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RESEARCH ARTICLE

Significance of Chromogranin A and Synaptophysin in Gastroenteropancreatic Neuroendocrine Tumors

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ABSTRACT

The two most commonly used immunohistochemical markers for neuroendocrine tumors are chromogranin A and synaptophysin. We had previously studied immunohistochemical staining on pancreatic neuroendocrine tumors: chromogranin A strongly positive tumors including gastrinomas, glucagonomas, pancreatic polypeptidomas were more malignant (>50%) than chromogranin A weakly positive tumors of insulinomas (<10%). With additional 40 cases of gastroenteric neuroendocrine tumors, formerly carcinoid tumors, we investigated chromogranin A and synaptophysin immunostaining: more aggressive neuroendocrine tumors of duodenum, small intestine and ascending colon were strongly positive for chromogranin compared to less aggressive neuroendocrine tumors of sigmoid colon and rectum. Immunohistochemical staining for chromogranin A represents a marker for the secretary granules with a possible marker of prognosis on all gastroenteropancreatic neuroendocrine tumors. Furthermore, serum CgA levels may be used as an indirect, independent diagnostic and prognostic marker for gastroenteropancreatic neuroendocrine tumors in three folds: First to diagnose gastroenteropancreatic neuroendocrine tumors. Secondly, to assess the degree of malignancy by tissue and serum CgA levels and thirdly, evaluate increasing serum CgA levels as a prognostic indicator. Since there is no difference of immunostaining for synaptophysin between more aggressive neuroendocrine tumors and less aggressive tumors, immunostaining for synaptophysin is not a marker for aggressive tumors.

Keywords: chromogranin A, gastroenteropancreatic neuroendocrine tumors, immunohistochemistry, synaptophysin

Introduction

Gastroenteropancreatic neuroendocrine tumors (GEPanNETs) were classified by the 2022 WHO classification as three entries, including neuroendocrine tumors (NETs), neuroendocrine carcinomas (NEC) and mixed neuroendocrine -non neuroendocrine neoplasm (MiNEN) (1). NECs and MiNENs are overtly, aggressive neoplasms which are generally diagnosed at advanced stages while NETs are unpredictable albite that they are potentially malignant depending on the locations, sizes of tumors and immunohistochemical staining status for chromogranin A (CgA) and synaptophysin (SPY), which are the two most common immunohistochemical markers for NETs (2-4). I have included classic gastrointestinal NETs, formerly carcinoid tumors, which are slow growing, indolent but potentially malignant tumors and the most common location in the GI tract is small intestine (38%), followed by rectum (34%), colon (16%), stomach (11%) (5) in addition to PanNETs (6). In small intestine, ileum is the most common site, followed by jejunum and distal duodenum and a 5-year survival is 85% if confined to the intestinal wall versus 5% if invaded through serosa (7-9). In large intestine, rectosigmoid (hindgut) is the most common site, followed by cecum and transverse colon (midgut)(9-11). Colorectal NETs are usually of low grade, slow-growing tumors with a 5-year survival of 90% (9-11). Small intestinal NETs are usually strongly positive for CgA while small NETs of the appendix are incidentally found in the appendectomy specimens, which are clinically benign incidentalomas with weak CgA immunostaining (1,9,10). Among colonic NETs, those of ascending colon (midgut) are usually large, generally more aggressive and are strongly positive for CgA while those of rectum (hindgut) are often small and relatively benign with weak CgA immunostaining (11,12). Thus, the strong CgA immunostaining for GENETs appear to be more aggressive than CgA negative tumors, thus CgA immunostaining may be used as an indirect, independent marker for invasive tumors. Pancreatic islets represent 2% of the pancreas and malignant PanNETs represent 2% of all pancreatic malignancies (13,14). β-cells are weaker stained for CgA compared to the non-β islet cells (2,6). β-cell tumors, insulinomas are less stained for CgA than the non-β cells and less than 10% of insulinomas invade the surrounding organs while non-βcell tumors, including gastrinomas, glucagonomas, somatostatinomas and pancreatic polypeptidomas are stronger immunostained to CgA and are more aggressive than insulinomas (14). Thus, CgA immuno-staining status appears to be one of independent markers for aggressive PanNETs (6). In this communication, added were GENETs of duodenum, small intestine, appendix and large intestine and rectum, regarding the locations, sizes of tumors and CgA and SPY immunostaining status. Despite the fact that both CgA and SPY have been widely used as neuroendocrine markers in NETs, no detailed comparative immunohistochemical studies with GENETs have not been reported in my knowledge, regarding immunohstochemical staining status for CgA and SPY, and this study was conducted following the similar studies with PanNETs, which I reported lately (6). Immunohistochemical markers for GEPanNETs include CgA, SPY, Leu 7, PGP 9.5 (14) and insulinoma-associated 1 (Insm 1), the latter is the more recently recognized as a second-generation marker for NETs (1,15).

Materials and Methods

A total of 40 cases of GENETs were from the University of Kansas Medical Center, collected between 1975 and 2001 during my tenure at the Medical Center. The GENETs included 4 duodenal NETs (gastrinomas) with Zollinger-Ellison syndrome, 14 ileal NETs, 5 incidental NETs from appendectomy specimens and 17 colonic NETs (5 ascending colon, 1 transverse colon, 1 descending colon, 4 sigmoid colon) and 6 rectal NETs. A total of 35 PanNETs were included in this study, consisting of 14 insulinomas, 4 gastrinomas, 2 glucagonomas, 6 pancreatic polypepdidomas (PPomas) and 5 non-functioning NETs (6). All the PanNETs are well differentiated NETs except Case 2 PPoma, which was originally a well differentiated NET but was transformed to small cell carcinoma after cancer chemotherapy (16). All the tissues were routinely fixed in buffered formalin and were embedded in paraffin. The archival paraffin blocks were freshly sectioned and deparaffined sections were treated with antigen retrieval procedure using citrate buffer pH 6.2. All the staining procedures were the same as previously reported for immunostaining insulin, glucagon, somatostatin, PP and gastrin (6), plus monoclonal anti-CgA (Dako, Clone DAK-A3) and rabbit polyclonal anti-SPY (Cell Marque, Cat. 336-76, Rocklin, CA) at 1 : 100 dilution. Each immunostaining was performed with 20 sections at each batch to yield good comparative immunostaining. The adjacent intestinal mucosa for GEPanNETs and normal pancreatic islets for PanNETs were set for +++ for pancreatic hormones and gastrin, CgA and SPY immunostaining, respectively and the less immunostaining was listed as ++ and +, and – as negative immune-staining. The clinical information GENETs on age, sex and locations of NETs is listed in Tables 3 and 4.

