoreactivities in Carcinoid Tumors

Original site	No. of tumors	No. metastatic	c-*myc*	*bcl*-2	c-*erb* B-2	c-*erb* B-3	c-*jun*	PCNA
Foregut								
Bronchus	22	2	16 (73%)	10 (45%)	8 (36%)	3 (17%)	7 (32%)	19 (86)
Mediastinum	1	0	1 (100%)	1 (100%)	1 (100%)	0 (0%)	1 (100%)	1 (100%)
Stomach	5	1	5 (100%)	2 (40%)	3 (60%)	0 (0%)	2 (40%)	4 (80%)
Midgut								
Jejunum and ileum	20	18	15 (75%)	3 (15%)	6 (30%)	1 (5%)	6 (30%)	18 (90%)
Appendix	30	1	14 (46%)[a]	4 (13%)[b,c]	4 (13%)[b]	1 (3%)	2 (7%)[d]	18 (60%)[e]
Cecum	2	0	2 (100%)	0 (0%)	1 (50%)	0 (0%)	0 (0%)	2 (100%)
Hindgut								
Rectum	7	3	7 (100%)	4 (57%)	4 (57%)	0 (0%)	2 (29%)	5 (71%)
Total	87	25	55 (63%)	24 (28%)	27 (31%)	5 (6%)	20 (23%)	67 (77%)

PCNA: proliferating cell nuclear antigen.
[a] $P = 0.032$.
[b] $P = 0.043$ vs. rectal carcinoid tumors.
[c] $P = 0.024$.
[d] $P = 0.046$ vs. lung carcinoid tumors.
[e] $P = 0.046$ vs. jejunum and ileum carcinoid tumors.

bcl-2 and c-*myc* immunoreactivities, in PNTs and bronchial carcinoid tumors.

Of the 29 PNTs studied, 13 tumors, which displayed *bcl*-2 immunoreactivity, also showed c-*myc* immunoreactivity (Table 1). Of 10 *bcl*-2 positive carcinoid tumors of the lung, 9 were also positive for c-*myc*. All 14 *bcl*-2 positive GI carcinoid tumors also demonstrated c-*myc* immunoreactivity. In addition, c-*myc*, *bcl*-2, and c-*erb* B-2 immunoreactivity were all present in four rectal carcinoid tumors, and among these c-*jun* immunoreactivity was also found in two.

Association with Clinicopathologic Findings

No significant correlation was observed between the immunolocalization patterns of c-*myc*, *bcl*-2, c-*erb* B-2, c-*jun*, and the clinicopathologic features of PNT patients. The presence of these oncoprotein immunoreactivities did not have any independent influence on the length of survival of these patients. Although there was a tendency of gastrinoma, glucagonoma, VIPoma, and nonfunctioning PNTs to show a higher incidence of positivity than that of insulinoma, the differences were not proven to be statistically significant.

However, there was a significant difference in oncoprotien immunoreactivities between carcinoid tumors of the appendix and rectum for c-*myc* ($P = 0.032$), *bcl*-2 ($P = 0.043$), and c-*erb* B-2 ($P = 0.043$) and between carcinoid tumors of the appendix and bronchus for *bcl*-2 ($P = 0.024$) and c-*jun* ($P = 0.046$). A high incidence of c-*myc*, *bcl*-2, and c-*erb* B-2 expres-

