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Endoscopic ultrasound fine-needle biopsy to assess DAXX/ATRX expression and alternative lengthening of telomeres status in non-functional pancreatic neuroendocrine tumors

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ABSTRACT

Background/objectives: Death domain-associated protein (DAXX) and/or α-thalassemia/mental retardation X-linked (ATRX) chromatin remodeling genes mutations and alternative lengthening of telomeres (ALT) activation are associated with more aggressive behavior of non-functional pancreatic neuroendocrine tumors (NF-PanNETs). We aimed to evaluate the reliability of such markers on endoscopic-ultrasound fine-needle biopsy (EUS-FNB) specimens.

Methods: Patients who underwent EUS-FNB and subsequent surgical resection for PanNETs between January 2017 and December 2019 were retrospectively identified. Immunohistochemistry (IHC) to evaluate DAXX/ATRX expression and fluorescence in situ hybridization (FISH) for ALT status were performed. Primary outcome was the concordance rate of markers expression between EUS-FNB and surgical specimens. Secondary aims were association between markers and lesion aggressiveness, their diagnostic performance in predicting aggressiveness, and agreement of preoperative and post-surgical Ki67-based grading.

Results: Forty-one NF-PanNETs (mean diameter 36.1 ± 26.5 mm) were included. Twenty-four showed features of lesion aggressiveness. Concordance of expressions of DAXX, ATRX, and ALT status between EUS-FNB and surgical specimens were 95.1% ($\kappa = 0.828$; $p < 0.001$), 92.7% ($\kappa = 0.626$; $p < 0.001$), and 100% ($\kappa = 1$; $p < 0.001$), respectively. DAXX/ATRX loss and ALT-positivity were significantly ($p < 0.05$) associated with metastatic lymphnodes and lymphovascular invasion. The combination of all tumor markers (DAXX/ATRX loss + ALT-positivity + grade 2) reached an accuracy of 73.2% (95%CI 57.1–85.8) in identifying aggressive lesions. Pre- and post-operative ki-67-based grading was concordant in 80.5% of cases ($k = 0.573$; $p < 0.001$).

Conclusion: DAXX/ATRX expression and ALT status can be accurately evaluated in a preoperative setting on EUS-FNB samples, potentially improving the identification of patients with increased risk and poorer prognosis.

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1. Introduction

Pancreatic neuroendocrine tumors (PanNETs) can be classified into functional and non-functional (NF-PanNETs) [1]. Clinical behavior of well-differentiated, sporadic, NF-PanNETs ranges from indolent to aggressive malignant ones [1]. Both imaging and pathological features associated with disease aggressiveness and recurrence have been reported [1–4]. Among pathological features, including size >2 cm, metastatic lymph nodes, tumor grade 2 or 3, lymphovascular invasion, and perineural invasion, only size measured at imaging and tumor grade estimated on Ki67 index value on endoscopic ultrasound (EUS) samples can be assessed preoperatively. Tumor grading on EUS-guided fine needle aspiration (EUS-FNA) or fine-needle biopsy (EUS-FNB) samples demonstrated a concordance with surgical specimens (SS) of 79.5% and 84.2%, respectively, with an undergrading rate up to 15% of cases [5–7]. Therefore, the identification of other pathological markers able to stratify the risk of aggressive lesions would be useful for a proper decision-making process [8].

Recently, whole-exome and whole genome sequencing studies revealed that the mutually exclusive inactivating mutations in death domain-associated protein (DAXX) and/or in α-thalassemia/mental retardation X-linked (ATRX) chromatin remodeling genes are associated with more aggressive disease [9–11]. The presence of DAXX/ATRX mutations can be assessed also with immunohistochemistry (IHC), which represents a reliable surrogate of their mutational status [12,13].

Both ATRX and DAXX encode homonymic nuclear proteins that regulate the deposition of histone variant H3.3 during the assembly of pericentromeric and telomeric chromatin [14]. Loss of DAXX/ATRX expression is strongly associated with the activation of the alternative lengthening of telomeres (ALT) pathway [12], a telomerase-independent telomere maintenance mechanism. The poor prognostic role of the activated ALT, which can be detected by fluorescence in situ hybridization (FISH), has been demonstrated in several studies with important clinical implications [12–16].

Most published studies investigated these biomarkers on SS [10–15] and only two small case series evaluated their feasibility on EUS-FNA samples [17,18]. However, EUS-FNB is replacing EUS-FNA in the current clinical practice due to the higher diagnostic performance for diagnosing solid pancreatic lesions and grading PanNETs, especially using new generation needles [19]. Therefore, we aimed to evaluate the reliability of IHC for DAXX/ATRX and FISH for ALT assessed on EUS-FNB samples in comparison with the corresponding SS in a cohort of primary NF-PanNETs. Secondary aims were the association between these biomarkers and clinical-pathological features of aggressiveness, their diagnostic performance in predicting lesion aggressiveness, and concordance between preoperative and postsurgical grading based on Ki67 index.

