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Alternative Lengthening of Telomeres and Loss of DAXX/ATRX Expression Predicts Metastatic Disease and Poor Survival in Patients with Pancreatic Neuroendocrine Tumors

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Abstract

Purpose: Pancreatic neuroendocrine tumors (PanNET) are a heterogeneous group of neoplasms with increasing incidence and unpredictable behavior. Whole-exome sequencing has identified recurrent mutations in the genes *DAXX* and *ATRX*, which correlate with loss of protein expression and alternative lengthening of telomeres (ALT). Both ALT and DAXX/ATRX loss were initially reported to be associated with a favorable prognosis; however, recent studies suggest the contrary. Our aims were to assess the prevalence and prognostic significance of ALT and DAXX/ATRX in both primary and metastatic PanNETs.

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Disclosure of Potential Conflicts of Interest
A. Slivka is a consultant/advisory board member for Boston Scientific. No potential conflicts of interest were disclosed by the other authors.

Experimental Design: Telomere-specific FISH and DAXX/ATRX IHC was performed on a multi-institutional cohort of 321 patients with resected PanNET and 191 distant metastases from 52 patients. These results were correlated with clinicopathologic features, including disease-free survival (DFS) and disease-specific survival (DSS).

Results: The prevalence of ALT and DAXX/ATRX loss in resected PanNETs was 31% and 26%, respectively, and associated with larger tumor size, higher WHO grade, lymph node metastasis, and distant metastasis ($P < 0.001$). The 5-year DFS and 10-year DSS of patients with ALT-positive and DAXX/ATRX-negative PanNETs were 40% and 50%, respectively, as compared with 96% and 89%, respectively, for wild-type PanNETs. Among distant metastases, ALT and DAXX/ATRX loss was 67% and 52%, respectively, and only occurred in the setting of an ALT-positive and DAXX/ATRX-negative primary PanNET. By multivariate analysis, both ALT and DAXX/ATRX loss were negative, independent prognostic factors for DFS.

Conclusions: ALT and DAXX/ATRX loss in PanNETs was associated with shorter DFS and DSS and likely plays a significant role in driving metastatic disease. Clin Cancer Res; 23(2); 600–9. 2016 AACR.

Introduction

Pancreatic neuroendocrine tumors (PanNETs) are the second most common neoplasms of the pancreas (1). Within the United States, the annual incidence is approximately 1 per 100,000 individuals per year, but autopsy studies have shown a much higher prevalence that ranges from 0.8% to 10% (2–5). Moreover, given the increased accessibility and sensitivity of abdominal imaging techniques, the incidence of PanNETs has steadily increased over the last 30 years (1, 3). The 5-year survival following resection of a PanNET is 65% and the 10-year survival is 45%(5). In addition, >50% of patients will develop metastases. However, although many patients develop infiltrative, widely metastatic disease, others may present with slowly progressive, indolent tumors (1, 5). Therefore, a significant challenge in prognostic stratification and management of patients with Pan-NETs is predicting their biological behavior.

Recent advances in sequencing technologies have uncovered the molecular basis of numerous cancers that has led to new prognostic classification systems and actionable targets. Whole-exome sequencing of PanNETs has identified recurrent mutations in the death domain-associated protein (*DAXX*) and a-thalassemia/mental retardation X-linked (*ATRX*) genes. Jiao and colleagues found 43% of PanNETs harbored mutations in either *DAXX* or *ATRX* (6). Both *DAXX* and *ATRX* encode nuclear proteins that regulate the deposition of histone variant H3.3 during the assembly of pericentromeric and telomeric chromatin (7). Mutations in these genes are associated with loss of nuclear expression of their respective proteins by IHC and correlate with alternative lengthening of telomeres (ALT), a telomerase-independent telomere maintenance mechanism, which can be assayed using telomere specific FISH (8). Interestingly, Jiao and colleagues reported patients with PanNETs containing DAXX/ATRX alterations had an improved overall survival as compared with patients with wild-type tumors. However, the authors did note their patient cohort size was small and required further validation on a larger series. In contrast, Marinoni and colleagues found loss of DAXX/ATRX nuclear expression in PanNETs was associated with metastasis, shorter disease-free survival (DFS), and shorter disease-specific survival (DFS; ref. 9). This discrepancy may be attributed to differences in the patient populations investigated. All of the patients evaluated by Jiao and colleagues had metastatic disease, as opposed to 18% of patients reported by Marinoni and colleagues. But once again, the number of patients with adequate follow-up within the study by Marinoni and colleagues was small and divided in two separate cohorts. In addition, correlative telomere-specific FISH to assess for ALT was performed only on a subset of PanNETs. Moreover, the status of ALT and DAXX/ATRX in metastatic foci in relationship to their corresponding primary PanNET is unknown.

The aims of this study were to (i) identify the prevalence of ALT by telomere-specific FISH and loss of DAXX/ATRX expression by IHC in PanNETs using a large, multi-institutional cohort; (ii) determine the prognostic significance of ALT and DAXX/ATRX loss in PanNETs; and (iii) assess the status of ALT and DAXX/ATRX within paired primary PanNETs and their corresponding distant metastases.

Materials and Methods

Study population

Study approval was obtained from the University of Pittsburgh (IRB# PRO13020493) and Washington University (St. Louis, MO; 201404143) Institutional Review Boards. The surgical pathology archives from the Departments of Pathology at the University of Pittsburgh Medical Center (Pittsburgh, PA) and Barnes-Jewish Hospital (St. Louis, MO) were queried for neuroendocrine neoplasms of the pancreas between 1995 and 2012 that underwent enucleation, central pancreatectomy, pancreati coduodenectomy, or distal pancreatectomy. Cases were cross-referenced with clinical and follow-up data obtained from patient paper and/or electronic medical records. The study inclusion criteria consisted of the following: a solitary, well-differentiated neuroendocrine neoplasm [confirmed with positive immunolabeling for neuroendocrine markers (e.g., synaptophysin and chromogranin A)] centered within the pancreas, surveillance and survival data of >2 years, absence of a genetic syndrome associated with pancreatic neuroendocrine neoplasms (e.g., multiple endocrine neoplasia type 1 syndrome, von Hippel–Lindau syndrome, neurofibromatosis type 1 syndrome, and tuberous sclerosis complex syndrome), and cases with sufficient material for ancillary studies. In total, 321 patients with a resected PanNET fulfilled the aforementioned criteria. In addition, the surgical pathology archives from the respective institutions were cross-referenced to identify corresponding distant metastases with sufficient pathologic material for ancillary studies. Among 93 patients with distant metastases, 52 patients had pathologic material available for telomere FISH and DAXX/ATRX IHC. In total, 191 distant metastases were identified from these 52 patients.

