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Original contribution

Loss of ATRX expression predicts worse prognosis in pulmonary carcinoid tumors ☆

Simone B.S.P. Terra, MD [a,1], Hao Xie, MD, PhD [b,1], Jennifer M. Boland, MD [a], Aaron S. Mansfield, MD [b], Julian R Molina, MD, PhD [b], Anja C. Roden, MD [a,*]

[a] *Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA*
[b] *Division of Medical Oncology, Department of Oncology, Mayo Clinic, Rochester, MN, USA*

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Summary Loss of alpha thalassemia/mental retardation syndrome X-linked (ATRX), a chromatin regulator, is associated with worse prognosis in pancreatic neuroendocrine tumors. We investigated ATRX expression in pulmonary carcinoid tumors (PCT) and its diagnostic and prognostic role in these patients. Resected PCTs (1997-2017) were reviewed. Tumors were staged according to 8th UICC/AJCC system. ATRX nuclear expression was recorded independently by 2 reviewers. A cutoff of ≤5% of nuclear ATRX expression was statistically established as loss of expression. One-hundred-fifteen patients (72 women [63%]; median age of 60.5 years [interquartile range, 50.8-71.5]) harbored 69 (60%) typical and 46 (40%) atypical PCTs. Median tumor size was 2.3 cm (interquartile range, 1.6-3.8 cm). Loss of ATRX expression was associated with atypical PCTs (OR 7.4 [95% CI, 2.6-23, $P < .001$]), when adjusted for lymphovascular invasion and perineural invasion. ATRX expression predicted atypical PCT with sensitivity of 37% (95% CI, 24%-52%), specificity of 92% (95% CI, 86%-98%), AUC of 0.62 (95% CI, 0.52-0.72). Loss of ATRX expression was associated with shorter disease-specific survival (HR = 11, 95% CI, 1.8-68, $P = .01$), after adjusting for lymphovascular invasion and presence of metastatic disease at time of diagnosis. Interobserver agreement on ATRX expression by two reviewers was substantial ($\kappa = 0.72$ [95% CI, 0.60-0.80]). ATRX expression is more commonly lost in atypical than in typical PCT, and is associated with more aggressive tumor characteristics and shorter disease-specific survival.

1. Introduction

Pulmonary carcinoid tumors (PCTs) are rare neuroendocrine neoplasms that account for 1% to 2% of all lung malignancies in adults and 25% of all neuroendocrine tumors [1]. PCTs are classified according to the World Health Organization (WHO) into typical and atypical subtypes based on the number of mitoses and the absence or presence of necrosis [2]. While most PCTs behave in an indolent fashion, some show a more aggressive behavior with recurrence and metastases, and rare patients will die from this disease. In

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* Corresponding author at: Department of Laboratory Medicine & Pathology, Mayo Clinic, Hilton 11, 200 First St SW, Rochester, MN, 55905.
E-mail address: Roden.anja@mayo.edu (A. C. Roden).

[1] Drs. Terra and Xie contributed equally to the study.

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general, atypical PCTs have a worse prognosis [1,3]. Based on the SEER database, the overall 10-year survival for typical PCT ranges from 98% for stage I tumors to 49% for stage IV tumors, while for atypical PCT 10-year survival ranges from 91% for stage I tumors to 18% for stage IV tumors [4]. Furthermore, 68% of typical PCT present at stage I while only 41% of atypical PCT present at that stage [4]. Seven percent of typical PCT have metastases at the time of diagnosis vs. 17% of atypical PCT. While the subclassification into typical and atypical PCT provides important information for prognosis

Table 1 Demographics of study population and morphologic and cell-kinetic features of pulmonary carcinoid tumors

