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Angaben zur Veröffentlichung / Publication details:

Grass, Albert, Atsuko Kasajima, Sebastian Foersch, Mark Kriegsmann, Alexander Brobeil, Maxime Schmitt, Daniel Wagner, et al. 2024. "PITX2 as a sensitive and specific marker of midgut neuroendocrine tumors: results from a cohort of 1157 primary neuroendocrine neoplasms." Modern Pathology 37 (4): 100442. https://doi.org/10.1016/j.modpat.2024.100442.

Mod Pathol 37 (2024) 100442

MODERN PATHOLOGY

USCAP UNITED STATES AND CANADIAN ACADEMY OF PATHOLOGY
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Journal homepage: https://modernpathology.org/

Research Article

PITX2 as a Sensitive and Specific Marker of Midgut Neuroendocrine Tumors: Results from a Cohort of 1157 Primary Neuroendocrine Neoplasms

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ARTICLE INFO

Article history:
Received 10 December 2023
Revised 12 January 2024
Accepted 25 January 2024
Available online 1 February 2024

Keywords:
midgut
neuroendocrine carcinoma
neuroendocrine neoplasms
neuroendocrine tumor
Pituitary Homeobox 2

ABSTRACT

As neuroendocrine tumors (NETs) often present as metastatic lesions, immunohistochemical assignment to a site of origin is one of the most important tasks in their pathologic assessment. Because a fraction of NETs eludes the typical expression profiles of their primary localization, additional sensitive and specific markers are required to improve diagnostic certainty.

We investigated the expression of the transcription factor Pituitary Homeobox 2 (PITX2) in a large-scale cohort of 909 NET and 248 neuroendocrine carcinomas (NEC) according to the immunoreactive score (IRS) and correlated PITX2 expression groups with general tumor groups and primary localization.

PITX2 expression (all expression groups) was highly sensitive (98.1%) for midgut-derived NET, but not perfectly specific, as non–midgut NET (especially pulmonary/duodenal) were quite frequently weak or moderately positive. The specificity rose to 99.5% for a midgut origin of NET if only a strong PITX2 expression was considered, which was found in only 0.5% (one pancreatic/one pulmonary) of non–midgut NET. In metastases of midgut-derived NET, PITX2 was expressed in all cases (87.5% strong, 12.5% moderate), whereas CDX2 was negative or only weakly expressed in 31.3% of the

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https://doi.org/10.1016/j.modpat.2024.100442

metastases. In NEC, a fraction of cases (14%) showed a weak or moderate PITX2 expression, which was not associated with a specific tumor localization.

Our study independently validates PITX2 as a very sensitive and specific immunohistochemical marker of midgut-derived NET in a very large collective of neuroendocrine neoplasms. Therefore, our data argue toward implementation into diagnostic panels applied for NET as a firstline midgut marker.

Introduction

Well-differentiated neuroendocrine tumors (NET) and poorly differentiated neuroendocrine carcinomas (NEC) are the 2 major entities grouped under the umbrella term neuroendocrine neoplasms (NEN).[1-3] Despite their divergent genetic background, distinct morphology, and significantly different biological behavior,[4-10] both groups share the important clinical feature that they frequently present as metastatic neoplasms at the time of initial diagnosis, often with an unknown primary.[1-3]

As NECs frequently express a variety of transcription factors independently of their lineage differentiation, the immunohistochemical delimitation of a possible primary is difficult or even impossible.[11] For NET, however, the situation is different. Because NET usually show a high degree of similarity to their tissue of origin, a combined expression profile of organotypic transcription factors (such as CDX2, TTF1, Islet-1, SATB2, or PAX8) and/or hormones (eg, insulin, glucagon, gastrin, or serotonin) can in many cases provide important clues regarding the location of the primary lesion. However, because there is still a fraction of NET that elude the typical expression profiles or show only weak expression of markers typical for a certain primary localization, additional sensitive and specific markers are required.[11]

Pituitary Homeobox 2 (PITX2) is a highly conserved transcription factor and its isoforms are not only involved in the embryonic development of the gut but also control gut tube rotation.[12-14] In a recent study, Soukup et al[15] investigated the expression of PITX2 in a focused cohort of 98 NETs and 47 NECs from different organs and noticed that expression of PITX2 was restricted to midgut NETs (jejunoileal/appendiceal/cecal), whereas they did not observe PITX2 positivity in foregut, hindgut, or pulmonary NET (typical/atypical carcinoids). These very intriguing data suggest that PITX2 might be a diagnostically promising marker for the identification of midgut NET. However, the exploratory cohort used in their study was comparatively small and contained limited numbers of midgut-derived NETs (eg, 19 small intestinal/9 appendiceal). Furthermore, the sensitivity and specificity of PITX2 in comparison with the well-known midgut-marker CDX2 has not been evaluated yet. Therefore, additional investigations on larger collectives are warranted to validate the proposed diagnostic usefulness of PITX2.

Therefore, our study independently investigated PITX2 expression patterns in a very large cohort of 1157 well and poorly differentiated NEN from various primary sites throughout the body (including 532 midgut-derived NETs) as well as metastases to validate the specificity and sensitivity of PITX2 for the diagnosis of midgut-derived NETs.

Material and Methods

Cohort

We established a multicentric cohort of 1157 primary NEN from 1007 patients who were surgically resected between 1994 and 2022 at the University Hospital Marburg, the University Hospital rechts der Isar of the Technical University of Munich, the University Hospital Heidelberg, the University Hospital Cologne, the University Hospital Regensburg, the University Hospital Erlangen-Nuremberg, and the University Hospital Augsburg. Four-hundred thirty-six patients were female (43%) and 571 were male (57%). The median age at diagnosis was 61 years. Survival data as well as clinicopathologic characteristics from all patients were extracted from local cancer registries or from hospital records. Data regarding the overall survival (OS) were available from 339 patients (34%), which we defined as a recorded death of any cause.