2. Materials and methods

2.1. Patients

The pathology archive of G.B. Rossi University Hospital, Verona, Italy, was queried for patients with a diagnosis of PanNET from January 2017 to December 2019. Approval for retrospective studies was obtained from the local Ethic Committee (Prog. N.1718CESC, 2018.04.04).

Inclusion criteria were: 1) >18-years old; 2) patients who underwent EUS-FNB and subsequent surgical resection. Exclusion criteria were: 1) metastatic disease at diagnosis based on imaging; 2) no availability of EUS-FNB sample or surgical specimen; 3) preoperative EUS-sampling performed at other institutions; 4) mixed types (e.g., mixed neuroendocrine-acinar/adenocarcinoma) or neuroendocrine carcinomas; 4) predominantly cystic lesions (more than 50% of the volume) on imaging; 5) systemic anticancer therapy before resection; 6) presence of multiples lesions; 7) genetic syndromes associated with PanNETs (i.e., multiple endocrine neoplasia type 1 syndrome, von-Hippel Lindau syndrome, etc.); 8) functional PanNETs.

2.1.1. EUS-FNB procedures

EUS-FNB was performed with patients under deep sedation by two expert endosonographers using a linear echoendoscope (EG3870UTK, Pentax Medical, Tokyo, Japan) with patients under deep sedation). EUS-FNB histology was carried out using either 22G or 25G end-cutting needles (SharkCore™, Medtronic, Minneapolis, USA). The slow-pull technique [20,21] was used in all cases and coupled, whenever possible, with the fanning technique [22]. in all cases. Rapid on-site evaluation was not performed [23] in any case and two to three needle passes were performed [24].

The acquired material underwent standard histologic handling. It was fixed in a 10% formaldehyde solution, embedded in paraffin, sectioned at 3 μm (μm), and then stained with hematoxylin and eosin (H&E).

2.1.2. Immunohistochemistry

Four μm formalin-fixed paraffin-embedded sections were immunostained with antibodies for Cytokeratin AE1/AE3 (AE1-AE3, 1:100 dilution, Novocastra/United Kingdom) Chromogranin A (DAK-A3, 1:2500, Dako/Denmark), and Synaptophysin (27G12, 1:100, Novocastra), Ki67 (MIB1, 1:100, Dako/Denmark), ATRX (1:400, Sigma-Aldrich) DAXX (1:200, Sigma-Aldrich) After antigen retrieval, immunostaining was performed in an automated Bond instrument (Vision-Biosystem, Leica, Milan, Italy) using a sensitive peroxidase-based 'Bond polymer Refine' detection system [25].

2.1.3. Fluorescence in situ hybridization

Deparaffinized slides were washed, hydrated, steamed for 20 min in citrate buffer, dehydrated d, and hybridized with Cy3-labeled peptide nucleic acid probe complementary to the mammalian telomerase repeat sequence (TelC Cy3, F1002 Lot No. ,100 nM, Panagene). The hybridization control centromere probe (Cent-FITC F3013 Lot No., 100 nM, Panagene) was included in the hybridization solution. Slides were imaged with an epifluorescence microscope (Leica DM600 B) equipped with appropriate fluorescence excitation filters. Images were captured with Cytovision Leica System, 7.7 version.

2.1.4. Outcomes

The primary outcome was the concordance rate between EUS-FNB and SS of DAXX/ATRX expression by IHC and ALT status by telomere-specific FISH.

Secondary outcomes were: 1) the association between DAXX/ATRX expression by IHC and ALT status by telomere-specific FISH on EUS-FNB samples with clinical-pathological features indicative of tumor aggressiveness; 2) the diagnostic performance of each marker and of the combination of all markers on EUS-FNB samples in identifying tumor aggressiveness; 3) the concordance of tumor grading based on ki67 index between EUS-FNB and surgical specimens.

2.1.5. Definitions

DAXX/ATRX expression was classified as negative in case of complete absent nuclear staining in the presence of unequivocal internal positive control provided by non-neoplastic cells with retained nuclear expression (cytoplasmic lymphocytes, endothelial cells). Cytoplasmic staining was considered nonspecific and disregarded (Fig. 1) [12,17].

ALT positivity was defined as large, ultra-bright intranuclear telomere FISH signals in at least 1% of tumor nuclei, and the total signal intensity for individual foci is > 10 fold than telomere signal intensities from normal stromal/endothelial cells in the same cases (Fig. 2) [17].

The Ki67 index was counted in at least 2000 cells in the areas with the highest labeling for EUS-FNB and SS [26,27]. For EUS-FNB samples not reaching 2000 cells, the highest number of cells was counted [28]. Grading was performed following the 2019 WHO classification based on Ki67 index [26].

