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ORIGINAL ARTICLE

Orthopedia homeobox protein (OTP) is a sensitive and specific marker for primary pulmonary carcinoid tumors in cytologic and surgical specimens

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Introduction Orthopedia homeobox protein (OTP) was recently demonstrated to be a pulmonary neuroendocrine marker showing specificity for pulmonary carcinoid tumors in histologic sections. Little is known of OTP performance and specificity for pulmonary neuroendocrine tumors in lung fine-needle aspiration (FNA) cell blocks (CBs), however.

Materials and Methods We evaluated OTP expression in lung non-neuroendocrine and neuroendocrine tumor CBs to determine its diagnostic utility in these specimens. Pulmonary typical carcinoid (TC) and atypical carcinoid (AC), small-cell lung cancer (SCLC) and large-cell neuroendocrine carcinoma (LCNEC), and squamous cell carcinoma (SQ) and adenocarcinoma (ADC) CBs were retrieved along with matched surgical cases and assessed for nuclear OTP expression with immunohistochemistry.

Results Nuclear OTP was seen in 82% TCs (9 of 11) and 83% ACs (10 of 12), but not in SCLC (0 of 9), LCNEC (0 of 9), SQ (0 of 10) or ADC (0 of 6) cytology cases. Similar to the cytologic specimens, nuclear OTP was seen in 82% TCs (9 of 11) and 80% ACs (8 of 10) but in none of the SCLC (0 of 8), LCNEC (0 of 7), SQ (0 of 8) or ADC (0 of 6) resections. Both AC and TC CBs showed a similar percentage of cells expressing nuclear OTP. By contrast, in resection specimens, 30% ACs (3 of 10) compared with 73% TC (8 of 11) cases showed >40% of cells nuclear OTP staining. Nuclear OTP demonstrated 80-83% sensitivity and

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100% specificity for pulmonary carcinoid tumors in cytology and surgical specimens.
Conclusion In the context of pulmonary malignancies, nuclear OTP immunohistochemistry is highly sensitive and specific in distinguishing carcinoid tumors from other pulmonary neuroendocrine and non-neuroendocrine malignancies in cytologic and surgical specimens.

Introduction

Lung cancer is the most common cause of death from cancer in the United States. The initial approach for a definitive diagnosis often involves cytologic evaluation of fine-needle aspiration (FNA) and small lung biopsies. In particular, establishing the presence of neuroendocrine differentiation is critical in the algorithm for the workup of a lung mass, as it alters downstream management. Among pulmonary malignancies, neuroendocrine neoplasms account for up to 25%, with small-cell lung cancer (SCLC) (20% of all primary lung neoplasms) being the most common, followed by large-cell neuroendocrine carcinoma (LCNEC; 3%), typical carcinoid (TC; 2%), and atypical carcinoid (AC; <1%).[1] Based on the World Health Organization classification scheme, pulmonary neuroendocrine tumors are categorized based on their morphologic features, number of mitoses and necrosis.[2] TCs lack mitoses (<2 mitoses per 10 high power fields [hpfs]) and necrosis and are considered low grade. ACs are considered intermediate grade with 2 to 10 mitoses per 10 hpfs with or without focal necrosis. Finally, high-grade pulmonary neuroendocrine carcinomas include both small-cell and large-cell neuroendocrine tumors that demonstrate >10 mitoses per 10 hpf and extensive necrosis.

Distinguishing between grade groups among lung neuroendocrine neoplasms is equally important for prognosis and therapeutic management. TCs have the best 5-year survival of 90%, followed by ACs with 60%, SCLCs with 31%, and LCNECs with 27%.[2,3] High-grade neuroendocrine tumors are more likely to respond to chemotherapy compared with low-grade neuroendocrine tumors that are more commonly resected.[4] Currently, a panel of cytoplasmic (chromogranin and synaptophysin), membranous (CD56), and nuclear (INSM1) markers are used to establish neuroendocrine differentiation in primary pulmonary tumors.[5,6] None of these markers, however, have specificity for a particular neuroendocrine subtype or primary site of origin, which can be challenging to distinguish in cytology specimens.