All neoplasms were diagnosed according to the criteria given by the WHO classifications, and all cases were revisited before they were included in this study.[1-3] NETs of the respective sites were diagnosed if an epithelial neoplasm with a well-differentiated neuroendocrine morphology (monomorphic nuclei with granular chromatin, lack of necrosis, organoid architecture) in combination with a strong expression of neuroendocrine markers such as synaptophysin or chromogranin A was evident (except for rectal NETs).[11] Grading of NETs was performed according to the ki-67 proliferation index (G1: <3%, G2: 3%-20%, G3: >20%). For pulmonary NETs (carcinoids/atypical carcinoids), the respective organ-specific criteria were applied.[2] NEC were also diagnosed according to the criteria given by the respective WHO classifications.[1-3] Poorly differentiated neoplasms of small- to medium-sized cells with scant basophilic cytoplasm, elongated hyperchromatic nuclei without distinctive nucleoli as well as high mitotic activity were diagnosed as small-cell NEC (SC NEC). Large-cell NEC (LC NEC) were diagnosed for cancers composed of solid sheets of medium- to large-sized tumor cells with rounded vesicular nuclei with prominent nucleoli. If a morphologically clearly recognizable NEC component was admixed with an adenocarcinoma component, a mixed adenoneuroendocrine carcinoma (MANEC) was diagnosed.

All cases from Marburg, Cologne, Munich, Regensburg, Augsburg, and Erlangen were assembled on a tissue microarray (TMA) comprising 2 tumor-carrying cores from the tumor center and the invasive front using the TMA grand master system (Sysmex/3DHistech). For the cases from the University Hospital Heidelberg, a TMA machine from AlphaMetrix Biotech was used to extract 1 core sample from each tissue donor block.[6,16] Tumors with insufficient fixation or insufficient tumor material on the TMA were excluded.

Immunohistochemical Analyses of PITX2 in the Main Cohort

A TMA comprising tissue cores from all 1157 NEN was stained with a PITX2 antibody (clone 2G6, dilution: 1:1000, Novus Bio-Tech) on a LINK48 autostainer (Agilent). PITX2 expression was evaluated manually by 2 pathologists (M.J., A.G.). The staining in the stromal cells of placental villi served as control tissue and only clear nuclear staining of PITX2 was considered specific[15] (Supplementary Fig. S1). The number of positive cells was assessed for each individual patient resulting in a cumulative percentage score (range: 0%-100%). The expression intensity was graded as strong (compared with normal placental stroma cells), moderate (clearly visible staining but notably weaker than in normal placental stromal cells), weak (barely perceptible and only notable in high magnifications), and negative (no staining reaction). Afterward, all NEN were assigned to different PITX2 expression groups according to the immunoreactive score (IRS)[17] derived from a sum score of the maximum staining intensity (score 0-3) as well as the percentage of expressing cells (score 0-4), which are then multiplied with each other. Afterward, 4 PITX2 expression groups were defined (PITX2 negative, IRS 0-1; PITX2 low, IRS 2-3; PITX2 moderate, IRS 4-8; PITX2 high, IRS 9-12).[18,19] The algorithm to determine the IRS as well as the resulting PITX2 expression groups are shown in detail in Table 1. To test interobserver variation, PITX2 expression in 110 midgut-derived jejunoileal and appendiceal NET was independently investigated by 2 additional observers (S.F., D.W.). Furthermore, PITX2 expression was investigated on whole slides of 15 NET to correlate the results obtained from the analysis on the TMA with whole tissue sections, where we observed a 100% concordance regarding the PITX2 expression groups according to the IRS score between TMA and whole slides.

Evaluation of CDX2 in Midgut-Derived NETs

CDX2 was evaluated in 180 midgut-derived NETs (primary jejunoileal NETs). A TMA with the tumors was stained with a CDX2 antibody (DAK-CDX2, Agilent Dako) on a LINK48 autostainer (Agilent). Non-neoplastic ileal mucosa and a colorectal adenocarcinoma with a strong CDX2 expression (IRS 12) were used as control tissue. Scoring of CDX2 expression as well as the grouping of CDX2 expression groups was performed similar to that of PITX2 according to the IRS.

Table 1
Algorithm to determine PITX2 expression scores according to the Immunoreactive Score

Immunoreactive score (IRS)	Staining intensity	Percentage of positive cells
Score[a]		
0	No staining reaction	0%
1	Weak staining reaction	<10%
2	Moderate staining reaction	10%-50%
3	Strong staining reaction	51%-80%
4		>80%
PITX2 expression groups		
IRS 0-1	PITX2 negative	
IRS 2-3	PITX2 low	
IRS 4-8	PITX2 moderate	
IRS 9-12	PITX2 high	

[a] IRS = score (staining intensity) × score (percentage of positive cells).

Evaluation of PITX2 and CDX2 Metastases from Midgut-Derived NET

A TMA containing 2 cores each from 32 metastatic lesions from 24 patients with jejunoileal NET (20 nodal metastases/12 hepatic metastases) was also evaluated regarding their expression of PITX2 and CDX2. PITX2 and CDX2 expression was evaluated similar to the primary tumors, and the results from the metastases were compared with the primary lesion.

Statistics

IMB SPSS Statistics, version 28 (IBM Corp) was used for statistical analyses. Hypothesis tests of associations were performed by χ^2 test and Fisher's exact test (2-sided). Univariable survival probabilities were probed with the Kaplan–Meier method, and log-rank tests were used to probe their statistical significance, $P \leq .05$ were considered statistically significant. Multivariable analyses were performed with the Cox Proportional Hazards Model.

Results

Clinicopathologic Features of the Main Cohort

As depicted in Fig. 1A, B, our cohort comprised 909 primary NETs (79%) and 248 primary NEC (21%). Specifically, our cohort included 732 unifocal NET from various sites and additionally included 177 NET from 27 individual patients suffering from multifocal jejunoileal NET.[20] Regarding the tumor grade, the 909 NETs were distributed into 613 NET G1 (68%), 119 NET G2 (13%) and 10 NET G3 (1%), in addition to 121 typical (13%) and 46 atypical carcinoids (5%) of the lung. Of the 518 midgut-derived NET, 460 were diagnosed as NET G1 (88.8%), along with 57 NET G2 (11%) and 1 NET G3 (0.2%), whereas of the remaining GEP-NET 153 were NET G1 (68%), 62 were NET G2 (28%) and 9 were NET G3 (4%) Of the 248 NEC, 104 were diagnosed as large-cell neuroendocrine carcinoma (LC NEC, 42%), 99 were diagnosed as small-cell carcinoma (SC NEC, 40%), and 45 were mixed adenoneuroendocrine carcinomas (MANEC, 18%). Regarding the primary tumor localization (Fig. 1C), the cohort comprised 435 jejunoileal neoplasms (37%), 66 appendiceal (6%), 31 cecal (3%), 44 other-colonic (noncecal/nonrectal, 4%), 37 rectal (3%), 176 pancreatic (15%), 18 duodenal (2%), 88 gastric/gastroesophageal junction (8%), and 262 pulmonary NEN (22%). As expected, patients with NEC had significantly worse OS compared with those with NET ($P < .001$, hazard ratio: 10.74).

