

# PCSK2 expression in neuroendocrine tumors points to a midgut, pulmonary, or pheochromocytoma–paraganglioma origin

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Neuroendocrine tumors (NETs) are often diagnosed from the metastases of an unknown primary tumor. Specific immunohistochemical (IHC) markers indicating the location of a primary tumor are needed. The proprotein convertase subtilisin/kexin type 2 (PCSK2) is found in normal neural and neuroendocrine cells, and known to express in NETs. We investigated the tissue microarray (TMA) of 86 primary tumors from 13 different organs and 9 metastatic NETs, including primary tumor-metastasis pairs, for PCSK2 expression with polymer-based IHC. PCSK2 was strongly positive in all small intestine and appendiceal NETs, the so-called midgut NETs, in most pheochromocytomas and paragangliomas, and in some of the typical and atypical pulmonary carcinoid tumors. NETs showing strong positivity were re-evaluated in larger tumor cohorts confirming the primary observation. In the metastases, the expression of PCSK2 mirrored that of the corresponding primary tumors. We found negative or weak staining in NETs from the thymus, gastric mucosa, pancreas, rectum, thyroid, and parathyroid. PCSK2 expression did not correlate with Ki-67 in well-differentiated NETs. Our data suggest that PCSK2 positivity can indicate the location of the primary tumor. Thus, PCSK2 could function in the IHC panel determined from screening metastatic NET biopsies of unknown primary origins.

Key words: Neuroendocrine tumors; primary origin; PCSK2; immunohistochemistry.

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The incidence of rare tumors of neuroendocrine origin is increasing (1). Hormone-producing neuroendocrine tumors (NETs) can occur anywhere in the body because NETs arise from dispersed or aggregated neuroendocrine (NE) cells. NE cells are found in the bronchus, the gastrointestinal (GI) tract, the islets of Langerhans of the pancreas, the

thyroid (as C cells), and the skin (as Merkel cells) (2). Pheochromocytomas (PHEOs) arise from the adrenal medulla, while paragangliomas (PGLs) arise from sympathetic or parasympathetic paraganglions (3). The most common NET sites are the GI tract and the lungs, while other locations such as the genitourinary tract and breast, are extremely rare (4). A general histological feature of neuroendocrine neoplasia that differentiates them from

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adenocarcinomas is the presence of cytoplasmic endocrine granules, a key feature of neuroendocrine specialization. Various hormones produced by NETs are stored in NE-specific granules (5). Recommended immunohistochemical (IHC) markers for the diagnosis of NETs are chromogranin A (CGA) and synaptophysin (SYP), the so-called common NE markers (6).

While radiological and nuclear imaging constitute first-line methods to diagnosing, staging, and the surveillance of NETs (7), primary tumors can be very small and undetectable upon imaging. Because the clinical presentation of NETs is often indolent, the diagnosis of malignant NETs is frequently made from a metastatic tumor sample. Knowledge of the primary tumor origin carries meaningful therapeutic significance to applying of adequate surgical and medical management, since the response to various treatments varies according to NET origin (8). In the Surveillance, Epidemiology, and End Results (SEER) 18 registry, the incidence of NETs with an unknown primary origin reached 0.84 per 100 000 population (1). The common NE markers CGA and SYP can indicate the NE nature of a metastatic tumor, but are uninformative regarding the tumor's location. Thus, organ-specific IHC markers play an important role in determining the tumor origin from tissue samples of an unknown primary tumor (9). For the diagnosis of metastatic NETs with an unknown origin, a proper antibody panel indicating the primary NET location is needed (10). Many organ-specific markers are proteins naturally occurring in specific cell types. Therefore, markers such as thyroid transcription factor-1 (TTF-1) indicating a lung and thyroid origin (10–12), caudal type homeobox 2 (CDX2) indicating a GI origin (10,12,13), and insulin gene enhancer-binding protein islet-1 (ISL1) and paired box 8 (PAX-8) indicating NETs of a pancreatic origin (14) have served to suggest the primary tumor location. New NE markers are identified from time to time (15). Antibodies are used in panels, since relying on a single primary antibody is never adequately specific or sensitive (16).

The proprotein convertase subtilisin/kexin type 2 (PCSK2) gene is located on chromosome 20, band p11.2 (17). The PCSK2 enzyme, a member of the serine protease family, is synthesized in the endoplasmic reticulum as inactive prePCSK2. Moving toward secretory granules, the prePCSK2 is proteolytically activated within the acidic compartments of the secretory pathway (18). PCSK2 is expressed in various neuroendocrine cell lines (19) and NE cells (20, 21). In NE cells, PCSK2 is a principal mediator of prohormones and propeptides (18, 21–23). Reports by Scopsi et al. 1995 (20), Kajiwara

et al. 1999 (24), Kimura et al. 2000 (25), and Tomita et al. 2001 (26) have described PCSK2 expression in human NE tissues and NETs (20, 24–27). The presence of the enzyme in NETs has been identified using various methods: IHC (20, 24–26), immunoblotting (20), electron microscopy (20), and mRNA *in situ* hybridization (24). PCSK2 has also been studied in adenocarcinomas (28).

Here, we aimed to investigate the expression levels and distribution of PCSK2 by relying on a normal NE cells, on a cohort of 86 primary tumors originating from 13 different organs, and in 9 metastatic NETs, and evaluate the usefulness of PCSK2 as a candidate for inclusion on a NET antibody panel. Strongly PCSK2 expressing NETs were investigated more precisely in larger tumor cohorts.

