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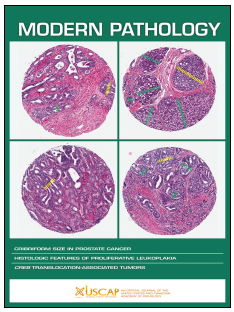

ARX, PDX1, ISL1 and CDX2 expression distinguishes five subgroups of PanNETs with correlations to histology, hormone expression and outcome

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Title:

ARX, PDX1, ISL1 and CDX2 expression distinguishes five subgroups of PanNETs with correlations to histology, hormone expression and outcome

Running title: PanNET transcription factor profiles

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Abstract

Many pancreatic neuroendocrine tumors (PanNETs) fall into two major prognostic subtypes based on DAXX/ATRX induced ALT phenotype and alpha and beta cell-like epigenomic profiles. However, these PanNETs are still flanked by other PanNETs that do not fit into either subtype. Furthermore, despite advanced genotyping, PanNETs are generally not well characterized in terms of their histological and hormonal phenotype. We aimed to identify new subgroups of PanNETs by extending the currently used transcription factor signatures and to investigate their correlation with histological, hormonal molecular and prognostic findings. 185 PanNETs (non-functioning 165, functioning 20) resected between 1996 and 2023 were classified into five subgroups (A1, A2, B, C, D) by cluster analysis based on ARX, PDX1, ISL1 and CDX2 expression and correlated with trabecular vs. solid histology, expression of insulin, glucagon, PP, somatostatin, serotonin, gastrin, calcitonin, ACTH, DAXX/ATRX, MEN1 and ALT status by FISH, and disease-free survival (DFS). A1 (46%, ARX+/ISL1+/PDX1-/CDX2-) and A2 (15%, ARX+/ISL1+/PDX1+/CDX2-) showed trabecular histology and glucagon/ PP expression, with A2 also showing gastrin expression. B (18%, PDX1+/ISL1+/ARX-/CDX2-) showed solid histology, insulin and somatostatin expression ($p<0.001$). It included all insulinomas and had the best outcome ($p<0.01$). C (15%, ARX-/PDX1-/ISL1-/CDX2-) showed solid histology and frequent expression of serotonin, calcitonin und ACTH. D (5%, PDX1+/CDX2+/ISL1-/ARX-) showed solid histology, expressed ACTH/serotonin and was an independent poor prognosticator ($p<0.01$). Differential expression of ARX, PDX1, ISL1 and CDX2 stratified PanNETs into five subgroups with different histology, hormone expression and outcome. Subgroups A1 and A2 resembled the alpha cell-like type, subgroup B the beta cell-like type. Subgroup C with almost a no transcription factor signature was unclear in cell lineage, while the PDX+/CDX2+ signature of subgroup D suggested a pancreatic/intestinal cell lineage. Assigning PanNETs to the subgroups may help to establish the diagnosis, predict the outcome, and guide the treatment.

Key words: PanNETs, subgroups, transcription factors, hormone expression, outcome

Introduction:

Pancreatic neuroendocrine tumors (PanNETs) are probably the most variable and complex of the gastroenteropancreatic (GEP) neuroendocrine neoplasms (NENs). The complexity affects every aspect of PanNETs, from their endocrinological facets, histological architecture, hormonal profile, genetic-epigenetic spectrum, to their clinical course. This variability complicates the prediction of patient outcome and the selection of treatment. Therefore, there are many approaches to improve prognostication. The first and still the most important prognosticator for patient survival is the Ki67 proliferation index. Together with the mitotic count, the Ki67 index was used to construct the grading system that has been propagated by the WHO classification since 2000. For PanNETs, this is currently a three-tiered system of malignancy grades that correlates with patient outcomes [1]. Another strong prognostic tool is the TNM classification, which has undergone many improvements over time [2]. However, these powerful tools cannot capture cellular and molecular heterogeneity. Therefore, a more refined system is needed to further improve the management of patients with PanNETs.

Recently, several studies have shown that PanNETs with mutations in DAXX and/or ATRX genes are associated with poor prognosis [3-6]. In addition, alternative lengthening of telomerase (ALT) was also found to be an indicator of poor outcome [3, 6-8], with ALT being independent from grade and stage [9].

In 2018, Chan et al. examined RNA expression in PanNETs and showed similarities to that of either alpha or beta cells of islet, suggesting that PanNETs may originate from these two islet cell types [10]. In addition, the expression of the transcription factors ARX and PDX1 were highly correlated with alpha and beta cell types, respectively [11].