PITX2 Expression in the Overall Cohort

PITX2 expression was found in 626/1157 (54%) of the primary NENs in our cohort, while 531 NEN were PITX2 negative (Fig. 1D). Of the 909 NETs (including typical carcinoids and atypical carcinoids), PITX2 expression (any degree) was evident in 591/909 NETs (65%). Within NECs, PITX2 expression (any degree) was detected in 35/248 tumors (14%, Fig. 1E). PITX2 expression was far more common in NETs than in NECs ($P < .001$). In all NET

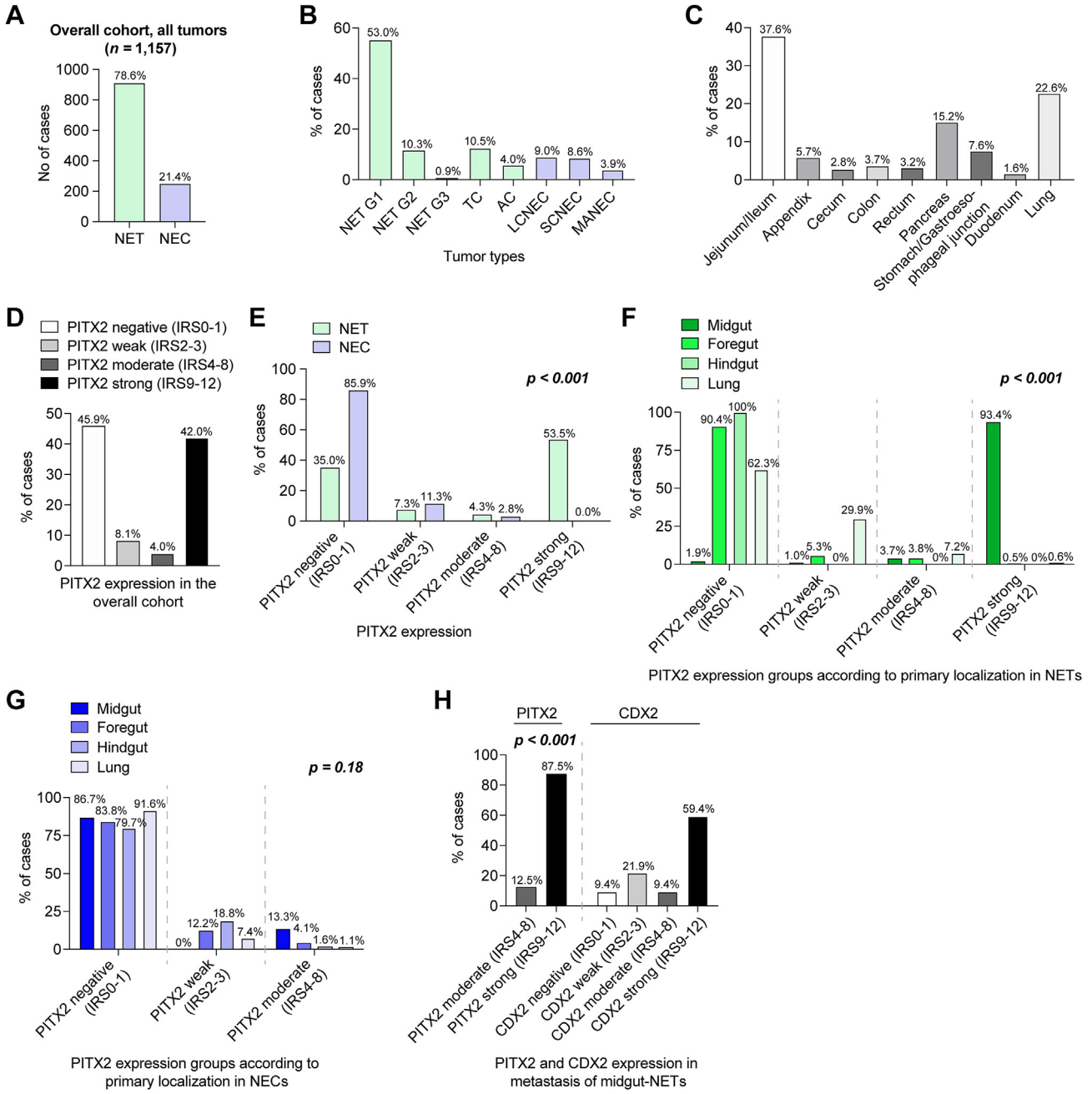

Figure 1.

Overview of the cohort and PITX2 expression in NET and poorly differentiated NEC. (A) Frequency of NET and NEC in the overall cohort of 1157 NEN. (B) Frequency of the specific types of NEN in the overall cohort. (C) Localization of the primary NEN investigated in the overall cohort. (D) Frequency of PITX2 expression groups in the overall cohort (all NEN). (E) General comparison of PITX2 expression in NET and NEC. (F) Frequency of PITX2 expression groups in NET according to their site of origin (foregut, midgut, hindgut, lung). (G) Frequency of PITX2 expression groups in NEC according to their site of origin (foregut, midgut, hindgut, lung). (G) Comparison of PITX2 and CDX2 expression groups in metastases of midgut-derived NET, note the much higher fraction of CDX2 negative or low lesions. NEC, neuroendocrine carcinomas; NEN, neuroendocrine neoplasms; NET, neuroendocrine tumors; PITX2, Pituitary Homeobox 2.

within the overall cohort, there was significant enrichment of PITX2 expression within G1 neoplasms ($P < .001$) due to the main expression in midgut-derived NET. In separate subgroup analyses within specific organ sites of NET, no association between general PITX2 expression and tumor grade was detected ($P = .65$ for midgut-derived NET; $P = .18$ in nonmidgut gastroenteropancreatic NET; $P = .62$ for pulmonary carcinoids [typical vs atypical]).