## MATERIAL AND METHODS

### Tumor material and normal neuroendocrine cell material

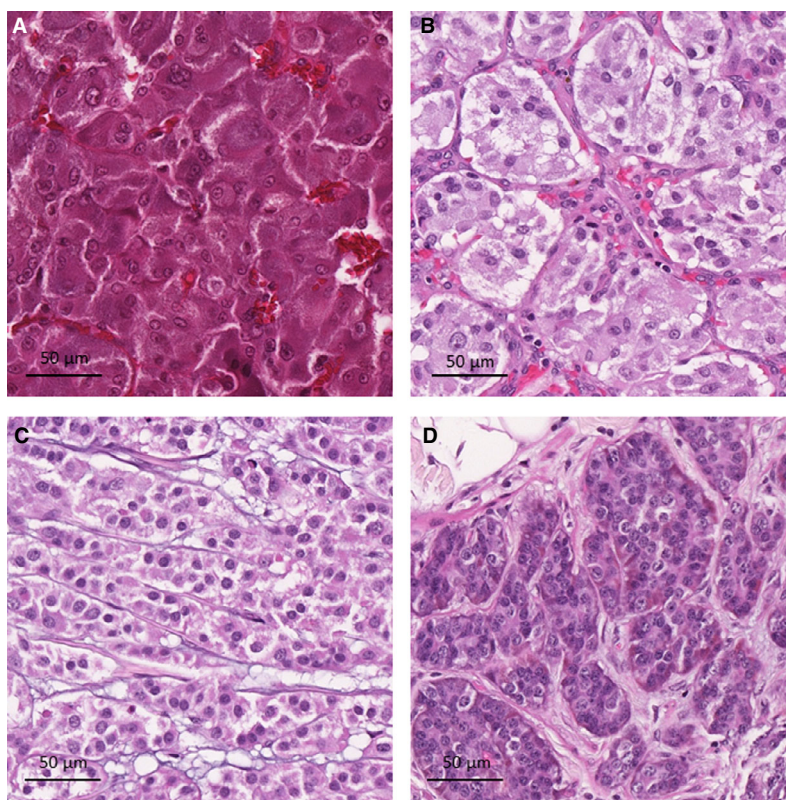
The study protocol adhered to the Helsinki Declaration of 1975 and was approved by the Ethics Committee of Helsinki University Hospital (3990/04/046/07). All of the methods followed the relevant guidelines and regulations.

Tumor material was collected from the archives of the Department of Pathology, Helsinki University Hospital. The tumor series consisted of 95 neuroendocrine tumors, consisting of primary (n = 86) and metastatic (n = 9) tumors of various grades from different organs (29). Histopathological diagnoses were re-evaluated from hematoxylin-eosin stained slides by an experienced endocrine pathologist J.A. (Fig. 1). Further analysis of the PCSK2 expression was performed on 32 PHEOs and 4 PGLs, as well as 20 small intestine and 38 pulmonary NETs. Adenocarcinomas from the pancreas (n = 27), gastric mucosa (n = 49) and colon (n = 24) served as parallel tumor material.

Normal NE tissue material (the bronchus, gastric mucosa, pancreas, small intestine, colorectum, thyroid, and adrenal medulla) was dissected from surgical patient materials. Both tumor and normal tissue materials were fixed in 10% formalin for 24–72 h, followed by normal laboratory processes (tissue processing, microtomy cutting, and hematoxylin-eosin stain) and interpretation by a pathologist.

### Tissue microarray blocks

Tissue microarray (TMA) blocks of multi-NETs, PHEOs, and PGLs, as well as adenocarcinomas of the pancreas, gastric mucosa, and colon were previously constructed; three parallel blocks with 1-mm double-punch cores were taken from the tumor area with a semiautomated TMA instrument (Beecher Instruments, Silver Spring, MD, USA). Pulmonary carcinoid TMA blocks were constructed with TMA Grandmaster (3DHitech, Budapest, Hungary) covering 1-mm double punches from the middle of the tumor, the tumor border, outside of the tumor, and from the bronchus, resulting in at total of seven punches per sample.



**Fig. 1.** Representative hematoxylin-eosin stainings of the PHEO (A), PGL (B), pulmonary carcinoid (C), and small intestine NET (D). Objective: 40 $\times$ .

### Immunohistochemistry

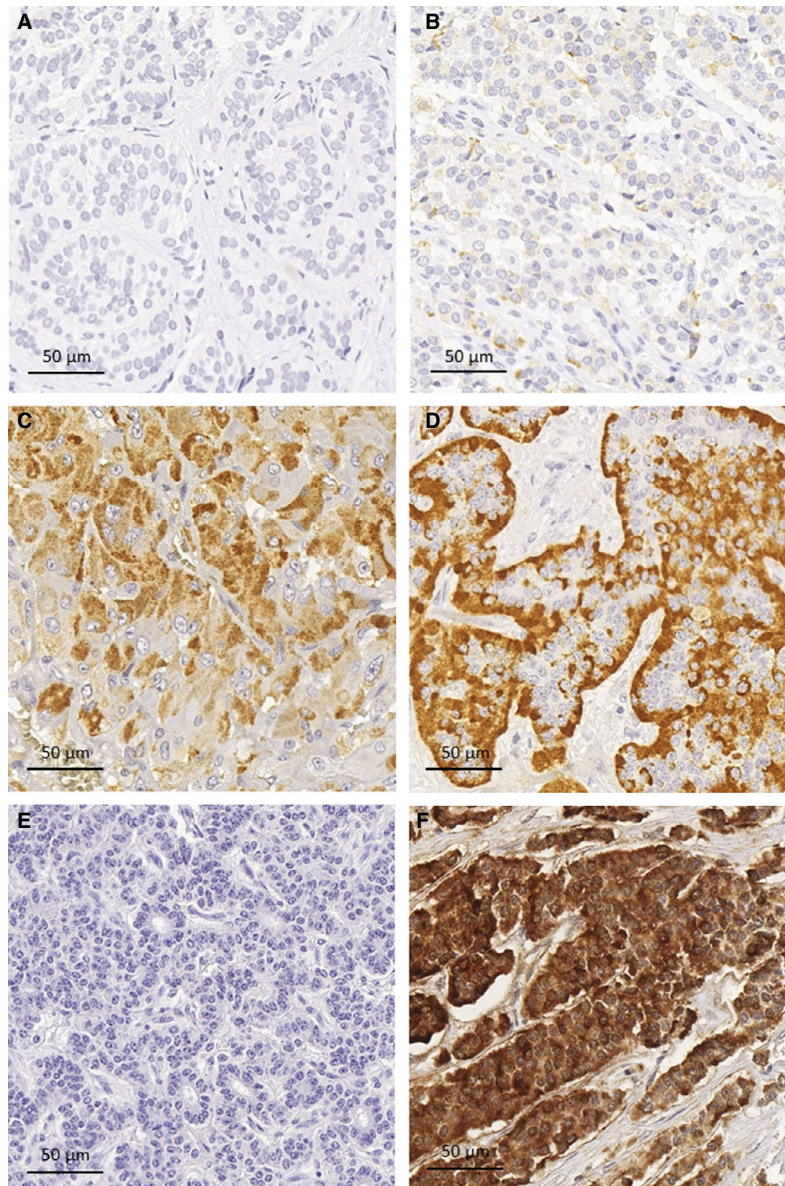
Tumor material was stained for PCSK2, chromogranin A, Ki-67, and serotonin. The slides were pretreated with the following reagents: (i) for PCSK2 (polyclonal, HPA048851, Sigma-Aldrich, St. Louis, MO, USA) and chromogranin A (polyclonal, A0430, Agilent, Santa Clara, CA, USA) in a pretreatment module (LabVision UK Ltd., UK) with a citrate buffer at pH 6.0; (ii) for Ki-67 in a microwave oven with Tris-EDTA at pH 9.0 (clone MIB-1, M7240, Agilent, Santa Clara, CA, USA); and (iii) serotonin (clone 5HT-H209, M0758, Agilent, Santa Clara, CA, USA) was stained without pretreatment. Endogenous peroxidase was blocked with the Dako REAL Peroxidase-Blocking Solution (Agilent, Santa Clara, CA, USA), and the antigens were visualized with the EnVision<sup>TM</sup> HRP conjugated polymer detection kit (Agilent, Santa Clara, CA, USA) in a LabVision Autostainer 480 (Thermo Scientific, Fremont CA, USA). PCSK2 (1:500), chromogranin A (1:2000), Ki-67 (1:100), and serotonin (1:10) were incubated for 30 min at room temperature. The slides were counterstained with Mayer's hematoxylin (Lillie's Modification) (Agilent, Santa Clara, CA, USA) and mounted with Eukitt<sup>®</sup> quick-hardening mounting medium (Sigma-Aldrich, St. Louis, MO, USA).