In 2020, a phyloepigenetic analysis by Di Domenico et al. linked DNA methylation profiles with hormonal, genomic, and transcription factor data and defined alpha-like and beta-like PanNETs 12. In addition, there was a large intermediate cluster with reduced similarity to alpha cells, comprising 58% of PanNETs, which was frequently associated with ARX positivity (83%), but remained unclear in its lineage differentiation and origin [12], as epigenetic signatures of other pancreatic cell types were lacking.

While genetic profiling of PanNETs has progressed, histological and functional segregation has remained relatively straightforward. Hormone expression has been reported only in tumors with corresponding clinical symptoms. More recently, histological patterns of non-functioning PanNETs have been associated with the expression of specific hormones. The following correlations have been described: trabecular-reticulated and often cystic patterns are associated with glucagon [13, 14], solid paraganglioma-like or glandular patterns with psammoma

bodies with somatostatin expression [15, 16], and nested solid cell strands embedded in sclerosing stroma with serotonin expression [17-19]. Regarding the hormonal phenotype of the alpha- and beta-cell-like PanNET subgroups, they have been shown to express glucagon or insulin in association with either ARX or PDX1 [12, 20]. For the other epigenetic PanNET subtypes that do not fall into the alpha- or beta-cell-like categories, their hormonal composition is unknown. Hormones such as somatostatin, pancreatic polypeptide, serotonin, calcitonin, gastrin, and ACTH are expected to be detected in these tumors and contribute to the phenotypic signature, with significant multihormonality expected [21].

In this comprehensive study, we used an expanded transcription factor panel to search for new PanNET subgroups in comparison to the already established subtypes. Our specific aims were (1) to define PanNETs based on the expression patterns of ARX, PDX1, Islet-1 (ISL1), and CDX2, (2) to correlate the obtained PanNET clusters with histological patterns and hormone expression, (3) to correlate the transcription factor clusters with the most frequent genomic types using DAXX, ATRX, and MEN1 loss and ALT status, and (4) to correlate the clinicopathological and genetic data with disease-free survival (DFS) of the patients.

Materials and Methods

Tissue and data assembling

We reviewed 185 resected primary PanNETs consecutively obtained between 1996 and 2023 from the in-house surgical pathology and consultation files (Consultation Center for Pancreatic and Endocrine Neoplasms) (N=143) of the Department of Pathology, University Hospital “rechts der Isar” of the Technical University Munich, School of Medicine and Health, and from the archives of the Department of Pathology, University of Regensburg (N=38), and University Hospital Augsburg (N=4). Small PanNETs (<1 cm) and PanNETs from patients with hereditary genetic syndromes, such as MEN1 or von Hippel-Lindau disease, were excluded. Also excluded were two cases of pancreatic metastases from ileal NET that were resected under the diagnosis of PanNET. In all cases, formalin-fixed paraffin-embedded (FFPE) tissue blocks, and representative slides were available. Diagnosis was made according to the current WHO classification [1].

The histological architecture of PanNETs was independently classified by A.K. and G.K. according to the dominant histological pattern. A pattern was considered dominant if it was present in more than 50% of the tumor area. Two general patterns were distinguished: a solid pattern and a trabecular pattern. In tumors with a predominantly solid pattern, neoplastic cells were arranged in small or large nests or sheets. This solid pattern was subclassified as solid nested if the neoplastic cells formed round ovoid cell groups of various sizes. In approximately 15% of cases, the solid structures resembled paraganglioma Zellballen with slightly pleomorphic

cells and nuclei. PanNETs with a trabecular growth pattern usually showed neoplastic cells arranged in cords and interconnected in a reticular pattern. Cystic structures were often present. Rarely, there were gyriform cell cords embedded in collagenized stroma or (pseudo) glandular patterns (when cell cords transform into glandular elements). Cytoplasmic and nuclear pecularities (such as oncocytic, clear cell, chromatin-rich) [22] were not considered in this study. Clinical data including sex, age, hormonal syndromes, and TNM status were obtained from patient records and are shown in Supplementary Table 1. Follow-up data on disease-free survival (DFS) and overall survival (OS) were obtained from the Bavarian Cancer Registry and/or patient records. Mean follow-up was 68 months (range 1 to 302 months). Follow-up analyses were performed in 167 patients after excluding 5 patients with less than 1 month of follow-up. The study was approved by the Ethics Committee of the Technical University of Munich (2022-396-S-DFG-SR).

Tissue microarray construction

Tissue microarrays (TMA) consisting of two cores, each 2 mm in diameter, from one FFPE block per case were constructed using the TMA Grand Master (Sysmex/3DHistech, Budapest, Hungary). Cores were obtained from representative central and peripheral tumor areas selected by two pathologists (AK and AU).

