Although pancreatic neuroendocrine tumors (PanNETs) are rare neoplasms, this tumor type represents the second most common pancreatic malignancy and is gradually increasing in incidence. Representing a clinically challenging disease, PanNETs encompass a heterogeneous spectrum of tumors that vary in clinical presentation, natural history, and prognosis. For example, some patients present with a slow growing, indolent tumor, whereas other patients will ultimately develop extensive metastatic disease. Although the overall 5-year relative survival rate is 54% for all patients, this rate varies dramatically with the Surveillance, Epidemiology, and End Results stage of localized, regional, or distant disease (93%, 77%, and 25%, respectively).

Prognostic biomarkers routinely used for PanNETs are tumor size and stage. In addition, the World Health Organization (WHO) suggests grading of PanNETs based on proliferative activity. However, measurements of the mitotic index and Ki-67 on small biopsies and cytology specimens are susceptible to sampling issues and interpretation errors and may not truly reflect the clinical behavior of these tumors. Because of the association with metastatic disease, the current recommendation is to surgically resect PanNETs > 2.0 cm in size with negative margins together with regional lymphadenectomy. Conversely, the potential overtreatment of patients diagnosed with a PanNET has been debated, and a surveillance approach may be warranted for a subset of patients with small tumors (≤2.0 cm in size). Importantly, although current prognostic stratification and clinical management still remain imprecise, recent discovery and validation studies have highlighted promising new prognostic biomarkers for this disease.

Germ-line mutations in the \textit{MEN1} gene, which encodes for the tumor suppressor \textit{menin}, predispose patients to develop multiple endocrine neoplasia type 1 syndrome, an autosomal dominant syndrome that initiates tumor development involving the islet cells of the pancreas, pituitary gland, and parathyroid gland. Somatic \textit{MEN1} mutations are also commonly observed in sporadic PanNETs. In addition to mutations in \textit{MEN1}, whole exome and genome sequencing studies of PanNETs have identified frequent, inactivating somatic mutations in 2 chromatin remodeling genes: \textit{α}-thalassemia mental retardation, X-linked (\textit{ATRX}) and death domain-associated protein 6 (\textit{DAXX}). These somatic mutations are mutually exclusive and strongly correlate with the alternative lengthening of telomeres (ALT) pathway, a telomerase-independent telomere maintenance mechanism. ATRX and DAXX form a histone chaperone complex that functions to deposit the histone variant H3.3 in heterochromatic regions of chromosomes containing highly repetitive elements, particularly pericentromeric and telomeric regions. Because of altered chromatin dynamics and telomere deprotection that lead to increased DNA damage and replicative stress, ALT-positive cancers maintain telomere lengths through a homology-directed DNA repair mechanism. Additional recurrent somatic mutations occur in varying degrees in the mTOR pathway (\textit{TSC2, PTEN}, and \textit{PIK3CA}), in the DNA damage repair pathway (\textit{MUTYH, CHEK2, and BRCA2}), and in chromatin remodeling (\textit{SETD2}). More recently, whole transcriptomic and epigenetic landscape profiling has revealed that differential gene or methylation expression profiles can separate different PanNET subtypes.

In particular, some studies have identified subtypes that partially resemble normal islet α cells or β cells and thereby potentially represent distinct cells of origin or highlight the ability of these tumors to transdifferentiate into distinct cellular states. These different subtypes can be represented by differential gene expression in transcription factors, for example, aristaless-related homeobox gene (\textit{ARX}) and pancreatic and duodenal homeobox 1 (\textit{PDX1}). Early sequencing studies identified an increased frequency of \textit{MEN1, DAXX, ATRX}, and mTOR pathway gene alterations in PanNETs; 43% of PanNETs harbored inactivating mutations in \textit{DAXX} or \textit{ATRX}. Since then, numerous groups have independently validated the prognostic significance of ALT and ATRX/DAXX nuclear protein loss in large cohorts of primary PanNETs. Overall, the presence of ALT and/or ATRX/DAXX loss in primary PanNETs is independently associated with aggressive clinicopathologic behavior and reduced recurrence-free survival; this emphasizes the significant roles that these alterations play in driving metastatic disease.
Although the use of next-generation sequencing (NGS) can allow for the identification of ATRX and DAXX inactivating mutations, along with an assessment of alterations in a larger panel of genes, alternative methods have been developed to identify ATRX/DAXX alterations and ALT activation. ALT can be assessed with a robust telomere-specific fluorescence in situ hybridization (FISH) assay using a fluorescently labeled peptide nucleic acid probe complementary to the guanine-rich telomere repeat sequence, and ATRX/DAXX nuclear protein loss can be easily assessed by immunohistochemistry (IHC). Characteristics of ALT-positive cancers include dramatic cell-to-cell telomere length heterogeneity and the presence of large, ultrabright nuclear foci of telomeric FISH signals marking ALT-associated telomeric DNA in interphase nuclei. Therefore, ALT-positive cases can be visually assessed and classified as ALT-positive if these large, ultrabright intranuclear foci are present in at least 1% of cancer nuclei. Immunolabeling for ATRX and DAXX can be considered preserved if the cancer cells retain nuclear staining. Conversely, cases can be considered negative if nuclear expression is completely lost (even despite retention of cytoplasmic expression) and adequate internal positive controls are present.