Variable	All patients (n = 115)	Typical carcinoid tumors (n = 69)	Atypical carcinoid tumors (n = 46)	*P*
Age at time of surgery, years, median [IQR]	60.5 [50.8, 71.5]	58.5 [47.8, 70.0]	62.6 [56.8, 73.8]	.1
Sex, female, N (%)	72 (62.6)	41 (59.4)	31 (67.4)	.4
Tumor size, cm, median [IQR]	2.3 [1.6, 3.8]	2.1 [1.5, 3.0]	2.6 [1.8, 4.7]	.02
Number of mitoses, median [IQR]	1 [0, 2]	0 [0, 1]	3 [2, 4]	<.001
Necrosis present, N (%)	13 (11.3)	0 (0.0)	13 (28.3)	<.001
Loss of nuclear ATRX expression, N (%)	23 (20.0)	6 (8.7)	17 (37.0)	<.001
Surgical treatment [a], N (%)				
Wedge resection	13 (11.4)	8 (11.8)	5 (10.9)	.3
Lobectomy	74 (64.9)	45 (66.2)	29 (63.0)	
Bilobectomy	3 (2.6)	1 (1.5)	2 (4.3)	
Pneumonectomy	5 (4.4)	1 (1.5)	4 (8.7)	
Sleeve resection	6 (5.3)	5 (7.4)	1 (2.2)	
Complete resection	112 (98.2)	67 (98.5)	45 (97.8)	1.0
Pathologic T stage, N (%)				.1
1a	11 (9.6)	9 (13.0)	2 (4.3)	
1b	40 (34.8)	24 (34.8)	16 (34.8)	
1c	25 (21.7)	18 (26.1)	7 (15.2)	
2a	17 (14.8)	10 (14.5)	7 (15.2)	
2b	10 (8.7)	5 (7.2)	5 (10.9)	
3	10 (8.7)	3 (4.3)	7 (15.2)	
4	2 (1.7)	0 (0.0)	2 (4.3)	
Pathologic TNM stage, N (%)				.007
IA1	9 (7.8)	8 (11.6)	1 (2.2)	
IA2	36 (31.3)	24 (34.8)	12 (26.1)	
IA3	15 (13.0)	11 (15.9)	4 (8.7)	
IB	9 (7.8)	7 (10.1)	2 (4.3)	
IIA	6 (5.2)	2 (2.9)	4 (8.7)	
IIB	20 (17.4)	12 (17.4)	8 (17.4)	
IIIA	14 (12.2)	5 (7.2)	9 (19.6)	
IIIB	1 (0.9)	0 (0.0)	1 (2.2)	
IIIC	0	0	0	
IVA	0	0	0	
IVB	5 (4.3)	0 (0.0)	5 (10.9)	
Additional therapy, N (%)	15 (13.0)	2 (2.9)	13 (29.3)	<.001
Adjuvant chemotherapy and radiation	2 (1.7)	0	2 (4.3)	
Salvage chemotherapy	3 (2.6)	0	3 (6.5)	
Salvage sandostatin	9 (7.8)	2 (2.9)	7 (15.2)	
Salvage everolimus	1 (0.9)	0	1 (2.2)	
Recurrence, N (%)	15 (13)	4 (5.8)	11 (23.9)	.009
Follow up time, median, years [IQR]	4.2 [1.2, 9.4]	4.9 [1.6, 10.2]	3.3 [0.6, 6.5]	.2
All-cause overall survival, median [95% CI]	19.1 [11.9-NR]	19.1 [12.9-NR]	9.2 [6.3-NR]	.009
Death, N (%)	29 (25.2)	12 (18.9)	16 (34.8)	.002
Died of disease	8 (7.0)	0	8 (17.4)	
Died of other cause	7 (6.1)	5 (7.2)	2 (4.3)	
Cause of death unknown	14 (12.2)	8 (11.6)	6 (13.0)	

Abbreviations: IQR: interquartile range; NR: not reached.

[a] Information missing in 1 patient.

[5,6], there are differences in outcome amongst tumors of the same subtype; in addition, there is histologic overlap between these two entities. Therefore, additional biologic markers are sought to better predict which PTCs might behave more aggressively to better adjust the management and treatment of these patients.