Adjustment of the IHC protocol for PCSK2 (30, 31) and evaluation of the primary antibody specificity was based on a literature review of the enzyme location in

normal NE tissues and NE cell components (5, 20, 21). In the accepted IHC setting, the enzyme was expressed in the expected histological (NE cells, islet of Langerhans) and cellular (cytoplasmic) locations (32). Nonspecific background staining or staining of the cell component assumed to be negative for the antibody used was not accepted (33, 34). Control tissues (small intestine and pancreas) were present in every IHC staining replicate as a patch-control slide. Stromal cell components (lymphocytes, fibroblasts) served as the internal negative controls (33, 34).

### Western blot analysis

The PHEO tumor was taken from deep-freeze ( $-70^{\circ}\text{C}$ ) in the Department of Pathology, Helsinki University Hospital. The tissue was homogenized and lysed with an LSB-buffer (Bio-Rad, Hercules, CA, USA). Samples were titrated as 2, 5, and 10  $\mu\text{L}$ , and subjected to 10% SDS-PAGE and immunoblotted onto an Immobilon-FL Transfer Membrane (Merck Millipore, Burlington, MA, USA). Blots were incubated overnight at  $+4^{\circ}\text{C}$  with polyclonal PCSK2 (HPA048851, Sigma-Aldrich, St. Louis, MO, USA) at a dilution of 1:1000 followed by Alexa Fluor 680-conjugated secondary goat anti-rabbit antibody (1:10 000) and enhanced with the Odyssey (LI-COR Biosciences, Lincoln, NE, USA) chemiluminescence detection.



**Fig. 2.** Immunohistochemical expression of PCSK2 in the NETs from the rectum (A), pancreas (B), PHEO (C), and small intestine (D). Scoring intensities for PCSK2 from 0–1 (A, B), and 2–3 (C, D) with the representative tumor areas. Serotonin expression in the NETs scored as negative (E, pulmonary) or positive (F, small intestine). Objective: 40 $\times$ .

### Scoring

PCSK2 was scored for cytoplasmic positivity as follows: 0 = none, 1 = mild, 2 = moderate, and 3 = strong (Fig. 2). For further analysis, 0 and 1 were considered negative, while 2 and 3 were considered positive. Staining for multi-NETs, PHEOs and PGLs was evaluated independently by S.R. and H.L., and those for the pulmonary carcinoids were evaluated by S.R. and T.V. The highest consensus score was selected for statistical analysis.

The Ki-67 proliferation index (35, 36) was assessed using the image analysis software ImmunoRatio

([jvsmicroscope.uta.fi/immunoratio/](http://jvsmicroscope.uta.fi/immunoratio/)) for all multi-NET TMAs and the whole-slide small intestine NETs (37).

Positivity for chromogranin A confirmed the neuroendocrine nature of the NETs. Serotonin staining was interpreted as positive or negative (Fig. 2).

### Statistical analysis

We used the Fisher's exact test in the statistical analyses and considered  $p < 0.001$  as statistically significant. Statistical analyses were performed using the SPSS 17.0 software program (SPSS Inc., Chicago, IL, USA).

## RESULTS

### The characterization of PCSK2 antibody

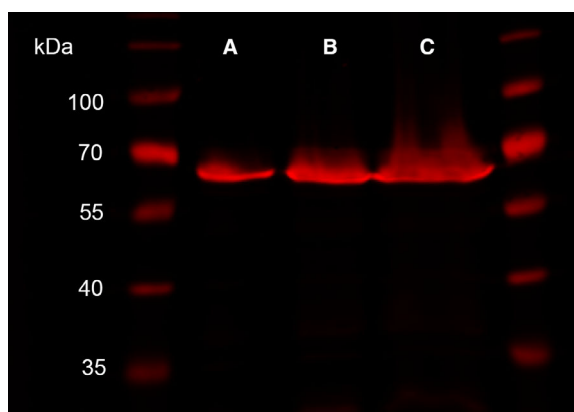
The specificity of the PCSK2 antibody was monitored using Western blot analysis on fresh frozen PHEO tumor tissue. In crude extracts from the tumor material, titrated as 2, 5, and 10  $\mu\text{L}$ , PCSK2 revealed a clear band migrating at an expected size of 70 565 Da (Fig. 3).