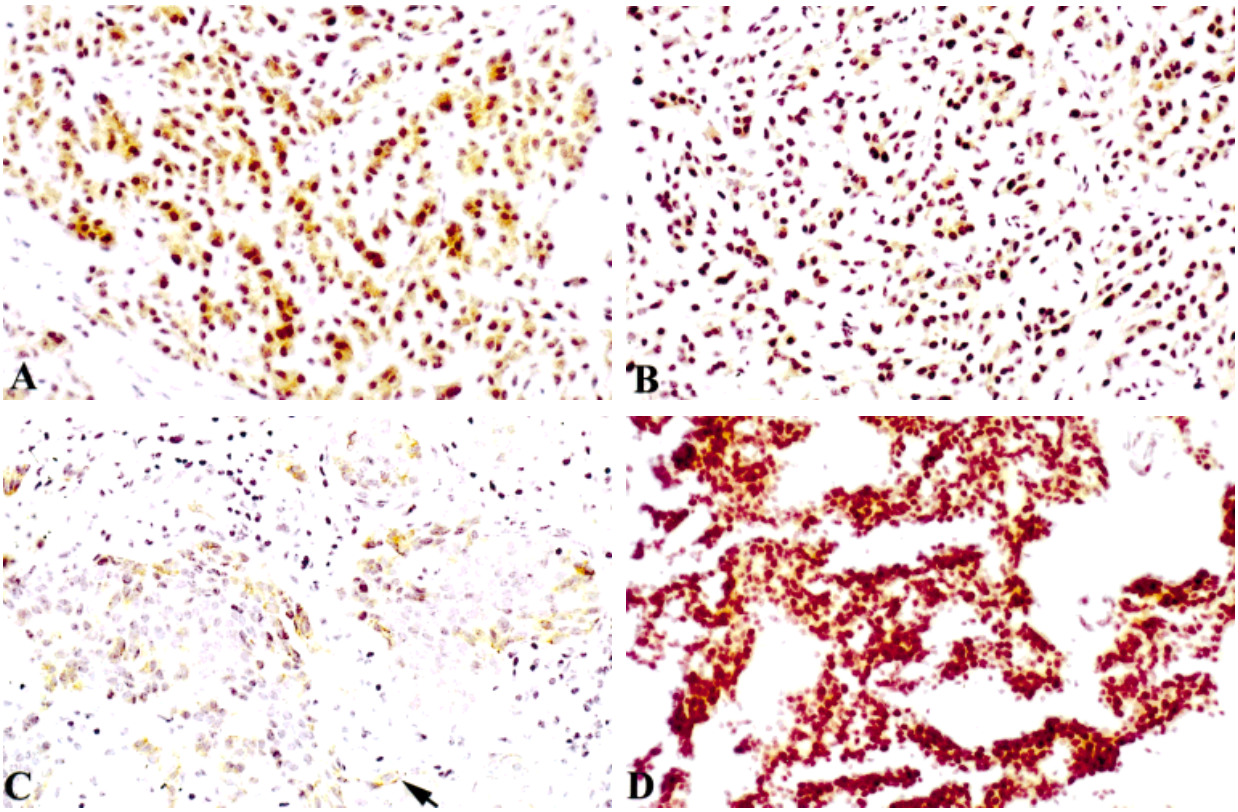

FIGURE 1. (A) c-*myc* immunoreactivity in a pancreatic neuroendocrine tumor (PNT) is shown (original magnification ×250). (B) *bcl*-2 immunoreactivity in the same tumor sample as (A) is shown (original magnification ×250). (C) c-*erb* B-2 immunoreactivity in a PNT is shown; the arrow indicates membrane staining (original magnification ×250). (D) c-*jun* nuclear immunoreactivity in a PNT is shown (original magnification ×250).

sion in carcinoid tumors from sites other than the appendix was also observed, although the difference did not reach statistical significance. Whereas only 7% of appendiceal carcinoid tumors showed c-*jun* positivity compared with 30% of jejunum and ileum carcinoid tumors, the difference was also not proven to be statistically significant.

Eleven of 13 patients with malignant PNTs and 15 of 26 patients with malignant GI carcinoid tumors (including 1 of 5 stomach, 13 of 18 jejunoileal, and 1 of 3 rectal carcinoid tumors) had hepatic metastases. However, the presence of immunoreactivity to any of the 5 oncogenes examined was not significantly linked to the presence of distant metastases.

There was absolute concordance between PCNA immunoreactivity and PNT behavior. Thirty-nine percent of benign tumors showed immunoreactivity for PCNA, whereas in malignant PNTs immunoreactivity to PCNA was present in 91% of cases ($P = 0.018$). There was also a statistically significant difference in the incidence of PCNA immunoreactivity between carcinoid tumors of the jejunum and ileum and those of the appendix ($P = 0.046$).

DISCUSSION

Pancreatic neuroendocrine tumors are slowly growing neoplasms with malignant potential. Approximately 80–90% of insulinomas (which comprise 70% of the functioning endocrine pancreatic tumors) are benign, whereas only approximately 10–30% of gastrinomas, VIPomas, and nonfunctioning tumors are benign.[3] In the current study, c-*myc* protein immunoreactivity was detected in all tumor samples, and a marked association with *bcl*-2 expression was observed. Recently, Iwahashi et al.[26] demonstrated that *bcl*-2 prevents apoptosis induced by cytokines in pancreatic islet cells. Despite the apparent importance of c-*myc* in the induction of cell proliferation, expression of c-*myc* has recently been linked to the induction of cell death in different cells.[27,28] However, apoptotic cell death induced by c-*myc* is inhibited by *bcl*-2, whereas cell proliferation enhanced by deregulated and overexpressed c-*myc* is not reduced.[29,30] Moreover, a marked synergy between *bcl*-2 and c-*myc* in c-*myc*/*bcl*-2 double-transgenic mice during tumorigenesis has been demonstrated.[31,32] The presence of both *bcl*-2 and c-*myc* immunoreactivities in PNTs would indicate that a dis-

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