For the purpose of the study all specimens were independently reviewed by two expert pathologists (AR and EM with more than 10 years of experience in pancreatic pathology) and controversial cases (if present) were solved after discussion.

Diagnostic sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV) of DAXX/ATRX loss, positive ALT status, and grade 2 on EUS-FNB samples in predicting tumor features of aggressiveness were calculated.

Tumor aggressiveness was defined as the presence of metastatic lymph node and/or lymphovascular invasion and/or perineural invasion and/or grade 2 on SS and/or local recurrence/appearance of lymph node/distant metastases on imaging during follow-up.

2.2. Statistical analysis

Patients' characteristics were summarized using conventional statistics, like mean ± standard deviation (SD) or median and interquartile range (IQR) for continuous variables and absolute frequencies and percentages for categorical data.

The agreement between matched samples was calculated using underweighted Cohen's Kappa, whereby a value of 0.1–0.20 indicated slight concordance, 0.21 to 0.40 fair, 0.41 to 0.60 moderate, 0.61 to 0.80 substantial, and 0.81 to 0.99 almost perfect concordance. Categorical variables were compared either with the chi-square test (with Yates' correction when appropriate) or Fisher's exact test. When comparing two groups normally distributed continuous variables will be analyzed by using a two-sample Student's t-test, whereas the Mann-Whitney *U* test will be used for not normally distributed variables. A p-value <0.05 will be considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics for Windows (IBM Corp., Armonk, N.Y., USA).

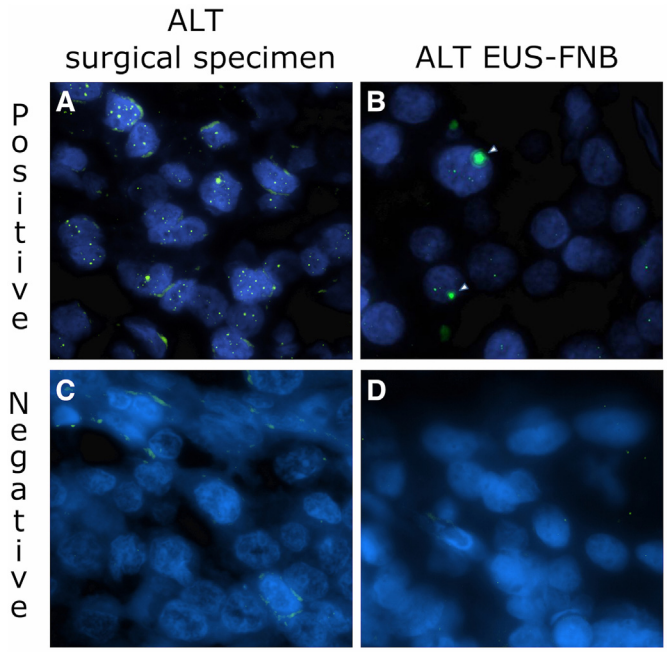


Fig. 2. Telomere-specific fluorescence in situ hybridization (FISH) in both EUS-FNB and surgically excised PanNETs. ALT-positivity in neoplastic cells is similarly visible, on biopsy and surgical tissue, as ultrabright, intranuclear fluorescence dots (A–B). In ALT-negative cells, no fluorescent dots are in the nuclei (B-C).

3. Results

3.1. Patients

The study flowchart is represented in Fig. 3. Forty-one patients (41.5% males, 53 ± 11.0 years) fulfilled inclusion/exclusion criteria and were included in the study. The clinicopathologic characteristics of the patients are reported in Table 1. On SS, 27 (65.9%) were grade 1 PanNETs and 14 (34.1%) were grade 2. Loss of expression of DAXX/ATRX and positive ALT status were observed in 12 (29.3%) and in ten (24.4%) lesions, respectively. DAXX/ATRX loss was mutually exclusive in all but one case showing negativity of both markers. Eleven patients had small (≤2 cm) NF-pNETs and underwent resection in response to the patient's preference in five cases [1], due to tumor