Immunohistochemical staining and evaluation

Immunohistochemical staining was performed using a fully automated slide preparation system (Benchmark XT, Ventana/Roche, Arizona, USA) and evaluated by three observers (EM, AU, AK). Ki67, cytokeratin 18, synaptophysin, chromogranin A, somatostatin receptor 2 (SSTR2), glucagon, pancreatic polypeptide (PP), insulin, somatostatin, serotonin, calcitonin, gastrin, and adrenocorticotropic hormone (ACTH) were immunostained on whole 3 micrometer thick tumor sections from FFPE blocks. TMA sections were used for immunohistochemical analysis of ISL1, ARX, PDX1, CDX2, DAXX, ATRX, MEN1, p53 and retinoblastoma 1 (RB1). Details of immunohistochemical staining are shown in Supplementary Table 2. For ARX, ISL1, PDX1 and CDX2, strong nuclear immunoreactivity was detected in > 10% of neoplastic cells as positive as described [9]. Expression of DAXX and ATRX was considered to be maintained when > 5% of tumor cells showed nuclear staining [9]. Loss of staining for DAXX or ATRX (DAXX/ATRX loss) had to be complete with the presence of intact internal staining in non-neoplastic cells. Complete loss of nuclear expression of MEN1 was considered negative. In the case of controversial results, consensus was reached by joint discussion. For case-to-case comparison, ISL1, ARX, PDX1, CDX2 were stained and evaluated on wholemounts of 15 randomly selected cases and their expression on TMA and wholemount slides was compared. Positive cytoplasmic expression of cytokeratin 18, synaptophysin, chromogranin A, and hormones was considered focally positive if up to 30% of tumor cells were stained and diffusely positive if > 30% were

stained. Membranous expression of SSTR2 was scored as previously described. Tumors with scores of 2+ or 3+ were considered positive, and those with scores of 0 and 1+ were considered negative [23, 24].

Telomere fluorescence in situ hybridization

Staining was performed on 4 micrometer TMA sections as previously described [7]. Briefly, after deparaffinization and rehydration, slides were boiled in normal saline citrate and 0.05% Tween 20 for 30 minutes. A peptide nucleic acid probe (telC-Alexa488; Pagagene, Daejeon, Korea) was diluted 1:10. The samples were denatured at 85 degrees for 4 minutes and incubated for 2 hours at room temperature in the dark. Anti-promyelocytic leukemia (antibody PG-M3; Santa Cruz, Heidelberg, Germany) 1:100 was incubated for 1 hour at room temperature, and secondary antibody (goat anti-mouse Alexa568; Cell Signaling, Danvers, MA) 1:500 was diluted and incubated for 1 hour at room temperature in a dark chamber. FISH was evaluated by EM, GK, and AK with the assistance of two experts (IM and AP) using an Olympus VS110 fluorescence scanner (Olympus, Vokestwik, Switzerland). At least two cells with clear hyperbright telomeres on one TMA spot was the minimum requirement for ALT classification.

Statistical analysis

JMP Pro version 17.1.0 software (SAS Institute, Inc., Cary, NC, USA) was used for all statistical analyses. The expression of four transcription factors on TMA and whole tissue slides were compared using the correlation probability test and the concordance correlation coefficient (R) was provided. All our PanNETs were grouped according to the transcription factor profile by the percentage distribution of the expression of the four transcription factors in 185 tumors (Ward´s method). This analysis resulted in 5 clusters grouped as A1, A2, B, C and D. Multiple groups were compared using Pearson's chi-squared test or Fischer´s exact test. The Kruskal-Wallis test was used to compare continuous values or scores between multiple groups that were not normally distributed by the Shapiro-Wilk test. The probability of differences in DFS and OS was determined using the Kaplan-Meier method with a log-rank test for significance. Multivariate survival analysis was performed using the proportional hazards model. A p-value of <0.05 was considered statistically significant.

Results

Subtypes according to transcription factor signatures

Hierarchical clustering based on the expression of the four transcription factors ARX, ISL1, PDX1 and CDX2 identified five subgroups in our cohort of 185 PanNETs (Supplementary Fig., Table 1). The dominant subgroup A1 comprised 46% (86/185) of the PanNETs which were positive for ARX (99%) and ISL1 (100%) (Fig. 1A-C) and almost or completely negative for