### PCSK2 expression in normal tissue

The NE cells of the small intestine and the adrenal medulla were strongly positive for PCSK2. The NE cells of the gastric mucosa, bronchus, islets of Langerhans, and thyroid C cells also stained positively, but at a lower intensity. Parietal cells of the gastric mucosa were also positive for PCSK2. We found no positivity in the NE cells of the colon mucosa (Fig. 4).

### PCSK2 expression in neuroendocrine tumors

PCSK2 was positive in all midgut NETs (small intestine and appendix; 12/12), in half of the typical and atypical lung carcinoids (4/8) and pituitary adenomas (2/4), and in none of the rectal NETs (0/5). The PHEOs (4/5) and PGLs (3/5) stained strongly. None of the NETs from the thymus, gastric mucosa, pancreas, thyroid, and parathyroid glands exhibited PCSK2 expression or showed a weak expression, which we considered negative (Table 1).



**Fig. 3.** Western blot analysis of the specificity of the PCSK2 antibody using PHEO in 2  $\mu\text{L}$  (A), 5  $\mu\text{L}$  (B), and 10  $\mu\text{L}$  (C). Antibody selectively detects the correct protein at the expected level without cross-reaction with other proteins in the tissue extract. About 10% of the empty margins of the original gel photograph are cropped. The picture is rotated three degrees CCW. The contrast levels are those obtained by the gel scanner.

Furthermore, PCSK2 positivity did not correlate with the Ki-67 proliferation index in any well-differentiated NET. PCSK2 expression associated with the serotonin expression in the small intestine NETs ( $p < 0.001$ ).

Because of the strong PCSK2 expression in the small intestine NETs, pulmonary NETs, PHEOs, and PGLs, the correctness of the staining was studied further using larger tumor cohorts. The results were concordant with those of the original multi-NET TMA. Small intestine NETs (20/20) expressed PCSK2 across the entire tumor area. Among the pulmonary NETs, half of the typical (13/19) and atypical lung carcinoids (9/19) were positive. In the majority of PHEOs, both non-metastatic (22/29) and metastatic (2/3) as well as PGLs (3/4) all stained strongly (Table 2).

### PCSK2 expression in neuroendocrine metastases

Table 3 summarizes the PCSK2 expression in primary tumor-metastases pairs. Most of the metastatic tumors showed comparable PCSK2 expression to the primary tumors.

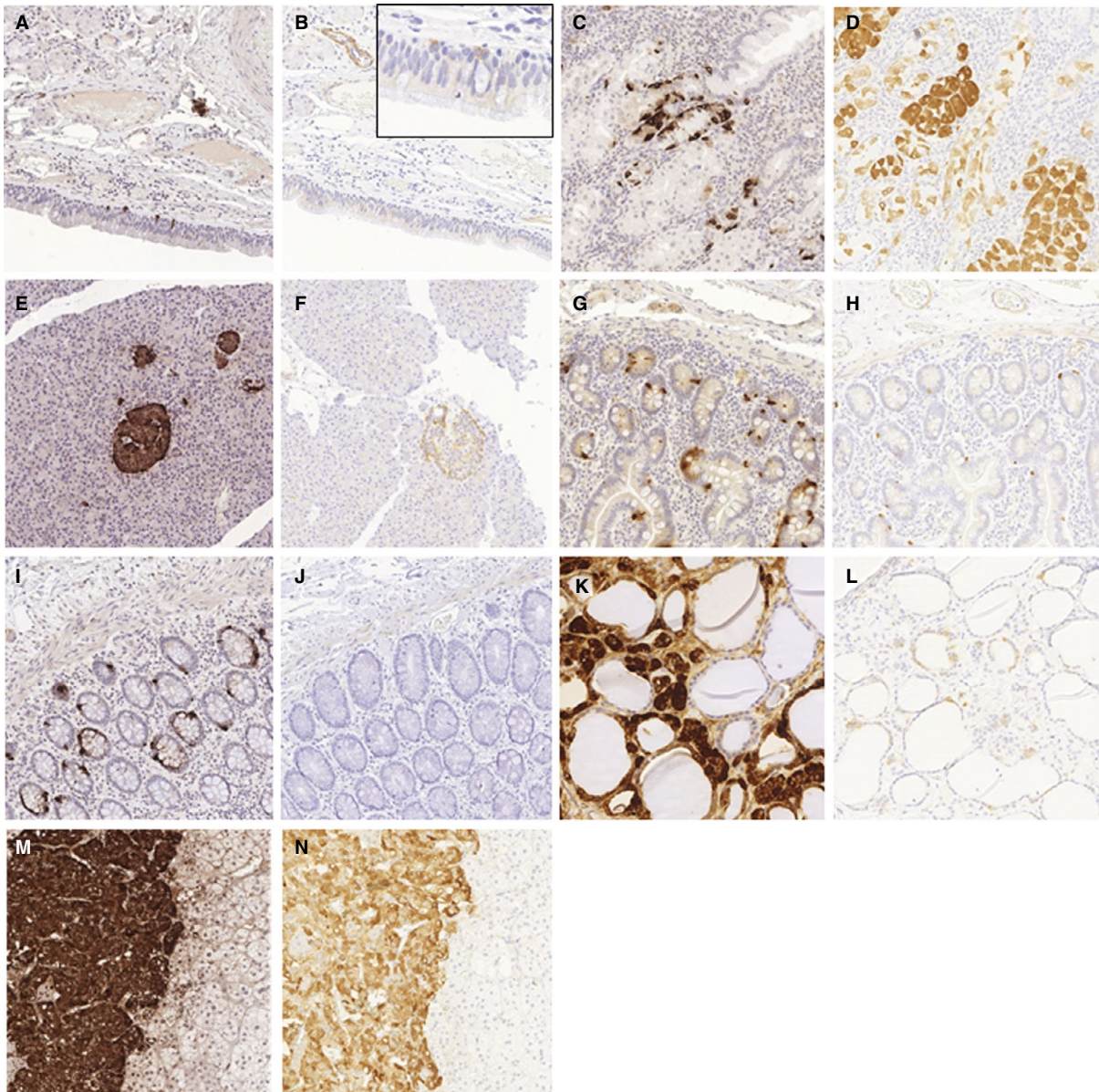
### PCSK2 in adenocarcinomas

PCSK2 staining was negative in 27 pancreatic and 49 gastric adenocarcinomas. Colon carcinomas showed a focal cytoplasmic positivity in 33% of cases, as summarized in Table 4.

