POU2F3 in SCLC: Clinicopathologic and Genomic Analysis With a Focus on Its Diagnostic Utility in Neuroendocrine-Low SCLC

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ABSTRACT

Introduction: POU2F3 is a recent marker of a small cell lung carcinoma (SCLC) subtype related to chemosensory tuft cells (SCLC-P). The characteristics of SCLC-P have not been fully defined, and the data on POU2F3 expression in other lung tumors are scarce.

Methods: We screened 254 SCLC for POU2F3 expression and comprehensively analyzed histopathologic, genomic, and clinical characteristics of POU2F3-positive tumors. We also explored POU2F3 expression in other major lung cancer types (n = 433) and a targeted set of potential diagnostic mimics of SCLC (n = 123).

Results: POU2F3 was expressed in 30 of 254 (12%) SCLC and was strongly associated with low expression of standard neuroendocrine markers (synaptophysin, chromogranin A, CD56, INSM1). Notably, POU2F3 was expressed in 75% of SCLC with entirely negative or minimal neuroendocrine marker expression (15/20) and was helpful in supporting the diagnosis of SCLC in such cases. Broad targeted next-generation sequencing revealed that SCLC-P (n = 12) exhibited enrichment in several alterations, including PTEN inactivation, MYC amplifications, and 20q13 amplifications, but similar rates of RB1 and TP53 alterations as other SCLC (n = 155). Beyond SCLC, POU2F3 expression was exclusively limited to large cell neuroendocrine carcinoma (12%) and basaloid squamous cell carcinoma (22%).

Conclusions: This is the largest cohort of SCLC-P clinical samples to date, where we describe the diagnostic utility of POU2F3 in a challenging subset of SCLC with low or absent expression of standard neuroendocrine markers. The distinct genomic alterations in SCLC-P may offer a novel avenue for therapeutic targeting. The role of POU2F3 in a narrow subset of other lung cancer types warrants further study.

Keywords: POU2F3; Small cell lung carcinoma; SCLC-P; Neuroendocrine-low

Introduction

POU2F3 is a master transcriptional regulator of tuft cells—a rare cell type thought to have a chemosensory function...
and immunomodulatory function. These cells are sparsely present in a wide variety of epithelia and are alternatively known as brush cells in the lung airways.

Tuft cell-like variant of SCLC was first described by Huang et al. as tumors having gene expression signature of tuft cells, with POU2F3 representing a lineage oncogene essential for tumor cell survival (SCLC-P). This initial and several subsequent studies have described the highly distinctive transcriptomic and epigenetic profiles of SCLC-P, with POU2F3 expression found to be mutually exclusive of other major SCLC transcriptional subtype markers, ASCL1 and NEUROD1. SCLC-P tumors were found to have distinct therapeutic vulnerabilities in model systems and recently in patients, nominating POU2F3 as an attractive marker for potential personalized treatment of SCLC. Given its recent discovery and relatively low prevalence among SCLC (7%-14%), there are only limited data on clinicopathologic and genomic characteristics of SCLC-P.

A notable characteristic of SCLC-P identified in prior studies was the low or absent expression of neuroendocrine (NE) phenotype markers (so-called NE-negative or NE-low SCLC). This was initially observed by mRNA. In a recent immunohistochemistry (IHC)-based study, we found that these tumors were also associated with low protein expression of all four standard NE markers used in diagnostic pathology (synaptophysin, chromogranin A, CD56/NCAM, INSM1). Considerable variation in the extent of NE marker expression in SCLC has long been documented at both protein and mRNA levels. By mRNA, approximately 20% of SCLC are regarded as NE low/negative, and a similar proportion of SCLC exhibit low expression of NE markers by IHC. The biological characteristics of NE-low SCLC have largely remained a mystery until the identification of POU2F3 expression as a defining characteristic in many of these tumors. In practice, SCLC with extremely low or negative NE markers can present a diagnostic challenge, and identification of a novel diagnostic marker to define this subtype would represent a significant diagnostic advance.

Recent identification of POU2F3 as a marker associated with NE-low subset of SCLC has therefore prompted us to consider whether POU2F3 could have a practical application as an additional marker in the diagnosis of SCLC.