PDX1 (1%) and CDX2 (0%). Subgroup A2 (15%, 28/185, Fig. 1D-F) was very similar in characteristics to A1, differing only in the additional expression of PDX1. It consistently expressed ARX (100%) and frequently ISL1 (71%) and PDX1 (61%) and rarely CDX2 (18%). Subtype B (18%, 33/185, Fig. 2 A-D) was characterized by PDX1 (100%) and ISL1 (73%) expression and very low expression of ARX (6%) and CDX2 (9%). Subtype C (15%, 28/185, Fig. 3 A-D) showed no expression of PDX1 (0%) and CDX2 (0%) and low expression of ARX (14%) and ISL1 (4%) and subgroup D, the rarest subgroup (5%, 10/185, Fig. 4 A-D), was positive for PDX1 (100%) and CDX2 (100%) and rarely for ARX (30%) and ISL1 (30%) (Table 1). Expression of the four transcription factors on TMA and whole-mount tissue was correlated and showed strong concordance ($p<0.001$, $R>0.95$ for all).

Correlation with histology and hormone expression

Subgroups A1 and A2 were associated with trabecular histology (81% and 54%, respectively, Figure 1A), while subgroups B, C, and D showed predominantly solid histological patterns (82%, 79%, and 100%, respectively, $p<0.001$, Fig. 2A, 3A, 4A). A PG-like pattern, a subtype of solid patterns, was identified in 12/33 (36%), and 8/12 (67%) were grouped in either B or C ($p<0.001$, Fig. 3A). All hormones except PP were at least focally expressed in the tumors of the five subgroups. Diffuse expression for glucagon/PP was mainly found in tumors of subgroup A1 (see Table 2 for details, Fig. 1D). Diffuse insulin expression was observed in all insulinomas (N=17), which grouped in B (Fig. 2C). In addition, there were 16 non-functioning tumors in B, which frequently showed somatostatin expression (5 diffuse, 10 focal, Fig. 2D) combined with diffuse or focal insulin expression. Somatostatin, serotonin, gastrin and calcitonin were rarely diffusely expressed (Fig. 3C, D). ACTH was only focally expressed and found most frequently in subgroups C and D (Table 2, Fig. 4D). The most frequently expressed hormones in all subgroups were PP (98/185), glucagon (96/185), somatostatin (110/185) and insulin (60/185), whereas low rates were observed for calcitonin (29/185), gastrin (29/185), serotonin (20/185) and ACTH (13/185) (see Table 2 for details). Expression of more than one hormone per tumor (i.e., multihormonality) was observed in 75% of PanNETs (two hormones 28%, three hormones 27%, four hormones 9%, and more than five hormones 10%). The number of hormones expressed per group did not correlate with any subgroup.

Correlation with syndromes and other clinical features

There were 20/185 functioning PanNETs (11%), the most common being insulinoma (one with metachronous metastasis), and one each glucagonoma, VIPoma and ACTH-producing tumor with Cushing syndrome. All insulinomas clustered in subgroup B ($p<0.0001$). The glucagonoma and VIPoma were grouped in A1, while the ACTH-producing PanNET was in subgroup D. None of the PanNETs with diffuse serotonin (4 cases), gastrin (6 cases), and calcitonin (5 cases)

expression were syndromic. No correlation was found between the five subgroups and other clinical characteristics such as age, sex, size, or TNM classification (Supplementary Table 3).

Correlation with SSTR2, DAXX/ATRX, MEN1 and ALT status

Membranous SSTR2 expression was predominantly observed in subgroups A1, A2 and D (99%, 100% and 90%, respectively) and less frequently in subgroups B and C (77% and 71%, p<0.0001). Immunohistochemistry for DAXX/ATRX and MEN1 was available in 173 (95%) and 161 (87%) cases, respectively. Loss of either DAXX or ATRX (DAXX/ATRX loss) and MEN1 was detected in 74/173 (43%) and 67/161 (42%) cases, respectively. ALT FISH could be evaluated in 146/185 (79%) cases. ALT positivity was detected in 63/146 (43%) PanNETs and was significantly associated with DAXX/ATRX loss (<0.001) (Supplementary Table 1). DAXX/ATRX loss and ALT positivity were observed in all five subgroups, with DAXX/ATRX loss/ALT positivity frequent in A1, A2 and C and less frequent in B and D (Table 2). MEN1 loss was observed in subgroups A1, A2, C and D but not in subgroup B (Table 2). Loss of DAXX/ATRX and MEN1 was significantly associated with ARX-positive PanNETs (p=0.002 and <0.0001, respectively), while preserved expression of DAXX/ATRX and MEN1 was associated with PDX1 expression (p<0.001 for both).

