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PGP 9.5 immunocytochemical staining for pancreatic endocrine tumors

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Aims/hypothesis: Protein gene product 9.5 (PGP 9.5) is a marker for neuroendocrine cells but has not been used for pancreatic islet cells and pancreatic endocrine tumors (PETs). Antibodies for PGP 9.5 are now commercially available for immunocytochemical study, with which immunostaining may be able to differentiate between benign and malignant PETs.

Results: All 4 kinds of normal islet cells were positively immunostained for PGP 9.5—moderately positive for β-cells and strongly positive for δ-cells, whereas ganglion cells were immunostained more strongly than islet cells. Nine of 12 insulinomas were moderately to strongly positive for PGP 9.5. Two glucagonomas, 3 of 6 pancreatic polypeptidomas (PPomas), 3 of 9 gastrinomas, and 2 of 4 non-functioning PETs were negative for PGP 9.5.

Materials and Methods: Thirty-four PETs were immunocytochemically stained for PGP 9.5 using a rabbit polyclonal antibody together with immunostaining for 4 pancreatic hormones, chromogranin A (CgA), and gastrin. PETs consisted of 12 insulinomas, 2 glucagonomas, 1 somatostatinoma (SRIFoma), 6 PPomas, 9 gastrinomas, and 4 non-functioning PETs.

Conclusion/Interpretation: PGP 9.5 immunostaining was universally positive for 4 kinds of islet cells and was moderately to strongly positive for 9 of 12 (75%) insulinomas. All 22 non-β-cell PETs were negative or weakly positive for PGP 9.5, and thus negative or weakly positive PGP 9.5 immunostaining may be used as a marker for potential malignancy and poor prognosis for non-β-cell PETs.

Introduction

PGP 9.5 is a ubiquitin-carboxyl hydrolase that is expressed in nerve tissues from mice brains at all stages of differentiation,[1-6] and thus it has been regarded as an universal cytoplasmic marker for neuroendocrine cells and neuroendocrine tumors (NETs) since the 1980s.[1-6] However, PGP 9.5 has not gained popularity as a diagnostic and prognostic marker in the past because of the limited supply of the antibody, and the immunostaining characteristics for 4 kinds of islet cells were not known. Commercial PGP 9.5 antibodies are now available for both polyclonal rabbit and monoclonal antibodies; rabbit anti-PGP 9.5 was used for pancreatic endocrine tumors (PETs) in this study. PETs have been classified by the widely accepted WHO 2004 and 2010 classifications for NETs including PETs in the gastroenteropancreatic system.[7,8] This study aimed to correlate PGP 9.5 immunocytochemical staining with both of the 2004 and 2010 WHO classifications for gastroenteropancreatic NETs.[7,8]

Results

In normal pancreatic islets, all islet cells, including β cells for insulin, α cells for glucagon, δ cells for SRIF, and PP cells for PP, were moderately to strongly positive for PGP 9.5, showing diffuse cytoplasmic staining for all islet cells and strong staining for the peripherally located islet cells, which corresponded to σ cells (**Fig. 1A–D**). Moderately positive for PGP 9.5, β cells were the major islet cells and were mainly located in the middle of islets, whereas σ cells were mainly located at the periphery of islets and the outer margin of islet lobules (**Fig. 1A and C**). δ cells were located in middle of islets adjacent to β cells and were moderately positive for PGP 9.5 (**Fig. 1B and C**). In the well-preserved tissues of the pancreas, scattered fine nerve fibers were identified in the interacinar and abundantly in the perivascular connective tissues. Periductal, peri-islet, and inter-islet fine nerve fibers were also positively immunostained together with scattered, strongly immunostained ganglion cells with plump cytoplasm (**Fig. 1E**; **Fig. 2D and F**). Eight insulinomas (75%), excluding 2 benign cases (Cases 4 and 7) and 1 G_2 malignant case (Case 9), were at least moderately positive for PGP 9.5 (**Fig. 2**; **Table 1**). Two glucagonomas were negative for PGP 9.5, and 1 SRIFoma was positive (**Fig. 3A and B**; **Table 1**). Three of 6 PPomas (Cases 2–4), 3 of 9 gastrinomas (Cases 4, 6, and 7), and 2 of 4 non-functioning PETs (Cases 1 and 3) were negative for PGP 9.5 (**Fig. 3**; **Table 1**). Using the 2004 WHO classification, 11 of 12 insulinomas (92%) were WDNET, 1 case of primary insulinoma