PITX2 Expression in Midgut-Derived NET (Jejunum/Ileum/Appendix/Cecum)

As depicted in Figure 1F, PITX2 was expressed in 98.1% of the 518 midgut-derived NETs in our cohort and the overwhelming majority showed a strong and diffuse PITX2 expression (484/518 93.4%, IRS 9-12), whereas 3.7% showed a moderate (19/518, IRS 4-8) and 1% (5/518, IRS 2-3) showed a weak PITX2 expression. Only

Table 2
Detailed distribution of PITX2 expression groups for each tumor localization in NET

Localization	Overall	PITX2 negative (IRS 0-1)	PITX2 weak (IRS 2-3)	PITX2 moderate (IRS 4-8)	PITX2 strong (IRS 9-12)
Jejunum/ileum	434 (100%)	6 (1.4%)	2 (0.5%)	17 (3.9%)	409 (94.2%)
Appendix	65 (100%)	4 (6.2%)	3 (4.6%)	0 (0%)	58 (89.2%)
Cecum	19 (100%)	0 (0%)	0 (0%)	2 (10.5%)	17 (89.5%)
Rectum	16 (100%)	16 (100%)	0 (0%)	0 (0%)	0 (0%)
Pancreas	171 (100%)	159 (93%)	6 (3.5%)	5 (2.9%)	1 (0.6%)
Stomach/gastroesophageal junction	19 (100%)	19 (100%)	0 (0%)	0 (0%)	0 (0%)
Lung	167 (100%)	104 (62.3%)	50 (29.9%)	12 (7.2%)	1 (0.6%)
Duodenum	18 (100%)	10 (55.6%)	5 (27.8%)	3 (16.7%)	0 (0%)

IRS, immunoreative score; NET, neuroendocrine tumors.

1.9% (10/518) of midgut-derived NETs showed no detectable PITX2 expression (6 jejunoileal and 4 appendiceal NETs). An exploratory analysis of interobserver variation of 110 cases between 3 pathologists showed an excellent concordance regarding the IRS-scoring groups between different observers (kappa score: Observer 1 vs Observer 2: 0.95; Observer 1 vs Observer 3: 0.93). In patients with multifocal NET, all of the individual tumors expressed PITX2 (177/177, 100%), with only slight variations regarding the expression intensity (strong vs moderate) between the multifocal primaries in 4 patients. The detailed expression of PITX2 in NET for the specific sites of all organs is provided in Table 2, and examples of PITX2 expression patterns in midgut-derived NET are illustrated in Figure 2.

CDX2 Expression in Midgut-Derived NET (Jejunum/Ileum/Appendix/Cecum)

CDX2 was evaluated in 180 primary jejunoileal NET and a general expression (any degree) was observed in 90.6% (163/180) of the tumors, whereas 17 were completely negative (9.4%). Regarding the CDX2 expression groups, 59.4% of tumors (107/180) were strongly positive, 23.9% of tumors (43/180) showed a moderate, and 7.2% (13/180) showed a weak CDX2 expression. Examples of the different CDX2 expression groups are given in Supplementary Figure S1.

Expression of PITX2 and CDX2 in Metastases from Midgut-Derived NET

In concordance with the respective primary tumors, all of the investigated metastatic lesions (20 nodal metastases/12 hepatic metastases) expressed PITX2 (32/32, 100%), with 28/32 metastases showing a strong and diffuse positivity (87.5%), whereas the other remaining metastases showed a moderate expression (4/32, 12.5%). CDX2 was expressed in 29 of the 32 metastatic lesions (90.6%), 3 tumors did not show CDX2 expression. Regarding the expression intensity, only 19/32 tumors showed a strong CDX2 expression (59.4%), which was significantly lower than the rate observed for PITX2 ($P < .001$), and the remaining CDX2-positive cases showed a moderate (3/32, 9.4%) or weak (7/32, 21.9%) expression (Fig. 1H and Supplementary Fig. S2).

PITX2 Expression in Foregut-Derived NET (Pancreas/Duodenum/Stomach)

We observed PITX2 expression in 9.6% of foregut-derived NET (20/208), whereas the remaining 90.4% (188/208) were entirely negative. Most PITX2-expressing tumors were found in the duodenum, where we observed a weak (5/18) or moderate positivity (3/18) in 44.4% of duodenal NET. In the pancreas, 93% of tumors were negative (159/171) and most PITX2-expressing cases showed a weak (6/171, 3.5%) or moderate positivity (5/171, 2.9%). A singular pancreatic NET showed a strong and diffuse expression (1/171, 0.6%). The detailed expression of PITX2 for NET of the specific sites of all organs is provided in Table 2 and examples of PITX2 expression patterns in nonmidgut NETs are illustrated in Figure 3.

PITX2 Expression in Hindgut-Derived NET (Distal Colon/Rectum)

In the 16 rectal NETs included in our cohort, no PITX2-positive cases were detected. The detailed expression of PITX2 for the specific sites of all organs is provided in Table 2, and examples of PITX2 expression patterns in nonmidgut NETs are illustrated in Figure 3.

PITX2 Expression in Pulmonary NET (Typical/Atypical Carcinoids)

Of 167 pulmonary NETs, PITX2 expression was observed in 37.7% of the cases (63/167). Most of the PITX2-expressing pulmonary NETs showed a weak expression (50/167, 29.9%), whereas some showed a moderate positivity (12/167, 7.2%). Again, a singular case showed a strong and diffuse expression of PITX2 (1/167, 0.6%). No statistical difference between atypical and typical carcinoids was evident ($P =$ n.s.). The detailed expression of PITX2 for the specific sites of all organs is provided in Table 2 and examples of PITX2 expression patterns in nonmidgut NETs are illustrated in Figure 3.