Correlation with patient outcome

Patients in subgroup B had the longest 5-year DFS rate (89%), while patients in subgroup D had the shortest 5-year DFS rate (33%, p=0.002, Fig. 5). Similar DFS rates were found in A1 (71%), A2 (74%) and C (71%). There was no significant difference in OS among the five subtypes. Other clinicopathological factors such as WHO grade (p=0.0001), larger tumor size (=>2.5 cm, p=0.004), tumor spread (pT1-4, p=0.01), presence of lymph node metastasis (pN1/2, p=0.002), presence of distant metastasis (p=0.005), DAXX/ATRX loss (p=0.02) and ALT-positivity (p=0.02) were associated with shorter DFS. In multivariate analysis including the above clinicopathological factors, subgroup D and tumor size were identified as independent poor prognosticators for DFS (p<0.05, Supplementary Table 4). ALT positivity (p=0.007) and DAXX/ATRX loss (p=0.005) were significantly associated with poor outcome in subgroup A2 (p=0.04) but not in other subgroups.

Correlation of MEN1 loss with clinicopathological factors in A1/A2 PanNETs

Among 94 A1 and A2 subgroup PanNETs, 21 PanNETs had MEN1 loss without DAXX/ATRX loss (MEN1 loss only). These tumors were smaller in size (median 1.9 cm) than the other A1/A2 tumors (median 3.0 cm, p<0.0001). The Ki67 index (median 1%) was slightly lower in MEN1 loss only PanNETs than in other A1/A2 PanNETs (median 2.5%), without statistical significance. Patient outcome did not differ between A1/A2 PanNETs with MEN1 loss only and other A1/A2 PanNETs (Supplementary Table 5).

Discussion

Based on the differential expression of four transcription factors, we identified five -subgroups of primary PanNETs in a cohort of 185 patients followed for a mean of 68 months. The four transcription factors included not only ARX and PDX1, as in most other studies subtyping PanNETs, but also ISL1 and CDX2. ISL1 is a transcription factor that binds to an islet-specific enhancer element in the insulin gene. It is expressed in all adult pancreatic neuroendocrine cell types [25]. CDX2 controls the differentiation of the intestinal cells into enterocytes, goblet cells, Paneth cells, and neuroendocrine cells and has been reported in rare serotonin-positive cells in the mouse pancreas [26]. The five transcription factor-subgroups, designated A1, A2, B, C and D, correlated significantly with histological patterns, hormone expression, and patient outcome.

Subgroup A1 with the signature ARX+/ISL1+/PDX1-/CDX2- comprised almost half (46%) of the PanNETs in our cohort, suggesting that they represent the most common neuroendocrine tumors in the pancreas. These tumors were easily recognized by their phenotype. Histologically they were characterized by a typical trabecular reticulated architecture which associated with the expression of glucagon, often accompanied by PP, and frequently with small or large cystic changes, as has been described previously [13]. The trabecular pattern is reminiscent of the reticular arrangement of alpha cells, that may be seen in large pancreatic islets which can be observed irregularly distributed in the normal pancreas or in the islet aggregates that occur in advanced chronic pancreatitis [27]. The PP cells were either intermingled with the glucagon cells or formed separate broad cords, as seen in the so-called PP islets in the ventral lobe of the pancreas in older individuals. The fact that both glucagon and PP cells occur so closely together suggests that their embryological development is related [28] and involved in the origin of these PanNETs.

Subgroup A2 was similar to A1 in that it also showed a trabecular reticulated pattern, cystic changes, and frequent expression of glucagon and PP. However, A2 differed from A1 by the gastrin positivity of some of its tumors and also by positivity for PDX1. There is no ready explanation why predominantly gastrin-positive tumors cluster with glucagon/PP tumors and ARX and PDX1.

A common feature of A1 and A2 was that they frequently showed loss of ATRX/DAXX and MEN1, were positive for ALT, and had survival data similar to those reported for ARX-positive-tumors [3-7]. Because not all A1 and A2 tumors with loss of ATRX/DAXX also had loss of MEN1, we investigated whether A1 and A2 PanNETs with loss of MEN1 but not ATRX/DAXX (MEN1 loss only PanNETs) differed clinicopathologically from those with loss of ATRX/DAXX. We found that the MEN1 loss only PanNETs were smaller in size (median 1 cm) and had a lower Ki67 index (median 1.9%) than the other A1/A2 PanNETs, but no differences were observed in

survival (Supplementary Table 5). Also, regarding the possibility that A2 tumors were more aggressive than A1 tumors, we did not find any differences between A1 and A2 tumors in terms of DAXX/ATRX or ALT status, WHO grade, Ki67, and DFS.

Comparing the data of subgroups A1 and A2 with the main findings of PanNETs identified in recent studies (using genomic, epigenetic, transcriptomic and immunohistochemical methods) [3-7] as tumors with an “alpha cell-like signature” characterized by ARX expression, it is clear that the PanNETs in subgroups A1 and A2 share many similarities with the reported alpha cell-like tumors. In particular, our hormone expression data strongly suggest that the alpha cell-like tumors originate from the alpha cell lineage (which appears to be related to the PP cell lineage).