In this study, using the largest cohort of SCLC-P to date (n = 30), we set out to comprehensively analyze the clinicopathologic and genomic characteristics of this SCLC subtype. To evaluate potential diagnostic utility of POU2F3, we specifically focused on SCLC with minimal or negative NE marker expression, some of which presented a diagnostic challenge. To explore the specificity of POU2F3, we surveyed the expression of POU2F3 by IHC in a large set of other lung cancer types (N = 433 tumors in total), including lung adenocarcinoma, squamous cell carcinoma (SCC), large cell NE carcinoma (LCNEC), lung carcinoids, and SMARCA4-deficient undifferentiated tumor (SMARCA4-UT), and evaluated POU2F3 expression in various tumors that may mimic SCLC pathologically (N = 123 total), such as melanoma, lymphoma and sarcoma. Notably, given the recently proposed close developmental relationship of tuft cells and basal cells in lung airways, we included in the analysis a rare lung carcinoma variant with presumed relationship to basal cells known as basaloid SCC. Last, we analyzed the expression of POU2F3 in conjunction with standard NE markers in normal lung tissue.

Materials and Methods

Sample Selection and Study Design

The study was performed with the approval of the institutional review board of the Memorial Sloan Kettering Cancer Center. SCLC specimens included in the analysis comprised 254 consecutive clinical samples of SCLC reviewed at the Memorial Sloan Kettering Cancer Center primarily between January 2017 and October 2021. Of these, 218 were from whole-tissue sections and 36 were from a previously constructed tissue microarray. The set contained 140 cases (up to January 2020) that were included in a prior study.

Other lung cancer types included in the analysis comprised 100 lung adenocarcinomas, 63 SCCs, and 167 carcinoids (136 typical and 31 atypical); these were analyzed using previously constructed TMAs. In addition, 52 LCNECs (35 from whole-tissue sections and 17 from a previously constructed TMA) and 32 whole-tissue sections of basaloid SCC were analyzed.

Potential diagnostic mimics of SCLC included in the study comprised 29 thoracic lymphomas (20 pulmonary mucosa-associated lymphoid tissue lymphomas and nine primary mediastinal B-cell lymphomas), 25 melanomas, 49 Merkel cell carcinomas, and 20 round cell sarcomas. Lymphomas and sarcomas were analyzed in whole-tissue sections, whereas melanomas and Merkel cell carcinomas were analyzed using previously constructed multitissue arrays and TMAs, respectively.

All histologic diagnoses were made using the WHO 2021 criteria, which for SCLC versus LCNEC relied largely on morphologic differences (larger cell size, more abundant cytoplasm, and more prominent nucleoli for LCNEC) and for SCLC versus basaloid SCC relied on morphologic features (such as abrupt keratinization and basement membrane material deposition) and/or labeling for squamous marker p40 for the latter.

POU2F3 IHC was performed on all the above-mentioned tumors (N total = 810). All POU2F3-positive SCLC and a control group of 142 POU2F3-negative SCLC were further analyzed for the expression of the four
standard NE markers (synaptophysin, chromogranin A, CD56/NCAM, and INSM1). SCLC transcriptional subtype markers (ASCL1, NEUROD1), retinoblastoma protein (Rb), and if sufficient tissue was available, TTF-1, Ki-67, and MYC. Pan-keratin (AE1/AE3 and/or Cam5.2) and p40 expression was also evaluated in a subset of SCLC.

**IHC Methods and Scoring Criteria**

For POU2F3, epitope retrieval was performed using Leica Bond III ER2 for 40 minutes on the stainer. The sections were incubated with the mouse monoclonal antibody to POU2F3 (Santa Cruz, CA, clone 6D1; 1:500 dilution) for 30 minutes, and detection was carried out using Bond Polymer Refine DAB IHC detection kit (Supplementary Table 1). Detailed IHC protocols and scoring criteria for other markers are summarized in Supplementary Table 1. Briefly, labeling for POU2F3, NE markers, ASCL1, NEUROD1, and MYC was scored using the intensity of labeling (1 = weak, 2 = moderate, 3 = strong) and percentage of positive tumor cells (1%–100%), with the product of these two parameters yielding a histoscore (H-score). When tumors were dichotomized as positive versus negative, tumors with H-score less than or equal to 10 were regarded as negative, unless stated otherwise. Ki-67 proliferative index was expressed as a percentage of positive tumor cells, and TTF-1 was recorded as positive (any amount of nuclear labeling) or negative. For Rb, only complete lack of nuclear staining in the presence of a positive internal control was interpreted as the loss of expression. The extent of NE marker expression (NE-score) was derived as an average H-score of the four standard NE markers. For descriptive purposes, tumors were grouped as NE-low (NE-score ≤150) versus NE-high (NE-score >150). Within the NE-low group, tumors with NE-score of 0 to 50 were defined as a NE-extremely low/negative subgroup.