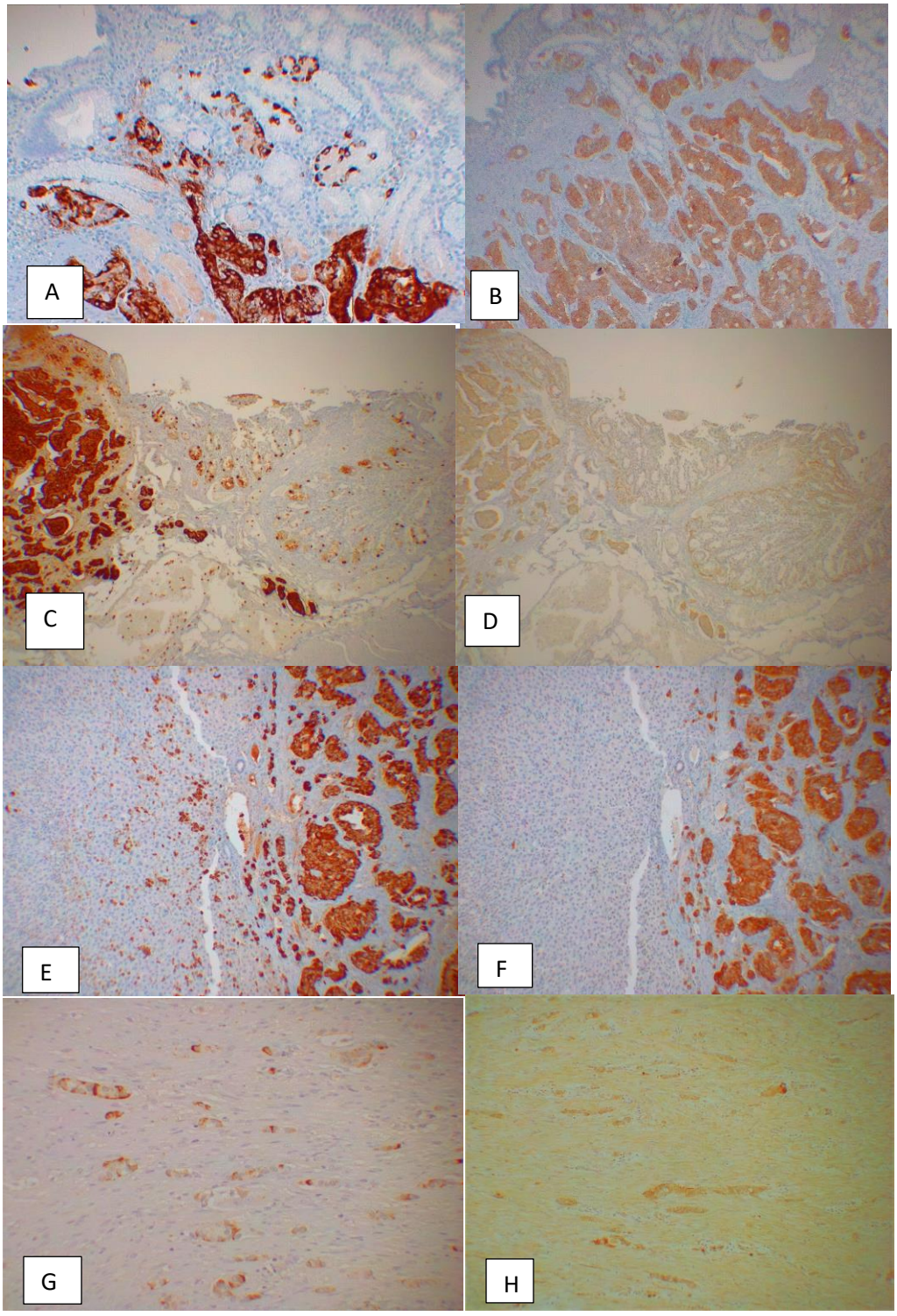

Figure 1. Duodenal gastrinoma, Case 2 ileal NET Case 8, metastatic ileal NET to liver in Case 4 and appendiceal NET, Case 4. Gastrinoma, Case 2, measuring 0.6 x 0.5cm, invaded into both mucosa and smooth muscle layer and was strongly positive for CgA and moderately for SPY (A and B). Ileal NET, Case 8 measuring 2.5 x 1.0 x 1.0cm, invaded into the intestinal wall and was strongly positive for CgA and weakly positive for SPY (C and D). A metastatic ileal NET to liver in Case 4 was strongly positive for CgA including tumor cells in the sinusoid, which were not positive for SPY despite strong positive staining in the main metastatic tumor cells (E and F). Appendiceal NET, Case 4, measuring 0.5cm, was a mixed tubular-globular pattern and partially positive for CgA in the tumor cell cytoplasm and diffusely, weakly positive for SPY (G and H). A,C,E and G: CgA, B,D,F and H: SPY immunostained

Results

GENETs

The majority of intestinal NETs were trabecular-lobular histologic pattern of smaller sizes (<2.0cm) and a mixture of solid pattern in mid-tumor and trabecular -lobular pattern at the tumor periphery in larger tumor (>2.0cm) (Tables 1 and 2). The duodenal NETs were all gastrinomas, which clinically presented with Zollinger-Ellison syndrome and the tumors were located in the distal duodenal submucosa, all of which were less than 2cm and were strongly positive to CgA and SPY (Fig. 1 A and B). Among 12 primary small intestinal NTs, 9 cases were smaller than 2cm (Table 1). Small intestinal NETs from ileum were all strong for CgA and weaker or negative for SPY in 10 cases (Table 1, Fig. 1 C and D). Case 4, a metastatic small intestinal tumor to the liver was strongly positive for CgA and SPY (Table 1, Fig. 1 E and F). Appendiceal NETs were incidentally found in appendectomy specimens at the tip of appendiceal submucosa and smooth muscle layers, measuring 0.2 to 1.0cm, and weakly positive for CgA and SPY (Table 1, Fig. 1. G and H). Among 17 colorectal NETs, 5 cases were from the ascending colon, one case from the transverse colon and 4 cases were from the sigmoid colon and 6 cases were from rectum (Table 2). Among 16 primary colorectal NETs, 9 cases were ≤0.5cm while four cases were ≥1.0cm.

Table 1. GastroenteroNTs of Duodenum, Small Intestine and Appendix: Chromogranin A and Synaptophysin Immunohistochemical Staining Duodenum (4)

	Age/Sex	Size (cm)	Histological Pattern	CgA	SPY
1	29/M*	0.8 x 0.5 cm	Trabecular	+++	+++
2	31/M*	0.6 x 0.5	Trabecular > Solid	+++	++
3	47/F	1.2 x 1.0	Solid	+++	+++
4	52/M	1.2 x 1.0	Solid > Trabecular	+++	+++
	Small Intestine (14)				
1	34/M	1.0 x0.5 cm	Solid > lobular	+++	+
2	34/M	0.3	Lobular	+++	+
3	41/M	0.3	Lobular > Trabecular	+++	-
4	41/F	Liver (Metastasis)	Lobular	+++	+++
5	50/M	0.5 x 0.4	Lobular > Solid	+++	-
6	58/M	3.0 x 2.5 x 2.0	Solid	+++	-
7	59/M	0.5	Lobular	+++	-
8	60/F	2.5 x 1.0 x 1.0	Solid	+++	+
9	65/M	0.8 x 0.5	Lobular > Solid	+++	-
10	66/M	Omentum (Metastasis)	Solid	+++	+++
11	70/M	2.0 x 1.0 x 0.5	Lobular > Solid	+++	-
12	71/F	1.0 x 0.6	Lobular > Trabecular	+++	-
13	76/M	1.2 x 1.0	Solid	+++	++
14	80/F	1.0 x 1.0	Lobular	+++	++
	Appendix (5)				
1	21/ F	0.2 cm	Tubular	+	+
2	22/F	0.2	Tubular	+	+
3	25/F	0.3	Tubular	+	+
4	38/M	0.5	Tubular > Globular	+	+
5	62/M	1.0 x 1.0	Lobular > Trabecular	++	+

Among 3 cases larger than 2.0cm, two cases were strongly positive for CgA, and another case was negative for CgA (Table 2). Among 5 cases from ascending colon, one case each from transverse colon and descending colon, all five cases were strongly positive for CgA and were weakly to strongly for SPY (Table 2). Among 4 cases of sigmoid colon, 2 cases were moderately to strongly positive for CgA and two small cases were negative for CgA and modera-tely to strongly for SPY (Table 2). Case 10, 0.2 cm in size from sigmoid colon, was the smallest tumor and was negative for CgA but moderately positive for SPY (Fig. 2 A and B). Case 2, 0.4 cm in size, from ascending colon, was negative for CgA and weakly positive for SPY (Fig. 2 C and D). Case 3, a polypoid tumor, measuring 1.5 x 1cm, from the ascending colon was strongly positive for CgA and SPY (Fig. 2 E and F) while all other small tumors, Cases 10,11,12,13,16, and 17, measuring ≤0.5cm, were negative for CgA and positive for SPY (Table 2).