## DISCUSSION

We found strong positivity for PCSK2 in midgut NETs, PHEOs, and PGLs. Half of the typical and atypical pulmonary carcinoids were also strongly positive, yet NETs from other organs were negative. The staining patterns and intensities were similar in primary tumors and their corresponding metastases. PCSK2 expression did not correlate with the proliferation index of well-differentiated tumors.

PCSK2 localizes in the granules of NE cells (5). Our findings showed a strong cytoplasmic positivity in appendiceal and small intestine NETs—the so-called midgut NETs—which agrees with previous reports (20, 24, 25, 26). The majority of PHEOs and PGLs, and half of the lung carcinoids and pituitary adenomas, were positive for PCSK2, supporting earlier observations of PCSK2 positivity in NETs (20, 24, 27). NETs originating from the thymus, gastric mucosa, pancreas, and colorectum were negative for PCSK2. Yet, our findings on PCSK2 expression in foregut and hindgut NETs differed from previous reports (20, 24, 25, 26).



**Fig. 4.** The expression of chromogranin A (A, C, E, G, I, M), calcitonin (K) and PCSK2 (B, D, F, H, J, L, N) in normal tissue NE cells from the bronchus (A, B), gastric mucosa (C, D), pancreas (E, F), small intestine (G, H), colorectum (I, J), thyroid C cells (K, L), and adrenal medulla (M, N). Objective: 20 $\times$ . The insert shows the bronchial NE cells; objective: 40 $\times$ .

Interestingly, Kajiwara et al. 1999 (24) described weak or negative mRNA (*in situ hybridization*) levels for PCSK2 in hindgut NETs, mirroring our IHC results. We found no positivity in thyroid medullary carcinomas, unlike reports from Scopsi et al. 1995 (20) and Kajiwara et al. 1999 (24). However, parathyroid adenomas and carcinomas were negative as previously indicated by Scopsi et al. 1995 (20). Table 5 summarizes the staining of PCSK2 in different studies.

Partial differences in the staining results may be explained by differences in the technical details and the selection of tumor material. The IHC staining results vary according to the choice of antigen retrieval methods, detection kits and selected antibodies (38). Previous studies used biotin as the reactive molecule for color production (20, 24, 25, 26) together with a high-temperature antigen retrieval (20, 26). Advances in the polymer detection technology allow for sensitive detection without the

**Table 1.** PCSK2 expression in NETs from 13 different organs

Tumor location	Organ	Diagnosis (n)	Gender (M:F)	Ki-67 (Range)	PCSK2 (POS)
Foregut	Pulmonary	Typical carcinoid (n = 6)	1:5	1.4–3.4%	3 (50%)
		Atypical carcinoid (n = 2)	2:0	4.1–5.8%	1 (50%)
		Large cell neuroendocrine carcinoma (n = 2)	0:2	63.6–77.6%	0%
	Thymus	Small cell lung carcinoma (n = 4)	1:3	61.6–97.5%	0%
		Well-differentiated neuroendocrine carcinoma (n = 1)	0:1	5.1%	0%
	Gastric mucosa	NET G2 (n = 3)	2:1	5.8–18.9%	0%
		NEC G3 (n = 3)	2:1	64.6–97.4%	0%
	Pancreas	NET G1 (n = 3)	0:3	0.8–1.3%	0%
		NET G2 (n = 7)	3:4	2.1–13.7%	0%
		NEC G3 (n = 1)	1:0	69.6%	0%
Midgut	Appendix	NET G1 (n = 4)	1:3	1.0–1.6%	4 (100%)
		NET G2 (n = 2)	0:2	3.5–4.0%	2 (100%)
	Small intestine	NET G1 (n = 3)	3:0	1.1–1.9%	3 (100%)
		NET G2 (n = 3)	1:2	2.3–11.3%	3 (100%)
Hindgut	Rectum	NET G2 (n = 4)	0:4	2.1–9.6%	0%
		NEC G3 (n = 1)	1:0	79.9%	0%
		Other	Thyroid	Medullary thyroid carcinoma (n = 5)	2:3
Other	Parathyroid	Parathyroid carcinoma (n = 2)	1:1	2.1–2.6%	0%
		Parathyroid adenoma (n = 3)	0:3	0.4–2.0%	0%
		Adrenal medulla	Pheochromocytoma (n = 5)	3:2	1.4–3.7%
	Paraganglia	Paraganglioma (n = 5)	2:3	2.0–3.6%	3 (60%)
	Skin	Merkell cell carcinoma (n = 4)	3:1	38.3–89.2%	1 (25%)
	Pituitary	Adenoma (n = 4)	4:0	1.0–1.8%	2 (50%)
	Total		n = 77		

**Table 2.** PCSK2 expression in large cohorts of the small intestine, pulmonary, adrenal, and extra-adrenal NE tumors

Tumor location	Diagnosis (n)	Gender (M:F)	Ki-67 (Range)	PCSK2 (POS)
Small intestine	NET G1 (n = 13)	4:9	0.3–1.8%	13 (100%)
	NET G2 (n = 7)	4:3	2.1–3.6%	7 (100%)
Pheochromocytoma	Non-metastatic (n = 29)	11:18	0.0–8.0%	22 (76%)
	Metastatic (n = 3)	3:0	3.0–10.0%	2 (66%)
Paraganglia	Non-metastatic (n = 0)	0:0	-	-
	Metastatic (n = 4)	3:1	1.0–8.0%	3 (75%)
Pulmonary	Typical carcinoid (n = 19)	10:9	<1.0–8.0%	13 (68%)
	Atypical carcinoid (n = 19)	8:12	<1.0–10.0%	9 (47%)
Total	n = 94			n = 69