**Next-Generation Sequencing**

A total of 167 SCLC samples, including 12 POU2F3-positive and 155 POU2F3-negative, were analyzed using MSK-IMPACT (hybrid capture-based next-generation sequencing platform) for somatic mutations in up to 505 cancer genes, as previously described.26

**Statistical Analysis**

JMP version 14.0 software (SAS Institute Inc., Cary, NC) was used for statistical evaluation of the IHC data. Two-tailed t test was used for analysis of continuous variables, whereas the likelihood-ratio chi-square and Fisher’s exact tests were used for analysis of categorical data. Survival analysis was performed using IBM SPSS Statistics for Windows, version 27.0 software (IBM Core Inc., Armonk, NY). Univariate Kaplan-Meier methodology was used to estimate the probability of overall survival (OS) in each group, for all stages, and stratified by extensive- versus limited-stage disease.

**Results**

**Prevalence and Extent of POU2F3 Expression in SCLC**

The screen of 254 SCLC revealed POU2F3 expression in 30 cases (12%). Of 30 POU2F3-positive SCLC, most (n = 28) had robust nuclear staining in more than 50% of tumor cells (mean 82% of nuclei labeling) and 2 to 3+ intensity was found in 90% of the cases (Supplementary Table 2). The mean H-score of POU2F3 expression was 177 (range: 40–300). The presence of rare scattered POU2F3-positive tumor cells was noted in three SCLC cases; those cases were considered negative for further analysis.

**Immunohistochemical Characteristics of SCLC-P**

Comparison of POU2F3-positive SCLC (n = 30) and a control group of 142 POU2F3-negative SCLC confirmed prior observation of the markedly lower level of NE marker expression in these tumors (Fig. 1A and Supplementary Table 3). Both the number of NE markers expressed and extent of each NE marker reactivity were significantly lower in SCLC-P compared with other SCLC (p < 0.0001 for all comparisons; Fig. 1A). The NE-scores (average H-score for all 4 NE markers combined) for SCLC-P versus other SCLC were 56 and 184, respectively (p < 0.0001). Similarly, TTF-1 expression was substantially lower in SCLC-P compared with other SCLC (7% versus 82%, respectively, p < 0.0001). Conversely, Ki-67 proliferation was comparably high in SCLC-P and other SCLC. Pan-keratin expression was also similar in SCLC-P (93% positive [14 of 15], 13% weakly or focally; Supplementary Table 2) and other SCLC (94% positive [34 of 36], 19% weakly or focally). All SCLC-P were negative for squamous marker p40 (with the exception of squamous components in combined SCLC-P; Supplementary Table 2).

In line with prior studies, expression of POU2F3 was mutually exclusive of ASCL1 and NEUROD1 (Fig. 1A), except for two cases containing immunophenotypically distinct tumor cell populations (subclones) with separate areas positive for POU2F3, ASCL1, or NEUROD1; one of these cases was described in our prior study.12

Detailed IHC results for all 30 SCLC-P are summarized in Supplementary Table 2.

**POU2F3 in SCLC Grouped by the Extent of NE Marker Expression**

To further explore the relationship between POU2F3 and NE marker expression, we divided SCLC...
into NE-high (n = 107) versus NE-low (n = 65) on the basis of the NE-score of more than 150 or less than or equal to 150, respectively. Of the 30 SCLC-P, only one case was NE-high, whereas all other tumors were NE-low (Fig. 1B). These NE-low SCLC generally lacked synaptophysin and chromogranin A expression and were only positive for CD56 and/or INSM1 (Supplementary Table 2), in line with prior studies on the relative sensitivities of these markers in SCLC.\(^\text{14}\)

Within the 29 NE-low SCLC-P, 20 cases had extremely low or negative NE marker expression (NE-score 0–50); of these, nine cases were either entirely negative or nearly negative (labeling in only rare cells, generally with only 1 NE marker) (Fig. 1B). Of 20 NE-extremely low/negative cases, 75% were positive for POU2F3. Notably, within the subgroup with entirely absent (or nearly absent) NE markers, all nine cases were POU2F3 positive (Figs. 1B and 2).