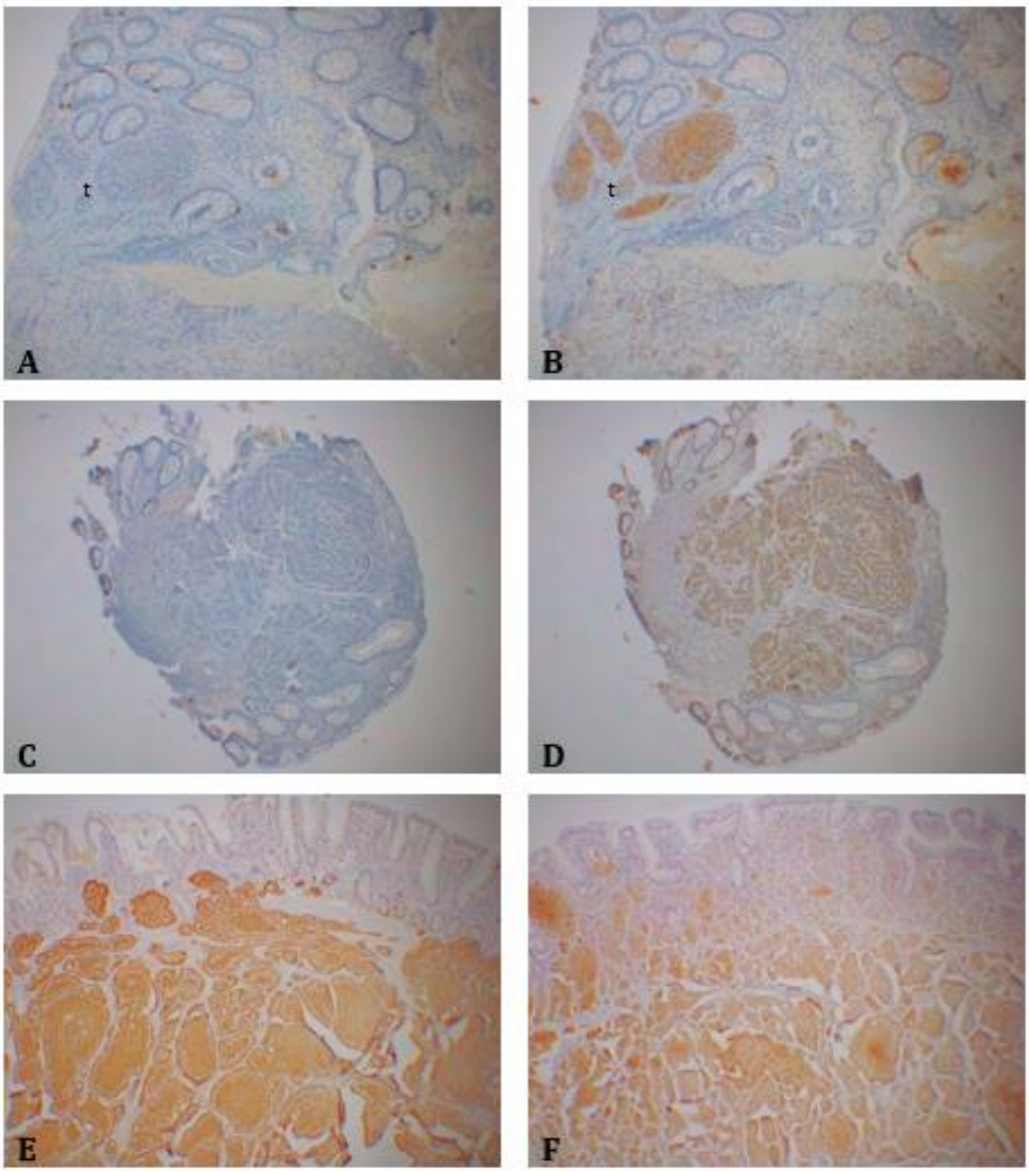

Figure 2. Colorectal NETs, small sizes Case 10, measuring 0.2cm, from sigmoid colon, was negative for CgA and strongly positive for SPY (A and B). Case, 2, measuring 0.4 cm, from ascending colon, was negative for CgA in the presence of CgA positive cell in the normal mucosa and was weakly positive for SPY (C and D). Case 3, measuring 1.5 x 1.0cm, from ascending colon was strongly positive for both CgA and SPY (E and F). A,C and E: CgA, B,D and F: SPY immunostained

Case 6, measuring 2.0 x 1.5 x 1.0cm, from the transverse colon infiltrated through the wall, and was strongly positive for CgA and negative for SPY (Fig. 3 A and B, Table 2). Case 9, measuring 1.5 x 1.0cm, from sigmoid colon was positive for CgA in 1% of tumor cells and diffusely and strongly positive for SPY (Fig. 3 C and D). This tumor invaded into the smooth muscle layer of the sigmoid colon and the invading tumor cells were >10% positive for CgA and diffusely, strongly positive for SPY (Fig. 3 E and F). Among 6 rectal tumors, all six cases were negative for CgA including Case 14, a large tumor measuring 4.8 x 2.3 cm and all rectal tumors were weakly to strongly positive for SPY (Table 2). Case 14 from the rectum was the largest tumor, measuring 4.8 x 2.3 x 2.3 cm with a solid pattern and was negative for CgA but strongly positive for SPY (Fig. 3 G and H, Table 2).

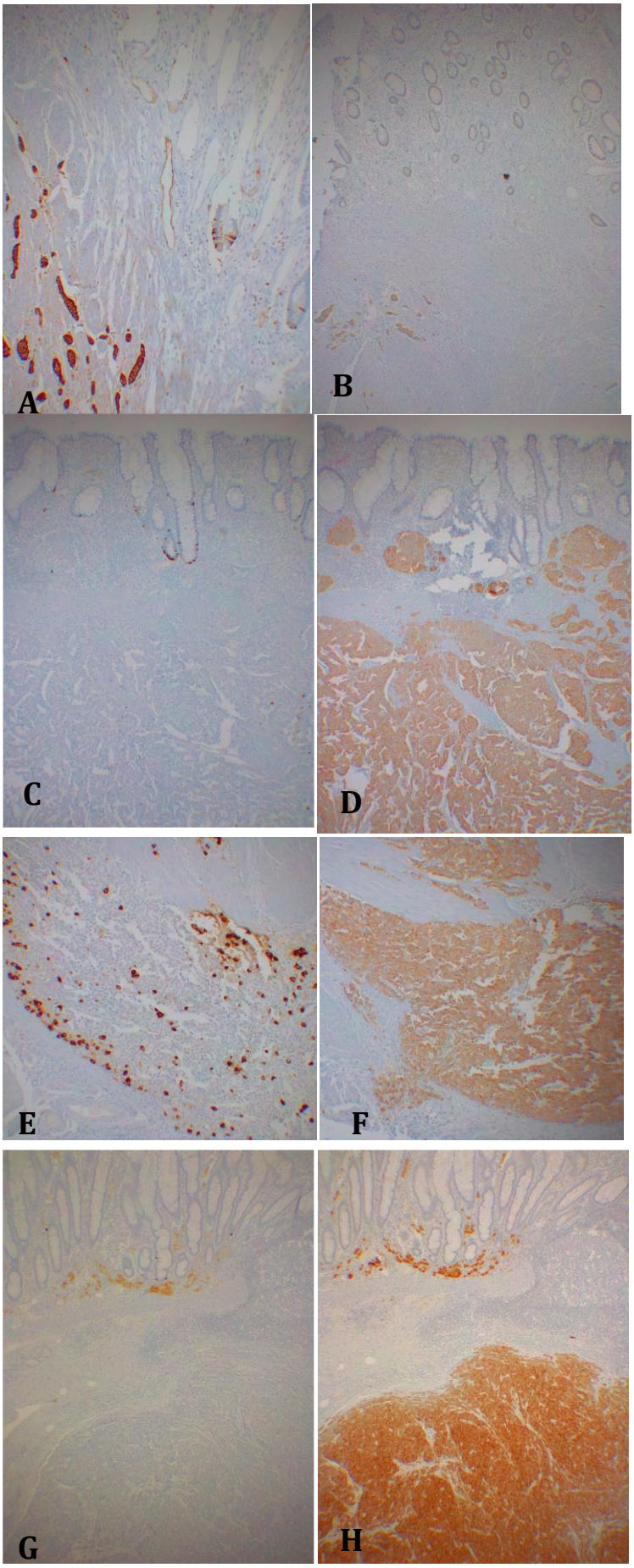

Figure 3. Colorectal NETs Case 6, measuring 2.0 x 1.5 x 1.0cm, from transverse was strongly positive for CgA and weaky positive for SPY (A and B). Case 9, measuring 1.5 x 1.0cm, from sigmoid colon was positive for CgA in 1% of tumor cells and was diffusely, strongly positive for SPY (C). The invading tumor cells in this Case 9 in the smooth muscle layer were 10% strongly positive for CgA and diffusely, strongly positive for SPY (D). Case 14, 4.8 x 2.3 x 2.3cm, from rectum was negative for CgA and diffusely, strongly positive for SPY (G and H). A,C,E and: CgA, B,D,F and H: SPY immunostained

Table 2. GastoenteroNETs of Colorectum: Chromogranin A and Synaptophysin Immunohistochemical Staining

Case	Age/Sex	Size (cm)	Histopathology	CgA	SPY
1	45/M A	0.4 cm	Trabecular	+++	+
2	54/M A	0.4	Trabecular	_	+
3	65/ F A	1.5 x 1.0	Lobular > Solid	+++	+++
4	67/M A	0.8 x0.5	Lobular > Solid	+++	+
5	70/F A	0.5	Trabecular	+++	++
6	80/F T	2.0 x 1.5 x 1.0	Lobular	+++	_
7	65/F D	Liver (Metastasis)	Lobular > Solid	+++	+++
8	70/M S	0.7 x 0.5	Solid	++	++
9	73/F S	1.5 x 1.0	Lobular > Solid	+++	+++
10	74/F S	0.2	Solid	_	+++
11	76/M S	0.5	Solid	_	++
12	47/M R	0.4	Trabecular	_	+
13	54/F R	0.4	Trabecular	_	+++
14	63/F R	4.8 x 2.5 x 2.3	Solid	_	+++
15	70/M R	0.8 x 0.5	Solid	_	+++
16	74/M R	0.5	Trabecular	_	++
17	76/M R	0.5	Trabecular	_	+

A: Ascending Colon, D: Descending Colon, S: Sigmoid Colon, R: Rectum, T: Transverse Colon

PanNETs

In the normal islets, the major β-cells (about 70%) were granularly and weakly to moderately (+ to ++) stained in the plump cytoplasm for CgA while the second major α-cells (10-20%) were densely (+++) stained in the compact cytoplasm and were located mostly at the margin of the islet lobules (Fig. 4 A and B). The δ-cells (<10%) with the slightly plump cytoplasm, located adjacent to β-cells and slender PP cells (<5%), the fewest islet cells with the compact cytoplasm, located both within and outside the islets (Fig. 4 C and D). The minor islet cells including three kinds of non β-cells were strongly positive for CgA while β-cells representing the major islet cells, were weaker stained for CgA (Fig. 4 E). All four kinds of islet cells were diffusely and moderately positive for SPY (Fig. 4 F).