Clinical and demographic data were reviewed for each case. Corresponding pathology gross reports and hematoxylin and eosin–stained slides were also reviewed for the following pathologic features: tumor size, location, lymphovascular invasion, perineural invasion, extension outside of the pancreas, and regional lymph node metastasis. Each PanNET was graded using the 2010 World Health Organization (WHO) classification system for pancreatic neuroendocrine neoplasms (10). Briefly, on the basis of mitotic rate and Ki67

IHC, the following criteria were used: grade 1 (G1), <2 mitoses/10 high-power fields (hpf) and Ki-67 of <3%; grade 2 (G2), 2 to 20 mitoses/10 hpf or Ki67 of 3% to 20%; and grade 3 (G3), >20 mitoses/10 hpf or Ki67 of >20%. The mitotic rate was derived from evaluation of multiple sections in 50 hpf (400, field diameter 0.55 mm^2) and expressed as mitoses/10 hpf. For Ki67, at least 500 neoplastic nuclei were counted in the highest staining region for each case with careful exclusion of nonneoplastic cells (11). A labeling index was calculated and expressed as a percentage. For cases with discordant mitotic rate and Ki67 measurements, the highest grade was assigned. Pathologic primary tumor classification was determined according to the American Joint Committee on Cancer (AJCC) Staging Manual, seventh edition (12). Follow-up information was extracted from the patient's paper and electronic medical records to include data on surveillance, disease recurrence/distant metastasis, and survival.

IHC

Immunohistochemical labeling was performed on 4-mm unstained whole slide sections from formalin-fixed, paraffin-embedded (FFPE) tissue blocks for each PanNET and distant metastases. Slides were deparaffinized with serial xylene treatments and subjected to antigen retrieval using heated citrate solution (pH 9.0) at 100 C for 10 minutes. Immunolabeling for Ki67 (mouse monoclonal, prediluted, Ventana Medical Systems), synaptophysin (rabbit polyclonal, prediluted, Cell Marque), chromogranin A (mouse monoclonal, prediluted, Ventana Medical Systems), DAXX (HPA008736 rabbit polyclonal, dilution 1:50, Sigma Aldrich), and ATRX (HPA001906 rabbit polyclonal, dilution 1:100, Sigma Aldrich) were performed on the automated Ventana Benchmark XT system using the biotin-free Ventana OptiView DAB IHC Detection Kit (Ventana Medical Systems).

Assessment of DAXX and ATRX was done blinded to any patient data, including outcome. Preserved or “positive” expression of DAXX and ATRX was defined as nuclear staining within tumor cells, using stromal cells as a positive internal control (Fig. 1). Loss or “negative” staining was scored in cases where the tumor lacked nuclear immunolabeling, but preserved expression within stromal cells was still identified. Intratu-moral heterogeneity or heterogeneous staining was defined as the clear presence of two distinct populations of tumor cells demonstrating preserved and loss of nuclear staining (Fig. 2). Each component (positive and negative nuclear staining) should comprise at least 10% of the neoplastic tissue. For subsequent statistical analysis, these cases were scored as loss or negative staining.

Tissue microarray construction and FISH

For telomere-specific FISH, high-density tissue microarrays (TMA) were constructed using archival FFPE tissue blocks from both resected PanNETs and distant metastases. Three 1.0 mm- sized cores were punched from representative areas of each tumor and harvested into recipient blocks. TMAs were cut at 4-mm sections. Sections were incubated for 30 minutes at 55 C, washed three times for 5 minutes in xylene, rinsed in successive 100%, 95%, and 70% ethanol baths, and washed in ddH_2O and 1% Tween before being placed in antigen unmasking solution in a boiling steamer for 30 minutes. Next, slides were rinsed in ddH_2O and dehydrated in successive ethanol washes of 70%, 95%, and 100%. Slides were incubated at 72 C for 10 minutes with an Alexa-488 telomeric-C PNA probe and hybridized overnight in a dark humidity chamber. Slides were washed with PNA wash buffer and PBST and incubated for 10 minutes in DAPI solution. After washing in ddH_2O, slides were mounted with prolong anti-fade mounting medium. Images were taken on a Leica fluorescent light microscope (13).

Scoring for ALT was performed by assessing at least 250 nuclei from all three tissue cores for each case (at least 750 tumor nuclei). Using previously described criteria, ALT-positive cases were defined by the presence of large, ultrabright intranuclear foci consistent with telomere FISH signals in at least 1% of tumor nuclei and the total signal intensity for individual foci >10 fold than telomere signals from stromal cells (8, 14–16). Among ALT-positive PanNETs, although a 1% inclusion criterion was used, the percentage of tumor cells that were ALT-positive (percentage of tumor nuclei with large, ultrabright signals) ranged from 5.2% to24.3% (mean, 10.3%; median, 10%). Of note, areas of necrosis were excluded from evaluation. Among ALT-negative PanNETs, no large, ultrabright, intranuclear signals were found in over 750 tumor nuclei that were screened.

Statistical analysis

χ^2 analysis or Fisher exact tests were used to compare categorical data, and ANOVA was used to compare continuous variables. Survival curves were constructed using the Kaplan–Meier method, and differences between groups were evaluated by the log-rank test. DFS was calculated from the date of surgery to the date of first distant metastasis/recurrence after surgery or to the date of last follow-up (in patients without distant metastasis/recurrence) for cases without synchronous distant metastasis. DSS was calculated from the date of surgery to the date of death due to disease or date of last follow-up (if death did not occur). The prognostic significance of clinical and pathologic characteristics was determined using univariate Cox regression analysis. Multivariate analyses of significant risk factors by univariate analysis were performed using Cox proportional hazard regression to identify independent risk factors for both DFS and DSS. All statistical analyses were performed using the SPSS Statistical software, version 22 (IBM), and statistical significance was defined as a P value of <0.05.