**Table 3.** PCSK2 expression in primary tumor-metastases pairs

Primary tumor location		Metastases			
PSCK2 Expression		0–1 + (NEG)		2–3 + (POS)	
Pulmonary (n = 5)	Typical carcinoid (n = 2)	2–3 + (POS)	1	Typical carcinoid (n = 1)	4
	Atypical carcinoid (n = 3)			Atypical carcinoid (n = 3)	
Pulmonary (n = 6)	Typical carcinoid (n = 2)	0–1 + (NEG)	4	Typical carcinoid (n = 1)	2
	Atypical carcinoid (n = 4)			Atypical carcinoid (n = 3)	
Small intestine (n = 1)		2–3 + (POS)	0		1
Pancreas (n = 4)		0–1 + (NEG)	4		0
Total n = 16			n = 9		n = 7

associated problems characteristic of endogenous biotin (39, 40). The selection of primary antibody plays a central role in IHC. All of the studies with PCSK2 rely on polyclonal primary antibodies (20,

24, 25, 26). The staining results using polyclonal antibodies are partly tied to the batch-to-batch variation of the antibody production and the purification steps (31). In addition, the antibody's ability

**Table 4.** PCSK2 expression in adenocarcinomas

Adenocarcinomas (n)	PCSK2	PCSK2
	0–1 + (NEG)	2–3 + (POS)
Pancreas (n = 27)	100% (n = 27)	–
Gastric (n = 49)	100% (n = 49)	–
Colon (n = 36)	66.6% (n = 24)	33.3% (n = 12)
Total n = 112	n = 100	n = 12

to recognize the target antigen depends on the peptide fragments used for immunization. The polyclonal primary antibody represents a mixture of different antibodies recognizing different epitopes of the target protein (40). Thus, the polyclonal antibody is a good choice for recognizing antigens with low expression levels, heterogeneity, or post-translational modifications (31). In our study, we used commercially produced PCSK2 with standardized production and purification steps. Furthermore, the binding specificity of the PCSK2 antibody to the target protein was confirmed using Western blot analysis. The antibody used by Scopsi et al. 1995 (20) was raised against a peptide fragment corresponding to the prePCSK2 variant of the enzyme. All of these methodological differences may explain the incoherencies we detected between results. Because PCSK2 is known to process hormones in NE cells (21), the selection of the tumor material may affect the overall staining. In our NET material, we were blinded to the NET hormonal activity, particularly in pancreatic NETs, which have mostly been hormonally active in other studies (20, 25).

Tissue markers suitable for the detection of tumors of unknown origin must be stable. Lineage-specific transcription factors, such as CDX2 and TTF-1, are considered highly specific and sensitive markers capable of indicating a tumor's GI and

pulmonary origin (11–13), respectively. PCSK2 represents an integral part of the dense-core granules found naturally in NE cells (5, 20). In our NET material, the intensity and expression profile of PCSK2 was similar in the primary tumors (pulmonary, pancreas, and small intestine) and the corresponding metastases (liver, lymph node, pleura, and soft tissue). All pancreatic and gastric adenocarcinomas were negative for PCSK2 although colon carcinomas showed a focal positivity, as previously established (28). In the diagnosis of unknown primary tumors, PCSK2 appears suitable for indicating a tumor's NE nature. If PCSK2 is used in an antibody panel, it appears to indicate the primary tumor location, particularly those with a midgut, pulmonary, or pheochromocytoma-paraganglion origin. However, the clinical value and usefulness of PCSK2 requires further examination.

The proliferation index specifies the NET grades (35, 36). Well-differentiated and poorly differentiated NETs differ in their expression profile for IHC markers (6, 10). It seems that PCSK2 is a stable antigen independent of the grade. In our tumor material, PCSK2 did not correlate with Ki-67, supporting an earlier observation (27). Similarly, the percentage of typical and atypical PCSK2-positive carcinoids remained rather uniform, further indicating that proliferation does not correlate with PCSK2 expression. These results suggest that PCSK2 may be a valuable addition to an IHC panel as a grade-independent marker of NETs, at least in well-differentiated NE tumors with a proliferation of 1–20%.

The staining pattern of PCSK2 was cytoplasmic, with a polarization and occasional granules. In small intestine NETs, pulmonary NETs, PHEOs, and PGLs, PCSK2 polarization was seen in single cells or in peripheral tumor areas. NETs secrete

**Table 5.** PCSK2 staining in parallel studies

References	Lung/bronchus A) Typical B) Atypical	Thymus	Gastric mucosa	Pancreas	Appendix	Small intestine	Colorectal	Thyroid	Parathyroid A) Adenoma B) Carcinoma	Pheochromocytoma	Paraganglioma
Scopsi et al. 1995 (20) n = 123	6/7 <sup>1</sup>	–	4/8	11/11	2/2	6/6	3/6	9/9	A) 0/7 B) –	6/6	6/6
Kajiwara et al. 1999 (24) n = 26	7/9 <sup>1</sup>	–	2/4	–	–	–	2/4	1/1	A) 3/4 B) –	2/2	–
Kimura et al. 2000 (25) n = 61	–	2/2	1/7	10/16	–	2/2	6/29	–	–	–	–
Tomita et al. 2001 (26) n = 35	–	–	3/3	–	7/7	11/11	10/10	–	–	–	–
Present study n = 164	A) 16/25 B) 10/22	0/1	0/6	0/11	6/6	26/26	0/5	0/5	A) 3/4 B) –	28/37	6/9

<sup>1</sup>No definition of the classification used.



various hormones, and PCSK2 is part of the hormone maturation process (21–23). Most likely, the amount and biological variation in the hormone production and processing explains the variation in the staining intensity seen in our normal NE cell tissues and the NET cohort.