In pancreatic neuroendocrine neoplasms (Pan-NET), loss of alpha thalassemia/mental retardation syndrome X-linked ATRX expression has been associated with worse prognosis [7]. ATRX is part of the SWItch/Sucrose Non-Fermentable (SWI/SNF) family of chromatin remodeling proteins [8]. Interestingly, acquired *ATRX* mutations have not only been

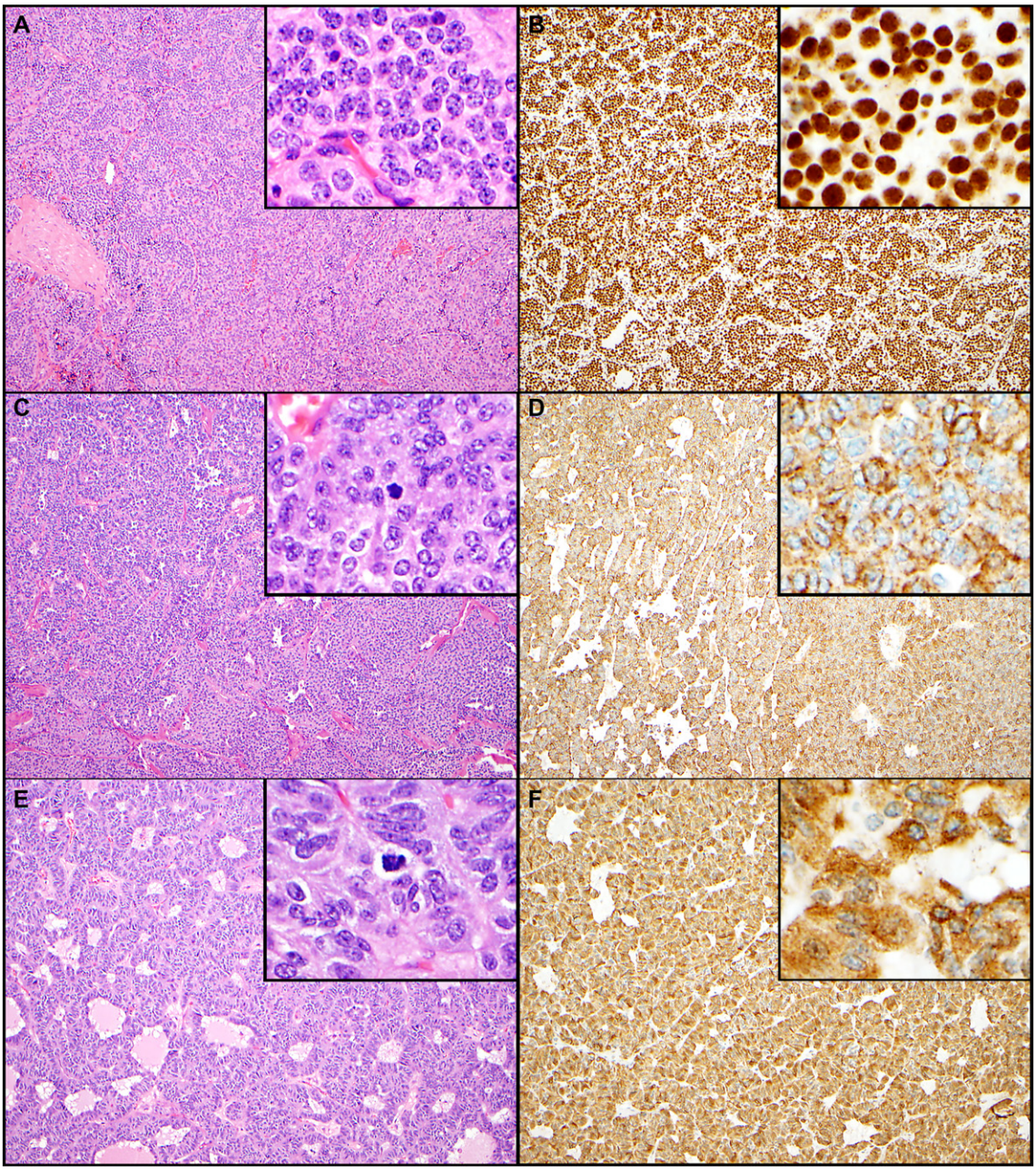

Fig. 1 ATRX expression in pulmonary carcinoid tumors. A and B, Typical carcinoid tumor with nuclear expression of ATRX. A. Typical carcinoid tumor as characterized by a nested growth of bland, round and oval nuclei with salt and pepper chromatin (insert A) and no mitotic activity or necrosis. B, ATRX is expressed in virtually all tumor cell nuclei (insert B). After complete resection of the tumor (pTNM stage IA2) the patient is alive without disease at 5 months follow up. C and D, Atypical carcinoid tumor as characterized by increased mitotic activity (2 mitoses/2 mm^2, C, insert C) with diffuse loss of nuclear expression of ATRX in tumor cells (D, insert D). After complete resection (stage pIIB) the patient developed metastasis/recurrence 7 years later and died of disease 13 years after initial resection. E and F, Atypical carcinoid tumor as characterized by 2 mitoses/2 mm^2 (E, insert E). One reviewer scored ATRX as loss of expression, the other scored it as expressed (F, insert F). After complete resection (stage pIA2) the patient developed metastasis/recurrence 11 years later and is alive with disease 15 years after initial resection. Magnification x 100 (A-F), x 400 (inserts A-F).

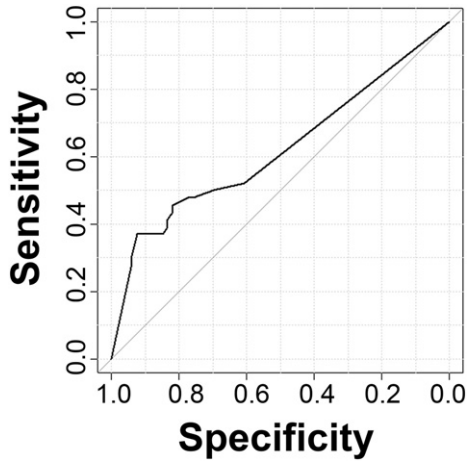

Fig. 2 ROC curve for ATRX nuclear expression to predict histological subtype of pulmonary carcinoid tumors.

identified in Pan-NET but also numerous other cancers including neuroblastoma, glioma, and pediatric osteosarcoma. In addition, reduced ATRX expression has been detected in neuroendocrine tumors, glioma, osteosarcoma and melanoma [8].