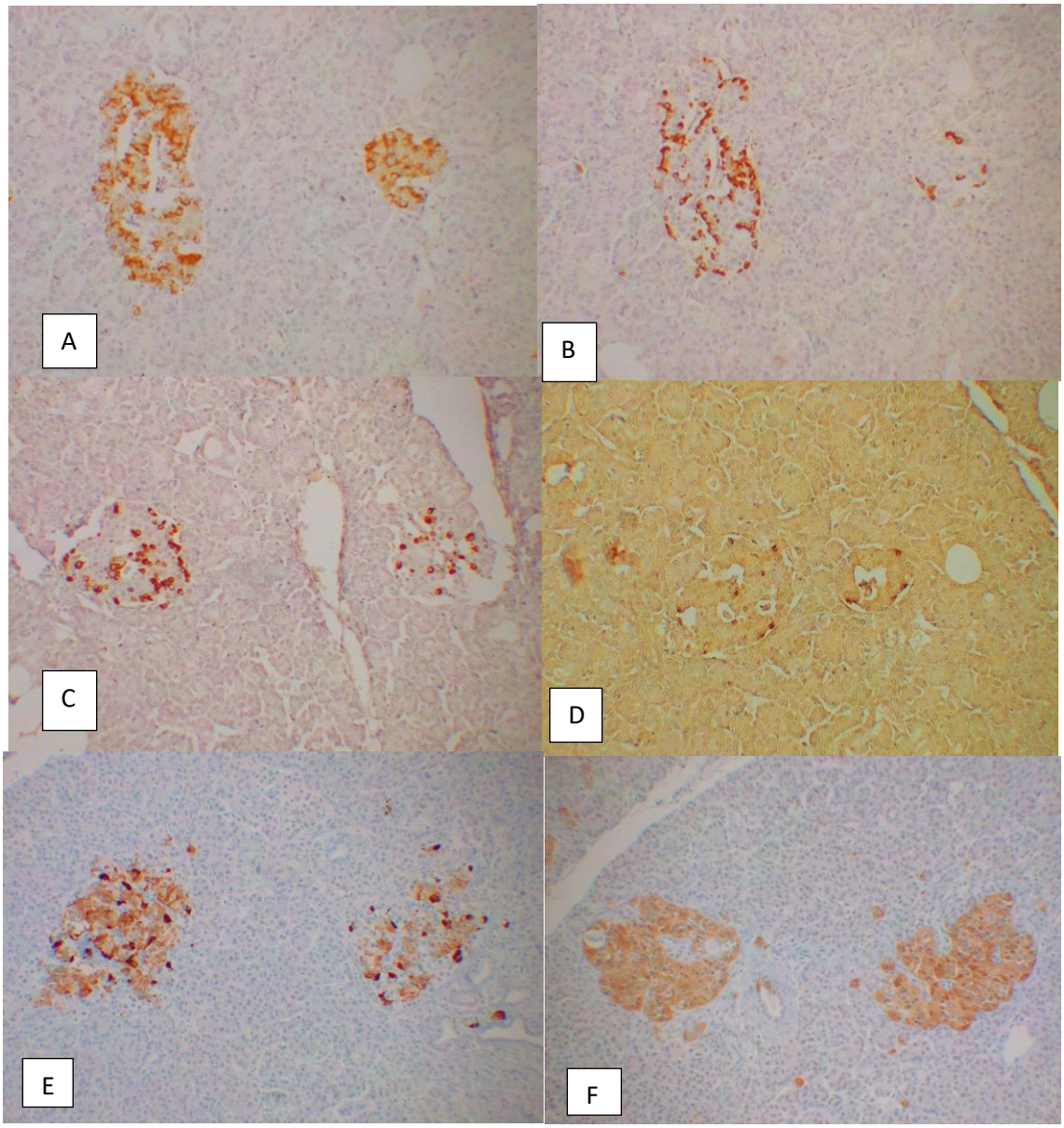

Figure 4. Normal Pancreatic Islets The major β-cells (about 70% of islet cells) contained plump cytoplasm and were strongly stained for insulin (A) while the second major α-cells (about 10-20% of islet cells) contained compact cytoplasm, which were located at the outer margin of islet cell lobules and were densely stained for glucagon (B). The δ-cells (< 5-10 %) contained slightly plump cytoplasm (C) and slender PP cells, the latter representing the fewest cells (D)(< 1-2%) contained compact cytoplasm (D) and were located both within and outside the islets (D). The CgA strongly positive cells corresponded to three kinds of non-β-cells and all four kinds of islet cells were diffusely, moderately positive for SPY. A: Insulin, B: Glucagon, C: Somatostatin, D: PP, E: CgA and F: SPY immunostained.

The majority of PETs were mixed lobular, trabecular and solid histopathological patter patterns (Tables 3 and 4). The majority of Pan-NET cells were less or the same staining intensity of the corresponding normal pancreatic endocrine cells or gastrin cells in the duodenum due to autonomous, faster hormone secretion by the tumor cells than normal endocrine cells (Tables 3 and 4). Among 14 insulinomas, 10 cases were less stained for insulin than normal β-cells, and the other four cases, were as strongly stained for insulin as much as the adjacent normal islet β-cells, including Case 3, which was weakly positive for CgA in <1% of tumor cells and strongly positive for SPY (Figure 5 A to C). The majority of benign insulinoma cells were of about the same size of normal islet cells with granular, less staining for insulin and CgA while SPY staining was moderately to strongly and diffusely positive in the entire cytoplasm as seen in Case 3 (Table 3). Case 9 metastasized to the liver, two years after enucleation, which consisted of predominantly solid pattern and showed less insulin and strong, scattered CgA staining in 10% of tumor cells and diffuse strong staining for SPY (Fig. 5 D to F).

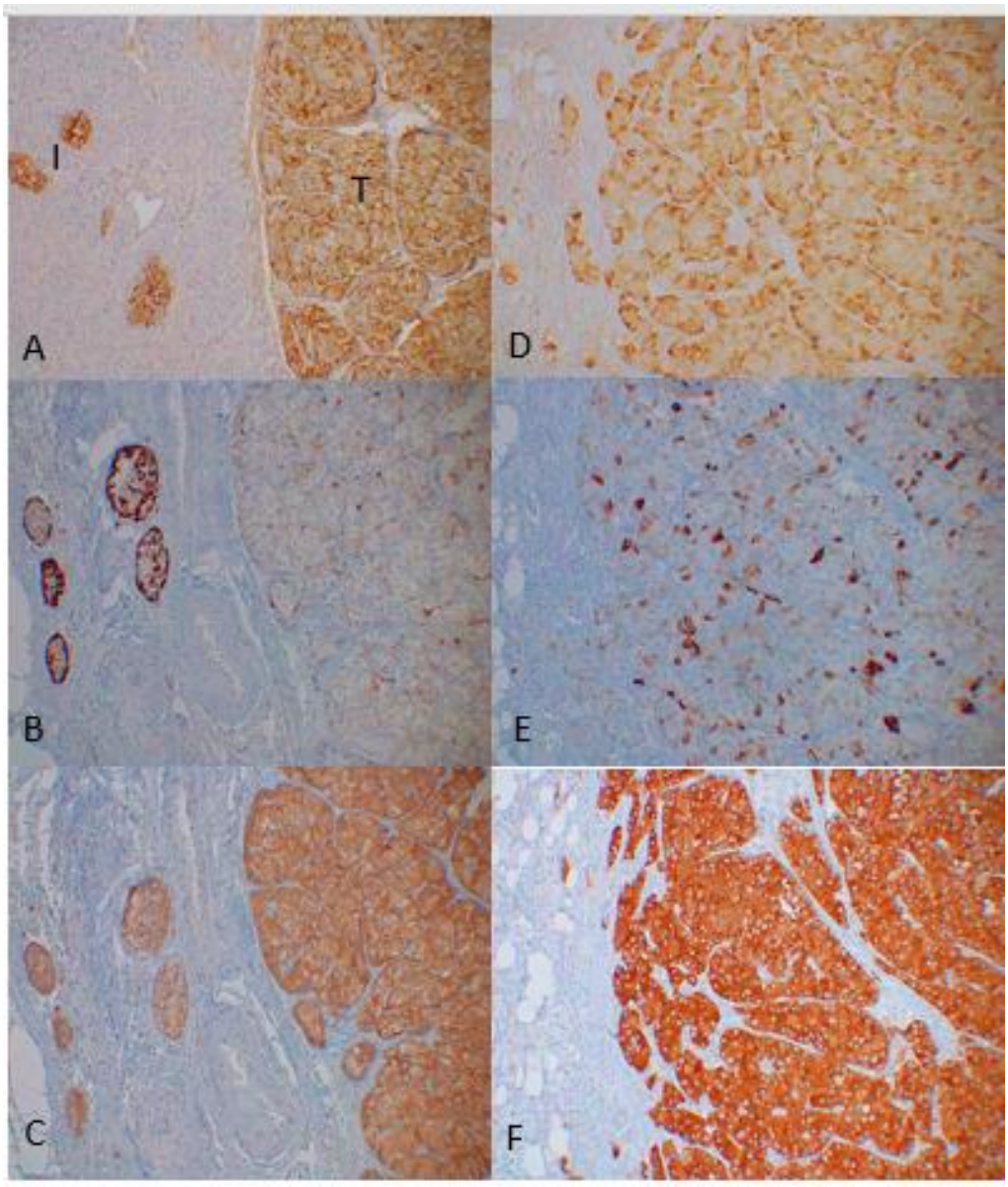

Figure 5. Insulinomas, Case 3 and 9 Case 3 Insulinoma, measuring 1.2 x 1.1cm, consisted of solid to trabecular pattern of normal-sized, a few cell-layered tumor cells with granularly, strongly positive for insulin and scattered, weakly positive for CgA than normal islet cells while they were diffusely, strongly positive for SPY (A, B and C). Case 9 metastasized malignant insulinoma to the liver two years after the initial resection consisted of mostly solid lobular pattern with diffusely, moderately stained for insulin and scattered, moderately positive for CgA at 10% of tumor cells and diffusely, strongly positive for SPY (D- Fl). I: Islet, T: Tumor A and D: Insulin, B and E: CgA, C and F: SPY immunostained.