PITX2 Expression in Small-Cell NEC, Large-Cell NEC, and MANEC (All Sites)

Of the 248 poorly differentiated NEN (NEC and MANEC), 20/103 LC NEC (23.1%), 8/99 SC NEC (8.1%), and 3/42 MANEC (6.7%) showed some degree of PITX2 expression. Of the 35 PITX2-expressing NEC, 28/35 showed a weak positivity (80%), whereas 7/35 showed moderate staining (20%). A strong expression was not observed. The detailed expression of PITX2 in NEC for the specific sites of all organs is provided in Table 3 and examples of PITX2 expression patterns in NECs are illustrated in Figure 4.

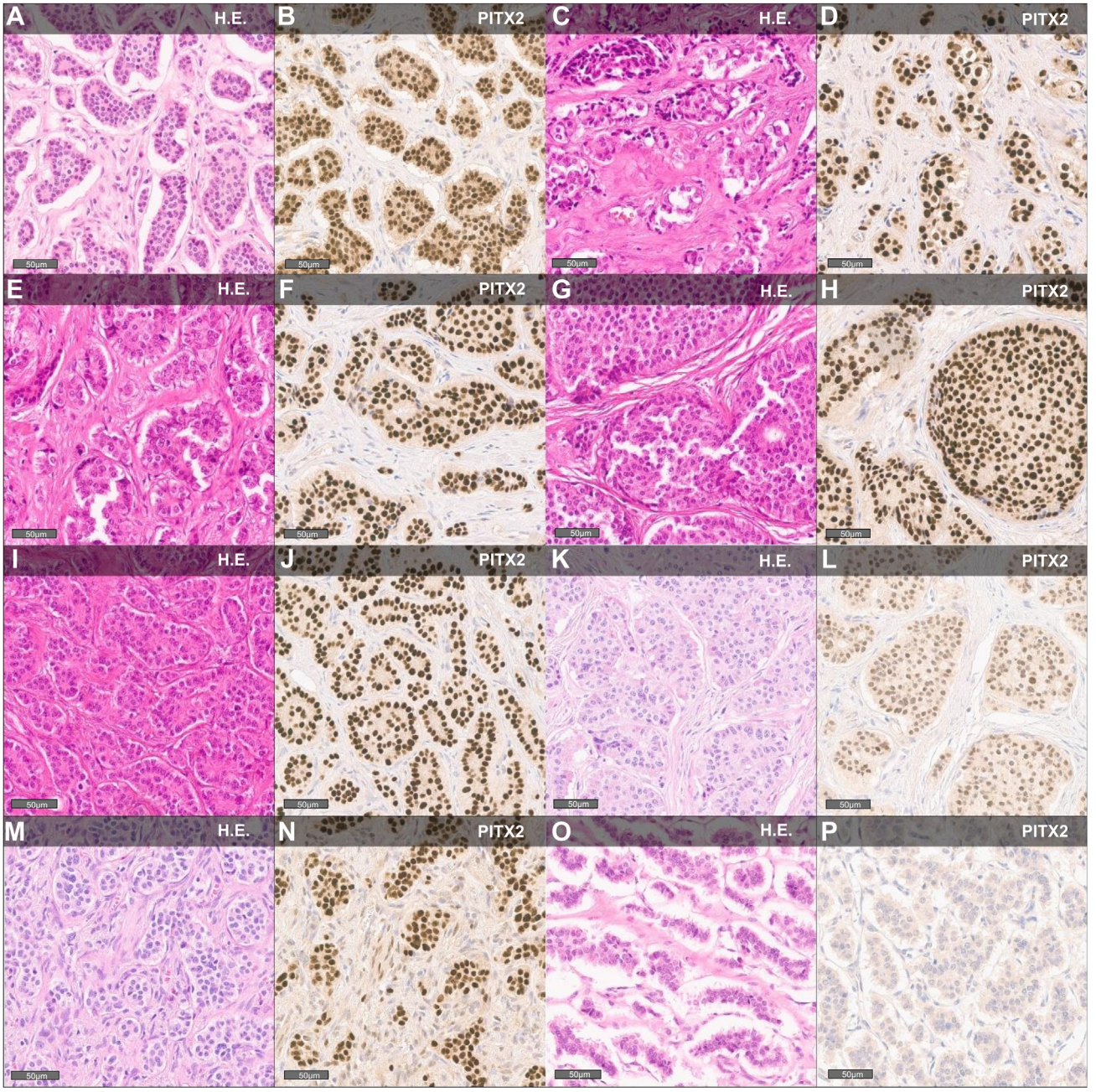

Figure 2.
Expression of PITX2 expression in midgut-derived NET. (A, B) Ileal NET (HE, A) with strong staining intensity in almost all of the tumor cells (B, IRS 12), reflecting a strong PITX2 expression. (C-H) Three individual ileal tumors (C, E, G, HE) from a patient with multifocal ileum NET. Each NET shows a strong staining intensity for PITX2 in almost all of the tumor cells (D, F, H; IRS 12). (I, J) Example of a hepatic metastasis from an ileal NET (H&E, I) with a strong staining intensity in almost all of the tumor cells (J, IRS 12). (K, L) Cecal NET (K, H&E) with an up to strong expression of PITX2 in more than 50% of the tumor cells (L, IRS 9). (M, N) Appendiceal NET (M, HE) with a strong staining intensity in almost all of the tumor cells (N, IRS 12). (O,P) Ileal NET (H&E, O) without expression of PITX2 (P, IRS 0). H&E, hematoxylin and eosin; IRS, immunoreactive score; NET, neuroendocrine tumors; PITX2, Pituitary Homeobox 2.

Sensitivity and Specificity of PITX2 Expression for the Primary Tumor Localization of NEN

Among NET, we observed a very significant association between midgut-origin and PITX2 expression ($P < .001$). A strong expression of PITX2 (IRS 9-12) had a sensitivity of 93.4% and a specificity of 99.5% for midgut-derived NETs, as only 2 nonmidgut NETs showed a strong PITX2 expression (1 pancreatic/1 pulmonary). When all degrees of PITX2 expression in NETs were considered, the sensitivity rose to 98.1%, whereas the specificity dropped to 78.8%, as a weak or moderate expression of PITX2 was not uncommon in nonmidgut NETs. In NEC, we did not observe any association between tumor localization and PITX2 expression ($P = .18$).

