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ATRX loss is an independent predictor of poor survival in pancreatic neuroendocrine tumours

Angela Chou, PhD FRCPA,^{1,2,3,4} Malinda Itchins, MBBS FRACP,^{2,5} Philip R de Reuver, MD PhD,^{6,7} Jennifer Arena, BSc,⁷ Adele Clarkson, BSc,^{1,8} Amy Sheen, BSc,¹ Loretta Sioson, BSc,¹ Veronica Cheung, MChD,^{1,8} Aurel Perren MD PhD,⁹ Christopher Nahm, MBBS FRACS,^{2,7} Anubhav Mittal, PhD FRACS,^{2,7} Jaswinder S Samra, DPhil FRACS,^{2,7} Marina Pajic PhD,⁴ Anthony J Gill, MD FRCPA^{1,2,8}

1 Cancer Diagnosis and Pathology Group, Kolling Institute of Medical Research, Royal North Shore Hospital St Leonards NSW 2065 Australia.

2 University of Sydney, Sydney NSW 2006 Australia.

3 Department of Anatomical Pathology, SYDPATH, St Vincent's Hospital, Darlinghurst NSW 2010 Australia.

4 The Kinghorn Cancer Centre and Garvan Institute of Medical Research, Darlinghurst NSW 2010 Australia.

5 Department of Medical Oncology, Royal North Shore Hospital, St Leonards, NSW, Australia

6 Department of Surgery, Radboud University Medical Center, Nijmegen, The Netherlands

7 Department of Upper Gastrointestinal Surgery, Royal North Shore Hospital St Leonards NSW 2065 Australia.

8 NSW Health Pathology, Department of Anatomical Pathology, Royal North Shore Hospital, St Leonards NSW 2065 Australia

9 Institute of Pathology, University of Bern, Bern, Switzerland

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Address for Correspondence:

Anthony J Gill

Department of Anatomical Pathology

Royal North Shore Hospital

St Leonards NSW 2065 Australia

Phone: +61 2 9926 5983

Fax: +61 2 9926 4084

Email: affgill@med.usyd.edu.au

ABSTRACT

Pancreatic neuroendocrine tumours (PanNETs) are rare neoplasms accounting for 1-2% of all pancreatic tumours. The biological behaviour of PanNETs is heterogeneous and unpredictable, adding to the difficulties of clinical management. The *DAXX* (*death domain associated protein*) and *ATRX* (*alpha-thalassemia/mental retardation syndrome X-linked*) genes encode proteins involved in SWI/SNF-like chromatin remodelling. Somatic inactivating mutations in *DAXX* and *ATRX* are frequent in PanNETs, mutually exclusive, and associated with telomere dysfunction resulting in genomic instability and alternate lengthening of telomeres. We sought to assess the clinical significance of the loss of the ATRX and DAXX proteins as determined by immunohistochemistry (IHC) in patients with PanNET.

From an unselected cohort of 105 patients, we found ATRX loss in 10 tumours (9.5%) and DAXX loss in 16 (15.2%). DAXX and ATRX loss were confirmed mutually exclusive and associated with other adverse clinicopathological variables and poor survival in univariate analysis. In addition ATRX loss was also associated with higher AJCC stage and infiltrative tumour borders. However only ATRX loss, lymphovascular invasion and perineural spread were independent predictors of poor overall survival in multivariate analysis.

In conclusion, loss of expression of ATRX as determined by IHC is a useful independent predictor of poor overall survival in PanNETs. Given its relative availability, ATRX loss as determined by IHC may have a role in routine clinical practice to refine prognostication in patients with PanNET.

Keywords: ATRX, DAXX, Pancreatic neuroendocrine tumour

INTRODUCTION

Pancreatic neuroendocrine tumours (PanNETs) are relatively rare neoplasms of the pancreas, accounting for 1 to 2% of all pancreatic neoplasms and occurring in 1 per 100,000 individuals per year [1]. PanNETs are usually sporadic, however they can be associated with inherited predisposition syndromes, most commonly multiple endocrine neoplasia 1 (MEN1), von Hippel-Lindau syndrome (VHL), neurofibromatosis type 1 (NF-1) and tuberous sclerosis [2]. PanNETs may be either 'functional' and secrete hormones that cause clinical symptoms/signs, or 'non-functional' and grow silently.