Table 3. PanNETs: Immunohistochemical Staining for CgA and SPY in Clinically Symptomatic PanNETs

Case	Age	Sex	Tumor size	Histopathology	Cell size	Insulin	CgA	SPY
Insulinomas (14)								
1	17	F	1.5.x 1.5 cm	Solid > Trabec	Same size	++	+	+
2	20	F	1.5 x 1.5	Solid > Organoid	Same size	++	++	+++
3	52	M	1.2 x 1.1	Solid > Trabec	Same size	+++	++	+ -++
4	64	F	7 x 7	Trabec > Solid	Same size	+	+	++
5	67	F	1.7x 1.5	Trabec > Solid	Same size	+	+	++
6	68	F	1.2 x 0.7	Lobular > Solid	Large Oncocy	+++	+1%	+++
7	68*	F	0.8 x 0.8	Trabec > Solid	Same size	++	++	+++
8	69	M	0.6 x 0.5	Solid	Same size	+	+	++
9	70*	F(Liver)	Metastasis	Solid > Lobular	Same size	++	+10%	+++
10	70	F	1.1 x 0.5	Trabecular	Same size	+++	+	+++
11	71	F	1.2 x 1.1	Trabec> Lobular	Same size	+++	++	++
12	71	M	1.4 x 1.2	Trabecular	Same size	++	++	++
13	79	F	1.5 x 1.4	Trabec>Organo	Same size	+	+	++
14	81	F	1.0 x 1.0	Solid	Same size	++	++	++
Gastrinomas (4)						Gastrin		
1	44#	F	0.8 x 0.5 cm	Lobular > Trabec	Same size	+	+	+++
2	45#	F(Liver)	Metastasis	Solid	Same size	+	+	++
3	68	M	5.0 x 4.5	Solid	Same size	++	+++	+++
4	69	M	4.0 x 3.0	Solid	Same size	+++	+++	++
Glucagonomas (2)						Glucagon		
1	44	F	14 x 10x 8 cm	Solid > Lobular	Same size	++	++	+++
2	60	F	11 x 6 x 5	Solid > Lobular	Large size	+	++50%	+++

*: Same patient, Cases 7 and 9 #: Same patient , Cases 3 and 4, Oncocy: Oncocytic, Trabec: Trabecular 1% : 1% of tumor cell cytoplasm positive, 10% : 10% of tumor cells positive, 50% : 50% of tumor cells positive

In Case 3 gastrinoma, measuring 4 x 3 cm, tumor cells were granular, strongly stained for gastrin and CgA and diffusely, moderately for SPY (Fig. 6 A to C). Case 3 was initially lobular pattern and metastasized to the liver one year after surgery, and the metastasized tumor was mostly solid pattern (Figure not shown).

In Case 2 glucagonoma, measuring 11 x 6 x 5 cm, tumor cells were diffusely, weaker stained for glucagon than normal α-cells but focally, moderately stained for CgA in 50% of tumor cell cytoplasm and diffusely, strongly positive for SPY but slightly weaker than normal islet (Fig. 6 D to F). The adjacent normal pancreas contained two types of islets, namely normal islet (I) and islet composed of neoplastic cells (TI), the latter formed islet-like structure and was slightly weaker stained for CgA and SPY than normal islet cells (1) (Fig. 6 E and F).

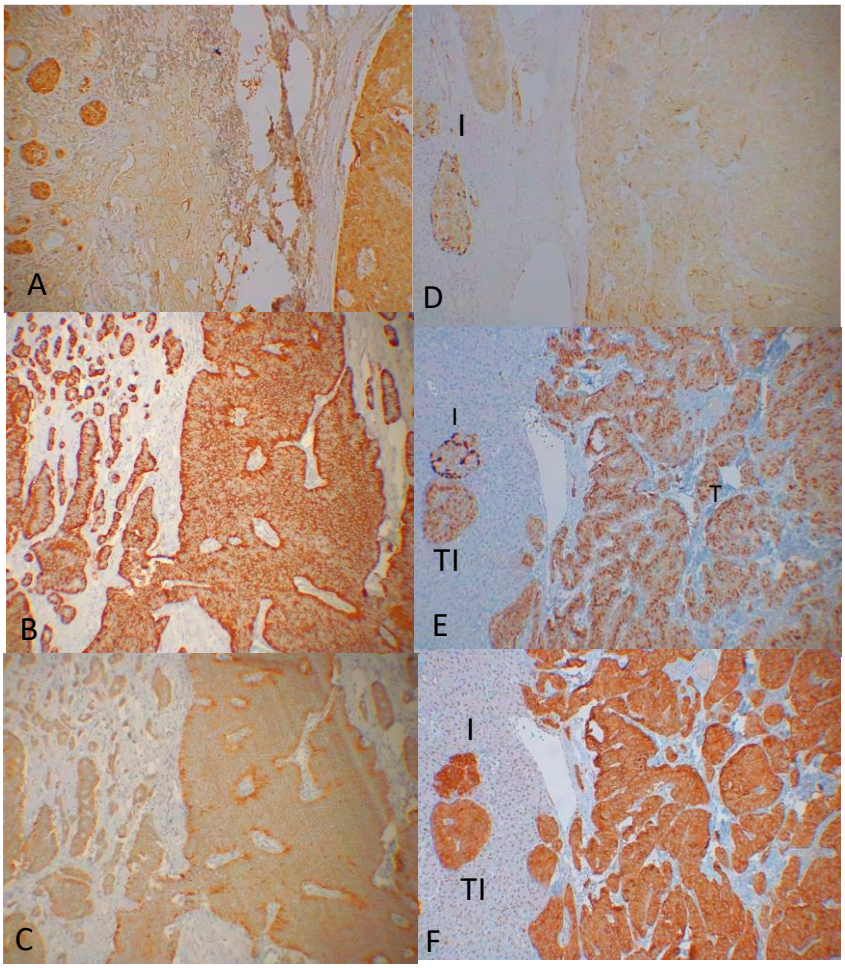

Figure 6. Gastrinoma Case 3 and Glucagonoma Case 2 In Case 3 gastrinoma, measuring 0.8 x 0.5cm, tumor cells were granularly and moderately stained for gastrin and diffusely, strongly stained for CgA while they were diffusely, weaker stained for SPY (A-C). In Case 2 glucagonoma, measuring 11 x 6 x 5 cm, lobular tumor cells were diffusely, weaker stained for glucagon (D) and were diffusely, moderately stained for CgA in 50% of tumor cell cytoplasm while they were diffusely, strongly stained for SPY but slightly weaker stained than normal islet cells (E and F). There were two types of islets in the adjacent pancreas in this case: one was a normal islet with normal-sized islet cells in Fig. E (I) and the other tumor islet with less stained for CgA and SPY in Fig. E (TI) than normal islet cells. I: Islet, M:

Case 3 benign PPoma, measuring 0.8 x 0.7 cm, consisted of a few cell trabecular pattern, which were negative for insulin, glucagon and somatostatin and were strongly positive for only PP with the same staining intensity of a few normal PP cells in the adjacent islets (Fig. 7 A to D) and tumor cells were diffusely, weakly stained for CgA and diffusely, strongly stained for SPY (Fig. 7 E and F).

One malignant PPoma Case 1, measuring 15 x 14 x 13cm, from a multiple endocrine neoplasia-1 (MEN-1) family was solid pattern with moderately and granular staining for PP and ended up small cell carcinoma 2 and half year after chemotherapy with negative staining for PP, CgA and SPY (Figure not shown, Table 4).

Table 4. PanNETs: Immunohistochemical Staing for CgA and SPY in Clinically Non-Symptomatic PanNTs PPomas (6)

Case	Age	Sex	Tumor size	Histopathology	Cell size	PP	CgA	SPY
1	33+	M	15x13x14 cm	Solid	Same size	+	+++1%	+++
2	35+	M(Liver)	Metastasis	Solid	Small cell	-	-	-
3	58	M	0.8 x 0.7	Gyriform,Trabecular	Sama size	++	++	++
4	70	F	2.0 x 1.6	Solid	Same size	+	+	++
5	74	F	1.3 x 1.2	Organoid	Same size	+	++50%	++
6	86	F	1.5 x 1.0 cm	Solid>Trabecular	Same size	++	++	++
Non-functioning Tumors (5)				Hormones				
1	42	F	11 x 6 x 5 cm	Trabecular>Solid	Same size	-	+50%	++
2	43	F	5.5 x 3.5	Solid>Trabecular	Same size	-	+	++
3	66	M	0.8 x 0.4	Solid >Lobular	Same size	-	++	++
4	70	F	1.3 x 1.2	Trabecular>Solid	Same size	-	++40%	++
5	80	F	1.5 x 1.0 cm	Solid>Trabecular	Same size	-	++	++

+: Same patient, Cases 1 and 2. 1% : 1% of tumor cells positive, 40% : 40% of tumor cells positive, 50% : 50% of tumor cells positive