Orthopedia homeobox protein (OTP) is a nuclear transcription factor that has well-established roles in neurodevelopment in the embryo, particularly in the development of the hypothalamus and in the neuroblast differentiation pathway.[7,8] More recently, a few studies have shown that nuclear OTP may also serve as a novel prognostic factor associated with low-grade pulmonary carcinoid tumors.[9-14] In one study by Hanley et al on FNAs from neuroendocrine tumors (NETs), OTP immunostaining was found to be specific for pulmonary NETs but was not detectable in NETs of extrapulmonary origin. The current knowledge of OTP is minimal, however, and the aim of our study was to expand our understanding of the performance of this marker in cytology cell blocks derived from FNAs of primary pulmonary malignancies. This is an important question, given the frequent use of aspirates from lung tumors for diagnostic and molecular purposes. In this study, we evaluated the diagnostic utility of OTP for both neuroendocrine and non-neuroendocrine pulmonary tumors in cytology specimens and matched surgical resections.

Material and methods

Cases of pulmonary tumors diagnosed by FNA along with matched surgical resection specimens (when available) between 2006 and 2018 at New York Presbyterian Hospital-Weill Cornell Medicine were identified. Cases were selected after review of the hematoxylin and eosin−stained cell block (CB) and surgical resection slides using the current World Health Organization diagnostic criteria. Our initial cohort of 60 cases contained 11 TC, 12 AC, 10 SCLC, 9 LCNEC, 10 squamous cell carcinoma (SQ), and 8 adenocarcinoma (ADC) CBs with 58 available corresponding surgical cases. Three cytology cases (1 SCLC and 2 ADCs) and 8 surgical cases (1 TC, 1 SCLC, 1 LCNEC, 2 SQs, 3 ADCs) did not have sufficient tumor cells for OTP immunohistochemistry or blocks were unavailable. The remaining 57 CBs and corresponding 50 surgical resections were stained with OTP.

Immunohistochemical staining was performed using OTP antibody (1:800, Catalog number HPA039365, Sigma-Aldrich, St. Louis, MO) on paraffin embedded tissue sections from CBs and select blocks from resection specimens on a Leica Bond system (Buffalo Grove, IL) using the modified protocol F provided by the manufacturer. The section was pretreated using heat-mediated antigen retrieval with Tris-EDTA buffer (pH = 9, epitope retrieval solution 2) for 20 minutes and incubated with the antibodies for 15 minutes at room temperature. OTP were detected using a horseradish peroxidase conjugated compact polymer system and DAB as the chromogen. Each section was counterstained with hematoxylin and mounted with Leica Micromount. The immunostains along with the hematoxylin and eosin−stained slides were reviewed and the intensity of the nuclear OTP staining was graded as 1+, 2+, or 3+ and the percentage of staining within the tumor was quantified. OTP immunohistochemical staining was considered positive

Table 1 Clinicopathologic parameters for pulmonary malignancy cohort.