Although ATRX and DAXX expression is altered by inactivating gene mutations, PDX1 and ARX expression has been determined through gene expression studies. PDX1 promoter hypermethylation is associated with decreased PDX1 expression.\textsuperscript{17,26} Recent studies have found that nuclear protein expression of PDX1 or ARX, as assessed by IHC, can also determine the risk of metastatic disease.\textsuperscript{18,19,27} In general, PDX1 expression is associated with indolent clinical behavior, whereas the expression of ARX or the lack of both proteins correlates with an aggressive disease course. The status of these transcription factors in PanNETs has recently been reported to be a prognostic biomarker for recurrence-free survival that is independent of tumor size, WHO grade, and ALT.\textsuperscript{19}

Because of the importance of validating these prognostic biomarkers, an international, multi-institutional cohort of 561 nonsyndromic and nonfunctional PanNETs without distant metastases at surgical resection, including 196 cases that were \(\leq 2.0\) cm in size, was assessed for the status of ALT by telomere-specific FISH and ATRX, DAXX, ARX, and PDX1 via IHC.\textsuperscript{28} As with previous studies, the presence of ALT correlated with the protein loss of ATRX or DAXX and was strongly associated with known adverse prognostic features for nonfunctional PanNETs, such as large tumor size, high WHO grade, lymphovascular invasion, perineural invasion, advanced pathologic T stage, and regional lymph node metastases, and metachronous distant metastases/recurrences. Importantly, ALT and ATRX/DAXX loss were independent prognostic biomarkers for shorter recurrence-free survival among patients with nonfunctional PanNETs, especially those presenting with small tumors (\(\leq 2.0\) cm).\textsuperscript{28} Furthermore, within a separate cohort of nonfunctional PanNET metastases representing a variety of distant sites, the presence of ALT was highly enriched in these metastases (76 of 107; 71%) in comparison with the primary PanNET cohort (160 of 561; 28.5%). Although ARX and PDX1 protein expression correlated with some adverse prognostic pathologic features, the classification using these protein profiles did not independently correlate with recurrence-free survival. In contrast to a previous cohort that was enriched for patients with MEN1 syndrome,\textsuperscript{19} this current study included only non-syndromic cases and, therefore, leaves open the possibility of a role for these biomarkers in certain clinical scenarios.

The assessment of the ALT and ATRX/DAXX status may also be helpful in the preoperative setting. Current National Comprehensive Cancer Network guidelines for PanNETs state that the extent of surgical resection and lymphadenectomy could be limited in small PanNETs (\(\leq 2.0\) cm) because such small, nonfunctional PanNETs often follow an indolent natural history. However, studies including an analysis of the Surveillance, Epidemiology, and End Results database suggest that a subset of small PanNETs can develop a more aggressive disease course. The identification of a particular biomarker profile in preoperative biopsies could indicate

---

**Figure 1.** Representative images are shown from ALT-negative and ALT-positive pancreatic neuroendocrine tumors detected on fine-needle aspirates. Shown are (A,E) H&E, (B,F) ATRX, (C) retained nuclear expression and (G) loss of nuclear expression of DAXX, and (D,H) telomere-specific fluorescence in situ hybridization to detect ALT. Arrows highlight cells with ultrabright telomeric DNA foci indicative of ALT (\(4^\prime,6\)-diamidino-2-phenylindole nuclear counterstain, original magnification \(\times 400\) in panels A-H). ALT indicates alternative lengthening of telomeres; ATRX, \(\alpha\)-thalassemia mental retardation, X-linked; DAXX, death domain-associated protein 6; H&E, hematoxylin and eosin. Reprinted with permission from the American Cancer Society.
an increased risk of developing metastatic disease and, in turn, prompt a change in surgical management.