In contrast to subgroups A1 and A2, PanNETs in subgroup B with the signature ARX-/ISL1+/PDX1+/CDX2- were very similar to tumors with a beta cell-like signature characterized by PDX1 expression, ARX negativity and good prognosis [3-7]. In subgroup B, approximately half (52%) were insulinomas. The remaining tumors in this subgroup consisted of 16 non-functioning PanNETs. They were composed mostly of somatostatin cells but contained a number of other islet cell types such as insulin cells, glucagon, PP, calcitonin, gastrin, and ACTH, usually in a small fraction. The tumors showed a solid histology with a PG-like architecture when expressing somatostatin, as described previously [15]. A previous study reported that multi-hormonality in insulinomas was associated with malignant behavior or large tumor size [21], but these observations could not be confirmed here. Another study reported that metastatic insulinomas were predominantly ARX-positive (ARX+/PDX1+) [29] in contrast to benignly behaving insulinomas. In our cohort, there was one insulinoma with metachronous metastasis that was ARX-negative and PDX1/ISL1-positive and metastasized after 4 years.

The PanNETs in subgroup C, with its near-zero signature (due to very low or absent expression of ARX-/ISL1-/PDX1-/CDX2-), showed no similarity to the alpha- and beta cell-like subtypes, but may belong to a group of tumors intermediate between tumors with alpha or beta-like signatures, as recently described in a study classifying PanNETs on genetic and epigenetic features [12]. The subgroup C tumors showed a predominantly solid histology but were very heterogeneous in terms of hormone expression, with variable expression of serotonin, calcitonin, glucagon, PP, somatostatin, or gastrin. While the diffusely serotonin-positive tumors, including one in subgroup B, remained CDX2 negative, others with focal serotonin expression were labeled for CDX2. This dichotomy for CDX2 expression in serotonin-expressing PanNETs was also demonstrated in three other cohorts of serotonin-producing PanNETs [17-19], which showed that, in contrast to ileal serotonin-producing NETs, CDX2 expression is found in only a fraction of serotonin-positive tumors in the pancreas, suggesting that most pancreatic serotonin tumors may originate from serotonin cells derived from the pancreas [19].

Subgroup D PanNETs - the smallest group in our PanNET cohort - represent a novel subtype in many respects. It was distinguished from all other tumors by consistent CDX2 and PDX1 positivity, in the near absence of ISL1 and ARX expression. Subgroup D PanNETs predominantly showed solid nested histology and various combinations of focal positivity for somatostatin and the ectopic hormones calcitonin, gastrin, serotonin, and ACTH. These tumors had the shortest DFS, although most were ALT negative and rarely mutated for ATRX/DAXX. Interestingly, five of the 10 subgroup D PanNETs were identified in the periampullary region, suggesting a potential cell-of-origin in this specialized area of the pancreas.

Our study has limitations. We did not examine the full panel of known (mainly from mouse studies) pancreatic transcription factors and did not perform epigenetic analysis to allow accurate comparison with epigenetically defined subgroups. Collection of samples with different pre-analytics may artificially affect immunohistochemical detection, but we did not see a clear institution-dependent trend. We also did not see any significant differences in DFS between the patients from Regensburg and Munich in the period between 2013 to 2023 and between 1995 and 2012, respectively. Finally, as the series also includes a few consultation cases, a selection of unusual PanNETs cannot be excluded.

In conclusion, we demonstrated that the combination of ARX, PDX1, ISL1, and CDX2 signatures could discriminate five subgroups of PanNETs with correlation to histology, hormone expression, DAXX/ARTX/MEN1 and ALT status, and outcome. Two subgroups reflected alpha cell-like PanNETs and one subgroup beta cell-like PanNETs. The fourth subgroup with a “zero” signature remained undefined with respect to cell lineage and phenotype. The fifth subgroup with the signature ARX-/ISL1-/PDX1+/CDX2+ is novel. It has a solid histology, is associated with a poor prognosis, and may arise from an endocrine cell with intestinal features near the ampulla and duodenum. Figure 6 shows the relationship of the tumors with respect to transcription factor signatures, cell lineage, frequency, histology, hormone expression and patient outcome. In daily practice, the presented subgrouping of PanNETs may be useful for directing diagnosis, predicting outcome, and guiding treatment.

Ethics approval: The study was approved by our local ethics committee (2022-396-S-DFG-SR).