Results

Pancreatic neuroendocrine tumor study cohort

The study cohort consisted of 321 patients with a solitary PanNET treated by enucleation ($n = 18$), central pancreatectomy ($n = 15$), pancreaticoduodenectomy ($n = 109$), or distal pancreatectomy ($n = 179$) to include resection of identifiable metastases with curative intent. Patients ranged in age from 29 to 83 years (mean, 59.1 years) with a slight predominance in male gender (171 of 321, 53%). Thirty-six of 321 (11%) patients had a functional PanNET. The tumors were predominantly located within the pancreatic body and tail ($n = 194$, 60%) and ranged in size from 0.6 to 18 cm (mean, 3.4 cm). Although all PanNETs were morphologically well differentiated, on the basis of mitotic rate and Ki-67 proliferation index, PanNETs were classified into the following WHO grades: 185 (58%) grade 1 (G1), 132 (41%) grade 2 (G2), and 4 (1%) grade 3 (G3). Lymphovascular and perineural invasions were identified in 136 (42%) and 95 (30%) tumors, respectively. Using the AJCC prognostic staging system (seventh edition), the PanNETs were classified into the following pathologic tumor (pT) stages: 116 (36%) pT1, 99 (31%) pT2, and 106 (33%) pT3. Regional lymph nodes were submitted for histologic evaluation in 268 (83%) cases with involvement of 100 (of 268, 37%) cases. At the time of surgery, 51 (16%) patients were found to have synchronous distant metastases that were resected. Of the remaining 270 patients, metachronous distant metastases were identified in 42 (of 270, 16%) cases. The DFS rates for these 270 patients were 91% at 3 years and 86% at 5 years. For all 321 patients, the DSS rates were 91% at 5 years and 87% at 10 years.

Telomere-specific FISH and DAXX/ATRX IHC

The results of telomere-specific FISH for ALT and IHC for DAXX and ATRX are summarized in Table 1. Among 321 resected PanNETs, ALT was detected in 98 (31%) cases. Loss of nuclear expression for DAXX, ATRX, or both was identified in 39 (12%), 30 (9%), and 15 (5%) PanNETs, respectively (Fig. 1). Heterogeneous loss of expression was seen in 1 DAXX-negative and 3 ATRX-negative tumors (Fig. 2). While ALT correlated with DAXX/ATRX loss, 14 (6%) ALT-positive PanNETs had preserved expression for DAXX/ATRX. ALT-positive PanNETs were associated with a predilection for male patients (P 0.011), larger mean tumor size ($P < 0.001$), lack of functionality ($P = 0.002$), higher WHO grade (P < 0.001), lymphovascular invasion ($P < 0.001$), peri-neural invasion ($P < 0.001$), higher pathologic tumor (pT) stage (P < 0.001), regional lymph node (pN) metastasis ($P < 0.001$), synchronous distant metastasis ($P < 0.001$), and postoperative metachronous distant metastasis ($P < 0.001$). There was no statistically significant difference between ALT status and mean patient age ($P = 0.195$) or tumor location ($P = 0.300$). Theclinicopathologic characteristics of DAXX/ATRX-negative Pan-NETs were nearly identical to PanNETs with ALT.

Prognostic significance of ALT and loss of DAXX/ATRX expression in primary PanNETs

Patients whose tumors demonstrated ALT had shorter DFS and DSS. Among ALT-positive PanNETs, the DFS rates were 63% at 3 years and 40% at 5 years. The DSS rates were 81% at 5 years and 50% at 10 years. In comparison, patients with ALT-negative PanNETs had significantly longer DFS (99% at 3 years and 96% at 5 years; $P < 0.001$) and better DSS (93% at 5 years and 89% at 10 years, $P < 0.001$) rates (Fig. 3). No statistically significant differences in DFS and DSS between ALT-positive PanNETs and DAXX/ATRX-negative PanNETs were identified.

Results from univariate Cox regression analysis for DFS and DSS in relation to various clinicopathologic features, including ALT status are shown in Table 2. Shorter DFS and poor DSS were associated with tumor size >2.0 cm ($P < 0.001$ and $P < 0.001$), G2 to G3 WHO grade ($P < 0.001$ and $P < 0.001$, respectively), lymphovascular invasion ($P < 0.001$ and $P < 0.001$, respectively), perineural invasion ($P < 0.001$ and $P < 0.001$, respectively), advanced tumor stage ($P < 0.001$ and $P < 0.001$, respectively), lymph node metastasis ($P < 0.001$ and $P < 0.001$, respectively), and ALT ($P < 0.001$ and $P < 0.001$, respectively). Age also correlated with shorter DFS ($P = 0.006$), but not DSS (P =0.063). Multivariate analysis was used to determine the prognostic significance of ALT for DFS and DSS and included tumor size

>2.0 cm, WHO grade, and regional lymph node (pN) metastasis. Although ALT was an independent prognostic factor for DFS (HR = 7.12, $P < 0.001$), it was not for DSS (HR = 1.35, $P = 0.388$; Table 2). Similar results were seen with loss of DAXX/ATRX expression when substituted for ALT.