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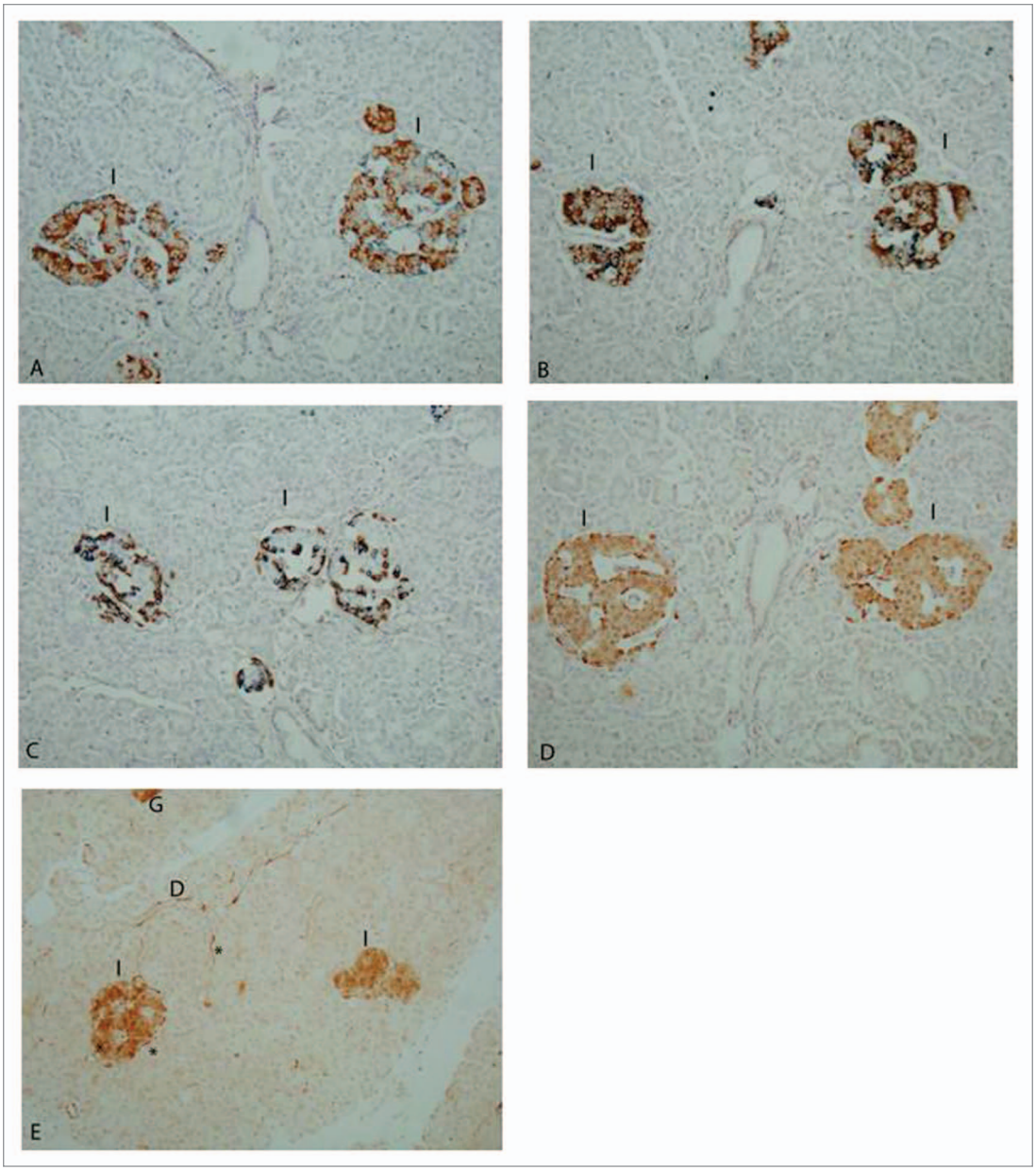