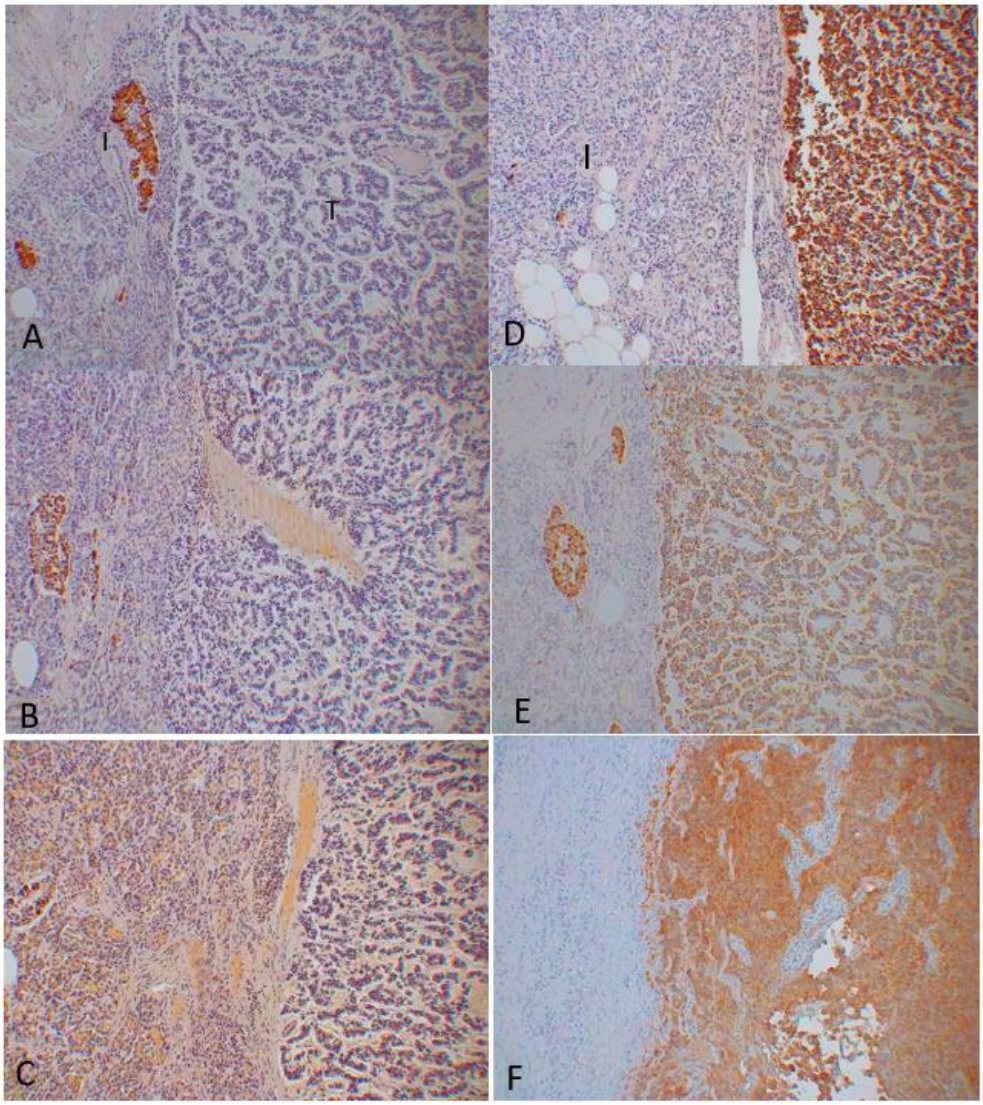

Figure 7. PPoma Case 3 PPoma, measuring 0.8 x 0.7cm, consisted of gyriform to trabecular pattern with mostly a few-cell-layered and was strongly positive for only PP (A-D) and weakly and granularly stained for CgA and strongly and diffusely stained for SPY (E and F). I: Islet, T: Tumor A: Insulin, B: Glucagon, C: Somatostatin, D: PP, E: CgA, F: SPY immunostained.

Case 3, the non-functioning tumor, measuring 0.8 x 0.4cm, was negative for four pancreatic hormones and gastrin with a few scattered PP-stained cells in the tumor (Table 4, Fig. 8 A to D). The mid part of tumor was diffusely, strongly positive for CgA, despite acutely infarcted condition, which was negative for SPY staining accompanied with the remaining outer margin of the tumor being weakly positive portion for SPY (Fig. E and F).

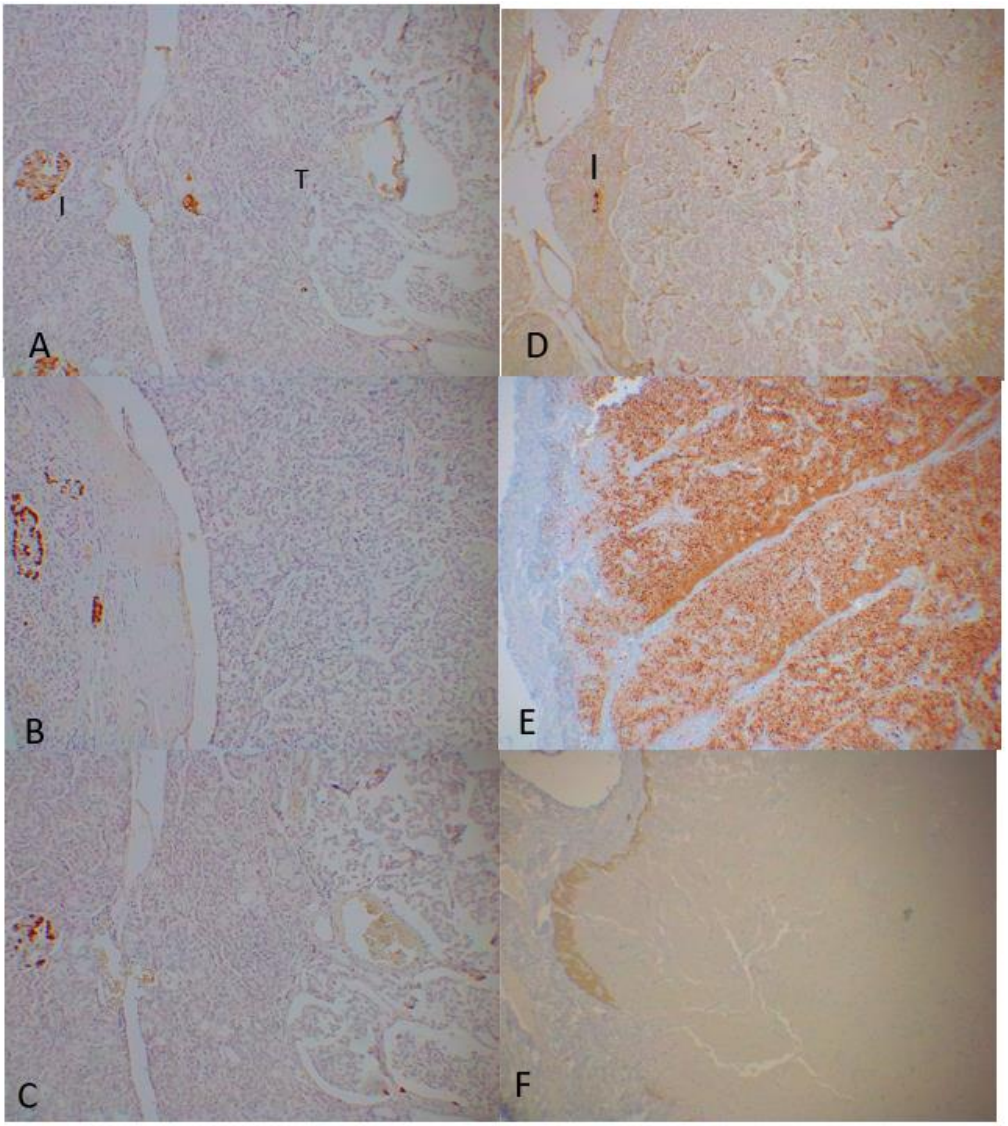

Figure 8. Non-symptomatic PanNET Case 3 non-symptomatic PanNET, measuring 0.8 x 0.4cm, was negative for insulin, glucagon, somatostatin and PP except a scattered weakly PP positive tumor cells (A to D). Tumor cells were solid, trabecular pattern with several cell layers and diffusely, moderately positive for CgA (E)and completely negative for SPY except the non-infarcted tumor margin with weakly stained for SPY (F). I: Islet, T: Tumor A: insulin, B: glucagon, C: somatostatin, D: PP, E: CgA, F: SPY immunostained

Discussion

The 2022 WHO classification included neuroendocrine tumors of gastrointestinal and pancreatic NETs collectively designated as GEPanNETs and we included both GENETs and PanNETs together. Among GENETs, midgut NETs of small intestine may present carcinoid syndrome including flush, diarrhea, bronchial constriction and right heart failure due to serotonin, tachykinins, bradykinins and prostaglandin secretion at a low percentage of 5-7% (17). Appendiceal NETs are benign if the tumors are less than 2cm including our 5 cases (5,9,10,18) (Table 3). Hindgut NETs of descending colon, sigmoid colon and rectum are often non-functioning with no hormone-related clinical symptoms (5,8,17,18). Compared to colonic adenocarcinomas, colorectal NETs appear to be smaller than adenocarcinomas at the time of diagnosis and follow better prognosis by early resection through colonoscopy. A 5-year survival was reported for duodenal NET (80%), small intestinal NETs (43%), appendiceal NETs (100%), colonic NETs (40%) and rectal NETs (100%) (7-10). The aggressive small intestinal NETs were strongly positive for CgA, supporting CgA positive staining as a marker for possible aggressiveness. Among 16 primary colorectal NETs, only two cases were larger than 2cm and seven of nine small cases (≤1.0cm) were negative for CgA but positive for SPY (Table 4). There was consistently CgA positive immunostaining for midgut NETs including 4 duodenal gastrinomas, 12 primary small intestinal, four ascending colonic NETs, two small intestinal

metastatic NETs (Table 3). Among hindgut NETs, NETs of transverse colon, descending colon, sigmoid colon and rectum, two of sigmoid colon and all 6 rectal NETs were negative for CgA and positive for SPY, supporting CgA negative tumors as better prognostic tumors.