PanNETs are most commonly low grade malignancies and the 10-year overall survival for patients with PanNET has been reported to range from 40% to 50% [3,4]. However, the biological behaviour of PanNETs in individual patients is quite variable. Whilst some tumours may grow slowly and not recur even after marginal excision by enucleation alone, others may behave aggressively and rapidly metastasise [5]. Widely accepted predictors of long-term survival include histological grade based on the proliferative rate as determined by mitotic count and Ki67 proliferative index, functional status and TNM staging [6,7]. These tools are useful at a population level and currently implemented into routine clinical practice, however it remains difficult to predict at the individual patient level which tumours will recur and behave aggressively.

Recent genomic studies have shed light on the molecular pathogenesis of PanNETs and hold promise to predict outcome [8]. Mutually exclusive somatic

inactivating mutations in either the *DAXX* (*death-domain-associated protein*) or *ATRX* (*alpha-thalassemia/mental retardation syndrome X-linked*) genes have been reported in up to 33% to 43% of PanNETs in some series [8,9]. The ATRX and DAXX proteins encoded by these genes bind with each other to form a histone chaperone complex involved in depositing histone variant H3.3 at the telomeres and pericentric heterochromatin regions of the genome [10,11]. Loss of function of either of these proteins leads to telomere dysfunction and results in impaired non-homologous end joining, alternate lengthening of telomeres (ALT) and general genomic instability [12-14].

Somatic mutations of *ATRX* or *DAXX* may be either homozygous or heterozygous, but heterozygous mutation (that is, mutation of a single allele) is associated with loss of protein expression as determined by immunohistochemistry (IHC) [9]. The hypothesised mechanisms of second allele inactivation in heterozygous mutation include epigenetic silencing of *DAXX* and X chromosome inactivation involving *ATRX*. Recent literature on the prognostic value of DAXX and ATRX protein expression as determined by IHC has been somewhat contradictory [9,15-18]. Although the majority of studies have suggested that DAXX and ATRX loss alone or the combination of either DAXX or ATRX loss is associated with poor outcome, this has not been a consistent finding and others have not found this association [15]. ATRX IHC is becoming increasingly available in routine clinical practice, because of its role in subclassifying glioblastoma. If ATRX or DAXX loss can be proven to be consistently associated with poor survival in independent cohorts, IHC for these proteins may be rapidly deployed into routine clinical

practice. We therefore sought to investigate the clinical and pathological associations of ATRX and DAXX expression as assessed by IHC in a large, unselected and independent cohort of patients with PanNET.

MATERIAL AND METHODS

We searched the computerised database of the Department of Anatomical Pathology, Royal North Shore Hospital, Sydney Australia for all patients undergoing surgical resection for PanNETs between January 1992 and August 2016. Cases which underwent needle biopsy alone rather than resection by encucleation or standard pancreatectomy procedures were excluded. All cases were independently reviewed by a pathologist (AG) experienced in the diagnosis of PanNET to definitively confirm the diagnosis and grade according to the 2017 World Health Organization (WHO) classification [7]. High grade pancreatic neuroendocrine carcinomas (that is Grade 3 PanNEC under the WHO 2017 classification) were excluded. However cases with low grade cytological features but high proliferative indices (that is Grade 3 PanNET under the WHO 2017 classification) were included. Mixed tumours with a pancreatic ductal adenocarcinoma, squamous or acinar component (MiNEN under the WHO 2017 classification) were also excluded. For patients with multiple tumours, the largest tumour was assessed.

Tissue microarrays (TMA) were created from formalin fixed paraffin embedded (FFPE) blocks so that they contained two separate 1 mm cores of tumour tissue. Overall survival was obtained from medical records and

publicly available death notices and defined as the duration alive from the time of definitive tissue diagnosis until the date of audit in July 2017.

Immunohistochemistry for DAXX and ATRX was performed on TMA slides using commercially available rabbit polyclonal antibodies. For DAXX (Sigma Aldrich, HPA 008736, dilution 1:100) and for ATRX (Sigma Aldrich, HPA 001906, dilution 1:200) antibodies were used after antigen retrieval in a high pH solution at 97°C for 30 mins for DAXX and 40 mins for ATRX. The TMA slides were sectioned at 4µm thickness onto positively charged slides (Superfrost plus; Menzel-Glaser, Germany) and the slides were stained on an automated platform, Leica Bond AR9640. A biotin-free polymer based detection system (Leica Bond Polymer Define Detection, Cat. No. DS9713) was used.