Assessment and correlation of ALT and loss of DAXX/ATRX expression in distant metastases

Considering ALT and loss of DAXX/ATRX expression correlated with the development of distant metastases, the status of ALT and DAXX/ATRX was assessed in paired resected primary and distant metastases from 52 patients (Table 3) within the study cohort that had sufficient pathologic material for further ancillary studies. Twenty-eight (54%) patients had synchronous metastases, 15 (29%) had metachronous metastases, and 9 (17%) had both. In total, 191 distant metastases were evaluated and consisted of 111 (58%) synchronous and 80 (42%) metachronous metastases. The sites of metastases varied widely and included 167 (87%) liver, 14 (7%) nonregional lymph nodes, 3 (2%) diaphragm, 2 (1%) omentum, 1 remnant pancreas, 1 small bowel serosa, 1 ovary, 1 adrenal gland, and 1 epidural space. ALT and loss of DAXX/ATRX expression was detected in 35 (67%) and 27 (52%) of patients with metastatic PanNETs, respectively (Supplementary Fig. S1). No differences in the status of ALT and DAXX/ATRX expression were found among metastatic PanNETs from the same patient, regardless of whether they were synchronous and/or metachronous metastases. A comparison of ALT and DAXX/ATRX status between metastases and corresponding primary PanNET from the same patient identified 4 discordant cases. In these 4 cases, the primary PanNET was ALT negative and had preserved expression for DAXX/ATRX, while the metastases were ALT positive and had loss of DAXX and/or ATRX. However, evaluation of DAXX/ATRX IHC and telomere FISH using additional sections of the patient’s primary PanNET revealed heterogeneous ALT positivity and DAXX/ATRX loss (Fig. 2).

Similar to their primary counterparts, ALT-positive metastatic PanNETs were associated with a predilection for male patients ($P = 0.038$), but no statistically significant difference between ALT status and mean patient age ($P = 0.921$), mean primary tumor size ($P = 0.294$), primary tumor WHO grade ($P = 1.000$), metastatic tumor(s) WHO grade (P 0.204), chronologic presentation with respect to the patient’s primary PanNET (synchronous vs. meta-chronous vs. both; $P = 0.051$), and metastatic site ($P = 0.932$). DAXX/ATRX-negative metastatic PanNETs had essentially identical clinicopathologic features as ALT-positive metastatic PanNETs. In addition, no differences in patient DSS rates were detected with respect to ALT and DAXX/ATRX status as assessed from the time after resection of the patient’s primary PanNET (Supplementary Fig. S2), but the number of cases within this cohort may be too small to be conclusive.

Discussion

The activation of a telomere maintenance mechanism is a central hallmark of human cancers (17). While the majority of cancers rely on the reverse transcriptase telomerase, a significant proportion of neoplasms maintain their telomere lengths utiliz ing the homologous recombination–based mechanism, known as ALT (18). A characteristic finding of ALT is the accumulation of large amounts of telomeric DNA, which is the basis for the telomere-specific FISH assay. Loss of nuclear expression for DAXX and ATRX coincides with ALT, and thus, both proteins are considered to be suppressors of ALT (8).

Within our study, the prevalence of ALT and loss of DAXX/ATRX expression in resected, primary PanNETs was 31% and 26%, respectively. All DAXX/ATRX-negative PanNETs were ALT positive, but 14% of ALT-positive cases had preserved expression for DAXX/ATRX. This finding suggests the presence of other suppressors of ALT in PanNETs. We also found ALT and loss of DAXX/ATRX expression can have a heterogeneous distribution within PanNETs. ALT and DAXX/ATRX loss was associated with larger tumor size, advanced pathologic tumor stage, regional lymph node metastasis, and distant metastasis. Moreover, the prevalence of ALT and DAXX/ATRX loss in metastatic PanNETs was 2-fold higher than in primary PanNETs. Considering de Wilde and colleagues reported the absence of ALT in pancreatic neuroendocrine microadenomas, our observations would support that ALT and loss of DAXX/ATRX expression are late events in the pathogenesis of PanNETs (14). In addition, ALT and DAXX/ATRX loss within distant metastases correlated with ALT and DAXX/ATRX loss within the corresponding primary PanNET. In many of these patients, only a small subpopulation of neoplastic cells was ALT positive and DAXX/ATRX negative. Furthermore, no differences in the status of ALT and DAXX/ATRX were identified among meta-static PanNETs from the same patient, regardless of whether they were synchronous or metachronous distant metastases. On the basis of these findings, although ALT and loss of DAXX/ATRX expression in PanNETs are late events, they occur prior to the development of metastatic disease and likely play a significant role in driving tumor metastasis.

In addition to homologous recombination–mediated telomere maintenance, ALT-positive tumors, including PanNETs, are characterized by complex karyotypes with extensive numerical and structural chromosomal instability (19–22). These chromosomal alterations are often clustered at specific genomic sites (19). It is plausible that ALT may lead to secondary deletions in tumor suppressor genes and oncogenic gains or rearrangements, which can potentiate metastasis formation. Extensive epigenetic modifications are also a feature of ALT-positive tumors. Both DAXX and ATRX are components of a heterochromatic/chromatin remodeling complex involved in the deposition of histone H3.3 to nucleosomes at pericentromeric and telomeric regions (7, 23). Histone H3.3 enrichment coincides with histone methylation and transcriptional repression at these sites (24–26). Therefore, loss of DAXX/ATRX could result in transcriptional activation of putative oncogenes involved in metastatic spread.

Regardless of their role in tumor metastasis, the prognostic and therapeutic implications of ALT and loss of DAXX/ATRX expression in PanNETs should be underscored. Currently, the WHO recommends classification of neuroendocrine neoplasms into 3 grades (G1, G2, and G3), based on proliferative index using mitotic rate and Ki67 immunolabeling (10). The WHO grade of a neuroendocrine neoplasm provides important prognostic information that is independent of tumor stage (27–29). PanNETs are typically categorized as G1 or G2, whereas G3 neoplasms are synonymous with poorly differentiated neuroendocrine carcinomas (PanNEC). PanNETs and PanNECs are distinct neoplasms that differ in their etiology, genetics, treatment, and outcome, and therefore, the accurate measurement of mitotic rate and Ki67 immunolabeling is critical (6, 30, 31). However, scoring mitotic figures suffers from poor interobserver reproducibility and is time consuming in high volume centers (32–34). Ki67 staining is also labor intensive and less reflective of the true proliferation index because it not only stains neoplastic cells within the M phase, but those in S, G_1, and G_2 phases of the cell cycle as well (35, 36). In addition, it has become increasingly recognized that G3 pancreatic neuroendocrine neoplasms not only include PanNECs, but also PanNETs (37, 38). Considering G3 PanNETs in the absence of a G1 or G2 component can be morphologically indistinguishable from PanNECs, WHO grade alone is insufficient for disease assessment in pancreatic neuroendocrine neoplasms (38).