Lesions suspected to be PanNETs on imaging studies are typically first evaluated by small specimen biopsy during endoscopic ultrasound procedures. Fine-needle aspiration (FNA) specimens typically yield very cellular specimens and allow for the use of IHC stains to confirm the diagnosis. Several studies have shown that cell block preparations made from FNA specimens are sufficient for use in IHC and FISH methodologies to determine the neoplasm biomarker status.27,29 As shown in Figure 1, we previously reported that both ALT and loss of ATRX/DAXX could be accurately determined on FNA specimens, with 100% concordance between FNA and surgical specimens.29 Hackeng et al27 recently found that the ARX status could be determined in FNA specimens and demonstrated 100% concordance with surgical specimens. In contrast, PDX1 expression was more difficult to determine reliably because of expression of PDX1 in nonneoplastic pancreatic elements (eg, ductal epithelium and acinar cells). One false-negative case of ALT was identified that was due to insufficient sampling and tumor heterogeneity; this suggests that adequacy thresholds must be well defined in larger prospective studies.

These data suggest that the preoperative assessment of biomarker status—particularly ATRX/DAXX IHC and/or ALT—can be performed with FNA specimens to help to guide the decision to manage patients by surveillance rather than surgical resection, particularly those with small (<2.0-cm), nonfunctional PanNETs. Although the expression of ARX correlated with known adverse prognostic features such as a larger tumor size and a high WHO grade, there was a lack of association with recurrence-free survival. Because only a few small studies have examined biomarker expression in FNA specimens, additional larger studies could help to validate these findings and define minimum thresholds for adequacy for each marker. Furthermore, studies could evaluate the use of NGS panels to identify ATRX/DAXX inactivating mutations in FNA specimens. However, the IHC and/or FISH methodologies have the benefit of being relatively portable, inexpensive, and rapid methods for determining the neoplasm biomarker status in comparison with other molecular methods such as NGS.

FUNDING SUPPORT
No specific funding was disclosed.

CONFLICT OF INTEREST DISCLOSURES
The authors made no disclosures.