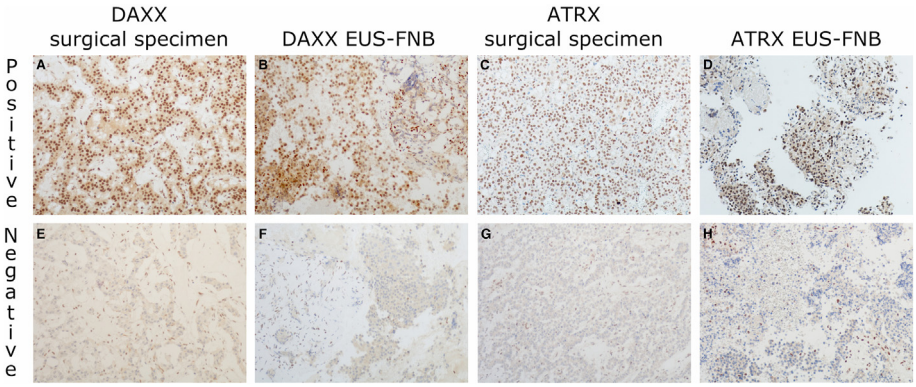
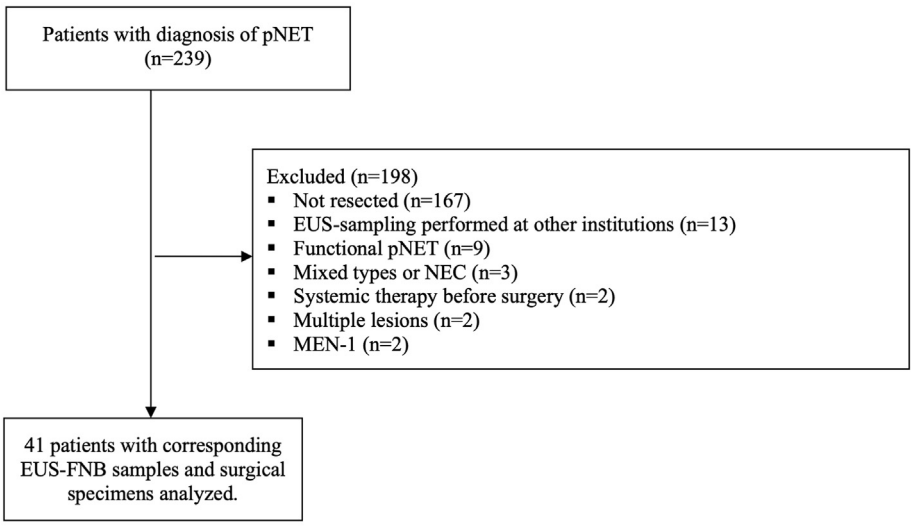


Fig. 1. DAXX and ATRX immunohistochemical staining in histological section of paired EUS-FNB and surgically excised PanNETs. In the first line series of images (A–D) the preserved nuclear expression for DAXX and ATRX is visible as brown dots highlighting the nuclear area in neoplastic cells. In the second line image series (E–H), examples of loss of expression of DAXX and ATRX in the nuclei of neoplastic cells that are homogenously blue. The preserved brown nuclear staining in endothelial cells represents the internal positive control of the staining procedure.



pNET, pancreatic neuroendocrine tumor, *EUS*, endoscopic ultrasound, *NEC*, neuroendocrine carcinoma; *MEN-1*, multiple endocrine neoplasia type 1.

Fig. 3. Study flow-chart.

Table 1
Demographical, technical, and pathological features of 41 NF-PanNETs. population.

Age, yr	
- Mean (SD)	53 (11.0)
Sex, N (%)	
- Male	17 (41.5)
- Female	24 (58.5)
Tumor site, N (%)	
- Head - Neck	16 (39.0)
- Body - Tail	25 (61.0)
Tumor size, mm	
- Mean (SD)	36.1 (26.5)
- Median (IQR)	30 (20–42.5)
Needle type and caliber, N (%)	
- SharkCore 22G	32 (78.0)
- SharkCore 25G	9 (22.0)
Time between EUS and surgery, days	
- Mean (SD)	86 (73.1)
- Median (IQR)	66 (41–98.5)
Type of surgery, N (%)	
- Duodenopancreatectomy	14 (34.1)
- Distal pancreatectomy	21 (51.2)
- Total pancreatectomy	2 (4.9)
- Enucleation	4 (9.8)
2019 WHO grade	
- Grade 1	27 (65.9)
- Grade 2	14 (34.1)
Primary tumor (pT) stage	
- T 1	11 (26.8)
- T 2	20 (48.8)
- T 3	10 (24.4)
Metastatic Lymphnode, N (%)	13 (31.7)
Lymphovascular invasion, N (%)	16 (39.0)
Perineural invasion, N (%)	10 (24.4)
Disease recurrence, N (%)	3 (7.3)
Total time of follow-up, months	
- Mean (SD)	38.2 (11.2)
- Median (IQR)	36 (30.5–48.5)

NF-PanNETs, non-functional pancreatic neuroendocrine tumors; *SD*, standard deviation; *IQR*, interquartile range; *WHO,* world health organization.

uptake on ^{18}FDG-PET in three cases [28], due to imaging overestimation of tumor diameters in two cases, and due to grade 2 on EUS-FNB sample in one case. Among them, five presented features of aggressiveness (three had metastatic lymph nodes and two had lymphovascular invasion, with two that were also grade 2).