Patient	Cell block (CB) or surgical resection (S)	Age, years	Sex	Tumor size	Source
Typical carcinoid					
1	Both	69	M	1.9	Lung
2	Both	66	M	2	Lung
3	Both	32	F	3	Lung
4	Both	55	M	1.5	Lung
5	Both	73	M	N/A	Lung
6	Both	80	F	1.3	Lung
7	Both	63	M	1	Lung
8	Both	78	F	1.5	Lung
9	Both	75	F	0.9	Lung
10	Both	64	F	1.5	Lung
11	Both	81	F	2.1	Lung
Atypical carcinoid					
12	Both	52	F	N/A	Lung
13	Both	75	M	1.9	Rib
14	Both	56	F	1.1	Lung
15	Both	71	F	2	Liver
16	Both	65	F	1	Lung
17	Both	56	M	0.7	Lung
18	Both	65	F	3	Liver
19	Both	52	F	2.7	Level 7 lymph node
20	Both	65	M	2.5	Lung
21	CB	78	F	2	Lung
22	Both	70	F	2.8	Liver
23	CB	66	M	2	Liver
Small-cell neuroendocrine carcinoma					
24	S	48	M	N/A	Right cervical lymph node
25	Both	51	M	4.9	Lung/mediastinum
26	Both	74	M	6.7	Liver
27	Both	72	F	6.5	Lung
28	Both	77	M	N/A	Level 7 lymph node
29	Both	70	M	N/A	Level 4,7 lymph nodes
30	Both	63	M	6.6	Lung
31	CB	59	M	8.3	Lung
32	Both	86	M	N/A	Lung
33	CB	80	F	1.3	Lung
Large-cell neuroendocrine carcinoma					
34	Both	80	F	3	Lung
35	Both	57	M	3.3	Lung
36	Both	71	M	2.5	Lung
37	Both	47	F	2.2	Peripancreatic lymph nodes
38	Both	66	M	8.2	Lung
39	Both	53	F	5	Lung
40	CB	66	M	1	Level 7 lymph node
41	Both	72	M	2	Liver metastasis
42	Both	79	F	2	Lung
Squamous cell carcinoma					
43	Both	57	M	3.6	Lung
44	Both	71	M	1.7	Lung
45	Both	59	F	5.3	Lung
46	Both	83	M	2	Lung
47	Both	62	M	5.2	Lung
48	Both	79	M	N/A	Lung
49	Both	63	M	5.8, 9.5	Lung
50	Both	50	F	4.5	Lung
51	CB	65	F	1.5	Lung
52	CB	86	M	1.6	Lung

(*continued on next page*)

Table 1 (*continued*)

Patient	Cell block (CB) or surgical resection (S)	Age, years	Sex	Tumor size	Source
Adenocarcinoma					
53	Both	72	M	2.1	Lung
54	S	63	F	1.3	Lung
55	S	83	F	6.1	Lung
56	Both	69	F	2.7	Lung
57	CB	56	M	3.4	Lung
58	Both	79	F	0.9	Lung
59	Both	73	F	2.1	Lung
60	CB	74	F	2.8	Lung

when tumors showed >1 + OTP expression in >5% of the tumor within the specimen. A surgical resection of a TC and neuronal tissue were used for optimization of OTP staining and subsequently as a positive control.

Results

From our archival pathology database, cytology CBs prepared from FNAs from 60 pulmonary tumors were retrieved along with 58 available matched surgical resection specimens encompassing both non-neuroendocrine and neuroendocrine tumors. Included within our cytology and surgical resection cohort of pulmonary tumors were 11 TCs, 12 ACs, 10 SCLCs, 9 LCNECs, 10 SQs and 8 ADCs. All tumors examined in this study were of pulmonary origin. All cases were primary lung tumors except 6 cases of ACs, 4 cases of SCLCs, and 3 LCNECs were secondary metastases to the lymph nodes or liver. The average age of the patients was 67 years (range: 32 to 86 years) with a male:female ratio of 1.1:1. The mean tumor size was 3 cm (0.7 to 9.5 cm). The clinicopathologic parameters for all the patients in each of the tumor types are summarized in Table 1.

Among the cytologic and surgical resection specimens stained, only select neuroendocrine tumors demonstrated positive OTP staining. Among the CB preparations, 82% of