References
1. Dasari A, Shen C, Halperin D, et al. Trends in the incidence, prevalence, and survival outcomes in patients with neuroendocrine tumors in the United States. JAMA Oncol. 2017;3:1335-1342.
2. Howlader N, Noone AM, Krapcho M, et al. SEER Cancer Statistics Review, 1975-2017. National Cancer Institute; 2020.
3. Lloyd RV, Osamura Ry, Klöppel G, Rosai J. WHO Classification of Tumours of Endocrine Organs. IARC Press; 2017.
4. Hwang HS, Kim Y, An S, et al. Grading by the Ki-67 labeling index of endoscopic ultrasound-guided fine needle aspiration biopsy specimens of pancreatic neuroendocrine tumors can be underestimated. Pancreas. 2016;47:1296-1303.
5. Falconi M, Eriksson B, Kaltasii G, et al. ENETS consensus guidelines update for the management of patients with functional pancreatic neuroendocrine tumors and non-functional pancreatic neuroendocrine tumors. Neuroendocrinology. 2016;103:153-171.
6. Howe JR, Merchant NB, Conrads T, et al. The North American Neuroendocrine Tumor Society consensus paper on the surgical management of pancreatic neuroendocrine tumors. Pancreas. 2020;49:1-33.
7. Sadot E, Reidy-Lagunes DL, Tang LH, et al. Observation versus resection for small asymptomatic pancreatic neuroendocrine tumors: a matched case-control study. Ann Surg Oncol. 2016;23:1301-1370.
8. Assi HA, Mukherjee S, Kunk PL, et al. Surgery versus surveillance for well-differentiated, non-functional pancreatic neuroendocrine tumors: an 11-year analysis of the National Cancer Database. Oncologist. 2020;25:e276-e83.
9. Li JW, Hua X, Reidy-Lagunes D, Untch BR. MEN1 loss as a tissue-specific driver of tumorigenesis. Mol Cell Endocrinol. 2018;469:98-106.
10. Jiao Y, Shi C, Edil BH, et al. DAXX/ATRX, MEN1, and mTOR pathway genes are frequently altered in pancreatic neuroendocrine tumors. Science. 2011;331:1199-1203.
11. Scarpa A, Chang DK, Nome K, et al. Whole-genome landscape of pancreatic neuroendocrine tumours. Nature. 2017;543:65-71.
12. Heaphy CM, de Wilde RF, Jiao Y, et al. ALTERed tumors in tumors with ATRX and DAXX mutations. Science. 2011;333:925.
13. Lovejoy CA, Li W, Reisenweber S, et al. Loss of ATRX, genome instability, and an altered DNA damage response are hallmarks of the alternative lengthening of telomeres pathway. Plus Genet. 2012;8:e1002772.
14. Dillely RL, Greenberg RA. Alternative telomere maintenance and cancer. Trends Cancer. 2015;1:145-156.
15. Raj N, Shah R, Stadler Z, et al. Real-time genomic characterization of metastatic pancreatic neuroendocrine tumors has prognostic implications and identifies potential germline actionability. JCO Precis Oncol. 2018;2018:PO.17.00267.
16. Lakis V, Lawler RT, Newell F, et al. DNA methylation patterns identify subgroups of pancreatic neuroendocrine tumors with clinical association. Commun Biol. 2021;4:155.
17. Chan CS, Laddha SV, Lewis PW, et al. ATRX or DAXX mutations. Nature. 2018;543:65-71.
18. Di Domenico A, Pipinikas CP, Stadler Z, et al. Epigenetic landscape of pancreatic neuroendocrine tumours reveals distinct cells of origin and means of tumour progression. Commun Biol. 2020;3:740.
19. Céspedes P, Drier Y, Drejerink KMA, et al. Enhancer signatures stratify and predict outcomes of non-functional pancreatic neuroendocrine tumors. Nat Med. 2019;25:1260-1265.
20. Kim JY, Brosnan-Cashman JA, An S, et al. Alternative lengthening of telomeres in primary pancreatic neuroendocrine tumors is associated with aggressive clinical behavior and poor survival. Clin Cancer Res. 2017;23:1508-1606.
21. Singh AD, Liu TC, Roncaioli J, et al. Alternative lengthening of telomeres and loss of DAXX/ATRX expression predicts metastatic disease and poor survival in patients with pancreatic neuroendocrine tumors. Clin Cancer Res. 2017;23:600-609.
22. Manirini I, Kurren AS, Vassella E, et al. Loss of DAXX and ATRX are associated with chromosome instability and reduced survival of patients with pancreatic neuroendocrine tumors. Gastroenterology. 2014;146:453-460.e6.
23. Pea A, Yu J, Marchionni L, et al. Genetic analysis of small well-differentiated pancreatic neuroendocrine tumors identifies subgroups with differing risks of liver metastases. Ann Surg. 2020;27:566-573.
24. Roy S, Laframboise WA, Liu TC, et al. Loss of chromatin-remodeling proteins and/or CDKN2A associates with metastasis of pancreatic neuroendocrine tumors and reduced patient survival times. Gastroenterology. 2018;154:2060-2063.e8.
25. Pipinikas CP, Libra H, Karpashvili A, et al. Epigenetic dysregulation and poor prognosis in DAXX-deficient pancreatic neuroendocrine tumours. Endocr Relat Cancers. 2015;22:113-118.
26. Booms G, Vandamme T, Ibrahimbekow M, et al. PDX1 DNA methylation distinguishes two subtypes of pancreatic neuroendocrine neoplasms with a different prognosis. Cancers (Basel). 2020;12:1461.
27. Hackeng WM, Morsink FH, Moore LMS, et al. Assessment of ARX expression, a novel biomarker for metastatic risk in pancreatic neuroendocrine tumors, in endoscopic ultrasound-guided fine-needle aspiration. Diagn Cytopathol. 2020;48:308-315.
28. Hackeng WM, Brosens LAA, Kim JY, et al. Non-functional pancreatic neuroendocrine tumours: ATRX/DAXX and alternative lengthening of telomeres (ALT) are prognostically independent from ARX/PDX1 expression and tumour size. Gut. Published online April 13, 2021. doi:10.1136/gutjnl-2020-322595.
29. Vanderbussche CJ, Aflatoon DB, Graham MK, et al. Alternative lengthening of telomeres and ATRX/DAXX loss can be reliably detected in FNA of pancreatic neuroendocrine tumors. Cancer Cytopathol. 2017;125:544-551.