Figure 1. Control islets. The majority of islet cells were β-cells (brown), and σ-cells (blue) were located at the periphery of islets and islet lobules (**A**). SRIF cells were minor islet cells, located in the middle of islets adjacent to β-cells (**B and C**). All of the islet cells were moderately immunostained for PGP 9.5 with the stronger staining for scattered ganglion cells and the peripheral islet cells, the latter corresponding to δ-cell (**D**). In the well-fixed tissues, fine nerve fibers were identified in intra- and inter-islet, inter-acinar and perivascular stroma together with stronger immunostained scattered ganglion cells containing plump cytoplasm (**E**). D, duct; G, ganglion cells; I, islet; *intra-islet, intra-, and inter-acinar and peri-ductal nerve fibers. (**A**) insulin and glucagon, (**B**) insulin and SRIF, (**C**) glucagon and SRIF double immunostained, (**D and E**) PGP 9.5 immunostained.

was originally WDNET, and a liver metastasis (Case 9) 3 y after tumor resection was WDNEC (**Table 1**). Two glucagonomas were WDNEC, and 1 SRIFoma was WDNET (**Table 1**). Among 6 PPomas, Cases 1, 2, and 3 were from the same patient, who presented initially with a large PPoma as WDNEC, which metastasized to the liver as the same WDNEC 2 y after hemipancreatectomy and subsequently involved the entire remaining pancreas diffusely and the liver 4 y after the initial surgery as PDNEC of small cell PDNEC (**Table 1**). In the remaining 3 cases of PPomas, 1 liver metastasis (Case 4) was WDNEC, and 2 cases of tumors smaller than 1.5 cm (Cases 5 and 6) were WDNET (**Table 1**). Among 8 primary gastrinomas, 6 cases of tumors smaller than 1.5 cm (Cases 1, 2, 3, 5, 8, and 9) and one 3-cm tumor (Case 6) were WDNET, and one case of a tumor larger than 3.5 cm (Case 7) and 1 liver metastasis (Case 4) were WDNEC (**Table 1**). Among 4 non-functioning PETs, 2 primary cases of tumors smaller than 1 cm (Cases 2 and 4) were WDNET, and 2 cases—one a large primary tumor (Case 1) and the other a lymph node metastasis (Case 3)—were WDNEC (**Table 1**).