ATRX expression has not been thoroughly studied in pulmonary neuroendocrine tumors. Therefore, we investigated the expression of ATRX in PCT, and its potential diagnostic and prognostic implications.

2. Material and methods

2.1. Cohort

Surgical pathology files of Mayo Clinic Rochester were searched for resected pulmonary carcinoid tumors (1997-2017). Because atypical PCT are rarer than typical PCT, cases were enriched for atypical PCT by including all atypical PCT that were resected during this time period. Typical PCT were randomly chosen. Most cases were reported previously [9]. Cases were reviewed by a thoracic pathologist (ACR) to confirm the diagnosis. Number of mitotic figures per 2 mm^2 was counted starting at a field with a mitotic figure, if present. The presence of necrosis, lymphovascular invasion, and perineural invasion was recorded. Cases were classified according to the current WHO classification into typical and atypical PCT [2]. Representative tissue blocks were selected for immunohistochemistry. Cases were staged according to the 8th edition of the UICC/AJCC staging (TNM classification) [10]. Clinical information was collected from medical records. The study was approved by the Mayo Clinic Rochester Institutional Review Board (#17-004558, date of approval: 6/13/2017).

2.2. Immunohistochemistry

Formalin-fixed, paraffin-embedded (FFPE) tissue blocks were cut at 4 microns. Consecutive slides were stained with hematoxylin-eosin (H&E), and antibody against ATRX (clone D-5, Santa Cruz Biotechnology, Inc, Dallas, TX, USA). ATRX expression was reviewed by a thoracic pathologist (ACR) and a pathology trainee (SBT) independently. Percent ATRX-positive tumor cells were recorded by each reviewer. Only cases that showed staining in the tumor cells, either cytoplasmic and/or nuclear, were included in the study.