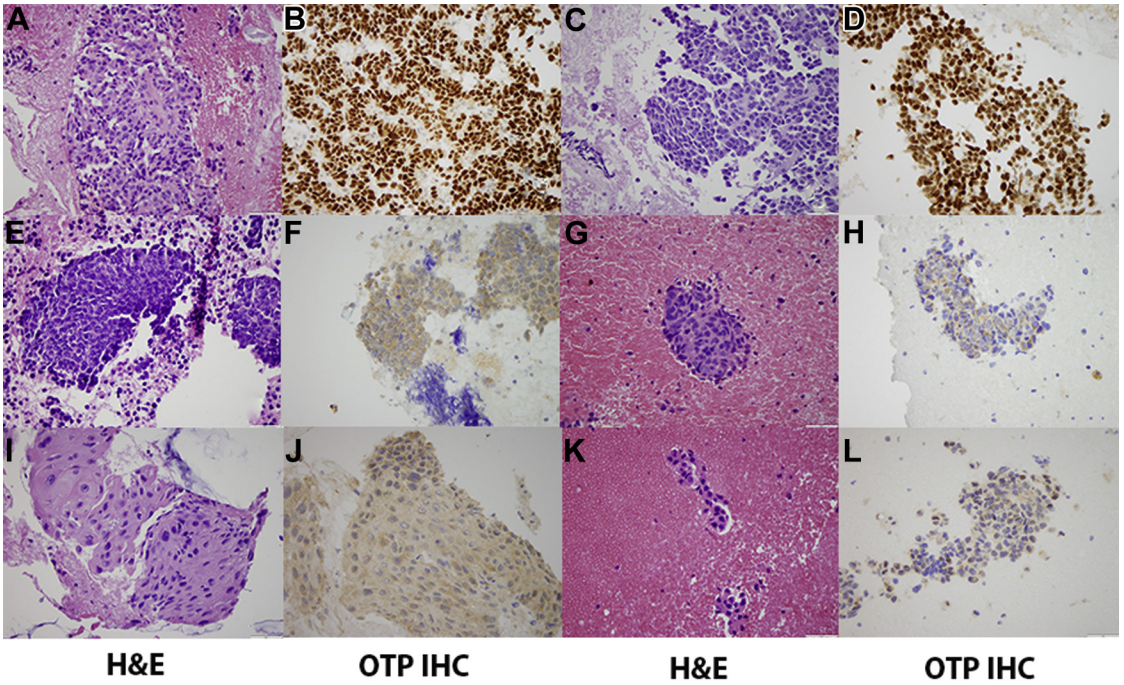

Figure 1 Nuclear OTP is expressed selectively in typical and atypical carcinoids in cytology specimens. Cell block preparations from fine-needle aspirates from typical carcinoid (A and B), atypical carcinoid (C and D), small-cell lung cancer (E and F), large-cell neuroendocrine carcinoma (G and H), squamous cell carcinoma (I and J), and adenocarcinoma (K and L) were stained with either hematoxylin and eosin or immunostained with OTP. Nuclear OTP is seen primarily in typical and atypical carcinoids but not in other pulmonary neuroendocrine or non-neuroendocrine malignancies in cytologic specimens. All images were captured at 400× magnification. H&E, hematoxylin and eosin; IHC, immunohistochemistry; OTP, orthopedia homeobox protein.

TCs (9 of 11) and 83% of ACs (10 of 12) showed strong OTP staining. No significant OTP staining differences were noted between the typical and atypical carcinoids in the cytology specimens. On the other hand, OTP staining was either not identifiable or demonstrated cytoplasmic granular staining in the high-grade SCLC or LCNEC cases. Similarly, with respect to the non-neuroendocrine cytology specimens, CB preparations from pulmonary SQs and ADCs did not demonstrate any nuclear OTP staining (Fig. 1). In cytology specimens, nuclear OTP staining showed a sensitivity of 82.6% and specificity of 100% for pulmonary carcinoid tumors (23 in total).

Similar to the cytologic specimens, nuclear OTP was seen in most pulmonary carcinoid tumors—82% of TCs (9 of 11) and 80% of ACs (8 of 10). In contrast, none of the resection specimens harboring SCLC, LCNEC, SQ, or ADC showed any demonstrable OTP staining (Fig. 2). In surgical resection specimens, OTP showed an 80.9% sensitivity and 100% specificity for pulmonary carcinoid tumors (21 in total).