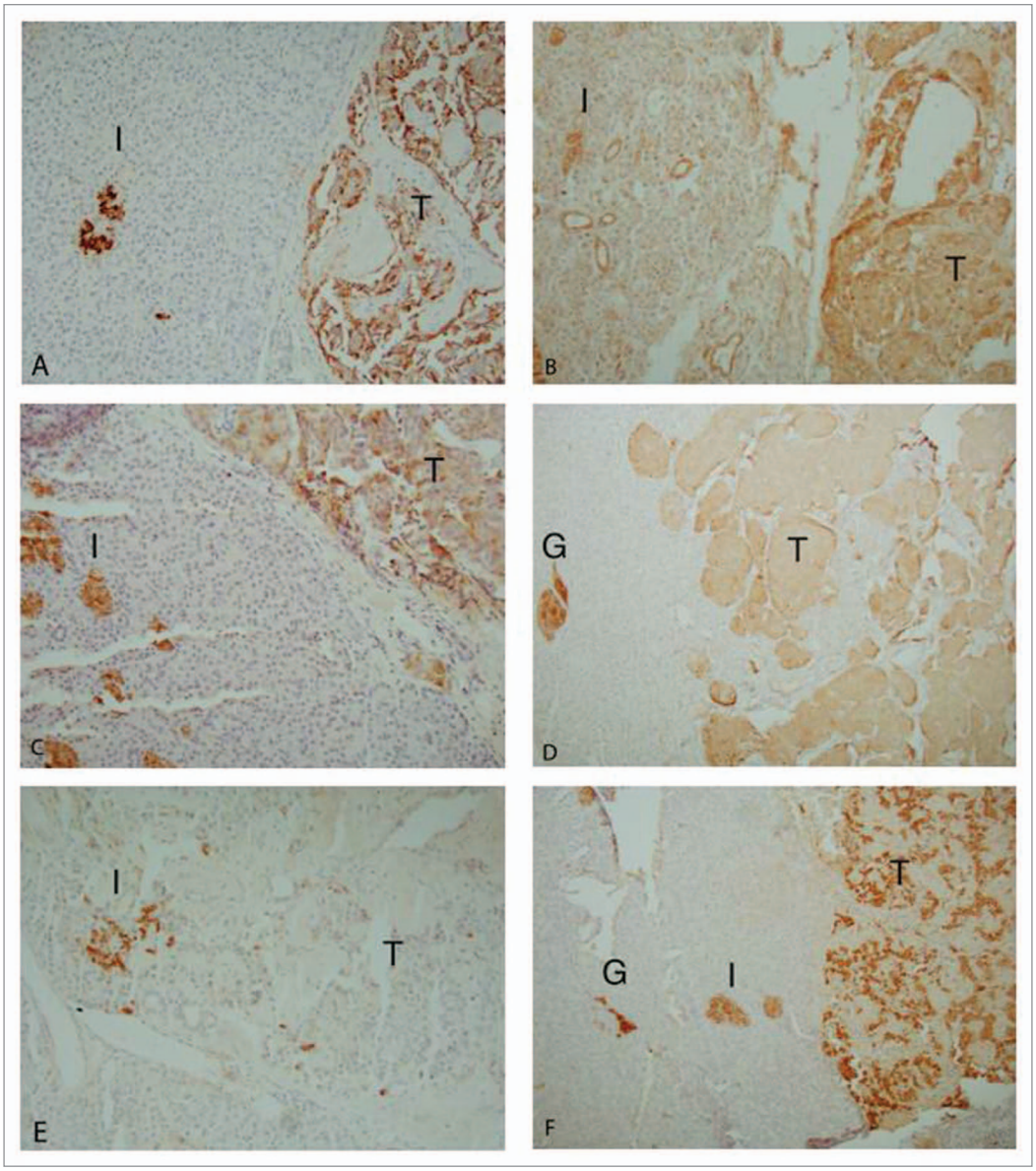

Figure 2. Insulinomas: Cases 10, 2, and 5. Case 10. This tumor was strongly positive for insulin (**A**) and moderately positive for PGP 9.5 (**B**). Case 2. This insulinoma was moderately positive for insulin as compared with normal β-cells embedded in the tumor (**C**) and was also moderately positive for PGP 9.5, whereas ganglion cells were strongly positive in the plump cytoplasm (**D**). Case 5. The tumor cells were weakly positive for insulin (**E**) but strongly positive for PGP 9.5 (**F**). Ganglion cells were more strongly immunostained for PGP 9.5 than the normal islet cells (**F**). G, ganglion cells; I, islet; T, tumor. (**A, C, and E**) insulin, (**B, D, and F**) PGP 9.5 immunostained.

Using the 2010 WHO classification, we used K_i-67 immunostaining; this provided more consistent results than mitotic figures, which appeared to be less consistent depending on the tissue preservation and fixation, and mitotic figures more than 10 to 20 per 10 high-power fields were not common even in the G_2 and G_3 cases. All of the WDNETs were G_1, all WDNECs were G_2, and 1 small cell PDNEC in 1 post-chemotherapy PPoma was G_3 (**Table 1**). Immunocytochemical staining revealed that 8 of 12 insulinomas were moderately positive (Cases 1, 2, 3, 6, 8, 10, 11, and 12) and 1 case (Case 5) was strongly positive for PGP 9.5 (9/12, 75%), whereas 2 cases (Cases 4 and 7) were weakly positive and 1 case was negative (Case 9) (3/12, 25%; **Fig. 2**; **Table 1**). Scattered ganglion cells with plump cytoplasm adjacent to the normal pancreas were much more strongly immunostained for PGP 9.5 than normal islet cells were (**Fig. 2D and F**). Two glucagonomas were negative, and 1 SRIFoma was weakly positive for PGP 9.5 (**Fig. 3A and B**; **Table 1**). Among 6 PPomas, 2 WDNETs, G_1 (Cases 5 and 6) and 1 WDNEC, G_2 (Case 1) were weakly positive for PGP 9.5, whereas 1 PDNEC, G_3 (Case 3) and 2 liver metastasis (Cases 2 and 4), WDNEC, G_2 were negative for PGP 9.5 (**Fig. 3**; **Table 1**). Among 9 gastrinomas, 6 WDNETs, G_1 (Cases 1, 2, 3, 5, 8, and 9) were weakly positive for PGP 9.5, whereas 1 WDNET (Case 7) and 2 WDNECs, G_2 (Cases 4 and 7) were negative for PGP 9.5 (**Fig. 3C and D**; **Table 1**).