2.3. Statistical analysis

Continuous variables were summarized as medians and interquartile ranges (IQR), and compared with Wilcox rank sum test. Categorical variables were summarized as frequency counts and percentages, and compared with Fisher's exact test. Logistic regression model with atypical versus typical PCT as the dependent variable and receiver operating characteristic

Table 2 Relationship between loss of ATRX expression and clinicopathologic features

Variable, no. of cases (% of expressed or loss of expression, respectively, unless specified otherwise)	ATRX Expressed	Loss of ATRX Expression	*P*
N (%)	92 (80)	23 (20)	
Age, years, median [IQR]	59.1 [50.8, 71.7]	62.9 [55.5, 70.8]	.5
Gender, female	64 (69.6)	8 (34.8)	.003
Tumor size, cm, median (IQR)	2.05 [1.50, 3.42]	3.70 [2.35, 5.10]	.001
Mitoses ≥2/ $2mm^2$	25 (27.2)	15 (65.2)	.001
Mitoses per $2mm^2$, median [IQR]	1 [0, 2]	2 [1, 3]	.001
Necrosis present	10 (10.9)	3 (13.0)	.7
Atypical morphology	29 (31.5)	17 (73.9)	<.001
T stage (T2-4)	25 (27.2)	14 (60.9)	.003
pTNM (>I)	32 (34.8)	14 (60.9)	.03
Disease-free survival in years, median [95% CI]	NR	10 [6.5-NR]	.07
Disease-specific survival in years, median	NR	NR [13-NR]	.03

Abbreviations: CI, confidence interval; NR, not reached.

(ROC) were used to establish the cut-off value for ATRX nuclear expression and to evaluate the diagnostic role of ATRX expression. Mitoses (≥2/2 mm^2) and necrosis were used to define atypical PCT, therefore, excluded from the logistic regression model. All logistic regression results were presented as odds ratio. Overall survival (OS) was calculated from the date of tumor resection to the date of death. Disease-free survival (DFS) was calculated from the date of tumor resection to the date of first local recurrence and/or metastasis. Time-to-event data were summarized using the Kaplan-Meier method with surviving patients censored at the date of last follow-up and compared using log-rank tests. Cox proportional hazards model was used for multivariable analyses of potential prognostic indicators. The results were presented as hazard ratios (HRs). Interobserver variability of ATRX expression between the two reviewers was quantified using pairwise kappa statistics. A κ value of 0.21 to 0.40 was regarded as fair agreement, 0.41 to 0.60 as moderate, 0.61 to 0.80 as substantial, and 0.81 to 1.00 as almost perfect agreement [11]. Data from reviewer 1 were used for final statistical analysis. All statistical tests were 2-sided. A $P < .05$ was considered statistically significant.

3. Results

3.1. Patient demographics, clinical characteristics, and morphologic findings

Our study included 115 patients with either typical (N = 69) or atypical (N = 46) PCTs. Demographics, treatment and outcome data are summarized in Table 1. Atypical PCTs were significantly larger ($P = .02$) than typical PCTs. Patients with atypical PCT were more likely to receive additional therapy after surgery ($P < .001$).

Follow up was available in all patients and was detailed in Table 1. Median follow-up time of the study