In addition to immunohistochemical staining for CgA, there is a good correlation between serum CgA and prognosis in patients with NETs. In patients with midgut NETs, the median survival of patients with serum CgA >5µg/ml was 33 months compared to 57 months in patients with serum CgA <5µg/ml (19). Patients harboring midgut NETs with elevated serum CgA, and liver metastasis were associated with significantly shorter survival when treated a long-acting somatostatin analogue while there was no correlation between survival and concentration of urinal 5-hydroxyindoleacetic acid (HIAA), the breakdown product of serotonin (19-21). The highest serum CgA levels were reported in jejunoileal NETs, particularly cases presenting clinical carcinoid syndrome (19-21). Significantly high serum CgA levels were associated with disseminated NETs than the limited cases (19-21). An exception was gastrinoma, in which serum CgA was high in the absence liver metastasis as supported by strong CgA staining in the tumors (22-25). Thus, serum CgA level is an indirect, independent marker for NETs, yet high serum CgA levels were recorded in patients with non-neoplastic gastric, renal and heart conditions (22-25). Nevertheless, high serum CgA levels were reported in 100% of gastrinomas, 89% of pheochromocytomas, 80% of small intestinal NETs, 69% of non-functioning PanNETs, and 50% of medullary thyroid carcinomas (19), corresponding to immunostaining status for these NETs. There has been no reported correlative study of tissue CgA levels and serum CgA levels of the basal and post-protein meal levels, which should be correlated with tumor CgA tissue levels.

The specific hormone production also influences the prognosis of PanNETs since over 90% of insulinomas are reportedly benign while non-β-cell tumors, including 60—90% of gastrinomas, 50—80% of glucagonomas, over 70% of somatostatinomas and 40—70% of vasointestinal polypeptidomas (VIPomas) are malignant (23-26) and PPomas are estimated as 60—90% malignant depending on the location and sizes of the tumors (24,25). In PanNETs, weakly CgA-stained insulinomas are mostly benign while strongly CgA-stained PanNETs including gastrinomas, glucagonomas, stomatostatinomas and PPomas are considered as malignant (23-26).Thus, CgA immunostaining status may distinguish CgA-weaker, mostly benign insulinomas from CgA-stronger more aggressive non-β-cell tumors. In clinically symptomatic insulinomas, tumors less than 2 cm, are generally curable by surgery while the mean size of PPoma with metastasis but no specific symptoms due to PP hypersecretion was 8.1 cm compared to 4.3 cm in size for those without metastasis since tumor sizes also are a factor for malignancy (20). Those tumors without positive immunostaining for three pancreatic hormones and gastrin were generally classified as non-functioning tumors without typical clinical symptoms of functioning PanNETs including PPomas, the latter do not present clinical symptoms albeit higher serum PP levels especially after a high protein-meal reported previously by us (16,28). In normal pancreatic islets, β-cells were lesser stained for CgA than the other three non-β-cells as described previously (2,16) (Fig. 4) and insulinomas were relatively weaker stained for CgA than non-β-cell tumors but as strongly stained for SPY as in non-β-cell tumors (Table 3). In our 13 primary insulinomas, 8 cases (Cases 1,4,5,6,8,9,10 and 13) were much weaker stained for CgA as reflecting the immunostaining nature of β-cell-derived tumors (2,6) (Table 3). Insulinoma Case 7, measured 0.8 x 0.8 cm, was histopathologically mixed trabecular and solid pattern, indistinguishable from the other benign insulinomas but this tumor metastasized to the liver 3 years after the initial resection and the metastatic tumor was predominantly solid pattern (Tables 3, Cases 7 and 9), corresponding to about 8% of malignancy in insulinomas, which was about the same reported rate of less than 10% malignancy for insulinomas (23-25) (Table 1). Cases 1 and 2 insulinomas occurred in young ages, 17 and 20 years-old, respectively, and both were from the multiple endocrine neoplasia-1 (MEN-1) families (Table 3). Case 6 insulinoma consisted of oncocytic histological pattern with plump clear cells of less invasive tendency, which were linearly and patchy immunostained for CgA in 1% of tumor cell cytoplasm, arranged parallel to the cell membrane, probably pushed by numerous mitochondria in the cytoplasm but diffusely stained for SPY (Figure not shown). Our PanNETs cases were diagnosed clinically and removed by our surgeon at the University of Kansas Medical Center, the late Dr Stan Friesen, who screened serum PP levels after a high-protein meal among the family members of MEN-1 (28), yielding a higher percentage of gastrinoma and PPoma cases than the other studies. Subjects with MEN-1 syndrome were reported to develop PanNETs in 60-70% of the cases and gastrinomas are the most common PanNETs, occurring at 40% of cases in the gastrinoma triangle (superiorly in the junction of cystic duct and common bile duct, inferiorly in the junction of the

second and third portion of the duodenum and medially in the junction of the neck and body of the pancreas), 60% in duodenum and 30% in the pancreatic head (27). Duodenal gastrinomas are usually small and multiple (< 1 cm in 77%, mean size--0.9 cm), which follow a good prognosis after resection while pancreatic gastrinomas are generally larger (< 1 cm 6%, mean—3.8 cm) and follow a worse prognosis (27,28). There have been similar PanNET statistics in the MEN-1 cases including the two well cited reports (29,30). Among 130 MEN 1 cases admitted to the National Institutes of Health Hospital, 86 cases (66 %) were found to have PanNETs , in which 61 cases (47 %) were gastrinomas, 15 cases (12 %) insulinomas and 5 cases (4 %) non-functioning PanNETs (29). A later study from the European hospital reported that 70 % of MEA 1 subjects had PanNETs including 40 % of gastrinomas, 10 % of insulinomas and 20 % of non-functioning PanNETs (30). Gastrinomas are potentially invasive and fatal tumors like other non-β-cell tumors, which metastasize to the liver at 60 to 90% (8,31-34) except small tumors in the duodenal submucosa (Table 3, Fig. 6 A to C), which clinically present an early peptic ulcer syndrome, Zollinger-Ellison syndrome and follow a better prognosis after resection than the same tumor in the pancreatic head (33,34). Insulinomas and gastrinoma cells were moderately to strongly stained for SPY, suggesting active SV involved in possible autonomous gastrin secretion through endocytosis (35). We found a disproportionally higher PPoma cases in our study measuring serum PP levels by radioimmunoassay after a high-protein meal and we performed immunostaining for PanNETs and PP tissue levels by radioimmunoassay with acid ethanol tissue extract (16,36). We believe that the real incidence of PPomas may be much higher than reported in the literature since not all PanNETs are routinely immunostained for PP at a regular pathology laboratory especially patients with no specific clinical symptoms (16,28,36). Our non-symptomatic cases included a total of 11 cases at 31% among 35 cases, consisting of 6 PPomas and 5 hormone-negative tumors, and non-symptomatic cases were second most common after 14 insulinoma cases (40%) (Tables 3 and 4). Case 1 PPoma was a huge tumor occupying the bulk of body and tail of the pancreas, 15 x 14 x 13 cm, and solid pattern, was probable malignant PanNET. The tumor metastasized to the liver after hemipancreatectomy and spread diffusely 2 and half years later to the remaining pancreas, liver, lungs and bone marrows after cancer chemotherapy, and the histopathology of the recurrent tumor was small cell anaplastic carcinoma, which was negative to PP, CgA and SPY (Figure not shown, Table 4) (16,36). Our other PanNETs were well-differentiated (28,36), and CgA immunostaining should be compared among the PanNETs of the same differentiation since less differentiated PET may not show strong CgA staining than well-differentiated ones such as CgA-negative small cell carcinoma (Table 3). The presence of CgA and SPY in the non-functioning tumors may represent mutated, inactive hormone secretory granules undetectable by specific anti-hormone antibodies or unknown hormones inside the secretory granules.

The synaptic vesicle (SV) of the readily releasable pool in the synapses is docked to the cell membrane and release neurotransmitters from the SV through endocytosis on stimulation in a similar mode of secretory granules secretion (37,38). It has been suggested that neuroendo-crine cells including pancreatic islet cells may secrete peptide hormone through mostly exocytosis of secretory granules fusing with the cell membrane, which represent the second phase of insulin secretion while the early spike of insulin secretion may be secreted through SV endocytosis since neuroendo-crine cells are equipped with both secretory granules for exostosis in a typical peptide hormone secretory mechanism and also through endocytosis with SV, the latter is the main secretory system for neurotransmitter, which takes place instantaneously in a matter of split-seconds (37). This early phase of glucose-induced insulin secretion is modulated through glucose-receptor before glucose is metabolized and is thought to be mediated via glucose-kinase in the β-islet cells (39,40). The stronger staining for SPY than CgA in insulinomas may also suggest robust SPY participation in insulin secretion through endocytosis. The other functioning PanNETs including gastrinomas and glucagonomas were also more strongly positive for SPY than CgA, suggesting active SV involvement on the early gastrin and glucagon section, respectively.