3.2. Concordance of markers expression between EUS-FNB samples and SS

All EUS-FNB specimens provided adequate material for DAXX and ATRX evaluation by IHC and ALT-status assessment by FISH. Concordance of the assessment of DAXX and ATRX between EUS-FNB samples and SS was 95.1% (almost perfect agreement, $\kappa = 0.828$; $p < 0.001$) and 92.7% (substantial agreement, $\kappa = 0.626$; $p < 0.001$), respectively. Overall, there were five discordant cases. No association was found between discordant cases and size of needles used [3/32 (9.4%) using a 22G needle vs 2/9 (22.2%) using a 25G needle, $p = 0.299$) or tumor size [3/31 (9.7%) for tumors >2 cm vs 2/11 (18.2%) for those ≤2 cm, $p = 0.593$]. Considering the eleven patients with small (≤2 cm) tumors, the concordance rate of DAXX/ATRX expression between EUS-FNB and surgical specimens was 81.8% (9/11) with two cases where the loss of expression of DAXX and/or ATRX was observed only on surgical specimens.

A perfect concordance (100%, $\kappa = 1$; $p < 0.001$) was observed for telomere-specific FISH for ALT between EUS-FNB samples and SS (Table 2).

Loss of expression of DAXX and/or ATRX was associated with ALT activation both in EUS-FNB samples and SS with substantial agreement ($\kappa = 0.716$; $p < 0.001$ and $\kappa = 0.628$; $p < 0.001$, respectively, Table 3). Seven (17.1%) ALT-positive EUS-FNB were associated with DAXX/ATRX loss, while three (7.3%) ALT-positive EUS-FNB were associated with DAXX/ATRX preserved expression. One of these three discordant cases, a large (80 mm) grade 2 tumor with positive nodes and lymphovascular and perineural invasion, lost the nuclear expression of DAXX in SS. The other two retained the expression of DAXX and ATRX in SS too. Both were >20 mm large tumors with positive nodes and presence of lymphovascular invasion, one grade 1 and one grade 2 with perineural invasion too.

3.3. Association between markers expression on EUS-FNB samples and features of lesion aggressiveness

Table 4 resumes the association between markers expression on EUS-FNB specimens and presence of features of lesion

Table 2
Concordance between ATRX and DAXX expression and ALT status between EUS-FNB and surgical specimens in 41 NF-PanNETs.

EUS-FNB	Surgical specimens			Concordance	*Kappa*	*P value*
	DAXX -	**DAXX +**				
DAXX -	6 (14.6)	0 (0)	6 (14.6)	95.1%	0.828	< ***0.001***
DAXX +	2 (4.9)	33 (80.5)	35 (85.4)			
	8 (19.5)	33 (80.5)	41 (100)			
	ATRX -	**ATRX +**				
ATRX -	3 (7.3)	1 (2.4)	4 (9.7)	92.7%	0.626	< ***0.001***
ATRX +	2 (4.9)	35 (85.4)	37 (90.3)			
	5 (12.2)	36 (87.8)	41 (100)			
	ALT -	**ALT+**				
ALT-	31 (75.6)	0 (0)	31 (75.6)			
ALT+	0 (0)	10 (24.4)	10 (24.4)	100%	1	< ***0.001***
	31 (75.7)	10 (24.4)	41 (100)			
	Grade 1	**Grade 2**				
Grade 1	27 (65.9)	8 (19.5)	35 (85.4)			
Grade 2	0 (0)	6 (14.6)	6 (14.6)	80.5%	0.573	**<0.001**
	27 (65.9)	14 (34.1)	41 (100)			

EUS-FNB, endoscopic ultrasound-guided fine-needle biopsy; *NF-PanNETs*, non-functional pancreatic neuroendocrine tumors; *ATRX*, α-thalassemia/mental retardation X-linked; *DAXX*, death domain-associated protein; *ALT*, alternative lengthening of telomeres.

aggressiveness. Overall, 24 (58.5%) lesions presented with at least one aggressive feature. A total of eight (19.5%) cases had loss of DAXX and/or ATRX expression and were associated with metastatic lymph nodes ($p = 0.007$) and lymphovascular invasion ($p = 0.039$). Moreover, a trend toward significant association was observed between DAXX/ATRX loss and tumor grade 2 ($p = 0.096$).

The presence of ALT in EUS-FNB was identified in ten (24.4%) cases and was associated with node positive status ($p = 0.0005$), lymphovascular invasion ($p = 0.006$), perineural invasion ($p = 0.044$), and pT stage ($p = 0.017$). Moreover, a trend toward significance was observed for age >50 years ($p = 0.059$) and tumor grade 2 ($p = 0.064$). Results were similar when DAXX/ATRX expression and ALT status were evaluated on surgical specimens (Supplementary Table 1).