Immunohistochemistry was interpreted by a single pathologist (AG) who was blinded to all clinical and pathological data at the time of analysis. DAXX and ATRX expressions (illustrated in Figure 1) were classified as either positive, defined as unequivocal nuclear staining in tumour cells; or negative, defined as completely absent nuclear staining in the presence of an unequivocal internal positive control provided by non-neoplastic cells with retained nuclear expression (for example lymphocytes, endothelial cells or stromal cells).

Cytoplasmic staining was considered non-specific and disregarded. If there was difficulty in confidently assessing the results of IHC on the TMA cores (for example if tumour cells were negative but there were no convincing internal positive controls) then staining was repeated on whole sections.

Of the 113 patients with PanNET, the results of DAXX and ATRX expression were available for 104 and 105 patients respectively. For 8 patients both DAXX and ATRX results were unavailable, due to the lack of tumour cells on the TMA sections or absent internal positive controls even on whole stained sections. For one patient ATRX was assessable, but DAXX status could not be assessed due to repeatedly absent internal positive controls on whole sections. For most cases (n= 83) the Ki67 proliferative index was based on whole sections reported at the time of diagnosis. For cases where the ki67 proliferative index was not evaluated at the time of routine reporting and only TMA specimens were available (n= 29) the ki67 index was determined based on the TMA cores only. For 2 cases the ki67 index could not be determined due to insufficient material present in the TMA.

Statistical analysis was performed using IBM SPSS Statistics v23 on OSX and *P* values of <0.05 were considered as statistically significant. Mean survival was estimated using Kaplan-Meier methods and the significance of the differences was tested using the log-rank test. Five and ten-year survival rate was estimated using life tables. Multivariable Cox regression proportional hazards analysis was used to explore the effect of DAXX and ATRX IHC status on overall survival adjusted for clinicopathological variables analysed with a *p* value of <0.05. This study was approved by the Northern Sydney Local Health District Human Research Ethics Committee – ref: LNR/13/Hawke/424

RESULTS

A total of 113 eligible patients who underwent surgical resections for PanNET were identified. The clinical and pathological features are presented in Table 1. Briefly, 47% were female; the median age at diagnosis was 58 years (range 18-87 years); 85% underwent partial pancreatectomy whereas 15% had enucleation surgery only; 59% were located in the body or tail and 35% in the head of the pancreas; 19% had multiple tumours resected; the median size of the tumour or largest tumour if multiple was 13mm (range 2-45mm); 5% showed coagulative necrosis; 56% were WHO 2017 grade 1, 42% grade 2 and 2% grade 3; 25% had nodal involvement at diagnosis; 65% were AJCC stage I or II tumours at presentation; 13% had known distant metastasis at presentation; 88% were completely resected with clear margins; 73% had a circumscribed/pushing tumour border; 30% had lymphovascular invasion; 7% had perineural spread; 21% were known to be hormone secreting clinically and 16% were associated with confirmed hereditary syndromes with predisposition to developing PanNET (usually MEN1).

The mean overall survival (OS) was 206 months. Features associated with worse overall survival included location in the head of pancreas ($p = 0.005$), the presence of coagulative necrosis ($p = 0.022$), an infiltrative tumour border ($p = 0.001$), the presence of lymphovascular invasion ($p = 0.028$), and the presence of perineural invasion ($p = 0.025$). Ki67 ($p = 0.788$), mitotic activity ($p = 0.893$), WHO grade ($p = 0.414$) and size ($p = 0.648$) were all not predictive of OS.

ATRX was found to be negative in 10 (10%) and positive in 95 (90%) cases. For DAXX, 16 (15%) cases were DAXX-negative, 88 (85%) cases were DAXX-positive. ATRX and DAXX loss were mutually exclusive in tumours and either ATRX or DAXX loss was present in 25% of tumours. The relationship between ATRX/DAXX loss and survival is presented in Table 2. Briefly, when analysed as a group, tumours which were either ATRX or DAXX-negative were associated with poorer overall survival (OS) than tumours with retained expression of both markers (mean 162 months versus 231 months, $p=0.045$, Figure 2A). However, when ATRX and DAXX were analysed separately, ATRX loss alone remained predictive of poor OS (103 months versus 226 months, $p=0.0001$, Figure 2B), whereas DAXX loss lost prognostic significance for OS (209 versus 208 months, $p=0.517$).