Using an arbitrary OTP staining cutoff of 40%, both TCs and ACs showed similar distributions in the percentage of cells expressing nuclear OTP in cytology CB preparations. By contrast, in resection specimens, only 30% of ACs (3 of 10) showed >40% of cells with nuclear OTP staining compared with 73% of TCs (8 of 11) that showed >40% of cells nuclear OTP staining (Fig. 3). Overall, nuclear OTP expression showed an 80% to 83% sensitivity and 100% specificity for pulmonary carcinoid tumors in cytology and surgical specimens (Table 2). The positive predictive value and negative predictive value of OTP immunohistochemistry for pulmonary carcinoid tumors were calculated to be as follows: 100% and 89.5% on cytology and 100% and 87.9% on surgical specimens, respectively.

Discussion

The distinction between non-neuroendocrine and neuroendocrine pulmonary malignancy is crucial as it impacts subsequent surgical management and downstream treatment.[3] Even within neuroendocrine tumors, however, the subdistinction between high-grade small-cell and large-cell neuroendocrine tumors from low- to intermediate-grade pulmonary carcinoid tumors will have significant impact on the prognosis of the patient and the type of chemotherapy regimen or surgical treatment that the patient receives. The diagnosis on neuroendocrine tumors on small lung biopsies and cytology specimens can be challenging and, for this reason, there is an important need for specific immunohistochemical markers that can assist in separating between the different subtypes of neuroendocrine tumors.

In the initial studies by Swarts et al, gene expression studies on carcinoid tumors with favorable and unfavorable prognoses revealed a set of downregulated genes in the unfavorable group that included OTP.[13,14] Among the 60