The proprotein convertase family is a nine-member protein family whose substrates are well-known cancer-associated proteins containing a cleavage site for proprotein convertases. The strong polarity of PCSK2 toward the stromal component in small intestine NETs may indicate some role in tumor invasion. Further studies are, however, needed since IHC only indicates the location of proteins, but cannot indicate their function.

A few limitations to our study should be noted. The use of TMA tissue material may compromise some of the PCSK2 positivity if the enzyme expression varies. We used parallel tissue cords to minimize this effect (41, 42), although interesting observations of PCSK2 positivity in tumors included in the TMA screening were further confirmed using larger tumor cohorts. As a method, IHC is not quantitative, and the semi-quantitative evaluation of IHC can be reliable only with carefully validated and standardized IHC protocol settings. The need for standardization should be realized when new, potential markers are discussed.

In conclusion, our results indicate that PCSK2 is a potential diagnostic marker for identifying NETs from the small intestine, appendix, and lung, as well as PHEOs and PGLs. PCSK2 did not correlate with Ki-67 and showed no loss of IHC reactivity in metastatic tumors, suggesting an antigenic stability in tumor differentiation. Like many IHC markers, PCSK2 was not completely specific for NETs. However, if added to an antibody panel, PCSK2 immunoreactivity could contribute to defining the NE nature and origin of unknown primary NETs.

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## CONFLICTS OF INTEREST

There are no conflicts of interest that may have affected the research reported.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## REFERENCES

1. Dasari A, Shen C, Halperin D, Zhao B, Zhou S, Xu Y, et al. Trends in the incidence, prevalence, and survival outcomes in patients with neuroendocrine tumors in the United States. *JAMA Oncol* 2017;3:1335–42.
2. Montuenga LM, Guembe L, Burrell MA, Bodegas ME, Calvo A, Sola JJ, et al. The diffuse endocrine system: from embryogenesis to carcinogenesis. *Prog Histochem Cytochem* 2003;38:155–272.
3. Tischler AS, de Krijger RR, Gill A, Kawashima A, Kimura N, Komminoth P, et al. WHO classification of tumours of the adrenal medulla and extra-adrenal paraganglia. In: Lloyd RV, Osamura RY, Klöppel G, Rosai J, editors. *WHO Classification of Tumours of Endocrine Organs*. Lyon, France: International Agency for Research on Cancer (IARC), 2017.
4. Oronsky B, Ma PC, Morgensztern D, Carter CA. Nothing But NET: a review of neuroendocrine tumors and carcinomas. *Neoplasia* 2017;19:991–1002.
5. Portela-Gomes GM, Hacker GW, Weitgasser R. Neuroendocrine cell markers for pancreatic islets and tumors. *Appl Immunohistochem Mol Morphol* 2004;12:183–92.
6. Perren A, Couvelard A, Scoazec JY, Costa F, Borbath I, Delle Fave G, et al. ENETS consensus guidelines for the standards of care in neuroendocrine tumors: pathology: diagnosis and prognostic stratification. *Neuroendocrinology* 2017;105:196–200.
7. Sundin A, Arnold R, Baudin E, Cwikla JB, Eriksson B, Fanti S, et al. ENETS consensus guidelines for the standards of care in neuroendocrine tumors: radiological, nuclear medicine & hybrid imaging. *Neuroendocrinology* 2017;105:212–44.
8. Alexandraki K, Angelousi A, Boutzios G, Kyriakopoulos G, Rontogianni D, Kaltsas G. Management of neuroendocrine tumors of unknown primary. *Rev Endocr Metab Disord* 2017;18:423–31.
9. Conner JR, Hornick JL. Metastatic carcinoma of unknown primary: diagnostic approach using immunohistochemistry. *Adv Anat Pathol* 2015;22:149–67.
10. Schmitt AM, Blank A, Marinoni I, Komminoth P, Perren A. Histopathology of NET: Current concepts and new developments. *Best Pract Res Clin Endocrinol Metab* 2016;30:33–43.
11. Agoff SN, Lamps LW, Philip AT, Amin MB, Schmidt RA, True LD, et al. Thyroid transcription factor-1 is expressed in extrapulmonary small cell carcinomas but not in other extrapulmonary neuroendocrine tumors. *Mod Pathol* 2000;13:238–42.
12. Bellizzi AM. Assigning site of origin in metastatic neuroendocrine neoplasms: a clinically significant application of diagnostic immunohistochemistry. *Adv Anat Pathol* 2013;20:285–314.
13. Moskaluk CA, Zhang H, Powell SM, Cerilli LA, Hampton GM, Frierson HF Jr. Cdx2 protein