**POU2F3 as a Novel Diagnostic Marker in NE-Low/Negative SCLC**

During the course of the study, we encountered three tumors that presented a diagnostic challenge (case identifications [IDs] 28–30; Supplementary Table 2). These tumors had either entirely absent labeling for NE markers (1 case) or minimal labeling in rare cells (2 cases). Two of the cases (case IDs 28 and 30) were biopsy specimens obtained at outside institutions, where they were both diagnosed as "poorly..."
differentiated carcinomas” with reports indicating insufficient IHC support for definitive tumor classification despite extensive IHC panels (15 and 12 stains, respectively). In all three cases, the presence of robust POU2F3 staining provided direct support for the diagnosis of SCLC despite the complete or near-complete absence of NE marker expression. Case 29 is illustrated in Figure 2 (right panel) and case 28 is illustrated in Figure 3.

Clinicopathologic Characteristics

As summarized in Figure 4A, patient characteristics (age, sex, smoking history) and stage at presentation

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<tr>
<th>Case ID</th>
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<td></td>
<td>NE-high</td>
<td>NE-low/negative</td>
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<th>H&amp;E</th>
<th>SYN</th>
<th>CHRA</th>
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<th>INSM1</th>
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Figure 2. Illustration of preferential POU2F3 expression in SCLC with low or absent expression of standard NE markers. Case in the left panel illustrates a NE-high SCLC (NE-score = 229) having diffuse expression of all conventional NE markers and TTF-1 and negative POU2F3. Other cases are examples of NE-low (case IDs 20, 23 and 25) or entirely negative (case ID 29) SCLC with robust expression of POU2F3. CHRA, chromogranin A; H&E, hematoxylin and eosin; ID, identification; NE, neuroendocrine; SYN, synaptophysin.
Figure 3. Illustration of a case in which POU2F3 supported the diagnosis of SCLC (case ID 28). High-grade tumor having histologic features of SCLC on H&E (high N:C ratio, nuclear molding, and finely granular chromatin) and high Ki-67 proliferative index (70%). Nevertheless, all NE markers and TTF-1 were negative. Various other markers were initially evaluated to exclude an alternative diagnosis (including p40 to exclude squamous cell carcinoma) and were negative. Subsequently performed POU2F3 provided direct support for the diagnosis of SCLC. CHRA, chromogranin A; H&E, hematoxylin and eosin; ID, identification; IHC, immunohistochemistry; N:C, nuclear-to-cytoplasmic; NE, neuroendocrine; SYN, synaptophysin.
were comparable for SCLC-P versus other SCLC. Specimen characteristics (sampling type and site) were also similar (Supplementary Table 4). SCLC-P showed a trend toward higher rate of combined histology with NSCLC compared with other SCLC (33% versus 18%, respectively, \( p = 0.06; \) Fig. 4A). In all combined carcinomas, POU2F3 expression was found exclusively or predominantly in SCLC components (Supplementary Fig. 1). Morphology of SCLC-P in pure and combined carcinomas was that of typical SCLC.

Clinical follow-up was available for all 30 patients with SCLC-P. Kaplan-Meier analysis revealed no significant difference in the OS between SCLC-P and a control group of 155 patients with other SCLC for all stages combined (median OS 19.5 versus 19.8 mo, respectively, \( p = 0.5; \) Fig. 4B) and stratified by stage (Supplementary Fig. 2).

### Table

<table>
<thead>
<tr>
<th>Gene/locus</th>
<th>POU2F3+ (N = 12)</th>
<th>POU2F3- (N = 155)</th>
<th>( p )-value</th>
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<tr>
<td>TP53</td>
<td>100%</td>
<td>97%</td>
<td>0.7</td>
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<tr>
<td>RB1†</td>
<td>83%</td>
<td>89%</td>
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<tr>
<td>NOTCH1-4</td>
<td>50%</td>
<td>26%</td>
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<td>20q13 locus*</td>
<td>42%</td>
<td>1%</td>
<td>1.6E-05</td>
</tr>
<tr>
<td>PTEN</td>
<td>42%</td>
<td>8%</td>
<td>0.0031</td>
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<tr>
<td>KMT2D</td>
<td>33%</td>
<td>19%</td>
<td>0.2</td>
</tr>
<tr>
<td>MYC</td>
<td>25%</td>
<td>3%</td>
<td>0.01</td>
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**Figure 4.** Clinicopathologic and genomic characteristic of SCLC-P. (A) Comparison of clinical and histologic features of SCLC-P (POU2F3 positive) versus other SCLC (POU2F3 negative). (B) Univariate Kaplan-Meier analysis comparing overall survival in patients with SCLC-P versus other SCLC. (C) Summary of molecular characteristics of SCLC-P. The most prevalent (>33%) genomic alterations in SCLC-P are illustrated, including genes with divergent rates in SCLC-P compared with other SCLC. †Rb IHC was performed on all SCLC cases, and the rate of illustrated RB1 alterations incorporates genomic and IHC findings. *Amplifications of the 20q13 locus involved several genes, including NCOA3 in all five cases, and AURKA and GNAS in three of five cases (case IDs 29, 22, and 16). (D) MYC expression by IHC in SCLC-P versus other SCLC. ADC, adenocarcinoma; ID, identification; IHC, immunohistochemistry; LCNEC, large cell neuroendocrine carcinoma; PY, pack years; SCC, squamous cell carcinoma.
Genomic Characteristics