In non-β-cell Pan-NETs, hormone immunostaining mostly correlates with that of CgA immunostaining, supporting that each hormone synthesis parallels with CgA synthesis while SPY immunostaining is quite different from the hormone and CgA immunostaining and this may support two secretory mechanisms in normal Islet cells and PanNETs: one through CgA in exocytosis and another through SV in endocytosis. In our cases, those with moderate CgA immunostaining (>++) in mixed more solid and less trabecular or lobular pattern may be considered as potentially malignant, which are more common in non-β-cell tumors than in insulinomas (Tables 1 and 2). Serum levels of CgA, neuron specific enolase and α-subunit of

glycoprotein hormones were elevated 50%, 43% and 24% of patients with NETs, respectively (41). Markedly elevated serum CgA levels, more than 300 ng/ml, were observed in only 2% of control patients compared to 40% of patients with NETs (42,43). Thus, serum CgA levels are the most specific among three markers, CgA, neuron specific enolase and α-subunit of glycoprotein hormones in patients with NETs (44).The baseline serum CgA levels were elevated in 103 of 208 patients (50%) with various NETs, including carcinoid tumors, insulinomas, gastrinomas, non-functioning PanNETs, pheochromocytomas, medullary thyroid tumors, neuroblastomas, Merkel cell tumors and pituitary adenomas (44). However, elevated serum CgA were rarely present in subjects with pituitary adenomas (13%), insulinomas (10%) and paragangliomas (8%) (45). The baseline serum CgA and PP were about the same at 100-150 ng/ml and elevated 30—90 min after a meal and reaches 2 –3 times above the baseline levels (45,46) and post protein-meal serum CgA would be much higher in subjects with NETs (45,46). Thus, the combined post protein-meal stimulated serum CgA and PP measurement will increase early detection of GEPanNETs (28,36,45,46). Elevated serum CgA levels were reported in 100% of gastrinomas, 89% of pheochromocytomas, 80% of carcinoids, 50% of medullary thyroid carcinomas and 69% of non-functioning PanNETs, respectively (19,20). Subjects with both functioning and non-functioning PanNETs showed up to 60—80 times higher serum CgA levels of the upper reference range (42-45). The mean serum CgA levels in the subjects with GENETs, insulinomas, gastrinomas and non-functioning Pan-NET were 688 ng/ml, 105 ng/ml, 772 ng/ml and 306 ng/ml, respectively, as compared to the control levels at about 100 ng/ml (19,20,42,45). The maximal serum CgA levels were reported in patients with GENETs, insulinomas, gastrinomas and non-functioning Pan-NETs at 5,200 ng/ml, 236 ng/ml, 1,900 ng/ml and 14,700 ng/ml, respectively (42). There was also a correlation between serum CgA levels and tumor progression: elevated serum CgA levels were reported in 83% of GEPanNETs and elevated serum CgA levels were present in 100% of cases with liver metastasis (20,42). In GEPanNETs, high serum CgA levels correlate with shorter survival and liver metastasis as reported in small intestinal NETs with up to 200 times above normal levels and in MEN-1 cases up to 150 times higher levels (19,42-44). Furthermore, a sudden increase in serum CgA was accompanied by rapid tumor growth and short survival (46).

In PanNETs, both functioning and non-functioning tumors showed serum CgA levels up to 60—80 times the upper normal levels, particularly in Zollinger-Ellison syndrome in MEN-1 cases with serum CgA levels being 80—100 time higher than the upper normal levels (42,45). So far, serum CgA levels are widely accepted as the marker for GEPNETs (19,42,43,45,46). These studies may support a good correlation between CgA tumor tissue levels and serum CgA levels in GEPanNETs where the strong CgA immunohistochemical staining appears to coincide with higher serum levels. A corroborative study between CgA immunohistochemistry of PanNET tissue and basal and protein-meal stimulated serum CgA levels had not been reported to date and such studies are warranted. In insulinomas, which contain less CgA than the other non-β-cell tumors, serum CgA levels are not increased in the patients, but measurement of serum CgA is a helpful indicator for tumor metastasis by the increasing CgA-secreting tumor mass (45,46).

Hijioka et al extensively studied serum CgA levels of patients with different pancreatic diseases using ELISA as follows: patients with PanNET: 297 ± 389 ng/ml, patients with pancreatic carcinoma: 155.9 ± 129.8 ng/ml, patients with chronic pancreatitis: 107.6 ± 66.9 ng/ml and patients with autoimmune pancreatitis: 98.5 ± 64.2 ng/ml, supporting the value of serum CgA levels among pancreatic diseases (47).

Noltig et al extensively analyzed the serum CgA levels from patients with PanNETs and midgut NETs: the sensitivity for PanNETs was 54% compared to patients with midgut NETs at 68%. The sensibility for liver metastasis with PanNETs was 63% compared to that with midgut NETs at 77% (48). Furthermore, faulty high serum CgA had been reported in other cancer patients (hepatocellular carcinoma, pancreatic carcinoma, colorectal carcinoma, breast carcinoma, ovarian carcinoma, prostatic carcinoma and other carcinomas), renal insufficiency, cardiovascular diseases and inflammatory diseases (48).

There are still no direct markers of GEPanNETs, and serum CgA level is an indirect marker for all GEPanNETs since CgA is co-secreted with other peptide hormones and components in the secretary granules (6). Thus, simple and reliable serum CgA levels may be used for an indirect, independent diagnostic and prognostic marker for GEPanNETs in three folds: first to diagnose neuroendocrine tumors, secondly, to evaluate the degree of malignancy for primary NETs and thirdly, to evaluate increasing serum CgA levels as an indicator of growing and metastatic tumors since elevated serum CgA levels suggest growing tumor sizes and metastatic tumors.

The disappearance of SPY immunostaining from the acute infarcted area of a PET further supports quick turnover of SV while still preserving the CgA-positive secretory granules as seen in the immunostained CgA in the infarcted PanNET cell cytoplasm (6) (Fig. 8 E and F). Since there were no differences of immunostaining for SPY between more aggressive GEPanNEts and less aggressive GEPanNETs, immunostaining for SPY is not a marker for aggressive tumors.

The 2022 WHO classification of GEPanNETs employed biomarkers of neuroendocrine lineage and differentiation such as INSM1 (1,4). ISNM1 gene codes a Zink-finger factor that was derived in an insulinoma DNA library (49,50). Pancreatic and intestinal endocrine cells express ISNM1 (1,49,50). ISNM1 gene encodes five DNA-binding with Zink-finger domain and conserves in evolution (50) and subsequently found to be expressed in a large number of neuroendocrine cells and their tumors (49-52). ISNM1 is essential for the differentiation for not only pancreatic endocrine cells but for the differentiation for intestinal endocrine cells (49). ISNM1 gene promotes the development of other pancreatic endocrine cells and further succeeds in promoting the development of all GEN cells (49-52).

The second generation of neuroendocrine markers include ISNM1, ISL-1 and secretagogin (53). ISL-1 binds to the insulin gene promoter and regulates insulin gene expression and had been expressed in pancreatic, duodenal, colonic and rectal neuroendocrine cells as well as Merckel's carcinoma, pheochromocytoma and medullary thyroid carcinoma as a marker in the nucleus (53). Secretagogin 1 is a calcium-binding protein, originally selectively expressed in the cytoplasm of pancreatic islets and was found to be expressed in other neuroendocrine cell cytoplasm (53). Thus, the development of insulin gene paves a way for the gene development of all GEPan neuroendocrine and neuron cells.

The first-generation endocrine markers including CgA, SPY, Leu 7, CD 56 may not stain poorly differentiated NETs, but nuclear immunostaining with ISNM1 is more profound in the less and poorly differentiated NETs (1,4). This is a major advantage of immunostaining GEPanNETs for ISNM1 but the nuclear marker of ISNM1 is not a prognostic marker. This leaves CgA as a sole potential prognostic marker for immunostaining the secretory granules in GEPanNETs and other NETs.

Conclusion

GEPanNETs store and secrete CgA into blood at different amounts. Among GENETs, midgut NETs (duodenum, small intestine, ascending colon) generally store and secrete more CgA than hindgut NETs (transverse, descending and sigmoid colon, rectum). Among PanNETS, non-β cell NETs (gastrinoma, glucagonoma, somatostatinoma, pancreatic polypetidoma) store and secrete more CgA. Clinically, those CgA strongly immunopositive tumors are often more aggressive than CgA-negative or weaky positive tumors. CgA immunopositive staining represents CgA storage in tumors. Thus, CgA immunopositive staining helps: 1) to diagnose GEPanNETs, 2) CgA strong immunostaining suggests more aggressive tumors, 3) elevated serum CgA levels are a marker for metastasis. Tissue levels of CgA and post-protein meal serum CgA levels are warranted for further collaborative study.

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