Table 1. Case summary and immunocytochemical staining results

Insulinoma (12)	Size of tumor	WHO 2004	WHO 2010	Insulin	PGP 9.5
1. 19/F	1.5 × 1.0 cm	WDNET	G_1	+	++
2. 20/F	1.5 × 1.5	WDNET	G_1	++	++
3. 52/M	1.5 × 1.1	WDNET	G_1	+	++
4. 64/F	7 × 7	WDNET	G_1	+	+
5. 68/F*	0.6 × 0.5	WDNET	G_1	+	+++
6. 68/F	1.7 × 1.6	WDNET	G_1	+++	++
7. 69/M	3.5 × 2.5	WDNET	G_1	+	+
8. 71/M	1.4 × 1.2	WDNET	G_1	++	++
9. 71/F*	Liver metastasis	WDNEC	G_2	+	–
10. 79/F	1.5 × 1.4	WDNET	G_1	+++	++
11. 81/M	1.5 × 1.4	WDNET	G_1	++	++
12. 84/F	1.5 × 1.0	WDNET	G_1	++	++
Glucagonomas (2)				Glucagon	PGP 9.5
1. 43/F	20 × 14 × 8 cm	WDNEC	G_2	+	–
2. 60/F	Liver metastasis	WDNET	G_2	+	–
Somatostatinoma (1)				Somatostatin	PGP 9.5
1. 42/F	1.5 × 1.0 cm	WDNET	G_1	+	+
PPomas (6)				PP	
1. 33/M†	15 × 14 × 13 cm	WDNEC	G_2	+	+
2. 35/M†	Liver metastasis	WDNEC	G_2	+	–
3. 37/M†	Panceas, diffuse	PDNEC	G_3	±	–
4. 70/F	Liver metastasis	WDNEC	G_2	+	–
5. 74/F	1.3 × 1.2	WDNET	G_1	+++	+
6. 86/F	1.5 × 1.0	WDNET	G_1	+	+
Gastrinomas (9)				Gastrin	PGP 9.5
1. 29/F	0.8 × 0.5 cm	WDNET	G_1	+	+
2. 45/F	0.8 × 0.5	WDNET	G_1	+	+
3. 47/F	1.5 × 1.1	WDNET	G_1	++	+
4. 52/N	Liver metastasis	WDNEC	G_2	+	–
5. 54/M	0.6 × 0.5	WDNET	G_1	++	+
6. 58/F	3 × 2 × 2	WDNET	G_1	+	–
7. 67/M	4 × 3 × 3.5	WDNEC	G_2	+	–
8. 68/M	1.2 × 0.7	WDNET	G_1	+	+
9. 71/M	1 × 1, Duodenum	WDNET	G_1	++	+
Non-functioning PETs (4)				Hormones	PGP 9.5
1. 42/F	11 × 6 × 5 cm	WDNEC	G_2	–	–
2. 66/M	0.5 × 0.4	WDNET	G_1	–	+
3. 73/F	LN metastasis	WDNEC	G_2	–	–
4. 80/F	1.0 × 0.6	WDNET	G_1	–	+

*Insulinoma Cases 5 and 9 were the same subject. †PPoma Cases 1, 2, and 3 were the same subject. LN, lymph node. WHO classification 2004: WDNET, WDNEC, and PDNEC. WHO classification 2010: G_1, G_2, and G_3.

Among 4 non-functioning PETs, which were positive for CgA but negative for all 4 pancreatic hormones and gastrin, 2 WDNETs, G_1 (Cases 2 and 4) were weakly positive for PGP 9.5, and 2 WDNECs, G_2 (Cases 1 and 3) were negative for PGP 9.5 (**Fig. 3E and F**; **Table 1**).