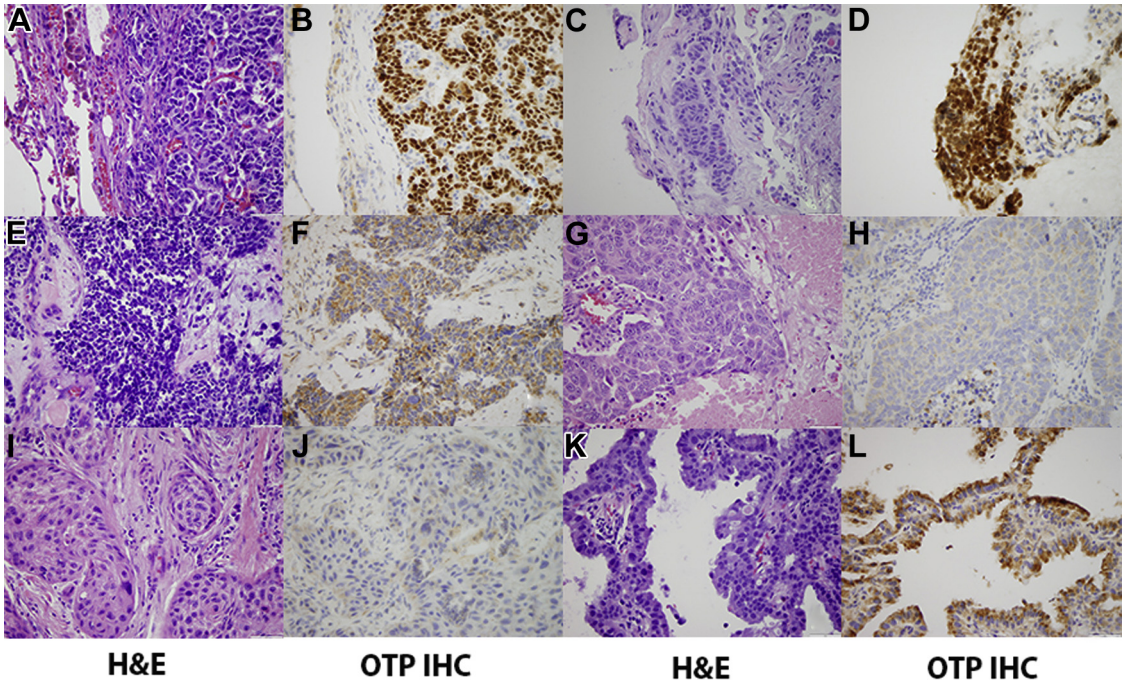

Figure 2 Nuclear OTP is expressed selectively in typical and atypical carcinoids in surgical resections. Representative sections from surgical resection specimens of typical carcinoid (A and B), atypical carcinoid (C and D), small-cell lung cancer (E and F), large-cell neuroendocrine carcinoma (G and H), SQ (I and J) and adenocarcinoma (K and L) were stained with either hematoxylin and eosin or immunostained with OTP. Similar to cytologic specimens, nuclear OTP is seen primarily in typical and atypical carcinoid. All images were captured at 400× magnification. H&E, hematoxylin and eosin; IHC, immunohistochemistry; OTP, orthopedia homeobox protein.

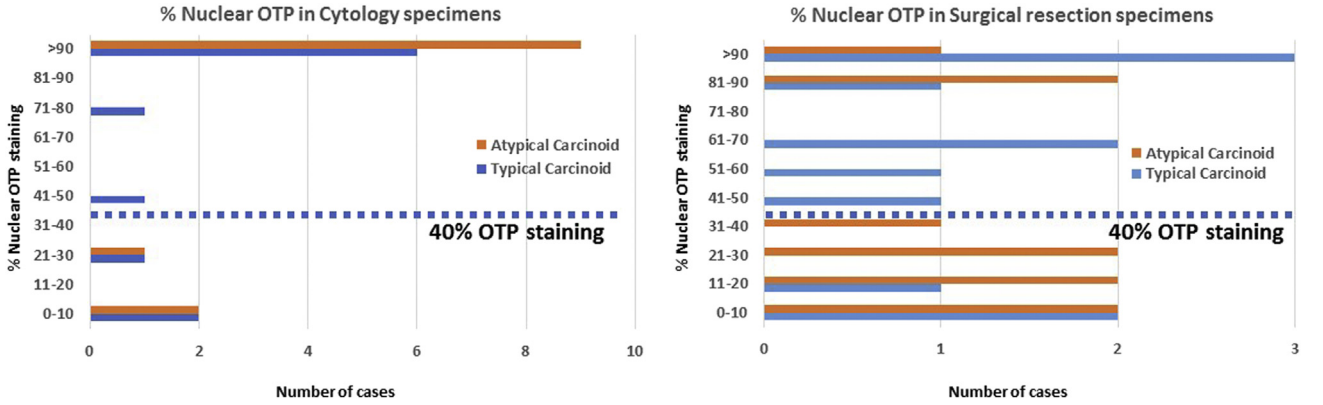

Figure 3 Typical carcinoids show a greater degree of nuclear OTP expression compared with atypical carcinoids in surgical resection specimens. The percentage of tumor demonstrating nuclear OTP expression in each cytologic and surgical case of typical and atypical carcinoid was quantified and plotted. At an arbitrary cutoff of 40% staining, no significant difference in the extent of nuclear OTP expression was noted between typical and atypical carcinoid. By contrast, 73% of typical carcinoid showed >40% nuclear OTP expression, whereas only 30% of atypical carcinoid showed >40% nuclear expression. OTP, orthopedia homeobox protein.

neuroendocrine tumors examined in their first study, loss of or decreased OTP was independently associated with decreased survival and increased risk of metastases; however, surprisingly, nuclear OTP was primarily noted only in low-grade carcinoid tumors and was lost or decreased in intermediate to higher-grade neuroendocrine carcinomas. In the same set of studies and in a separate study by Papaxoinis et al, in addition to the downregulation of OTP, loss of CD44 expression was also associated with biologically aggressive carcinoid tumors and loss of both OTP and CD44 correlated with a worse outcome compared with loss of either one alone[12-14]; thus, the combination of OTP and CD44 could also be promising to test in cytologic specimens. Similarly, in a more recent study Papaxoinis et al stratified 166 pulmonary carcinoid cases into 3 clusters based on nuclear TTF-1 and nuclear OTP staining.[11] TTF-1+/OTP + carcinoid tumors comprised cluster 1, which had a female-predominant distribution, a more peripheral location, was associated with neuroendocrine hyperplasia, and a slightly worse time to relapse compared with TTF-1-/OTP+ tumors in cluster 2, which were more central and not associated with neuroendocrine hyperplasia. By comparison, TTF-1-/OTP- cluster 3 tumors, including atypical carcinoids, were more aggressive and had a far worse time to relapse compared with both cluster 1 and cluster 2 carcinoid tumors. Thus, OTP appears to have prognostic implications in the context of pulmonary carcinoid tumors.[9,10,12-14] Therefore, it was important to test whether the OTP findings that have primarily been in surgical resection specimens could be translated over to cytologic specimens.