In addition to the quantification of mitotic rate and Ki67, telomere-specific FISH for ALT and DAXX/ATRX IHC represent useful adjunct tests to the assessment of PanNETs. Consistent with the results published by Marinoni and colleagues, the presence of large, ultrabright telomere FISH signals indicative of ALT and loss of DAXX/ATRX expression in PanNETs within our study cohort correlated with shorter DFS and DSS. The 5-year DFS and 10-year DSS of patients with ALT-positive and DAXX/ATRX-negative PanNETs were 40% and 50%, respectively, as compared with 96% and 89%, respectively, among patients with ALT-negative and DAXX/ATRX-positive PanNETs. In multivariate analysis, ALT and DAXX/ATRX loss were negative, independent prognostic factors for DFS. Moreover, as opposed to other prognostic parameters and markers, ALT and DAXX/ATRX status reflects the underlying molecular pathogenesis of these neoplasms. Yachida and colleagues performed comparative molecular and immunohistochemical analysis of PanNETs and PanNECs and demonstrated preserved expression for DAXX/ATRX in PanNECs, while loss in a subset of PanNETs (30). However, in contrast to Ki67, the presence of ALT and loss of DAXX/ATRX was not an independent prognostic factor for DSS. Thus, the assessment of ALT and DAXX/ATRX expression in conjunction with WHO grading can further refine current prognostic classification of pancreatic neuroendocrine neoplasms and, in cases of G3 neuroendocrine neoplasms, improve selection of appropriate treatment. In fact, based on the results reported herein and those by Marinoni and colleagues, DAXX/ATRX IHC has been integrated into the routine evaluation of resected pancreatic neuroendocrine neoplasms at our institution.

Nonetheless, the current study is not without limitations. It is retrospective by design and not all patients received the same form of treatment. While a pancreaticoduodenectomy or distal pancreatectomy was performed in the majority of cases, 9% of patients underwent an enucleation or central pancreatectomy, and thus, regional lymph node sampling may be inadequate. Removing these patients from our analysis would have little impact on the statistical associations and prognostic findings of ALT and DAXX/ATRX loss. Of note, enucleation and central pancreatectomy procedures are typically done in the setting of small PanNETs (≤ 2.0 cm) because these tumors often have anindolent natural history (39). However, studies including an analysis of the SEER database suggest that a subset of small PanNETs can pursue a more aggressive course (40–42). In fact, within our study cohort, 7% of PanNETs that measured 2.0 cm in size were ALT positive and showed loss of DAXX/ATRX expression. Although further studies are required, the identification of ALT and DAXX/ATRX loss in preoperative biopsies could indicate an increased risk of developing metastatic disease and, in turn, prompt a change in surgical management to ensure complete regional lymph node dissection. Another point of contention is that *DAXX* and *ATRX*, within this study, were evaluated by IHC rather than mutational analysis. In many scenarios, protein expression does not accurately mirror the status of the corresponding gene. Heaphy and colleagues demonstrated a strong correlation between *DAXX/ATRX* mutations and loss of DAXX/ATRX protein expression (8). Furthermore, DAXX/ATRX loss can occur in the absence of detectable genetic alterations, suggesting the presence of other inhibitory mechanisms, such as promoter methylation, and thus, the assessment of protein expression is likely the ideal method of evaluating these genes (8). Regardless, telomere-specific FISH for ALT was analyzed in all cases.

In summary, we report the comprehensive assessment of ALT and DAXX/ATRX status in a large, multi-institutional cohort of primary and metastatic PanNETs. Patients with ALT-positive and DAXX/ATRX-negative PanNETs had shorter DFS and DSS. In addition, ALT and loss of DAXX/ATRX expression is a negative, independent prognostic factor for DFS. Furthermore, based on our analysis of paired primary PanNETs and their corresponding distant metastases, ALT and DAXX/ATRX loss are late events in the pathogenesis of PanNETs and occur prior to the development of metastatic disease. Although further studies are required, ALT and loss of DAXX/ATRX expression likely play a significant role in driving distant metastases in patients with PanNETs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

A significant challenge in the management of pancreatic neuroendocrine tumors (PanNET) is predicting their behavior. Clinicopathologic grading and staging systems and biomarker development for PanNETs have evolved considerably over the past few decades, but for a subset of cases, may be subjective in interpretation and may not take into account the underlying biology of PanNETs. Whole-exome studies have identified recurrent mutations in the genes *DAXX* and *ATRX*. Mutations in these genes correlate with loss of protein expression by IHC and alternative lengthening of telomeres (ALT) by telomerespecific FISH. Both ALT and DAXX/ATRX loss in PanNETs are associated with shorter DFS and DSS. Therefore, telomerespecific FISH for ALT and DAXX/ATRX IHC is a useful adjunct to current prognostic classification systems and reflects the biological behavior of these neoplasms. As a result of this study, DAXX/ATRX IHC has been integrated into the routine evaluation of resected pancreatic neuroendocrine neoplasms at our institution.