Discussion

PETs are relatively rare tumors occurring in fewer than 1 in 100,000 populations,[9-11] representing 1–2% of all pancreatic neoplasms,[9] and the incidence in random autopsy studies has

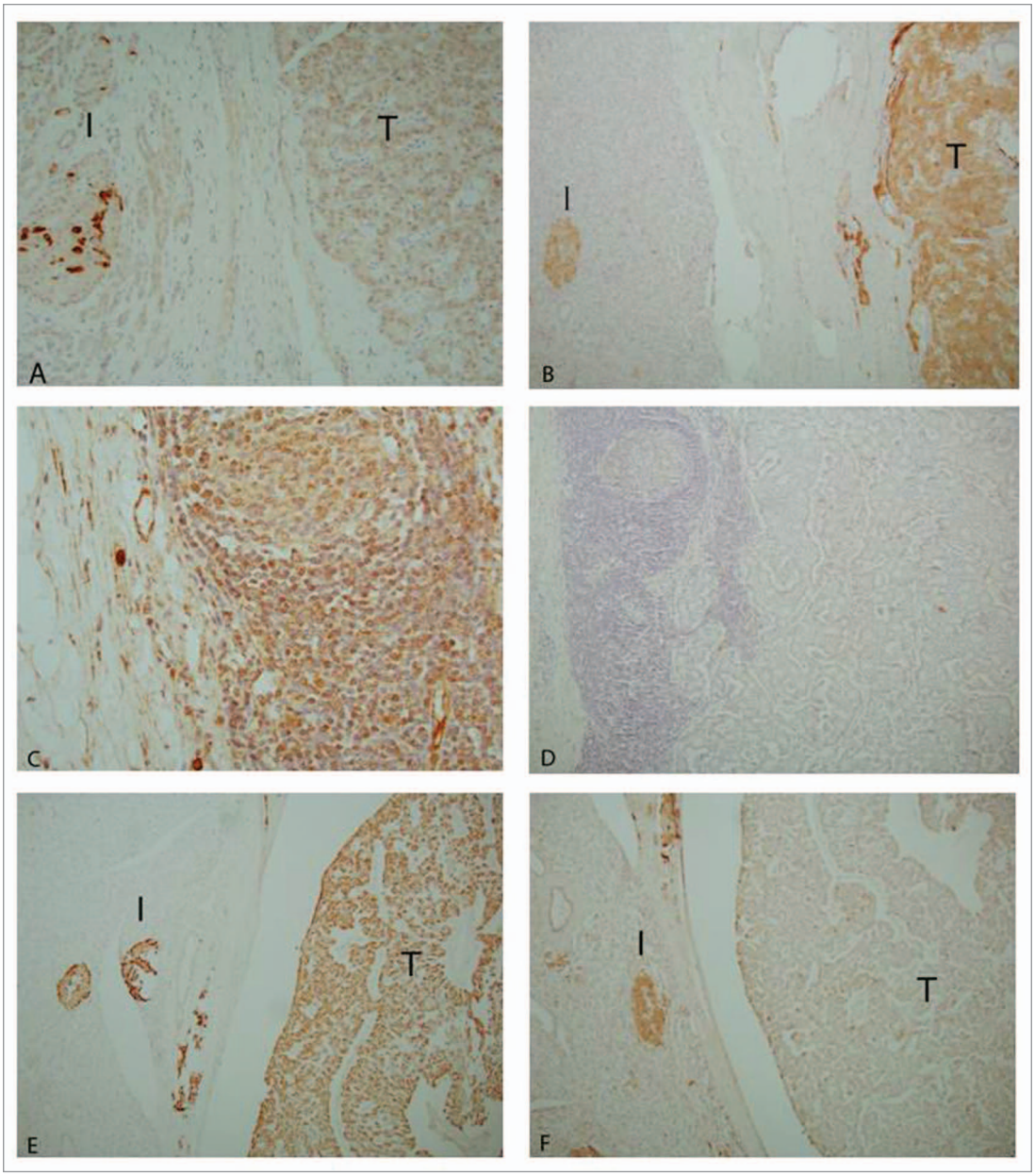

Figure 3. Non-β-cell PETs, SRIFoma Case 1, gastrinoma Case 7, and non-functioning PET Case 2 SRIFoma, Case 1. The tumor cells were weakly positive for SRIF (**A**) and moderately positive for PGP 9.5 (**B**). Gastrinoma, Case 7. The tumor cells were moderately positive for gastrin (**C**) and were negative for PGP 9.5 (**D**). Non-functioning PET Case 2. The tumor cells were positive for CgA (**E**) but negative for insulin, glucagon, SRIF, PP and were weakly positive for PGP 9.5 (**F**). I, islet, T, tumor. (**A**) SRIF, (**C**) Gastrin, (**E**) CgA, (**B, D, and F**) PGP 9.5 immunostained.