Table 2 OTP Immunohistochemistry performance summary in cytology and surgical specimens.

Tumor type	Negative, n	Positive, n	Total, n	% positive
Cytology specimens				
Typical carcinoid	9	2	11	82
Atypical carcinoid	10	2	12	83
Small-cell carcinoma	9	0	9	0
Large-cell neuroendocrine carcinoma	9	0	9	0
Squamous cell carcinoma	10	0	10	0
Adenocarcinoma	6	0	6	0
Total	53	4	57	
Surgical specimens				
Typical carcinoid	9	2	11	82
Atypical carcinoid	8	2	10	80
Small-cell carcinoma	8	0	8	0
Large-cell neuroendocrine carcinoma	7	0	7	0
Squamous cell carcinoma	8	0	8	0
Adenocarcinoma	6	0	6	0
Total	46	4	50	

To date, only 1 other study has explored the expression of OTP in pulmonary cytology specimens. Hanley et al assessed 63 neuroendocrine tumors from extrapulmonary and pulmonary sites.[9] OTP was found to be specific for NETs derived from pulmonary sites in 67% of the cases, whereas none of the NETs derived from extrapulmonary sites showed any evidence of OTP staining. Among 15 pulmonary TCs and ACs, 100% of the TCs were OTP-positive, compared with only 17% of the ACs on CBs. In our study, none of the non-neuroendocrine tumors (including primary lung SQ or lung ADC) stained with nuclear OTP; this has not been reported previously. Similarly, we also did not identify any nuclear staining in any case of SCLC or LCNEC. The only positive nuclear staining was observed in pulmonary carcinoids (82.6% of all

carcinoid tumors), thus confirming the high specificity of this marker for carcinoid tumors. However, we did not observe a significant difference in OTP staining between TCs and ACs in CBs. Although a range in the degree of staining (2+ to 3+ with varying percentages of staining) could be seen, overall, both TCs and ACs showed equal immunoreactivity towards OTP (82% and 80% of TCs and ACs, respectively). Both Hanley et al's study and our study relied on the same polyclonal OTP antibody, thus the differences in staining conditions between institutions and the aliquot of antibody used may explain the differences between our results.

We did note, however, that on the surgical resection specimens, there was a significant difference observed between the degree of OTP staining between TCs and ACs. Whereas 73% of TCs showed >40% OTP staining, only 30% of ACs demonstrated >40% staining. OTP expression has been shown to be decreased with higher-grade carcinoid tumors and is associated with poor outcomes. Both Hanley et al and Nonaka et al observed reduced OTP expression in ACs in cytology and surgical resections, respectively.[9,10] Given that we did not observe a significant difference between TCs and ACs in cytology specimens but are observing a trend towards decreased OTP expression in ACs in surgical resections, one possibility to consider is that our staining conditions may not be optimal enough to detect subtle changes in OTP between TCs and ACs in small specimens such as cell blocks. Interestingly, Papaxoinis et al, in a recent study addressing the clinical implications of using OTP immunohistochemistry, had recommended not using OTP as a marker to distinguish between TCs and ACs as it appeared to stain in a gradient that can cause significant overlap between the 2 and lead to misinterpretation if relying solely on OTP immunohistochemistry.[12] Other important points to consider include underlying genetic factors, which may contribute to the extent of OTP expression in our patient cohort, or varying degrees of formalin fixation in relation to the size of the specimen, which may also affect OTP staining. Thus, the differences in results observed between the studies are likely to be multifactorial.