Genomic analysis was performed using targeted next-generation sequencing (MSK-IMPACT) on 12 SCLC-P in comparison with 155 other SCLC (Fig. 4C and Supplementary Table 5). SCLC-P harbored a mean of 22 nonsynonymous mutations per sample (range: 8–49) with a mean coverage of 691× (range: 340–1005×). Tumor mutation burden was similar in SCLC-P versus other SCLC (median 7.0 versus 7.9, respectively), as was microsatellite instability score27 (median 2.3 versus 1.5, respectively) (Supplementary Fig. 3).

The most prevalent alterations in SCLC-P were TP53 mutations, present in 100% of the cases. RB1 alterations, defined as genomic alterations and/or loss of expression by IHC, were present in 83% of the cases. The frequencies of TP53 and RB1 alterations were similar to those found in other SCLC, as was the rate of mutations in NOTCH family genes (50%) and KMT2D chromatin modifier (33%). Notably, SCLC-P exhibited significantly higher rate of PTEN truncating mutations/losses (42% versus 8%, respectively, p = 0.0031) and MYC amplifications (25% versus 3%, respectively, p = 0.01) compared with other SCLC. In addition, amplification of 20q13 locus (which harbors AURKA, GNAS, and NCOA3 oncogenes) was found in 42% of SCLC-P compared with only 1% of other SCLC (p < 0.0001).

In an exploratory analysis, we assessed MYC protein expression by IHC in 14 SCLC-P versus 30 other SCLC and found that it was significantly higher in SCLC-P than in other SCLC (mean H-score 118 versus 28, respectively, p = 0.0005; Fig. 4D). Only nine SCLC-P had data for both MYC amplification and expression (Supplementary Table 2). Of those, 2/2 MYC-amplified SCLC-P had high levels of MYC expression (H-scores of 260 and 140), but SCLC-P without MYC amplification (n = 7) had a range of MYC expression (H-scores of 1–260; mean 115) with five cases having MYC H-scores of more than or equal to 100.

POU2F3 Expression in Other Major Lung Cancer Types and SCLC Mimickers

To evaluate POU2F3 expression in lung tumors other than SCLC, POU2F3 immunoreactivity was surveyed in other major lung cancer types (433 total tumors tested; Fig. 5A). All major conventional lung cancer types (adenocarcinoma n = 100, SCC n = 63, and carcinoids n = 167) were completely negative for POU2F3. We also