been reported as 0.5–1.6%.[13-15] Most PETs are well-differentiated, relatively low-grade neoplasms, but their association with characteristic paraneoplastic syndromes has drawn more attention to PETs than their prevalence in proportion to such syndromes.[15] Among all PETs, insulinomas and gastrinomas used to be the most common PETs presenting in 1 of 2 cases out of a million per year among MEA-1 family members, who present the typical hyperinsulinemia/hypoglycemia symptoms of insulinoma and the peptic ulcers of gastrinoma,[16] but non-functioning PETs with no clinical symptoms were reportedly the most common in the general autopsy cases.[15,16] The histopathological definition of NETs has been expanded in the WHO 2010 classification to include NECs in adenoendocrine carcinomas, which include small cell or large cell PDNECs in adenocarcinomas with no hormone-associated symptom as the major PETs including non-symptomatic hormone secreting PPomas.[16-19] The most common clinically symptomatic insulinomas were reported to be 90% benign and were detected early because of the typical symptoms,[14,15] whereas non-β-cell PETs usually followed a more aggressive clinical course and at least 50% of these tumors presented as biologically malignant,[14] which included all non-β-cell PETs (e.g., glucagonomas, SRIFomas, PPomas,

vasoactive intestinal polypeptidomas [VIPomas], gastrinomas, and non-functioning PETs).[14,15] Nine of 12 insulinomas (9/12, 75%) were moderately to strongly positive for PGP 9.5, whereas 2 glucagonomas (2/2, 100%), 3 of 6 PPomas (3/6, 50%), 3 of 9 gastrinomas (3/9, 33%), and 2 of 4 non-functioning PETs (2/4, 50%) were negative for PGP 9.5 (**Table 1**). Thus, among 22 non-β-cell PETs, 10 of 22 cases (10/22, 45%) were negative for PGP 9.5, and all 22 non-β-cell PETs were either weakly positive or negative for PGP 9.5, which matches well with the incidence of biological malignancy of non-β-cell PETs.[12] About 10% of insulinomas develop metastasis, whereas non-β-cell tumors develop higher percentages of metastasis: 60% for both gastrinomas and glucagonomas, 70% for VIPomas, and 50% for SRIFomas and non-functioning PETs.[17] The WHO 2004 and 2010 classifications are widely used for NETs of the gastroenteropancreatic system including classic gastrointestinal carcinoids tumors and PETs.[7,8] Pathology reports on PETs thus should include the 2004 and 2010 WHO classifications along with the hormone-secreting status, location, and size of each tumor as presented in this study.[7,8,14,15] An example was the Case 1 PPoma in a 33-y-old male from an MEA type 1 family who presented a large 13 × 14 × 15-cm tumor in the body and tail of his pancreas, a PPoma with more than 10 times the serum PP levels (stimulated by fasting and protein meals) than the age-matched control values.[16,17] This PET was WDNEC, G_2 with moderate PP immunostaining and 10 times more tissue PP levels than that of the control pancreatic tissue extracts.[18,19] The tumor metastasized to the liver 2 y after the initial hemipancreatectomy, which presented as WDNEC, G_2 with scant PP immunostaining and less tissue PP levels than that of the normal control pancreas.[19] The patient died of diffuse tumor involvement in the remaining pancreas and multiple metastases to the liver, lymph nodes, and bones 5 y after the initial surgery presented as PDNEC, G_3 of small cell PDNEC with a trace of PP immunostaining and tissue PP levels, ending up as a non-functioning small cell PDNEC.[18]

The new WHO 2010 classification allowed for more NETs to be added to the classic gastrointestinal carcinoids by including small cell and large cell PDNECs in adenoendocrine carcinomas within the entire digestive system. Thus, classic carcinoids used to occurred in the digestive tract extending only from the stomach to the rectum, but the newly classified NETs now include NECs anywhere within the tract from the oral cavity[20,21] to the rectum.