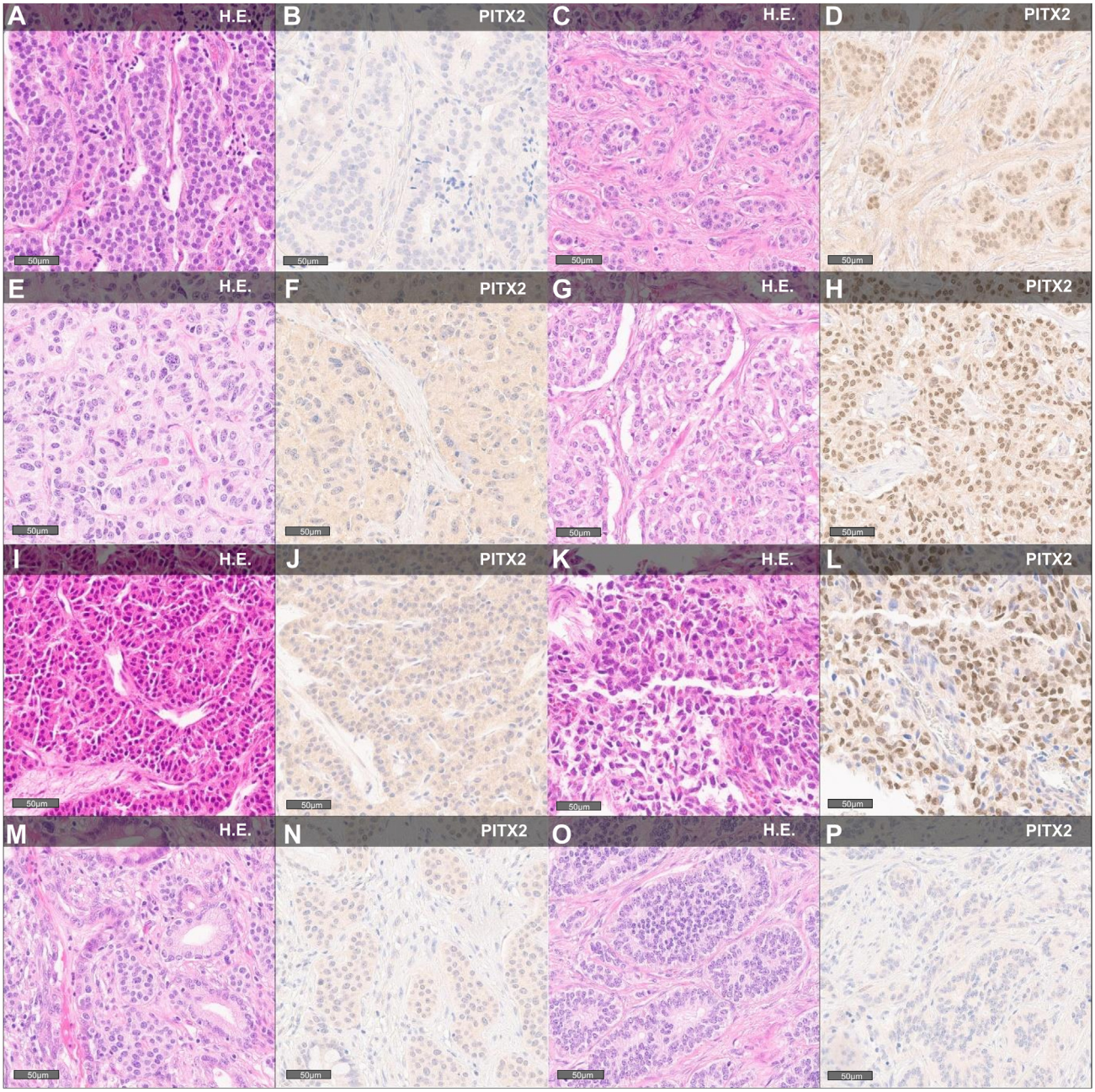

Figure 3.
PITX2 expression in NET of non-midgut origin. (A, B) Example of a duodenal NET (H&E, A) without expression of PITX2 (B). (C, D) Duodenal NET (C, H&E) with a moderate staining intensity in more than 50% of the tumor cells (IRS 6). (E, F) Example of a pancreatic NET (H&E, E) without expression of PITX2 (F). (G, H) Singular pancreatic NET (G, H&E) with an expression of PITX2 in almost all tumor cells with an up to strong staining intensity (H, IRS 12). (I, J) Pulmonary typical carcinoid (HE, I) without expression of PITX2 (J). (K, L) Singular tumor from the pulmonary carcinoid group (K, H&E) in our cohort with an up to strong staining intensity in almost all of the tumor cells (L, IRS 12). (M, N) Example of a gastric NET (H&E, M) without expression of PITX2 (N). (O, P) Example of a rectal NET (H&E, O) without expression of PITX2 (P). H&E, hematoxylin and eosin; NET, neuroendocrine tumors; PITX2, Pituitary Homeobox 2.

Discussion

Because NETs often present as metastatic tumors at the time of initial diagnosis, narrowing down the origin of a possible primary tumor is one of the central tasks of routine histopathology.[1-3] Although the growth pattern based on hematoxylin and eosin morphology can already provide important hints regarding the localization of the primary,[21] a reliable assessment is only possible through immunohistochemistry.[11]

Recently, Soukup et al[15] introduced Pituitary Homeobox 2 (PITX2) as a possible addition to the list of transcription factors such as CDX2, Islet 1, SATB2, or TTF1, which are commonly used for this purpose.[11] In their study of 98 NETs, all of the 20 jejunoileal and 8/9 appendiceal NETs were PITX2 positive, whereas they did not observe any PITX2 expression in nonmidgut–derived NETs. Therefore, the authors concluded, that PITX2 is a very sensitive and specific marker for midgut-derived NETs. Although these exploratory data are very intriguing, the authors recognized

Table 3
Detailed distribution of PITX2 expression groups for each tumor localization in neuroendocrine carcinomas

	Overall	PITX2 negative (IRS 0-1)	PITX2 weak (IRS 2-3)	PITX2 moderate (IRS 4-8)	PITX2 strong (IRS 9-12)
Jejunum/ileum	1 (100%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)
Appendix	1 (100%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)
Cecum	13 (100%)	11 (84.6%)	0 (0%)	2 (15.4%)	0 (0%)
Rectum	21 (100%)	17 (81%)	4 (19%)	0 (0%)	0 (0%)
Pancreas	5 (100%)	5 (100%)	0 (0%)	0 (0%)	0 (0%)
Stomach/gastroesophageal junction	69 (100%)	57 (82.6%)	9 (13%)	3 (4.3%)	0 (0%)
Lung	95 (100%)	87 (91.6%)	7 (7.4%)	1 (1.1%)	0 (0%)
Colon other	43 (100%)	34 (79.1%)	8 (18.6%)	1 (2.3%)	0 (0%)

the rather small number of investigated tumors as a major limitation of their study.