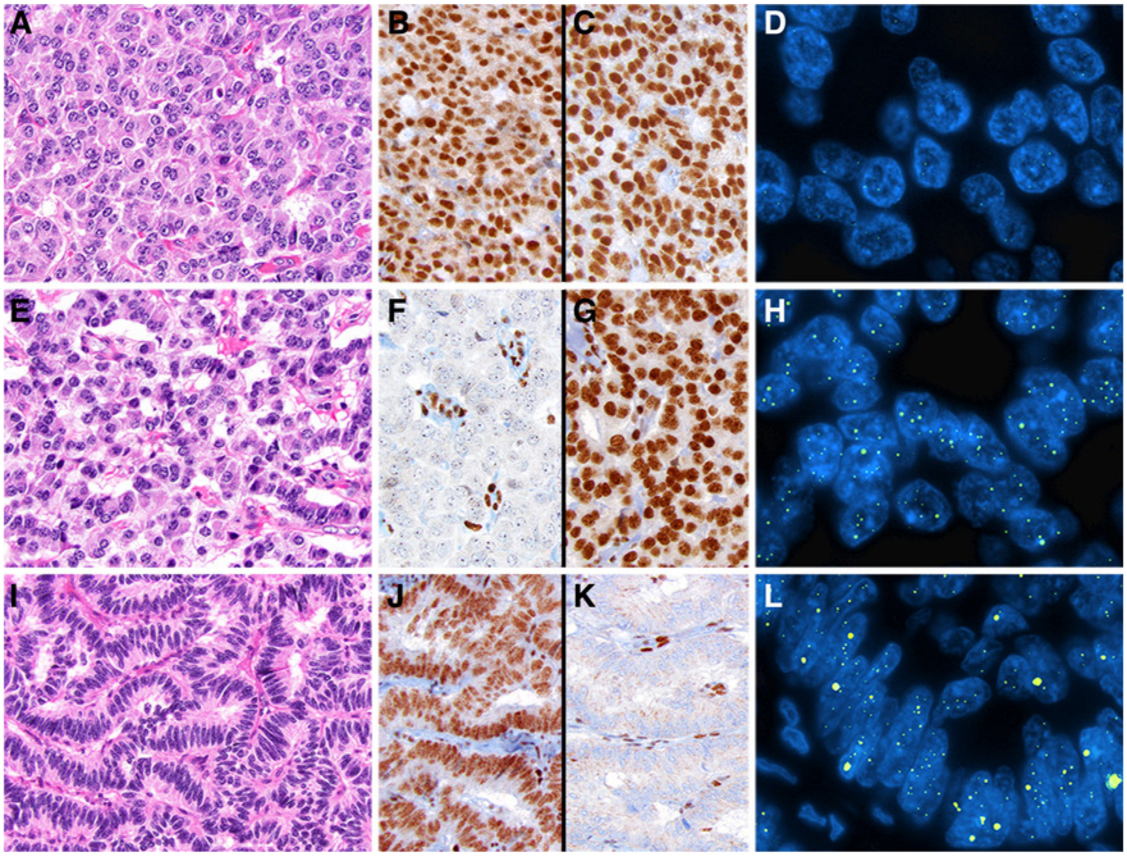

Figure 1.

Representative examples of PanNETs assessed by DAXX and ATRX IHC and telomere-specific FISH. **A**, PanNET with preserved nuclear expression for both DAXX (**B**) and ATRX (**C**) and absence of the ALT phenotype (**D**). E, PanNET with DAXX loss (**F**), but preserved expression for ATRX (**G**). The loss of DAXX expression correlated with the presence of large, ultrabright intranuclear foci by telomere-specific FISH, consistent with ALT (**H**). I, PanNET with preserved expression for DAXX (**J**), but ATRX loss (**K**) and ALT positive (**L**) by telomere-specific FISH.

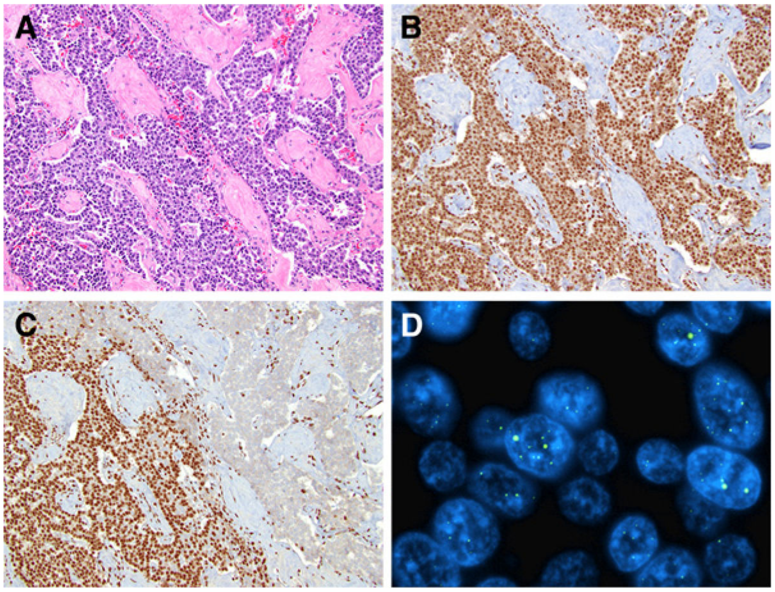

Figure 2.

Intratumoral heterogeneity for DAXX/ATRX protein expression and ALT in PanNETs. **A**, PanNET with preserved nuclear expression for DAXX (**B**), but ATRX loss (**C**, top right) within a subpopulation of neoplastic cells. Within areas of ATRX loss, telomere-specific FISH revealed large, bright signals consistent with ALT (**D**).