PGP 9.5, an ubiquitin carboxyl-terminal hydrolase[2] expressed in nerves and some neuroendocrine cell cytoplasm,[1-6] is a neuron-specific peptide.[22] PGP 9.5 is also used as a new member of all sensory nerve fibers including small-diameter fibers transmitting pain and large fibers transmitting proprioception.[23,24] At present, moderately to strongly positive PGP 9.5 immunostaining suggests the presence of a positive cytoplasmic marker for biological benignity; absent or weakly positive PGP 9.5 immunostaining in non-β-cell PETs suggests potentially biological malignancy; and negative staining that is more aggressive than weakly positive staining as similarly shown in negative nuclear immunostaining for caspase-3 suggests an aggressive marker in non-β-cell PETs.[25]

PGP 9.5 immunostaining is localized for the water-soluble proteins and is characterized by diffuse, homogenous staining in the entire cytoplasm with especially strong staining for ganglion cells (**Fig. 1E**; **Fig. 2D and F**),[26,27] which is reportedly not related to the cell type of hormone products.[1-5]

With cryosections, fine details of nerve fibers are clearly immunostained using monoclonal PGP 9.5 antibody but not rabbit polyclonal PGP 9.5 antibody (unpublished data). Using synapsin I/II and PGP 9.5, Rodriguez-Diaz et al.'s cryosections demonstrated that human islets have less innervation than mouse islets.[27] Similarly, using polyclonal LYVE-1 antibody and monoclonal D2-40 antibody for lymphatic vessel staining resulted in better immunostaining with frozen sections than with routine paraffin sections.[26] As shown in this study, PGP 9.5 immunostaining is a reliable and useful cytoplasmic marker for all NETs of the gastroenteropancreatic system, and negative and weakly positive immunostaining for PGP 9.5 may serve as a potentially biologically malignant marker especially for non-β-cell PETs as shown in all 22 cases of non-β-cell PETs (**Table 1**). PGP 9.5 immunocytochemical phenotype may be added as not only a diagnostic marker but also a prognostic marker.

Materials and Methods

A total of 34 cases of PETs obtained between 1974 and 2001 from the University of Kansas Medical Center were included in this study and were selected among the previously reported cases.[18,19,25,28,29] The PETs consisted of 12 insulinomas, 2 glucagonomas, 1 somatostatinoma (SRIFoma), 6 pancreatic polypetidomas (PPomas), 9 gastrinomas, and 4 non-functioning PETs. All primary tumor tissues including the adjacent normal pancreatic tissues were routinely fixed in 10% neutral formalin and embedded in paraffin. For immunocytochemical staining, all deparaffinized tissue sections were treated with 0.1 N citric buffer (pH 6.2) at 100°C for 10 min using a high pressure cooker (Biocare Medical). For PGP 9.5 immunostaining, rabbit anti-PGP 9.5 (Gene Tex Inc., GTX 17039) was used at 1:100 dilution for overnight incubation at 4°C. As previously reported by us,[28] K_i-67 immunostaining was performed as were insulin, glucagon, somatostatin (SRIF), pancreatic polypeptide (PP), gastrin, and chromogranin A (CgA) immunostaining.[29] The percentage of K_i-67-positive tumor cells was calculated in the cumulative 1,000 tumor cells in the 5 hot spots examined at 10 × 40 = 400× magnification.[28] For control islets, double immunostaining was performed to reveal the relative locations of each of the 2 pancreatic hormones, including insulin and glucagon, insulin and SRIF and glucagon and SRIF, respectively. The sections were initially immunostained for the first pancreatic hormone for brown color using diaminobendidine tetrahydrochloride; then the same sections were subsequently immunostained for the second pancreatic hormone for blue color using Vector SG (SK-4700, Vector Lab). We defined the strongest immunostaining in the adjacent strongest immunostained pancreatic islet cells as +++, moderate staining as ++, weak staining as +, and negative staining as 0.[29] PETs were histopathologically graded according to the 2004 and 2010 WHO classifications of the gastroenteropancreatic NET[4,5]

as follows: The 2004 classifications are well-differentiated NET (WDNET) with benign and uncertain behavior, well-differentiated neuroendocrine carcinoma (WDNEC), and poorly differentiated neuroendocrine carcinoma (PDNEC).[7] The 2010 classifications, in which mitotic figures per 10 high-power fields and the K_i-67 positive percentage index were used, are G_1, mitotic figures fewer than 2 per 10 high-power fields and the K_i-67 index less than 2%; G_2, mitotic figures 2–20 per 10 high-power fields and the K_i-67 index 3–20%; and G_3, mitotic figures greater than 20 per 10 high-power fields and the K_i-67 index greater than 20% of tumor cells.[8]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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