3.4. Diagnostic performance of tumor markers in predicting lesion aggressiveness

Diagnostic performances of DAXX/ATRX loss and positive ALT status assessed on EUS-FNB specimens were reported in Table 5. When considered individually, grade 2 demonstrated the lowest sensitivity (25.0%, 95% CI 9.8–46.7) whereas positive ALT status the highest (41.7%, 95% CI 22.1–63.4). Specificity was 100% for all markers. The combination of all tumor markers (DAXX/ATRX loss + positive ALT status + grade 2) reached the highest sensitivity (54.2%, 95% CI 32.8–74.5) without decreasing of specificity and resulting in an accuracy of 73.2% (95% CI 57.1–85.8). As reported in Supplementary Table 2, similar results were observed when DAXX/ATRX expression and ALT status were evaluated on surgical specimens. However, Ki67-based grade 2 demonstrated higher sensitivity when evaluated on surgical specimens (58.3%, 95% CI 36.6–77.9), confirming the risk of undergrading on EUS-FNB samples [5].

3.5. Concordance of preoperative and postoperative grading

All EUS-FNB samples were suitable for Ki67 index evaluation. Grading agreement between EUS-FNB samples and SS was observed in 33/41 (80.5%) cases (moderate agreement, $k = 0.573$; $p < 0.001$). Under-grading occurred in 8/41 (19.5%) cases. No case of over-grading was observed. Results of tumor grading concordance are shown in Table 2. Considering the 11 small (≤2 cm) tumors, the concordance rate of Ki67-based grade 2 between EUS-FNB and surgical specimens was 90.9% (10/11) with one case of undergrading.

4. Discussion

Presurgical assessment of PanNETs is crucial for proper patient management [1]. In this study, we demonstrated that the expression of DAXX/ATRX by IHC and the assessment of ALT status by FISH can be accurately evaluated on EUS-FNB specimens. To our knowledge, the present study is the first one evaluating these tumor markers on EUS-FNB specimens collected with new generation end-cutting EUS needles. Of note, all included samples had sufficient residual material for both DAXX/ATRX IHC and ALT FISH. The concordance between EUS-FNB and SS was approximately 95% and 93% for DAXX and ATRX, respectively. In 2017, VandenBussche et al. [17] performed a study comparing 20 EUS-FNA samples with the correspondent SS. The Authors reported 100% of concordance for both DAXX and ATRX markers, despite three EUS-FNA ATRX negative cases being eventually described as heterogeneous, not definitely negative, on resection specimens.

Similarly, we observed a perfect concordance of ALT status by FISH between EUS-FNB specimens and SS, in agreement with the results reported by VandenBussche et al. [17] on EUS-FNA samples. Differently, a slightly lower agreement rate (91%) was observed in another small study including 13 cases with EUS-FNA and corresponding SS [18]. Overall, both in the present and in the above-mentioned studies, it seems that ICH for DAXX/ATRX expression

Table 3
Association between ATRX and/or DAXX expression and ALT status in EUS-FNB samples and surgical specimens of 41 patients with NF-PanNETs.

		ATRX/DAXX expression N (%)			Kappa	p value
		Loss	**Preserved**			
EUS-FNB	**ALT status N (%)**					
	Positive	7 (17.1)	3 (7.3)	10 (24.4)		
	Negative	1 (2.4)	30 (73.2)	31 (75.6)		
		8 (19.5)	33 (80.5)	41 (100)	0.716	< ***0.001***
Surgical specimens	**ALT status N (%)**					
	Positive	8 (19.5)	2 (4.9)	10 (24.4)		
	ALT-negative	4 (12.9)	27 (65.8)	31 (75.6)		
		12 (29.3)	29 (70.7)	41 (100)	0.628	< ***0.001***

EUS-FNB, endoscopic ultrasound-guided fine-needle biopsy; *ATRX*, α-thalassemia/mental retardation X-linked; *DAXX*, death domain-associated protein; *ALT*, alternative lengthening of telomeres; *NF-PanNETs*, non-functional pancreatic neuroendocrine tumors.

Table 4
Association between ATRX/DAXX expression and ALT status on EUS-FNB samples with clinicopathological features in 41 patients with NF-pNETs.