<table>
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<th>Total tested</th>
<th>POU2F3+ N (%)</th>
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<tr>
<td>Lung tumors:</td>
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<tr>
<td>Adenocarcinoma</td>
<td>100 0</td>
</tr>
<tr>
<td>SCC, NOS</td>
<td>63 0</td>
</tr>
<tr>
<td>SCC, basaloid</td>
<td>32 7 (22%)*</td>
</tr>
<tr>
<td>LCNEC</td>
<td>52 6 (12%)**</td>
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<tr>
<td>Carcinoids</td>
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<tr>
<td>Typical</td>
<td>136 0</td>
</tr>
<tr>
<td>Atypical</td>
<td>31 0</td>
</tr>
<tr>
<td>SMARCA4-UT</td>
<td>19 0</td>
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<tr>
<td>Other tumors that can mimic SCLC#</td>
<td></td>
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<tr>
<td>Merkel cell carcinoma</td>
<td>49 0</td>
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<tr>
<td>Melanoma</td>
<td>25 0</td>
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<tr>
<td>Lymphoma</td>
<td>29 0</td>
</tr>
<tr>
<td>Round cell sarcoma</td>
<td>20 0</td>
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Figure 5. POU2F3 expression in other major lung cancer types and histologic mimics of SCLC. (A) Summary of the frequency of POU2F3 expression in other major lung cancer types. *Mean (range) POU2F3 H-score in basaloid SCC: 153 (15–275). **Mean (range) POU2F3 H-score in LCNEC: 204 (85–300). *Lymphomas included 20 mucosa-associated lymphoid tissue lymphomas and nine primary mediastinal B-cell lymphomas. Round cell sarcomas included 10 Ewing/Ewing family sarcomas, seven CIC-rearranged sarcomas, one NCOA2-rearranged sarcoma, one BCOR-CCNB3 sarcoma, and one desmoplastic round cell tumor. (B) Illustration of representative cases of basaloid SCC (left panel) and LCNEC (right panel) with POU2F3 expression. Confirming the diagnosis of basaloid SCC is diffuse expression of p40 and the lack of NE markers. LCNEC is distinguished from SCLC on the basis of standard morphologic criteria. LCNEC, large cell neuroendocrine carcinoma; NOS, not otherwise specified; SCC, squamous cell carcinoma; SMARCA4-UT, SMARCA4-deficient undifferentiated tumor.
tested 19 thoracic SMARCA4-UT—the primitive undifferentiated tumors that can mimic SCLC; all of those were completely POU2F3 negative. Conversely, POU2F3 was expressed in 12% (6 of 52) of LCNEC and 22% (7 of 32) of basaloid SCC (Fig. 5A and B). Expression of standard NE markers in POU2F3-positive LCNEC was lower than in other LCNEC (data not shown). For POU2F3-positive basaloid SCC, the diagnosis was confirmed by diffuse p40 immunoreactivity in all cases. In both LCNEC and basaloid SCC, POU2F3 immunoreactivity was strong and diffuse (mean H-scores of 153 and 204, respectively).

In addition, we explored the expression of POU2F3 in various tumors that can pathologically mimic SCLC, including Merkel cell carcinomas, lymphomas, round cell sarcomas, and melanomas (N total = 123; Fig. 5A). All these tumors were POU2F3 negative, although one Ewing sarcoma had weak nuclear staining in rare tumor cells (H-score = 7).

POU2F3 Expression in Normal Lung

Last, POU2F3 expression was evaluated in randomly selected sections of the benign lung tissue containing airways from five lobectomy specimens for tumor resections. Nuclear staining for POU2F3 was detected in rare cells within airways but not in alveolar lung tissue. POU2F3-positive cells were located primarily in the distal airways, whereas the proximal lobar and segmental airways were largely negative. On manual quantification, the number of POU2F3-positive cells ranged from 0 to 3 cells per distal bronchiolar cross-section, with most bronchioles having none (average < 1). Triple staining for POU2F3, synaptophysin, and p40 highlighted that POU2F3-positive cells were located suprabasally and were synaptophysin-negative. Instead, synaptophysin highlighted separate scattered intra-bronchial NE cells that lacked the expression of POU2F3 (Fig. 6).

Discussion

POU2F3 expression and tuft cell-like phenotype in SCLC have been described in several prior studies after the initial description in 2018. This is the largest study to date on this distinct subtype of SCLC, significantly expanding the clinicopathologic and genomic characteristics of this subset and providing first evidence for the utility of POU2F3 as an ancillary marker for the diagnosis of NE-low SCLC. This is also the first study to perform a survey of POU2F3 expression by IHC in large cohorts of all other major lung cancer types and several histologic mimics of SCLC (N = 556 tumors in total), confirming a very narrow and specific expression pattern for POU2F3.

We and others have previously found that SCLC-P are associated with a low “NE phenotype,” characterized by low expression of various standard markers of NE differentiation. In our prior study, which evaluated the distribution of ASCL1, NEUROD1, POU2F3, and YAP1 by IHC in SCLC, only 12 tumors were of SCLC-P type, whereas in the current study, this group was expanded to 30 cases, allowing a more granular examination of the characteristics of these tumors. In this expanded set, we have confirmed the strict exclusivity of POU2F3 with ASLC1 and NEUROD1 (except for rare SCLC with regional subclones exhibiting distinct transcriptional
regulators, as noted previously\textsuperscript{12} and strong association of POU2F3 with a NE-low phenotype. In addition, we specifically evaluated the tumors with extremely low or negative NE marker expression (NE-score 0–50; n = 20). We found that 75% of the tumors in this subgroup expressed POU2F3, with the rate of POU2F3 expression reaching 100% in tumors with negative or nearly negative labeling for NE markers. Therefore, the likelihood of POU2F3 expression in SCLC is exquisitely and quantitatively linked with the level of NE marker expression, although it can also be rarely found in SCLC with NE-high phenotype. These observations provide a rationale for including POU2F3 as a potential additional diagnostic marker in SCLC lacking or exhibiting minimal levels of standard NE marker expression.