Author Contributions: E.M., G.K, I.M., A.P., and A.K. performed study concept, design, histological evaluation, and data analysis. U.A., L.V., K.S. and C.M. performed immunohistochemical staining, evaluation. M.E. and B.M. provided material assembling, clinical

information. K.S., M.M., H.F., and A.W. provided clinical data acquisition and interpretation of data. All authors read and approved the final paper.

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Data Availability Statement: The Datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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Tables:

Table 1: Five subgroups of pancreatic neuroendocrine tumors based on the immunohistochemical signatures of the four transcription factors, ARX, ISL1, PDX1 and CDX2

Table 2: Function, histology and hormone expression and status of DAXX/ATRX, ALT and MEN1 expression in five subgroups of pancreatic neuroendocrine tumors

Figure Legends:

Figure 1: Histological and immunohistochemical images of subgroup A1 (A-C) and A2 (D-F) pancreatic neuroendocrine tumors. A) Trabecular reticulated growth pattern, B) a strong and diffuse nuclear expression of ARX and C) ISL1. D) strong glucagon expression and E) expression of PP and F) focal gastrin expression.

Figure 2: Histological and immunohistochemical images of subgroup B pancreatic neuroendocrine tumor. A) Solid growth pattern, B) a strong and diffuse nuclear expression of PDX. C) Expression of insulin and D) somatostatin.

Figure 3: Histological and immunohistochemical images of subgroup C pancreatic neuroendocrine tumor. A) Solid paraganglioma-like growth pattern, B) negative expression of ISL1. C) Expression of calcitonin and D) serotonin.

Figure 4: Histological and immunohistochemical images of subgroup D pancreatic neuroendocrine tumor. A) Solid growth pattern, B) strong nuclear expression of CDX2 and C) PDX1. D) Single cell positivity for ACTH.

Figure 5: Kaplan-Meier survival curves (diease free survival) of 167 patients with a pancreatic neuroendocrine tumor subgrouped by signatures of the four transcription factors ARX, ISL1, PDX1 and CDX2

Figure 6: Relationship of prognosis, transcription factor signatures, histology, hormone expression and cell lineage in five pancreatic neuroendocrine tumor subgroups

Table 1: Five subgroups of pancreatic neuroendocrine tumors (PanNETs) based on the immunohistochemical signatures of the four transcription factors (TFs), ARX, ISL1, PDX1 and CDX2

			A1	A2	B	C	D	p-value
TF	Total PanNETs, N (%)	185 (100)	86 (46)	28 (15)	33 (18)	28 (15)	10 (5)	
ARX	median (25%-75% quartile)	%	100 (90-100)	85 (80-100)	0 (0-0)	0 (0-8)	2.5 (0-38)	p<0.0001
	positive	N (%)	85 (99)	28 (100)	2 (6)	4 (14)	3 (30)	
	negative		1 (1)	0 (0)	31 (94)	24 (86)	7 (70)	
ISL1	median (25%-75% quartile)	%	100 (100-100)	60 (5-90)	90 (5-100)	0 (0-0)	1 (0-16)	p<0.0001
	positive	N (%)	86 (100)	20 (71)	24 (73)	1 (4)	3 (30)	
	negative		0 (0)	8 (29)	9 (27)	27 (96)	7 (70)	
PDX1	median (25%-75% quartile)	%	0 (0-0)	35 (0-78)	100 (100-100)	0 (0-0)	100 (30-100)	p<0.0001
	positive	N (%)	1 (1)	17 (61)	33 (100)	0 (0)	10 (100)	
	negative		85 (99)	11 (39)	0 (0)	28 (100)	0 (0)	
CDX2	median (25%-75% quartile)	%	0 (0-0)	0 (0-4)	0 (0-0)	0 (0-0)	80 (78-100)	p<0.0001
	positive	N (%)	0	5 (18)	3 (9)	0 (0)	10 (100)	
	negative		86 (100)	23 (82)	30 (91)	28 (100)	0 (0)	
Transcription factor signatures			ARX+/ISL+/ PDX1-/CDX2-	ARX+/ISL+/ PDX1+/CDX2-	ARX-/ISL+/ PDX1+/CDX2-	ARX-/ISL-/ PDX1-/CDX2-	ARX-/ISL-/ PDX1+/CDX2+	

Table 2: Function, histology, hormone expression and status of SSTR2, DAXX/ATRX, ALT and MEN1 expression in five subgroups of pancreatic neuroendocrine tumors (PanNETs)