The 5-year and 10-year survival rates for ATRX-negative tumours were 54% and 0%, compared to 88% and 69% for ATRX-positive tumours. Multivariate survival analysis demonstrated that lymphovascular invasion ($p = 0.03$), perineural invasion ($p = 0.011$), and loss of ATRX expression ($p = 0.012$) were independently associated with poorer overall survival. Combined ATRX/DAXX expression was not an independent predictor of overall survival ($p = 0.174$) in this model - summarised in Table 3.

The clinical and pathological associations of ATRX and DAXX loss are presented in Table 4. Tumours which were negative for either DAXX or ATRX were more common in patients >58 years of age ($p = 0.013$), were associated with higher Ki67 proliferative indices ($p = 0.004$), high grade ($p = 0.017$), nodal

involvement ($p = 0.001$), higher stage ($p = 0.001$), infiltrative borders ($p = 0.003$) and lymphovascular invasion ($p = 0.001$).

In view of a previous study suggesting that ATRX/DAXX loss was associated with better survival in patients presenting with metastatic disease [9], we performed further survival analysis on the 13 patients who initially presented with metastatic disease (2 of the 15 patients who initially presented with metastasis had unavailable ATRX/DAXX results; table 5). In this cohort, ATRX/DAXX-negative PanNETs with metastasis at presentation showed a trend towards improved overall survival (mean 169 months vs 83 months) compared to ATRX/DAXX-positive tumours, however the difference did not reach statistical significance ($p=0.889$). Metastatic ATRX-negative tumours demonstrated a non-statistically significant trend towards worse overall survival (mean 17 versus 215.8 months, $p=0.263$) compared to ATRX-positive tumours. All three patients with metastatic DAXX-negative tumours were alive with no metastasis at last follow up, however again this trend towards better survival did not reach statistical significance ($p = 0.418$ and 0.726).

DISCUSSION

In this large study of 113 unselected patients with PanNET, we demonstrated that loss of ATRX or DAXX as determined by IHC occurred in 25% of the 105 assessable patients and was associated with poor overall survival in univariate analysis, but not in multivariate analysis because of a strong correlation with other adverse prognostic features. Separate analysis of

ATRX and DAXX expression demonstrated that ATRX negativity was associated with poor overall survival (103 months versus 226 months) in both univariate ($p = 0.0001$) and multivariable analysis ($p = 0.012$) whereas DAXX loss by itself was not statistically significant. Remarkably, no patients with ATRX-negative PanNETs survived 10 years. Other independent variables associated with poorer survival in multivariate modelling included the presence of perineural invasion ($p = 0.011$) and lymphovascular invasion ($p = 0.03$).

Our findings are in keeping with four previous studies which have found ATRX/DAXX-negative PanNETs are associated with worse outcomes [16-19]. Marinoni et al. found this association in two independent cohorts consisting of 90 and 72 patients as did Singhi et al in a larger cohort of 321 patients [16, 17]. Of note, although Pipinikas et al. found a similar association of poor survival for combined ATRX or DAXX-negative PanNETs, when ATRX and DAXX loss were examined separately DAXX loss, but not ATRX loss, was associated with poor progression-free survival [18]. However we note that this was a study of a smaller cohort (53 PanNETs of whom 34 had clinical follow-up). More recently, Roy et al [19] found genomic alterations in ATRX/DAXX as well as chromatin remodelling genes (*ARID1A*, *SETD2*) and *CDKN2A* gene were frequent in metastatic PanNET. They subsequently, assessed alteration of these genes in a large cohort of primary PanNETs and found loss of function of at least one marker was detected in 81% of PanNETS and associated with poorer survival. Univariate survival analysis found ATRX/DAXX loss correlated with poorer survival, similar to our study; loss of

HEK36me3, deletion of *CDKN2A*, but not loss of *ARID1A* was also associated with poorer survival. However, similar to other three studies, Roy et al. did not address whether each gene alteration are independent prognostic factors of survival.