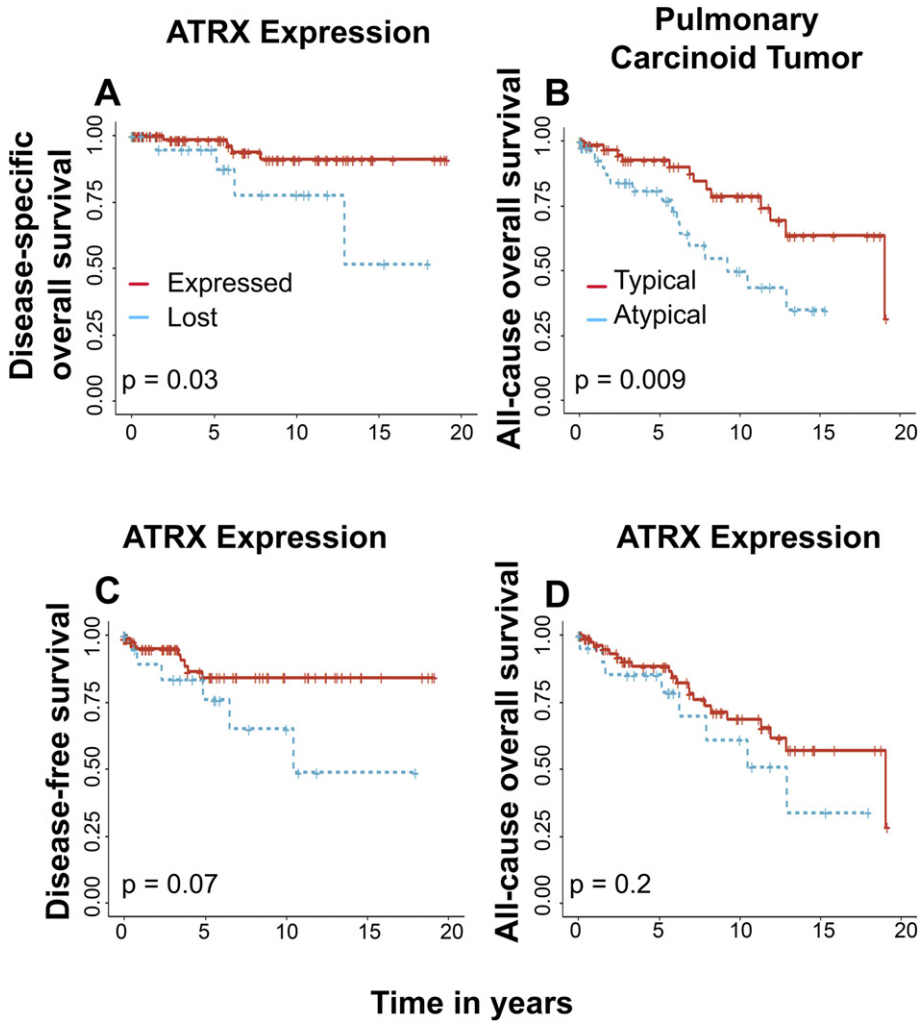

Fig. 3 Kaplan Meier curves. A, Loss of ATRX expression is associated with shorter disease-specific survival. B, Atypical carcinoid tumors are associated with shorter all-cause overall survival. Loss of ATRX expression is not associated with disease-free survival (C) or all-cause overall survival (D).

population was 4.2 years (IQR, 1.2 to 9.4). Patients with atypical PCTs had a worse prognosis including more common recurrences (P = .009), shorter DFS (P = .009), and higher likelihood of death due to disease (P = .002). Subsequent recurrences included metastases in 12 patients (3 of 69, 4.3% with typical PCT; 9 of 46, 19.6% with atypical PCT). The majority of metastases occurred in liver, mediastinal lymph nodes, and supraclavicular lymph nodes. All eight patients who died from disease had an atypical PCT.

3.2. Relationship between ATRX expression and clinicopathologic features

Univariate logistic regression model with histologic types (atypical vs typical PCT) as a dependent variable and ATRX nuclear expression as an independent variable determined a cut-off of 5%. Therefore, loss of ATRX nuclear expression was defined as ≤5% of tumor cell nuclei expressing ATRX. Loss of ATRX expression was more commonly identified in atypical than typical PCT (P .001) (Table 1, Fig. 1A-D) and was associated with atypical PCT with OR 7.4 [95% CI, 2.6-23, P < .001], after adjusting for lymphovascular invasion and perineural invasion. Loss of ATRX nuclear expression predicted atypical PCT with a sensitivity of 37% (95% CI, 24%-52%), specificity of 92% (95% CI, 86%-98%), AUC of 0.62 (95% CI, 0.52-0.72) (Fig. 2).