Although SCLC has been traditionally regarded as primarily a morphologic diagnosis that can be made on hematoxylin and eosin–stained slides alone, in recent years, with the increasing recognition of various poorly differentiated tumors that can mimic SCLC, the diagnosis is increasingly supported by IHC.\textsuperscript{13,14} In recent studies, the incorporation of IHC was found to increase accuracy and reproducibility of the SCLC diagnosis,\textsuperscript{29} with labeling for NE markers representing the key feature of SCLC. Nevertheless, even with the utilization of the full panel of NE markers, some SCLC have minimal or entirely negative expression of NE markers.\textsuperscript{15} Because of the lack of positive markers, such tumors may cause a diagnostic challenge and/or require an extensive IHC workup to exclude other entities. Having POU2F3 as a positive marker for these NE-minimal/negative SCLC constitutes a valuable addition to the IHC marker arsenal for the diagnosis of SCLC. Notably, we illustrate here several cases with NE-negative or NE-minimal profiles, where robust expression of POU2F3 served as supporting evidence for SCLC diagnosis.

In addition to proposing the diagnostic utility of POU2F3 in SCLC, in this study, we also examined the pattern of POU2F3 expression in other major lung cancer types (n = 433) and histologic mimics of SCLC (n = 123). We found that POU2F3 expression was consistently negative in all tested lung adenocarcinomas, conventional SCC, carcinoid tumors, and SMARCA4-UT, as well as lymphomas, melanomas, Merkel cell carcinomas, and various round cell sarcomas. As expected, on the basis of the overlap in the gene expression and genomic profiles between SCLC and LCNEC,\textsuperscript{15,30,31} the frequency of POU2F3 expression in LCNEC was similar to that of SCLC (12% in both). The characterization of POU2F3-expressing LCNEC warrants further study. From the diagnostic perspective, the distinction of POU2F3-positive SCLC versus LCNEC should be based on the standard morphologic criteria (see Materials and Methods section).\textsuperscript{13}

An unexpected and intriguing finding in this study was that of expression of POU2F3 in 22% of basaloïd SCC—primitive carcinomas of presumed basal/squamous cell lineage. This finding may be understood in the context of the recent data from in vivo lineage tracing studies that revealed basal cell origin of pulmonary tuft cells.\textsuperscript{18,20} Furthermore, a similarity between a NE-low subset of SCLC (SQ-P) and “primitive phenotype lung squamous cell carcinomas” was suggested previously on the basis of the shared methylation profiles.\textsuperscript{32} Generally, there may be a closer biological relationship between SCLC and basaloïd SCC than currently recognized. Notably, in a recent study, it was noted that basaloïd SCC exhibit up-regulated expression of various NE markers compared with conventional poorly differentiated SCC.\textsuperscript{33} From the diagnostic perspective, POU2F3-positive basaloïd SCC were distinct from SCLC by morphologic features (see Methods) and consistently diffuse expression of squamous marker p40, whereas SCLC-P were consistently p40-negative. Furthermore, in an exploratory analysis, the rate of Rb loss by IHC was significantly lower in POU2F3-positive basaloïd SCC than in POU2F3-positive SCLC (data not shown). Further studies will be needed to clarify the relationship between these tumors.

To the best of our knowledge, there is only one study to date—by Yamada et al.\textsuperscript{14}—that has evaluated POU2F3 expression in tumor types other than SCLC. That study identified POU2F3 expression and tuft cell-like signature in 72% of thymic carcinomas. In addition, the authors analyzed publicly available databases from The Cancer Genome Atlas and other studies and identified POU2F3 mRNA expression and tuft cell-like signature in 2% of lung SCC, all of which were poorly differentiated and less than 1% of adenocarcinomas. It was noted, however, that a thorough pathologic evaluation of tumors was not possible owing to the nature of publicly available data sets. In fact, our re-review of virtual images of the three POU2F3-positive adenocarcinomas suggests a possible morphologic overlap with LCNEC (Supplementary Fig. 4).