In contrast to our findings, two studies have suggested that ATRX/DAXX-negative PanNETs are associated with better survival [9,15]. These had some methodological and cohort differences which may explain the discrepant findings. For example in Jiao et al's study, patients with high stage disease (stage III/IV) were over-represented compared to our cohort, and indeed all patients with ATRX/DAXX-negative tumours (29 out of 68) presented with metastatic disease [9]. To investigate whether ATRX/DAXX loss may be associated with better prognosis in metastatic or advanced disease but worse prognosis in localized tumours, we therefore performed a subgroup analysis in the 13 patients with metastatic disease at presentation in our cohort and also found that there was a trend for ATRX/DAXX-negative tumours to be associated with longer survival. Although this finding did not reach statistical significance ($p = 0.889$), it raises the intriguing possibility that ATRX/DAXX loss may be associated with poor survival in low stage tumours but better survival in high stage or metastatic tumours. If this association is valid, we can only postulate that PanNETs diagnosed at advanced stage may be molecularly different to those diagnosed earlier and that other cancer pathways may play a more significant role in advanced PanNETs. Ultimately further studies would be required to confirm whether this is a true association and to investigate potential mechanisms.

Park et al [15] also found ATRX/DAXX-negative PanNETs (60 out of 76 tumours) were associated with better overall survival in their unselected cohort recruited from a major teaching hospital. However, compared to many other studies including our own which generally show a combined incidence of ATRX/DAXX loss of no more than 43% [8,9,16,17], the 79% incidence of ATRX/DAXX-negative PanNETs appears unusually high. We postulate that technical differences in antibody deployment and interpretation may account for this outlying result. For example Park et al [15] defined negative staining as 'the presence of positive cytoplasmic staining with negative nuclear staining in the presence of positive internal control'. In contrast in our, and most other studies, a negative result was interpreted as negative nuclear staining in the presence of positive internal control (endothelial, stromal or inflammatory cells), irrespective of cytoplasmic staining which we interpreted as non-specific and ignored. This method of interpretation is supported by Marinoni's study [16] which demonstrated a high correlation between ATRX/DAXX mutation status and loss of nuclear staining (irrespective of cytoplasmic staining) for ATRX/DAXX.

In our study, ATRX loss on its own but not isolated DAXX loss was prognostically significant in PanNETs. Previous study have found ATRX and DAXX were part of a common pathway of alternative lengthening of telomeres (ALT) associated with survival, hence most previous studies have not assessed ATRX and DAXX as independent prognostic factors [16,17]. It is worth noting that despite the initial recognition of DAXX as a pro-apoptotic

protein, it has recently been found to also have anti-apoptotic effects. Various studies using cancer cell lines (prostate and ovarian cancer), have demonstrated that *DAXX* knockdown can result in increased autophagy, decreased angiogenesis, and reduced cell proliferation in tumour cells resulting in lack of tumour growth and progression [20,21]. In contrast, *ATRX* knockdown in *in-vivo* models of glioblastoma has been demonstrated to accelerate tumour growth and reduce survival in mice [22]. Future mechanistic studies would therefore be helpful to understand the different roles *ATRX* and *DAXX* play in the biological progression of PanNETs.

We note that in this study the Ki67 proliferative index did not predict overall survival ($p=0.788$ for Grade 1 vs Grade 2 tumours). One potential reason for this and weakness of this study is that for $n=29$ (26%) of cases the proliferative index was only performed on TMA cores which may be less accurate than whole sections. We also note that our primary endpoint was overall survival which may be a less accurate measure of the biology of these tumours than disease free survival given the relatively indolent nature of low grade PanNETs. Interestingly in most large published cohorts, the overall (as opposed to the disease free) survival difference between Grade 1 and Grade 2 PanNETs is usually small and often commonly not significant in multivariate analysis [23,24]. In fact in one recent large study of 274 patients[24], there was no difference in survival between Grade 1 and Grade 2 PanNETs using the then established WHO criteria of a cut off of $\leq 2\%$ for proliferative index. In fact in that study the 10-year overall survival of patients with Ki67 proliferative

index $\leq 2\%$ was 80% - a trend to be worse than the 10 year survival of patients with a Ki67 2.1–5% which was 95% ($P=0.487$)[24].

In conclusion, in our study either ATRX or DAXX loss (which occurred in 25% of patients with PanNET) was associated with poor survival and adverse clinicopathological variables. However, only isolated ATRX loss and not isolated DAXX loss was independently associated with overall survival. Interestingly the adverse prognostic effect of ATRX/DAXX loss was not present in patients presenting with metastasis and, in fact, in these patients ATRX/DAXX loss was associated with a trend toward better survival. In view of its increasing availability in diagnostic pathology laboratories, ATRX immunohistochemistry in particular may therefore have a role as an independent prognostic biomarker in the routine management of patients with PanNET.