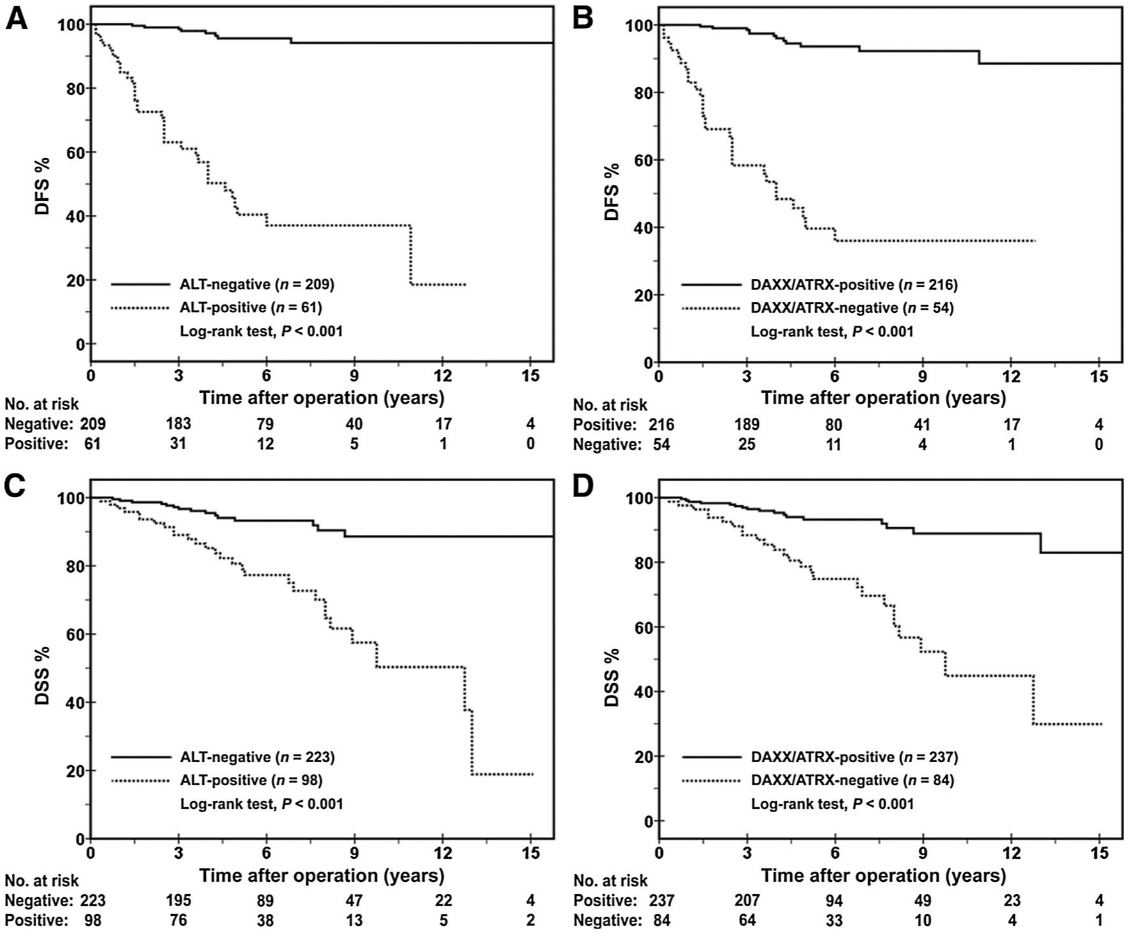

Figure 3.

Kaplan–Meier curves comparing the cumulative probabilities of DFS and DSS after surgical resection among PanNET patients with respect to ALT and DAXX/ATRX status. Patients with ALT-positive and DAXX/ATRX-negative PanNETs were associated with shorter DFS (**A**, **B**) and shorter DSS (**C**, **D**), as compared with patients with ALT-negative and DAXX/ATRX-positive PanNETs.

Table 1.

Clinical and pathologic comparison of ALT and DAXX/ATRX status in PanNETs

Patient or tumor characteristics	**ALT-positive, *n* = 98 (31%)**	**ALT-negative, *n* = 223 (69%)**	***P***	**DAXX/ATRX-negative, *n* = 84 (26%)**	**DAXX/ATRX-positive, *n* = 237 (74%)**	***P***
Gender						
Female	35 (35%)	108 (51%)	0.011[a]	29 (35%)	121 (51%)	0.011[a]
Male	63 (65%)	115 (49%)		55 (65%)	116 (49%)	
Mean age (range), years	60.5 (31–85)	58.6 (29–83)	0.195	61.4 (31–85)	58.4 (29–83)	0.050
Mean tumor size (range), cm	5.0 (1.0–15.0)	2.8 (0.6–18.0)	<0.001[a]	5.0 (1.0–15.0)	2.8 (0.6–18.0)	<0.001[a]
Functional						
No	95 (97%)	190 (85%)	0.002[a]	81 (96%)	204 (86%)	0.008[a]
Yes	3 (3%)	33 (15%)		3 (4%)	33 (14%)	
Location						
Head and uncinate	37 (38%)	90 (40%)	0.300	29 (35%)	98 (41%)	0.300
Body and tail	61 (62%)	133 (60%)		55 (65%)	139 (59%)	
WHO grade						
Low (G1)	28 (29%)	157 (70%)	<0.001[a]	25 (30%)	160 (68%)	<0.001[a]
Intermediate (G2)	66 (67%)	66 (30%)		56 (66%)	76 (32%)	
High (G3)	4 (4%)	0 (0%)		3 (4%)	1	
Lymphovascular invasion						
Absent	22 (22%)	163 (73%)	<0.001[a]	20 (24%)	165 (70%)	<0.001[a]
Present	76 (78%)	60 (27%)		64 (76%)	72 (30%)	
Perineural invasion						
Absent	55 (56%)	185 (83%)	<0.001[a]	35 (42%)	191 (81%)	<0.001[a]
Present	43 (44%)	38 (17%)		49 (58%)	46 (19%)	
Primary tumor (pT) stage						
T1	6 (6%)	110 (49%)	<0.001[a]	6 (7%)	110 (46%)	<0.001[a]
T2	28 (29%)	71 (32%)		26 (31%)	73 (31%)	
T3	64 (65%)	42 (19%)		52 (62%)	54 (23%)	
Regional node (pN) stage	*n = 96*	*n = 172*		*n = 83*	*n = 185*	

Patient or tumor characteristics	ALT-positive, *n* = 98 (31%)	ALT-negative, *n* = 223 (69%)	*P*	DAXX/ATRX-negative, *n* = 84 (26%)	DAXX/ATRX-positive, *n* = 237 (74%)	*P*
N0	39 (41%)	129 (75%)	<0.001[a]	32 (39%)	136 (74%)	<0.001[a]
N1	57 (59%)	43 (25%)		51 (61%)	49 (26%)	
Synchronous metastases						
Absent	61 (62%)	209 (94%)	<0.001[a]	54 (64%)	216 (91%)	<0.001[a]
Present	37 (38%)	14 (6%)		30 (36%)	21 (9%)	
Metachronous metastases	*n* = *61*	*n* = *209*		*n* = *54*	*n* = *216*	
Absent	28 (46%)	200 (96%)	<0.001[a]	25(46%)	203 (94%)	<0.001[a]
Present	33 (54%)	9 (4%)		29 (54%)	13 (6%)	
ALT						
Negative				0 (0%)	223 (94%)	<0.001[a]
Positive				84 (100%)	14 (6%)	

[a] Indicates that the value in question is statistically significantly better than the relevant control, where significance is defined by $P < 0.05$.

Table 2.