	Cases	ATRX/DAXX (N = 41)		p value	ALT (N = 41)		p value
	N = 41	Loss 8 (19.5)	Preserved 33 (80.5)		Positive 10 (24.4)	Negative 31 (75.6)	
Gender, N (%) rowhead							
Female	24 (58.5)	2 (15.4)	11 (84.6)		4 (16.7)	20 (83.3)	
Male	17 (41.5)	6 (21.4)	22 (78.6)	1.0[a]	6 (35.3)	11 (64.7)	0.269[a]
Age years, N (%) rowhead							
>50	25 (61.0)	6 (24.0)	19 (76.0)		9 (36.0)	16 (64.0)	
≤50	16 (39.0)	2 (12.5)	14 (87.5)	0.447[a]	1 (6.3)	15 (93.7)	*0.059*[a]
Tumor site, N (%) rowhead							
Head - Uncinate 16 (39.0)		5 (31.2)	11 (68.8)		5 (31.2)	11 (68.8)	
Body – Tail	25 (61.0)	3 (12.0)	22 (88.0)	0.225[a]	5 (20.8)	19 (79.2)	0.482[a]
Tumor size rowhead							
>20 mm	30 (73.2)	7 (23.3)	23 (76.7)		9 (30.0)	21 (70.0)	
≤20 mm	11 (26.8)	1 (9.1)	10 (90.9)	0.412[a]	1 (9.1)	10 (90.9)	*0.238*[a]
WHO grade 2019, N (%) rowhead							
G1	27 (65.9)	3 (11.1)	24 (88.9)		4 (14.8)	23 (85.2)	
G2	14 (34.1)	5 (35.7)	9 (64.3)	0.096[a]	6 (42.9)	8 (57.1)	*0.064*[a]
Lymphovascular invasion, N (%) rowhead							
Present	16 (39.0)	6 (37.5)	10 (62.5)		8 (50.0)	8 (50.0)	
Absent	25 (61.0)	2 (8.0)	23 (92.0)	**0.039**[a]	2 (8.0)	23 (92.0)	***0.006***[a]
Perineural invasion, N (%) rowhead							
Present	10 (24.4)	3 (30.0)	7 (70.0)		5 (50.0)	5 (50.0)	
Absent	31 (75.6)	5 (16.1)	26 (83.9)	0.411[a]	5 (38.7)	26 (83.9)	***0.044***[a]
Primary tumour (pT) stage, N (%) rowhead							
T1	11 (26.8)	1 (9.1)	10 (90.9)		1 (9.1)	10 (90.1)	
T2	20 (48.8)	3 (15.0)	17 (85.0)		3 (15.0)	17 (85.0)	
T3	10 (24.4)	4 (40.0)	6 (60.0)	0.234[a]	6 (60.0)	4 (40.0)	***0.017***[a]
Regional node (pN) stage, N (%) rowhead							
N0	28 (68.3)	2 (71.4)	26 (92.9)		2 (71.4)	26 (92.9)	
N1	13 (31.7)	6 (46.2)	7 (53.8)	**0.007**[a]	8 (61.5)	5 (38.5)	***0.0005***[a]

ALT, alternative lengthening telomerases; ATRX, alpha-thalassemia/mental retardation X-linked; DAXX, death domain-associated protein; EUS-FNB, endoscopic-ultrasound fine-needle biopsy.
[a] Fisher's exact test.

Table 5
Diagnostic performance in terms of sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV), and accuracy of DAXX/ATRX loss, positive ALT status, grade 2, and the combination of all tumor markers on EUS-FNB specimens for the prediction of features of aggressiveness in 41 NF-PanNETs.

	DAXX/ATRX loss	Positive ALT status	Ki67-based grading	DAXX/ATRX + ALT + Grade 2
	% (95%CI)	% (95%CI)	% (95%CI)	% (95%CI)
Sensitivity	33.3 (15.6–55.3)	41.7 (22.1–63.4)	25.0 (9.8–46.7)	54.2 (32.8–74.5)
Specificity	100 (80.5–100)	100 (80.5–100)	100 (80.5–100)	100 (80.5–100)
NPV	51.5 (44.5–58.5)	54.8 (46.4–63)	48.6 (42.8–54.3)	60.7 (50.0–70.5)
PPV	100	100	100	100
Accuracy	61.0 (44.5–75.8)	65.9 (49.4–79.9)	56.1 (39.8–71.5)	73.2 (57.1–85.8)

NPV, negative predictive value; *PPV*, positive predictive value; *NF-PanNETs*, non-functional pancreatic neuroendocrine tumors; *ATRX*, α-thalassemia/mental retardation X-linked; *DAXX*, death domain-associated protein; *ALT*, alternative lengthening of telomeres.

and FISH for ALT status are reliable when performed on either EUS-FNA and EUS-FNB specimens. However, larger comparative studies are needed to establish which sampling technique should be used for this purpose.

As previously reported, in our study, DAXX and/or ATRX loss were associated with positive ALT status in 80% of cases. However, a few non-concordant cases were found. Indeed, it is known that the sensitivity of DAXX/ATRX protein expression analysis is approximately 85% because it depends on the site of the mutation [30]. In our study, among ten EUS-FNB ALT positive cases, three showed preserved DAXX or ATRX expression. Moreover, among seven cases with ATRX/DAXX preserved expression but with metastatic lymph nodes/lymphovascular invasion, two showed positive ALT status. Therefore, despite the significant association between DAXX/ATRX loss and positive ALT, it seems useful to perform both analyses to reduce the risk of missing aggressive lesions.