- expression in normal and malignant human tissues: an immunohistochemical survey using tissue microarrays. *Mod Pathol* 2003;16:913–9.
14. Koo J, Mertens RB, Mirocha JM, Wang HL, Dhall D. Value of Islet 1 and PAX8 in identifying metastatic neuroendocrine tumors of pancreatic origin. *Mod Pathol* 2012;25:893–901.
  15. Fujino K, Yasufuku K, Kudoh S, Motooka Y, Sato Y, Wakimoto J, et al. INSM1 is the best marker for the diagnosis of neuroendocrine tumors: comparison with CGA, SYP and CD56. *Int J Clin Exp Pathol* 2017;10:5393–405.
  16. Gown AM. Diagnostic immunohistochemistry: what can go wrong and how to prevent it. *Arch Pathol Lab Med* 2016;140:893–8.
  17. Ohagi S, LaMendola J, LeBeau MM, Espinosa R, Takeda J, Smeekens SP, et al. Identification and analysis of the gene encoding human PC2, a prohormone convertase expressed in neuroendocrine tissues. *Proc Natl Acad Sci USA* 1992;89:4977–81.
  18. Mbikay M, Seidah NG, Chretien M. Neuroendocrine secretory protein 7B2: structure, expression and functions. *Biochem J* 2001;357:329–42.
  19. Li Q, Naqvi S, Shen X, Liu Y, Lindberg I, Friedman TC. Prohormone convertase 2 enzymatic activity and its regulation in neuro-endocrine cells and tissues. *Regul Pept* 2003;110:197–205.
  20. Scopsi L, Gullo M, Rilke F, Martin S, Steiner DF. Proprotein convertases (PC1/PC3 and PC2) in normal and neoplastic human tissues: their use as markers of neuroendocrine differentiation. *J Clin Endocrinol Metab* 1995;80:294–301.
  21. Portela-Gomes GM, Grimelius L, Stridsberg M. Prohormone convertases 1/3, 2, furin and protein 7B2 (Secretogranin V) in endocrine cells of the human pancreas. *Regul Pept* 2008;146:117–24.
  22. Doblinger A, Becker A, Seidah NG, Laslop A. Proteolytic processing of chromogranin A by the prohormone convertase PC2. *Regul Pept* 2003;111:111–6.
  23. Lloyd RV, Jin L, Qian X, Scheithauer BW, Young WF Jr, Davis DH. Analysis of the chromogranin A post-translational cleavage product pancreastatin and the prohormone convertases PC2 and PC3 in normal and neoplastic human pituitaries. *Am J Pathol* 1995;146:1188–98.
  24. Kajiwara H, Itoh Y, Itoh J, Yasuda M, Osamura RY. Immunohistochemical expressions of prohormone convertase (PC)1/3 and PC2 in carcinoids of various organs. *Tokai J Exp Clin Med* 1999;24:13–20.
  25. Kimura N, Pilichowska M, Okamoto H, Kimura I, Aunis D. Immunohistochemical expression of chromogranins A and B, prohormone convertases 2 and 3, and amidating enzyme in carcinoid tumors and pancreatic endocrine tumors. *Mod Pathol* 2000;13:140–6.
  26. Tomita T. Immunocytochemical localization of prohormone convertase 1/3 and 2 in gastrointestinal carcinoids. *Endocr Pathol* 2001;12:137–45.
  27. Iino K, Oki Y, Yamashita M, Matsushita F, Hayashi C, Yogo K, et al. Possible relevance between prohormone convertase 2 expression and tumor growth in human adrenocorticotropin-producing pituitary adenoma. *J Clin Endocrinol Metab* 2010;95:4003–11.
  28. Tzimas GN, Chevet E, Jenna S, Nguyen DT, Khatib AM, Marcus V, et al. Abnormal expression and processing of the proprotein convertases PC1 and PC2 in human colorectal liver metastases. *BMC Cancer* 2005;5:149.
  29. Pulkkinen V, Ezer S, Sundman L, Hagstrom J, Remes S, Soderhall C, et al. Neuropeptide S receptor 1 (NPSR1) activates cancer-related pathways and is widely expressed in neuroendocrine tumors. *Virchows Arch* 2014;465:173–83.
  30. Torlakovic EE, Cheung CC, D'Arrigo C, Dietel M, Francis GD, Gilks CB, et al. Evolution of quality assurance for clinical immunohistochemistry in the era of precision medicine - part 2: immunohistochemistry test performance characteristics. *Appl Immunohistochem Mol Morphol* 2017;25:79–85.
  31. Acharya P, Quinlan A, Neumeister V. The ABCs of finding a good antibody: How to find a good antibody, validate it, and publish meaningful data. *F1000Research* 2017;6:851.
  32. Hewitt SM, Baskin DG, Frevert CW, Stahl WL, Rosa-Molinar E. Controls for immunohistochemistry: the Histochemical Society's standards of practice for validation of immunohistochemical assays. *J Histochem Cytochem* 2014;62:693–7.
  33. Burry RW. Specificity controls for immunocytochemical methods. *J Histochem Cytochem* 2000;48:163–6.
  34. Torlakovic EE, Nielsen S, Vyberg M, Taylor CR. Getting controls under control: the time is now for immunohistochemistry. *J Clin Pathol* 2015;68:879–82.
  35. Klimstra DS, Klöppel G, La Rosa S, Rindi G. Classification of neuroendocrine neoplasms of the digestive system. In: Carneiro F, Chan JKC, Cheung NA, Cree IA, Fitzgibbons PL, Gasson S, editors. *WHO Classification of Tumours. Digestive System Tumours*. Lyon, France: International Agency for Research on Cancer (IARC), 2019.
  36. Klöppel G, Couvelard A, Hruban RH, Klimstra DS, Komminoth P, Osamura RY, et al. Neoplasms of the neuroendocrine pancreas. In: Lloyd RV, Osamura RY, Klöppel G, Rosai J, editors. *WHO Classification of Tumours of Endocrine Organs*. Lyon, France: International Agency for Research on Cancer (IARC), 2017.
  37. Remes SM, Tuominen VJ, Helin H, Isola J, Arola J. Grading of neuroendocrine tumors with Ki-67 requires high-quality assessment practices. *Am J Surg Pathol* 2012;36:1359–63.
  38. Kim SW, Roh J, Park CS. Immunohistochemistry for Pathologists: Protocols, Pitfalls, and Tips. *J Pathol Transl Med* 2016;50:411–418.
  39. Buchwalow IB, Böcker W. *Immunohistochemistry: Basics and Methods*. Heidelberg: Springer, 2010.
  40. Kammerer U, Kapp M, Gassel AM, Richter T, Tank C, Dietl J, et al. A new rapid immunohistochemical staining technique using the EnVision antibody complex. *J Histochem Cytochem* 2001;49:623–30.
  41. Elfving H, Mattsson JSM, Lindskog C, Backman M, Menzel U, Micke P. Programmed cell death ligand 1 immunohistochemistry: a concordance study between surgical specimen, biopsy, and tissue microarray. *Clin Lung Cancer* 2019;20:258–262.e1.
  42. Nolte S, Zlobec I, Lugli A, Hohenberger W, Croner R, Merkel S, et al. Construction and analysis of tissue microarrays in the era of digital pathology: a pilot study targeting CDX1 and CDX2 in a colon cancer cohort of 612 patients. *J Pathol Clin Res* 2017;3:58–70.