Genomic characteristics of SCLC-P are not well established, although prior studies documented that these tumors harbor a similar rate of TP53 and RB1 alterations as other SCLC.\textsuperscript{35} Here, we performed an analysis of genomic characteristics of 12 SCLC-P using targeted broad next-generation sequencing in comparison to 155 other SCLC. We confirmed that the prevalence of TP53 and RB1 was comparable in SCLC-P versus other SCLC. Notably, we identified that SCLC-P harbored a significant enrichment in PTEN and MYC alterations. The high rate of PTEN inactivation in SCLC-P (42% versus 8% in other SCLC) may be of interest therapeutically given that targeting of PTEN-deficient cancers is an area of active investigation.\textsuperscript{36,37} MYC amplification and
overexpression has been long noted to be associated with NE-low SCLC (so-called variant SCLC) in cell lines \cite{38,39} and more recently in SCLC mouse models. \cite{6,40} Association of high MYC mRNA specifically with SCLC-P has also been documented. \cite{4} To the best of our knowledge, this is the first demonstration of enriched MYC amplification and protein expression by IHC in SCLC-P patient samples. The presence of high MYC protein expression in some MYC-unamplified SCLC-P suggests an alternative mechanism for MYC upregulation in some of these tumors. The high rate (42%) of 20q13 locus amplification in SCLC-P is also a novel finding. Although this locus houses several oncogenes, AURKA may be of particular interest given the known activity of Aurora kinase inhibitors in SCLC, \cite{61-63} which in some studies was selective for tumors with high MYC expression. \cite{10,40,44-46} These data provide initial insight that SCLC-P exhibits enrichment in several potentially therapeutically exploitable genomic alterations; however, expanded analysis of a larger set of these tumors is warranted in future studies.

Current understanding of subtype-dependent clinical outcomes in patients with SCLC is limited, although it has been suggested that SCLC-P subtype is associated with poor prognosis. \cite{5} In our series, patients with SCLC-P had similar OS to those with other SCLC. Nevertheless, studies in larger cohorts will be needed to further evaluate the prognostic and treatment implications of SCLC-P relative to other SCLC subtypes.

The presence of tuft cells (also known as brush or caveolated cells) has been described by electron microscopy in human and animal lung tissues decades ago. \cite{3} Recently, these cells were visualized by immunofluorescence using POU2F3 antibody in mouse lung. \cite{4} Nevertheless, to our knowledge, this is the first immunohistochemical illustration of POU2F3-positive native cells in normal human lung tissue. As expected, POU2F3-positive cells were present as rare single cells in the lung airways. Interestingly, POU2F3-positive cells localized primarily to smaller, more distal airways, whereas these cells were largely absent in the larger proximal airways. This contrasts with the observations in mice, where it was found by immunofluorescence that the density of POU2F3-positive cells was higher in the proximal than in the distal murine airways. \cite{4} This could reflect functional differences of tuft cells in human versus murine lung. Furthermore, by performing triple IHC, we confirm that these cells are distinct from basal and NE cells. Nevertheless, whether tuft cells represent the cell of origin of SCLC-P or whether expression of POU2F3 and other tuft cell genes is attributable to aberrant transdifferentiation remains unclear. The rare examples of cases illustrated in our current and prior \cite{1-2} studies with co-existence of distinct POU2F3-, ASCL1-, or NEUROD1-expressing components within a single tumor imply that at least in some cases POU2F3 expression reflects transdifferentiation rather than tuft cell origin.

In summary, we illustrate the diagnostic utility of POU2F3 IHC in NE-low SCLC and identify distinct genomic characteristics of SCLC-P. We also demonstrate that POU2F3 expression in lung tumors extends beyond SCLC to include a fraction of LCNEC and basoloid SCC, whereas various other lung cancer types and potential histologic mimickers of SCLC are consistently POU2F3-negative. Given our limited understanding of the distribution and oncogenic function of POU2F3 in tumors other than SCLC, further examination of its expression in tumors of different histotypes and organ sites is needed. Understanding whether the distinct characteristics of SCLC-P extend to other tumors with POU2F3 expression may allow for common therapeutic strategies for POU2F3-positive malignancies beyond SCLC.

**CRediT Authorship Contribution Statement**

Marina K. Baine: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing—original draft.

Christopher A. Febres-Aldana: Formal analysis, Methodology, Writing—review and editing.

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Supplementary Data
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