Mutually exclusive mutations in DAXX and ATRX genes are present in up to 40% of PanNETs [31] and are associated with poor prognosis, a higher rate of metastatic disease, and shorter recurrence-free survival [9–12]. In our study, loss of DAXX/ATRX expression and positive ALT status on EUS-FNB specimens were significantly associated with the presence of features of tumor aggressiveness. Particularly, both DAXX/ATRX loss and ALT positivity were associated with lymphovascular invasion and metastatic lymph nodes, whereas ALT positivity correlated with perineural invasion and higher pT stage too. Similar results were demonstrated by Cives et al. [32] in a cohort of 56 resected PanNETs undergoing targeted next-generation sequencing. Mutations of DAXX/ATRX were significantly associated with nodal involvement, lymphovascular invasion, disease recurrence, and disease-free survival [32].

We also evaluated the concordance rate between preoperative and post-surgical grading based on Ki67 index evaluation. We found 80.5% of concordance with 19.5% undergrading, in agreement with previous literature [5].

Finally, we calculated markers' individual and cumulative diagnostic performance for identifying tumor aggressiveness features. Interestingly, all individual markers demonstrated 100%

specificity but with disappointing sensitivity. In particular, the commonly used Ki67-based grading showed the lowest sensitivity (25%) with an accuracy of 56.1%. The highest sensitivity was reached by ALT-positive status (41.7%) with an accuracy of approximately 66%. However, when all tumor markers were combined, the sensitivity increased to 54.2% and the accuracy to 73.2%, suggesting the potential clinical impact of implementing these new markers in clinical practice, as previously suggested [8,16].

The clinical implications of our findings are easily understandable. The capability of EUS-FNB of identifying more aggressive lesions could be crucial in a variety of clinical scenarios. Patients with unresectable tumors could be included in clinical trials for new targeted therapies targeting the ALT pathway [33], those with a borderline resectable or oligometastatic disease could be planned for neoadjuvant therapy before surgery, and those with small (≤2 cm) tumors properly addressed for surgical resection vs active surveillance or less invasive treatment [34]. The latter scenario seems one of the most important considering the discrepancy between an increasing number of incidentally discovered small lesions and the morbidity and mortality of pancreatic surgery [35]. In a large cohort study of resected PanNETs, it was demonstrated that loss of DAXX/ATRX and ALT positivity correlates with relapse-free survival in small (≤2 cm) NF-PanNETs [10]. In the present study, 11 small (≤2 cm), NF-PanNETs underwent resection and five showed features of tumor aggressiveness on surgical specimens. Overall, based on EUS-FNB markers, eight out of 11 lesions (72.7%) would have been correctly stratified with no false positive cases. However, it should be considered that ATRX/DAXX loss is considered a late event in the pathogenesis of PanNETs. Indeed, PanNETs with ATRX/DAXX loss are usually larger in size, with advanced tumor stage, and associated with lymph node or distant metastases [9–12]. Therefore, in case of negative markers on index EUS-FNB samples, resampling of those tumors showing increasing size during surveillance could be reasonable. Therefore, in case of negative markers at first EUS-FNB, resampling those tumors showing increasing size during surveillance could be reasonable. Moreover, features of aggressiveness at imaging, such as upstream dilation of the main pancreatic duct [2,36], infiltrative tumor margins [2,3], and 18FDG-PET positivity [29], should be evaluated and combined with biopsy findings at the time of diagnosis.

Our study had several limitations. First, the retrospective design could carry some selection bias. Second, despite to the best of our knowledge this is the larger study so far, the sample size is still relatively small. Third, a few patients underwent parenchyma-sparing resection (enucleation) with suboptimal lymphadenectomy increasing the risk of missing node metastases. Fourth, we were not able to perform survival analyses because of the short follow/up time (median 36 months) and the small number of disease recurrences.

In conclusion, the assessment of DAXX/ATRX protein expression by IHC staining and the evaluation of ALT status by FISH are feasible on EUS-FNB samples and reflect the results obtained on SS. The opportunity to detect ALT positivity and DAXX/ATRX loss in a preoperative setting can improve the identification of patients with increased risk of progression and poorer prognosis, impacting on the decision-making process. These implications prompt to design large prospective studies for the subsequent introduction of these biomarkers in clinical practice.

Author's contribution

MGM, EM, SFC: conception and design; MDB, AF, SFC: analysis and interpretation of the data; MGM, EM, SFC: drafting of the article; EM, AR, SP, LB, MCCB, LL, AP: performing endoscopic procedures, surgical procedures, and pathological analyses; MGM, SA, SFC: data collection; CL, MB, AG, AP, LL, AS, SFC: critical revision of the article for important intellectual content; All Authors: final approval of the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pan.2023.05.002.

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