The relationship between ATRX expression and clinical-pathologic features were summarized in Table 2. Loss of ATRX expression was associated with male sex (P =.003), increased mitotic activity (P = .001), larger tumors (P = .001), higher pT stage (P = .003), and higher pTNM stage (P = .03). The group of patients with PCT that showed loss of ATRX expression was comprised of a higher percentage of atypical PCT cases than the group of patients with PCT that expressed ATRX (74% vs 32%, respectively, P < .001).

3.3. Loss of ATRX nuclear expression was prognostic for shorter disease-specific survival

Loss of ATRX nuclear expression was associated with shorter disease-specific survival (P = .03) in the entire patient cohort (Fig. 3A), but in neither typical (P = 1) nor atypical (P = .5) PCTs. Multivariable survival analysis confirmed that loss of ATRX expression is associated with disease-specific survival after adjusting for lymphovascular invasion and presence of metastatic disease at time of diagnosis (HR = 11; 95% CI, 1.8-68, P = .01) (Table 3). The histological classification of atypical vs typical PCT appeared to remain to be the best prognostic factor (P = .009) with atypical PCT exhibiting worse overall survival (Fig. 3B). Loss of ATRX nuclear expression was not associated with either disease-free survival (P = .07) (Fig. 3C) or all-cause overall survival (P = .2) (Fig. 3D).

Table 3 Multivariable survival analyses of prognostic factors for disease-specific survival

Variables	HR (95% CI for HR)	P
Loss of ATRX nuclear expression	11.0 (1.8-68)	.01
Lymphovascular invasion	31 (2.0-474)	.01
Metastasis	60 (7.3-489)	<.001

3.4. Interobserver agreement of ATRX expression in pulmonary carcinoid tumors was substantial

Interobserver agreement of ATRX nuclear expression between two pathologists was substantial with kappa statistics of 0.72 [95% CI, 0.60-0.80] (Fig. 1E&F).

4. Discussion

In a series of 115 pulmonary carcinoid tumors, we have shown that nuclear ATRX expression is more commonly lost in atypical than in typical PCT, and is associated with more aggressive tumor characteristics such as larger tumor size, increased mitotic activity, higher T stage and higher pTNM stage. Moreover, loss of ATRX expression was associated with shorter disease-specific survival. Our findings suggested that loss of ATRX protein might play a role in the pathogenesis of at least a subset of PCT.

Little is known about genetic alterations in pulmonary neuroendocrine tumors, specifically in PCT. The overall mutation rate of PCT appears to be low, with reported 0.4 mutations per megabase [12]. Furthermore, a meta-analysis of specific genomic alterations in pulmonary neuroendocrine tumors suggested that chromosomal alterations are much less common in typical PCTs (mean, 2.8 aberrations/tumor) than in atypical PCTs (mean, 6.1) [13].

ATRX mutations have been strongly implicated in the pathogenesis of Pan-NET. Whole exome sequencing revealed mutations in *ATRX* in 18% of Pan-NET tumors [14]. ATRX has multiple functions, including chromatin remodeling during heterochromatin assembly at repetitive guanine-rich regions, where it is required for incorporation of the histone variant H3.3 [15-17]. Mutations in *ATRX* result in the loss of the nuclear ATRX protein, which leads to activation of the alternative lengthening of telomeres (ALT) pathway, a mechanism of telomerase-independent telomere maintenance. This eventually results in chromosomal instability, tumor heterogeneity and metastases [18]. In fact, abnormal telomeres were found in 61% of Pan-NET [16]. In addition, all Pan-NET with these abnormal telomeres had *ATRX* or *DAXX* (death-domain-associated protein6, another protein involved in the ALT pathway) mutations, or loss of nuclear ATRX or DAXX protein. These data suggested that an alternative telomere maintenance function may operate in tumors with alterations in the *ATRX* gene. Interestingly, surgical series of Pan-

NET have shown that patients with loss of ATRX or DAXX expression have a worse prognosis [18-20]. In fact, loss of ATRX expression was shown to be an independent parameter for poor overall survival in Pan-NET [19,20]. Furthermore, loss of ATRX and/or DAXX was associated with chromosomal instability, possibly explaining the presence of chromosomal instability in a subset of more aggressive Pan-NET [18,21,22]. Acquired *ATRX* mutations were also seen in various other cancers including gliomas [16].