For this reason, our study investigated the expression of PITX2 in an extremely large-scale cohort of 1157 NEN including 909 NET and 248 poorly differentiated NEC. Our study confirmed a strong expression of PITX2 (IRS 9-12) as a very sensitive (93.4%) and an extremely specific (99.5%) marker of midgut-derived NET, as a strong and diffuse positivity of PITX2 was only observed in 2/391 (0.5%) nonmidgut NET. In our exploratory analysis of midgut-NET metastases, all of the metastatic lesions were PITX2 positive and

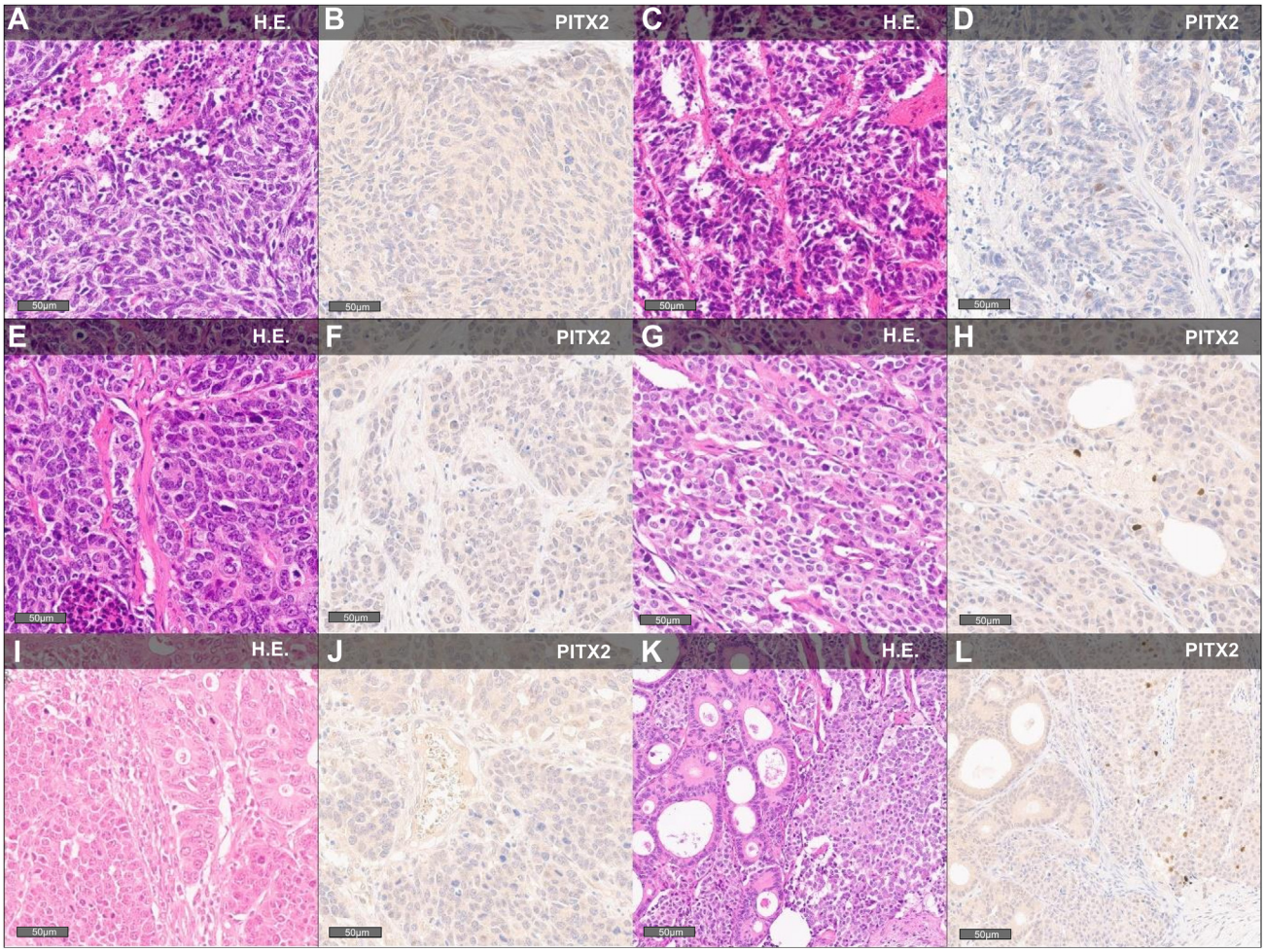

Figure 4.
PITX2 expression in poorly differentiated NEC. (A, B) Example of a pulmonary small cell NEC (A, H&E, ×40) without specific nuclear expression of PITX2 (B, ×40, PITX2). (C, D) Example of a weak expression of PITX2 (IRS 2) in a pulmonary small cell NEC (C, HE, ×40) with an up to moderate nuclear staining intensity in less than 10% of the tumor cells (IRS 2), therefore falling into the low expression group (D, ×40, PITX2). (E, F) Example of a colorectal large-cell NEC (E, HE, ×40) without specific nuclear expression of PITX2 (F, ×40, PITX2). (G, H) Weak expression of PITX2 (IRS 3) in a gastric large-cell NEC (G, HE, ×40), which shows an up to strong staining intensity for PITX2, which is however restricted to less than <10% of the nuclei, therefore falling into the low expression group. (I, J) Example of a gastric MANEC (I, HE, ×40) composed of a large-cell NEC combined with a tubular adenocarcinoma without specific nuclear expression of PITX2 in either of the components (J, ×40, PITX2). (K, L) Colorectal MANEC (K, HE, ×40) composed of a large-cell NEC combined with an adenocarcinoma NOS. The NEC component shows an up to strong staining intensity for PITX2, which is restricted to less than <10% of the nuclei (L, ×40, PITX2). The adenocarcinoma component remains completely negative. IRS, immunoreactive score; MANEC, mixed-adenoneuroendocrine carcinoma; NEC, neuroendocrine carcinomas; NOS, not otherwise specified; PITX2, Pituitary Homeobox 2.

usually showed a very strong and diffuse reaction as was observed in most primary midgut-derived NET. Therefore, our data strongly support the hypothesis that a clinically unknown primary tumor of a metastatic NET − if it shows a high expression of PITX2 − is almost certainly located in the ileum, appendix, or cecum, whereas other locations would appear highly unlikely in this setting.