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AUTHOR CONTRIBUTIONS

Design of the study: all authors

Writing of the manuscript: all authors

Data Collection: MI, PRR, JA, AS, CN, AM, JSS

Data analysis: ACh, AP, PRR, AJG

Figure legends

Figure 1: ATRX and DAXX immunohistochemistry (IHC).

A-C) Serial sections from an ATRX-positive (panel B) and DAXX-negative (panel C) tumour. Only nuclear staining is considered significant with cytoplasmic staining being ignored. For either DAXX or ATRX to be considered negative there is a requirement for unequivocal staining in non-neoplastic cells which act as internal positive controls. Original magnifications 400x.

D-F) Serial sections from an ATRX-negative (panel E) and DAXX-positive (panel F) tumour. Again a little non-specific cytoplasmic staining is ignored and the need for positive internal controls is emphasized. Original magnifications 400x.

Figure 2: Kaplan-Meier survival curves. A) Overall survival of ATRX/DAXX-positive versus -negative tumours. B) Overall survival of ATRX-positive versus -negative tumours. C) Overall survival of DAXX-positive versus -negative tumours.

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Table 1. Clinicopathological features of PanNET in the cohort

Clinicopathological variables	n = 113 (%)	Mean OS (months)	P value
Gender			
female	53 (47)	175	0.215
male	60 (53)	222	
Age (median 58 years, mean 57, range 18-87 months)			
≤58	59 (52)	211	0.461
>58	54 (48)	173	
Type of surgery			
enucleation	17 (15)	206	0.941
pancreatectomy	96 (85)	201	
Location			
head	39 (35)	152	0.005
body/tail	67 (59)	231	
unknown	7 (6)		
Number of tumours			
single	91 (81)	203	0.495
multiple (>1)	22 (19)	171	
Size (median 13mm, mean 20mm, range 2-45mm)			
≤13mm	24 (21)	193	0.648
>13mm	87 (77)	201	
unknown	2 (2)		
Tumour necrosis			
absent	107 (95)	212	0.022
present	6 (5)	111	
Mitosis			
<2/10 HPF ¹	84 (74)	204	0.893 ¹
2-20/10 HPF ¹	27 (24)	227	
>20/10 HPF	1 (1)	6	
unknown	1 (1)		
Ki67			
<3%	71 (63)	206	0.788 ²
3-20%	37 (33)	202	
>20%	3 (2.5)	26	
unknown	2 (1.5)		
2017 WHO grade			
grade 1	63 (56)	194	0.414 ³
grade 2	47 (42)	228	
grade 3	3 (2)	26	
Node involvement			
absent	84 (74)	209	0.306
present	28 (25)	174	

unknown	1 (1)		
Metastasis at presentation			
absent	97 (86)	208	0.172
present	15 (13)	197	
unknown	1 (1)		
2017 AJCC stage			
I-II	73 (65)	214	0.153
III-IV	39 (34)	185	
unknown	1 (1)		
Resection status			
clear	99 (88)	203	0.206
involved	10 (9)	147	
unknown	4 (3)		
Tumour border			
circumscribed	82 (73)	209	0.001
infiltrative	27 (24)	144	
unknown	4 (3)		
Vascular invasion			
absent	78 (69)	218	0.028
present	34 (30)	182	
unknown	1 (1)		
Perineural invasion			
absent	104 (92)	208	0.025
present	8 (7)	65	
unknown	1 (1)		
Hormone secreting			
absent	87 (77)	192	0.255
present	24 (21)	223	
unknown	2 (2)		
Associated with hereditary syndrome			
absent	95 (84)	207	0.821
present	18 (16)	169	

¹ Mitosis of >20/10 hpf were excluded from analysis due to low numbers and all cases were censored.

² Ki67 >20% was excluded from analysis due to low numbers and all cases were censored.

³ Grade 3 was excluded from analysis due to low numbers and all cases were censored.