ATRX expression, mutations, and associated prognostic implication have not been thoroughly investigated in pulmonary neuroendocrine tumors. While morphologic features of low grade Pan-NET and PCT are similar, it is unclear whether they have similar pathogeneses, or whether there might be site-specific differences. Therefore, we investigated whether loss of ATRX expression might play a role in pulmonary neuroendocrine tumors. We focused on PCTs rather than small cell or large cell neuroendocrine carcinomas, since the genetic pathogenesis of PCTs have not been well characterized given their generally low mutational burden. Moreover, a subset of PCTs behaves in a more aggressive fashion, which may occur even in typical PCTs and therefore is not always accurately predicted by the current WHO classification system. Therefore, there is a need for additional prognostic parameters to predict clinical behavior in PCTs. In our study, we have confirmed that atypical PCT are larger, are more likely to recur, and have a higher likelihood of causing death. Loss of ATRX expression was associated with features of more aggressive behavior, such as larger tumor size, increased mitotic activity, and higher T stage. However, while loss of ATRX expression showed a trend towards shorter overall survival, atypical PCTs were more significantly associated with shorter overall survival, therefore the histologic distinction between typical and atypical PCT appears to be a better predictor of outcome. In our study we identified 19% of PCT with loss of ATRX expression. However, this is likely an overestimate, given that our series was not comprised of consecutive PCT but instead enriched for atypical PCTs, which, given the higher frequency of ATRX loss, will falsely inflate that number. Our findings were similar to Pan-NET, in which 14% showed loss of expression of ATRX [19] and 18% harbored *ATRX* mutations [14]. However, another study of Pan-NET showed loss of ATRX expression in 72% of the cases [20]. It is not entirely clear why there is this discrepancy in the reported rate of ATRX loss in Pan-NET, but it might be, at least in part, due to the use of different antibodies. While we used a monoclonal ATRX antibody, the study by Park et al [20]. utilized a rabbit polyclonal antibody.

Our data raise the possibility that alterations in ATRX-associated pathway(s) might play a role in the pathogenesis of at least a subgroup of PCTs, similar to Pan-NET. However, our findings need to be validated in larger studies. While data regarding *ATRX* mutations in PCTs is not robust, in one whole exome sequencing study, only 1 (of 35) atypical PCT and none of 53 typical PCT harbored such a mutation [23]. Although it has been shown that *ATRX* mutations result in loss of expression of the ATRX protein, conceivably, the lack of a demonstrable *ATRX* mutation does not necessarily preclude the lack of ATRX protein expression.

Interobserver variability for the assessment of nuclear expression of ATRX was substantial, although not perfect. Difficulties in reproducibility mainly stemmed from cases that showed relatively strong cytoplasmic expression which sometimes masked the loss of nuclear expression.

Our study had several limitations. First, although this is a relatively large study given the rarity of the disease, we had to enrich the study population for atypical PCTs as those are much less common. Therefore we could not include consecutive patients. Second, we only included resection specimens to have whole tissue sections available for immunohistochemistry, as ATRX expression can be patchy; therefore we do not know if this test would be applicable to small biopsies. Third, the low number of cases with loss of ATRX nuclear expression prevented us from detailed subgroup analysis of outcomes in typical vs atypical PCT.

5. Conclusions

Loss of nuclear expression of ATRX is associated with clinical and morphologic features suggestive of more aggressive behavior in PCT. ATRX expression might be a useful marker for larger studies of pulmonary neuroendocrine tumors, possibly also including high grade neuroendocrine carcinomas. Alterations in ATRX-related pathways might play a role in at least a subset of PCT.

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