On small biopsy or cytology specimens, the differential for pulmonary carcinoids includes carcinoid tumorlets, neuroendocrine hyperplasia, and diffuse idiopathic pulmonary neuroendocrine cell hyperplasia (DIPNECH). In their cohort of 162 pulmonary tumors, Nonaka et al also included 31 cases of neuroendocrine hyperplasia and all of these cases were strongly immunoreactive for OTP.[10] Both neuroendocrine hyperplasia (without alveolar wall involvement), carcinoid tumorlets (defined as <0.5 cm and with involvement of alveolar walls[2]), and DIPNECH can show similar cytomorphologic features on aspiration, including uniform cells with round nuclei and the canonical salt-and-pepper chromatin. Given the OTP immunoreactivity, these entities are important pitfalls to consider.[15] Interestingly, the synaptophysin-positive Kulchitsky neuroendocrine cells interspersed within the normal bronchus and bronchioles do not demonstrate nuclear OTP reactivity, suggesting that the forms of neuroendocrine hyperplasia that are positive for OTP may represent precursors for the development of carcinoid tumors.[10] Ultimately, the use of radiologic imaging to identify a well-defined mass larger than 0.5 cm will help delineate the presence of a carcinoid tumor over other entities on the differential.

Few prior studies have shown that the OTP antibody can also demonstrate cytoplasmic staining.[9,10,13] In our study, we did identify multiple cases of non-carcinoid tumors with a similar staining pattern. Unlike the uniform positive nuclear staining that we saw with carcinoid tumors, the cytoplasmic staining with the OTP antibody had a coarse granular quality. As per The Human Protein Atlas, the cytoplasmic granular staining with the OTP antibody can be seen in multiple organs and in multiple common tumor types. However, none of the organs or the tumors with the granular OTP staining had detectable OTP mRNA levels.[10] OTP is a known homeobox transcription factor that primarily functions in the nucleus and drives differentiation through binding of the Hox genes and regulating gene expression.[7,8] Thus, the cytoplasmic granular staining seen in non-carcinoid tumors likely represents an artifact or cross-reactivity with other proteins due to the polyclonal nature of the antibody used and is therefore considered negative.

One important caveat must be considered with respect to the OTP specificity. In our study, we primarily focused on FNAs of common lung primary malignancies. We did not include secondary metastases of neuroendocrine tumors and Merkel cell carcinoma. Merkel cell carcinoma can be challenging to diagnose in cytology because of discohesive and uniform small-to-medium-sized cells that can mimic neuroendocrine cytology. Both Nonaka et al and Hanley et al showed that Merkel cell carcinoma can also strongly stain with OTP.[9,10] Another important pitfall to note is that although we did not identify nuclear OTP reactivity in SCLC, both of the other studies identified a small proportion of cases (approximately 1% to 3%) with OTP immunoreactivity.[9,10,13] Thus, caution is necessary before making a diagnosis of carcinoid tumor based on OTP immunohistochemistry, especially in small biopsies and cytology specimens. Although OTP is highly specific for pulmonary carcinoid tumors compared with other neuroendocrine and non-neuroendocrine tumors, the cytomorphologic features of the aspirate should considered in conjunction with the immunohistochemical results, clinical history, and radiologic findings to avoid diagnostic misinterpretation.

Conclusion

In summary, our data demonstrate that OTP is a promising, highly sensitive and specific marker for primary pulmonary carcinoid tumors. Although OTP could not be reliably used to distinguish between typical and atypical carcinoids in

cytology specimens, ultimately, the final classification of the tumor necessitates surgical excision. Future studies should be aimed at developing additional tools and markers, such as CD44, which, in conjunction with OTP, may enable more accurate subclassification and prognostication of these pulmonary carcinoid tumors in cytologic and surgical specimens. Additional studies are also necessary for understanding the interplay between the regulation of OTP expression and its biologic role in the downstream aggressive behavior of carcinoid tumors.

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