CDX2 is a well-known transcriptional factor expressed in intestinal neoplasms including midgut NETs. Previous studies reported a high sensitivity of CDX2 for midgut-NETs, ranging from 84 and even up to 100%, but the results were obtained from rather small cohorts.[11,22-24] When we stained the same metastatic lesions for CDX2, almost 30% of the lesions were either CDX2 negative or only showed a weak expression, which was contrasted by a usually strong or at least moderate PITX2 positivity of exactly the same lesions. We observed a similar picture regarding CDX2 and PITX2 when we compared their expression in primary midgut-derived NETs. Although CDX2 was generally expressed in the vast majority of tumors, a significantly higher degree of tumors was negative or showed a weak expression, which was in contrast to PITX2, where a negative or weak expression was exceptionally rare.

However, the picture in terms of sensitivity and specificity of PITX2 for midgut-derived NET shifts significantly if all degrees of immunohistochemical PITX2 expression are considered. Because complete negativity for PITX2 in midgut-NETs appears to be an absolute rarity and the non−strongly positive neoplasms showed moderate or at least weak expression, the sensitivity in this setting increased to 98.1%. In contrast, the specificity drops to 78.8%, as a weak or (rarely) moderate expression of PITX2 was also observed quite frequently in nonmidgut NETs, especially in the lung and the duodenum. This indicates that in contrast to a high PITX2 expression, a nonmidgut origin cannot be fully ruled out for NETs with a low or moderate PITX2 positivity, and additional stainings (hormones and other transcription factors) are warranted in this scenario. Although these results are in contrast to the results from the initial study from Soukup et al, we believe that they are likely to be explained through the very large size of our cohort and underline the importance of validation studies in large collectives.

According to our data, PITX2 outperforms the primarily used midgut-marker CDX2 regarding sensitivity and specificity for midgut-derived NET. On the basis of our large cohort, CDX2 appears to be less sensitive, as it is entirely negative or only weakly positive in a considerably higher fraction of primary tumors and/or metastases of midgut-derived NET.[11,20,22-26] In addition, CDX2 also has a disadvantage regarding specificity (compared with a high PITX2 expression), as it is very commonly expressed in various carcinomas with intestinal differentiation and also NETs.[1,27,28]

Immunohistochemistry plays a limited role in assigning a site of origin for poorly differentiated NECs because of their frequent aberrant expression of transcription factors (eg, TTF1)[11] that would be considered as sensitive or specific in NET. Comparable to other transcription factors, PITX2 expression was (mostly weakly) expressed in 14% of NEC without significant association with the location of the primary. Therefore, it is important to remember that the sensitivity and specificity for midgut-NEN of PITX2 only applies to NET and cannot be transferred to NEC.

Although our multicentric NEN cohort is very large, our study has the limitation that some primary sites such as duodenal, gastric, and rectal NET are underrepresented compared with the much larger numbers of jejunoileal/pancreatic and pulmonary NET. Therefore, additional studies with a larger amount of cases from these localizations (especially from the duodenum) should be performed, especially when 44% of the duodenal NET in our collective showed a weak or moderate PITX2 expression. Another issue that should be considered is that our study used a slightly stronger but still very specific concentration of the PITX2 antibody than the previous study by Soukup et al, to ensure a robust staining quality on our investigated TMAs.

In conclusion, our study confirms PITX2 as a highly sensitive and specific marker of midgut-derived NET and our data argue that PITX2 should be implemented into diagnostic panels for the site of origin assignment of NET as a firstline midgut marker. A high expression of PITX2 is extremely specific for midgut-derived NET, whereas PITX2 expression in general (including weak and moderate staining) appears to be very sensitive to detect a midgut lineage, but is not as specific, as these expression types are also quite commonly found in nonmidgut NET, especially from the lung and the duodenum.

Acknowledgments

The authors are grateful to Marion Kalden, Manuela Gerber, and Viktoria Wischmann from the University Hospital Marburg for excellent technical assistance.

Author Contributions

M.J., A.K., and D.K.B. designed this study. M.J., A.K., and A.G. wrote the manuscript with assistance from D.K.B., C.D., G.K., A.K., B.M., M.E. M.J., A.G., S.F., A.B., M.K., A.Q., M.E., and D.W. performed histopathologic analyses. E.M., J.P., T.M., H.W., A.K., E.M., Do.W., A.R., T.M.G., D.K.B., C.D., G.K., A.K., B.M., M.E. M.J., A.G., S.F., A.B., M.K., A.Q., M.E., and D.W. provided technical and material support. All authors read and approved the final paper.

Data Availability

Tissue and data from this manuscript are stored at the Institute of Pathology, Phillips University Marburg und University Hospital Marburg, Marburg, Germany, and are available from the corresponding author upon reasonable request.

Funding

This research project has not received any funding.

Ethics Approval and Consent to Participate

Our study was approved by the local ethics committees of the Technical University of Munich (reference numbers: 252/16 s and 2022-396-S-DFG-SR, combined ethical vote for all cases from Munich/Regensburg/Augsburg), the University Hospital Marburg (reference number: reference number: AZ 206/10 and AZ 43/21), the University Hospital Cologne (reference number:13-091), the University Hospital Erlangen-Nuremberg (reference number: 329_16B), and of the University Hospital Heidelberg (reference number: #S315−2020, NCT#2603).

Supplementary Material

The online version contains supplementary material available at https://doi.org/10.1016/j.modpat.2024.100442.

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