Table 2. Correlation of ATRX and DAXX IHC with overall survival

Immunohistochemistry profile	n = 105	Mean OS (months)	P value
DAXX			
negative	16	209	0.517
positive	88	208	
ATRX			
negative	10	103	0.0001
positive	95	226	
Combined ATRX & DAXX			
ATRX/DAXX negative	26	162	0.045
both positive	79	231	

Table 3. Multivariate cox regression model for overall survival

	Hazard ratio	95% Confidence interval	P value
Location			
head	1		
body/tail	0.668	0.195-2.287	0.52
Necrosis			
absent	1		
present	2.332	0.551-9.870	0.25
Border			
circumscribed	1		
infiltrative	0.996	0.948-1.046	0.878
Lymphovascular invasion			
absent	1		
present	3.694	1.132-12.051	0.03
Perineural invasion			
absent	1		
present	11.823	1.781-78.499	0.011
ATRX			
positive	1		
negative	16.982	1.882-153.238	0.012
ATRX/DAXX			
positive	1		
negative	1.851	0.042-1.773	0.174

Table 4. Comparison between ATRX/DAXXX negative and positive tumours

	ATRX/DAXX negative n=26	ATRX & DAXX positive n=79	P value	ATRX negative n=10	ATRX positive n=95	P value
Gender						
female	10	40	0.366	5	45	1.000
male	16	39		5	50	
Age (median 58 months, average 57, range 18-87 months)						
≤58	8	47	0.013	3	52	0.187
>58	18	32		7	43	
Type of surgery						
enucleation	2	15	0.229	1	16	1.000
pancreatectomy	24	64		9	79	
Location						
head	8	29	0.809	5	32	0.288
body/tail	16	46		4	58	
unknown	2	4		1	5	
Number of tumours						
single	24	62	0.147	9	77	0.685
multiple (>1)	2	17		1	18	
Size (median 13mm, average 20mm, range 2-45mm)						
≤13mm	2	17	0.147	1	18	0.685
>13mm	24	62		9	77	
tumour necrosis						
absent	23	76	0.160	9	90	0.460
present	3	3		1	5	
Mitosis						
<2/10 HPF ¹	17	62	0.291	8	71	0.902
2-20/10 HPF ¹	9	16		2	23	
>20/10 HPF	0	1		0	1	
Ki67						
<3%	10	56	0.004	5	60	0.433
3-20%	15	19		4	30	
>20%	0	3		0	3	

unknown	1	1		1	2	
2017 WHO grade						
grade 1	9	49		6	52	
grade 2	17	27		4	40	0.831
grade 3	0	3	0.017	0	3	
Node involvement						
absent	13	67	0.001	6	74	0.244
present	13	12		4	21	
Metastasis at presentation						
absent	20	72	0.083	7	85	0.107
present	6	7		3	10	
2017 AJCC stage						
I-II	9	61	0.001	3	67	0.015
III-IV	17	18		7	28	
Resection status						
clear	21	73		8	86	
involved	5	5	0.116	2	8	0.475
unknown	0	1		0	1	
Tumour border						
circumscribed	14	65		4	75	
infiltrative	12	12	0.003	6	18	0.013
unknown	0	2			2	
Lymphovascular invasion						
absent	11	63	0.001	6	68	0.477
present	15	16		4	27	
Perineural invasion						
absent	24	74	1.000	10	88	1.000
present	2	5		0	7	
Hormone secreting with clinical syndrome						
absent	23	59	0.178	9	73	0.687
present	3	20		1	22	
Associated with hereditary syndrome						
absent	24	66	0.348	8	82	0.633
present	2	13		2	13	

Table 5. Correlation of ATRX and DAXX IHC with overall survival in patients presented with metastasis

Immunohistochemistry profile	n = 13	mean OS (months)	P value
ATRX			
negative	3	17	0.263
positive	10	215.8	
DAXX*			
negative	3	N/A**	0.418
positive	9		
Combined ATRX & DAXX			
ATRX or DAXX negative	6	169	0.889
both positive	7	83	

* One case had unavailable DAXX result

**Mean survival was unable to be calculated because all 3 patients with DAXX negative tumour are still alive without recurrence.