	Subgroups		A1	A2	B	C	D	p-value[A]	p-value[B]
	N (%)		86 (46)	28 (15)	33 (18)	28 (15)	10 (5)		
Function	Non-functioning		84 (98)	28 (100)	16 (48)	28 (100)	9 (90)	0.0001	p = 0.01 B-D, p<0.0001 A1-B, A2-B, B-C
	Insulinoma		0	0	17 (52)	0	0		
	Others		2 (2)[g]	0	0	0	1 (10)[h]		
Histology	Trabecular		70 (81)	15 (54)	6 (18)	6 (21)	0 (0)	<0.0001	p<0.001 A1-B, A1/A2-D, B-D, p< 0.02 C-D, A1-A2
	Solid		16 (18)	13 (46)	27 (82)	22 (79)	10 (100)		
Hormone	Glucagon[a]	negative	28 (33)	15 (54)	18 (56)	20 (71)	7 (70)	0.0003	p<0.05 A1-A2, A1-B, A1-C, A1-D
		positive	58 (67)	13 (46)	14 (44)	8 (29)	3 (30)		
		focal	27	10	12	7	3		
		diffuse	31	3	2	1	0		
	PP	negative	24 (28)	10 (36)	23 (70)	20 (71)	10 (100)	<0.0001	p<0.01 A1-B, A1-C, A1-D, A2-B, A2-C, A2-D
		positive	62 (72)	18 (64)	10 (30)	8 (29)	0 (0)		
		focal	39	15	8	7	0		
		diffuse	23	3	2	1	0		
	Insulin	negative	66 (78)	23 (82)	6 (18)	23 (82)	6 (60)	<0.0001	p<0.001 A1-B, A2-B, B-C, P=0.048 B-D
		positive	19 (22)	5 (18)	27 (82)	5 (18)	4 (40)		
		focal	19	5	6	5	3		
		diffuse	0	0	21	0	1		
	Somatostatin[a]	negative	43 (51)	12 (43)	4 (12)	11 (39)	4 (40)	0.002	p<0.001 A1-B, p=0.01 A2-B, B-C
		positive	42 (49)	16 (57)	29 (88)	17 (61)	6 (60)		
		focal	37	15	23	16	6		
		diffuse	5	1	6	1	0		
	Serotonin	negative	83 (95)	26 (93)	30(94)	20 (71)	6 (60)	0.005	p<0.001 A1-C, A1-D
		positive	4 (5)	2 (7)	2 (6)	8 (29)	4 (40)		
		focal	4	2	1	5	4		
		diffuse	0	0	1	3	0		
	Calcitonin[a]	negative	77 (91)	24 (86)	27 (82)	19 (68)	9 (90)	NS	p<0.01 A1-C
		positive	8 (9)	4 (14)	6 (18)	9 (32)	1 (10)		
		focal	7	4	5	6	1		
		diffuse	1	0	1	3	0		
	ACTH	negative	83 (97)	27 (96)	30 (91)	24 (86)	8 (80)	NS	p<0.05 A1-C, A1-D
		positive	3 (3)	1 (4)	3 (9)	4 (14)	2 (20)		
		focal	3	1	3	4	2		
		diffuse	0	0	0	0	0		
	Gastrin	negative	78 (91)	20 (71)	28 (85)	23 (82)	7 (70)	NS	p=0.01 A1-A2
		positive	8 (9)	8 (29)	5 (15)	5 (18)	3 (30)		
		focal	7	4	5	4	3		
		diffuse	1	4	0	1	0		
SSTR2[b]	negative		1 (1)	0	7 (23)	8 (29)	1 (10)	< 0.0001	p < 0.01 A1/2-B/C
	positive		85 (99)	28 (100)	24 (77)	20 (71)	9 (90)		
ALT[c]	negative		37 (51)	10 (50)	18 (75)	11 (55)	7 (78)	NS	p=0.03 A1-B
	positive		36 (49)	10 (50)	6 (25)	9 (45)	2 (22)		
DAXX/ ATRX[d]	preserved		37 (45)	15 (58)	25 (81)	14 (56)	8 (80)	0.006	p<0.001 A1-B p=0.03 A1-D
	loss		44 (55)	11 (42)	6 (19)	11 (44)	2 (20)		
MEN1[e]	preserved		30 (41)	11 (48)	31 (100)	16 (64)	6 (67)	p<0.0001	p<0.001 A1-B, A2-B, B-C, p<0.05 A1-C
	loss		43 (59)	12 (52)	0	9 (36)	3 (33)		

Footnote: Data missing in a) 1, b) 2, c) 39, d) 12 and e) 24 cases. A: Pearson´s chis-quare test among five subtypes. B: Fisher´s exact text between two subtypes, g: 1 patient with VIPoma and Glucagonoma each, h: 1 patient with Cushing Syndrome