Univariate and multivariate Cox regression analysis for DFS and DSS

	Univariate Cox regression analysis				Multivariate Cox regression analysis			
Patient or tumor characteristics	**DFS HR (95% CI)**	***P***	**DSS HR (95% CI)**	***P***	**DFS HR (95% CI)**	***P***	**DSS HR (95% CI)**	***P***
Gender, male vs. female	1.12 (0.61–2.05)	0.726	1.03 (0.57–1.88)	0.914				
Age, years	1.04 (1.01–1.06)[a]	0.006[a]	1.02 (0.99–1.05)	0.063				
Functional vs. nonfunctional	0.17 (0.02–1.26)	0.084	0.58 (0.18–1.88)	0.367				
Location, head and uncinate vs. body and tail	0.45 (0.17–1.16)	0.098	0.57 (0.31–1.03)	0.062				
Tumor size, >2.0 cm vs. ≤2.0 cm	36.47 (5.02–265.24)[a]	<0.001[a]	26.84 (3.69–195.03)[a]	<0.001[a]	11.60 (1.57–85.89)[a]	0.016[a]	8.51 (1.15–63.15)[a]	0.036[a]
WHO grade, G2 or G3 vs. G1	4.69 (2.45–8.97)[a]	<0.001[a]	5.75 (2.86–11.54)[a]	<0.001[a]	1.65 (0.80–3.41)	0.175	2.87 (1.34–6.16)[a]	0.007[a]
Lymphovascular invasion, presence vs. absence	6.97 (3.53–13.75)[a]	<0.001[a]	12.42 (5.13–30.05)[a]	<0.001[a]				
Perineural invasion, presence vs. absence	5.99 (3.21–11.16)[a]	<0.001[a]	4.75 (2.58–8.75)[a]	<0.001[a]				
Tumor stage (pT), pT3 vs. pT1 and pT2	5.62 (3.04–10.40)[a]	<0.001[a]	7.87 (3.92–15.82)[a]	<0.001[a]				
Lymph node metastasis (pN), pN1 vs. pNO	6.35 (3.25–12.42)[a]	<0.001[a]	5.86 (2.95–11.64)[a]	<0.001[a]	2.49 (1.24–5.00)[a]	0.010[a]	3.09 (1.51–6.33)[a]	0.002[a]
ALT, positive vs. negative	20.55 (9.45–44.66)[a]	<0.001[a]	4.76 (2.53–8.94)[a]	<0.001[a]	7.12 (3.06–16.56)[a]	<0.001[a]	1.35 (0.68–2.66)	0.388

[a] Indicates that the value in question is statistically significantly better than the relevant control, where significance is defined by $P < 0.05$.

Table 3.

Clinical and pathologic comparison of metastatic PanNETs with respect to ALT and DAXX/ATRX status

Patient or tumor characteristics	ALT-positive, *n* = 35 (67%)	ALT-negative, *n* = 17 (33%)	*P*	DAXX/ATRX-negative, *n* = 27 (52%)	DAXX/ATRX-positive, *n* = 25 (48%)	*P*
Gender						
Female	13 (37%)	12 (71%)	0.038[a]	8 (30%)	17 (68%)	0.012[a]
Male	22 (63%)	5 (29%)		19 (70%)	8 (32%)	
Mean age at initial presentation (range), years	58.2 (31–82)	57.8 (32–77)	0.921	58.3 (31–82)	57.8 (32–77)	0.874
Mean primary tumor size (range), cm	6.3 (1.0–18)	5.2 (1.3–17.0)	0.294	6.0 (1.0–15.0)	5.9 (1.3–18.0)	0.952
Primary tumor, WHO grade						
Low (G1)	10 (29%)	4 (24%)	1.000	6 (22%)	8 (32%)	0.536
Intermediate (G2)	25 (71%)	13 (76%)		21 (78%)	17 (68%)	
Metastatic tumor(s), highest WHO grade						
Low (G1)	2 (6%)	4 (24%)	0.204	0	6 (24%)	0.030[a]
Intermediate (G2)	26 (74%)	10 (59%)		21 (78%)	15 (60%)	
High (G3)	7 (20%)	3 (17%)		6 (22%)	4 (16%)	
Primary tumor, ALT status						
Positive	35 (100%)[b]	0	<0.001[a]	27 (100%)[b]	8 (32%)	<0.001[a]
Negative	0	17 (100%)		0	17 (68%)	
Primary tumor, DAXX/ATRX status						
Negative	27 (77%)[b]	0	<0.001[a]	27 (100%)[b]	0	<0.001[a]
Positive	8 (23%)	17 (100%)		0	25 (100%)	
Timing of metastatic tumor(s)						
Synchronous	16 (46%)	12 (71%)	0.051	14 (52%)	14 (56%)	0.651
Metachronous	10 (29%)	5 (29%)		7 (26%)	8 (32%)	
Both	9 (25%)	0		6 (22%)	3 (12%)	
Metastatic sites	*n* = *135*	*n* = *56*		*n* = *108*	*n* = *83*	
Liver	114 (84%)	53 (94%)	0.932	89 (82%)	78 (94%)	0.719
Nonregional lymph node	12 (9%)	2 (4%)		10 (9%)	4 (5%)	

Patient or tumor characteristics	ALT-positive, *n* = 35 (67%)	ALT-negative, *n* = 17 (33%)	*P*	DAXX/ATRX-negative, *n* = 27 (52%)	DAXX/ATRX-positive, *n* = 25 (48%)	*P*
Diaphragm	2 (2%)	1 (2%)		2 (2%)	1 (1%)	
Omentum	2 (2%)	0		2 (2%)	0	
Remnant pancreas	1 (1%)	0		1 (1%)	0	
Small-bowel serosa	1 0%)	0		1 (1%)	0	
Ovary	1 (1%)	0		1 (1%)	0	
Adrenal gland	1 (1%)	0		1 (1%)	0	
Epidural space	1 (1%)	0		1 (1%)	0	

[a] Indicates that the value in question is statistically significantly better than the relevant control, where significance is defined by $P < 0.05$.

[b] For 4 PanNETs, a representative tumor section for each case was ALT negative and DAXX/ATRX positive; however, staining of additional tumor sections identified a small subclone that was both ALT positive and DAXX/ATRX negative.