Highlights

ATRX and DAXX loss are mutually exclusive and occur in 25% of PNETs

ATRX loss is associated with worse survival (103 months versus 226 months, $p=0.0001$)

No patients with ATRX negative tumours survived 10 years

ATRX and DAXX loss are associated with other adverse prognostic indicators

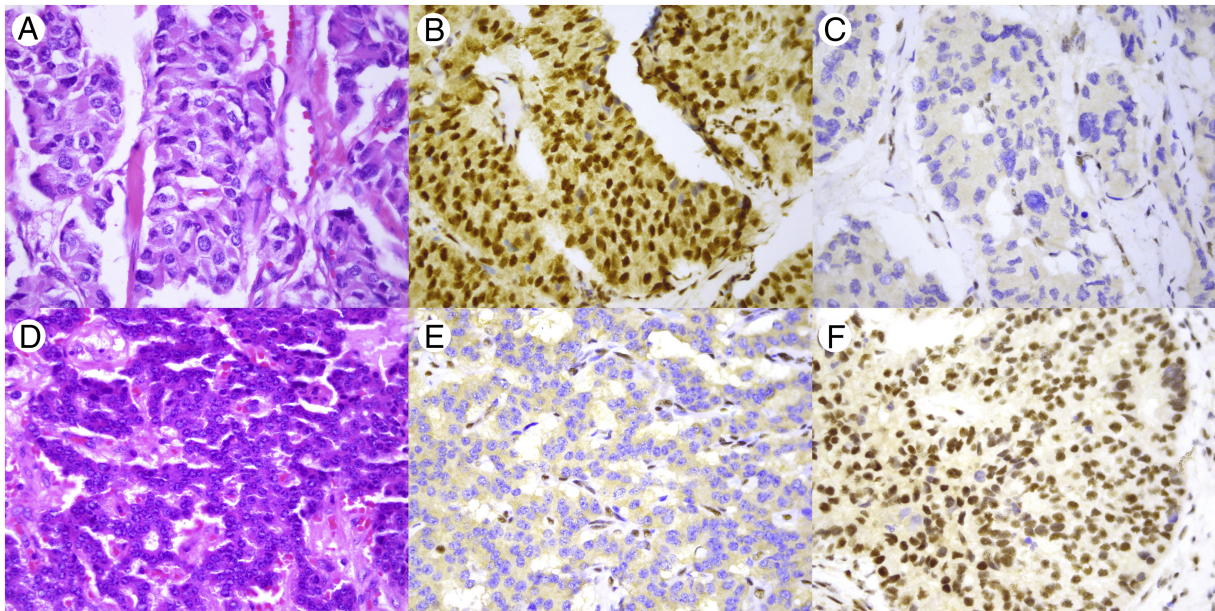


Figure 1

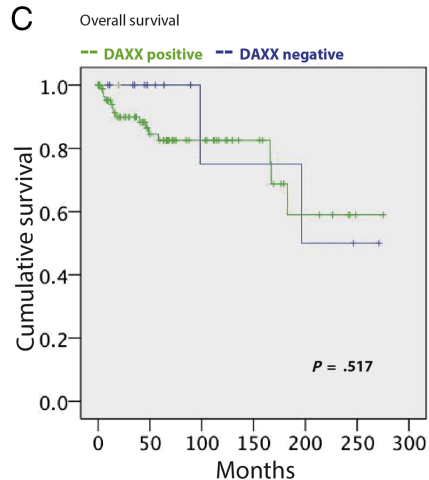
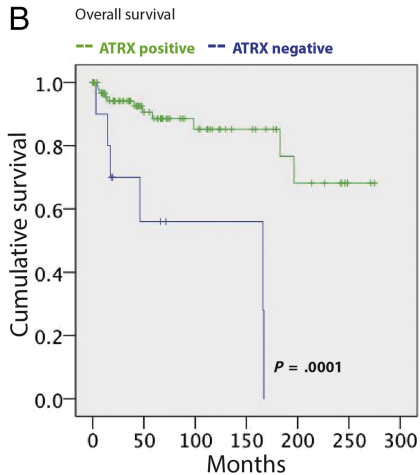
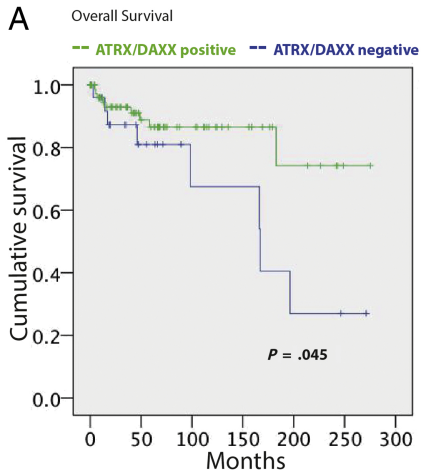


Figure 2