**ABSTRACT**

**Introduction:** Recent studies have identified subtypes of small cell lung carcinoma (SCLC) defined by the RNA expression of ASCL1, NEUROD1, POU2F3, and YAP1 transcriptional regulators. There are only limited data on the distribution of these markers at the protein level and associated pathologic characteristics in clinical SCLC samples.

**Methods:** The expression of ASCL1, NEUROD1, POU2F3, and YAP1 was analyzed by immunohistochemistry in 174 patient samples with SCLC. Subtypes defined by these markers were correlated with histologic characteristics, expression of classic neuroendocrine markers (synaptophysin, chromogranin A, CD56, INSM1), and other SCLC markers, including the neuroendocrine phenotype-associated markers TTF-1 and DLL3.

**Results:** ASCL1 and NEUROD1 expression had the following distribution: (1) 41% ASCL1+/NEUROD1--; (2) 37% ASCL1+/NEUROD1++; (3) 8% ASCL1−/NEUROD1++; and (4) 14% ASCL1−/NEUROD1−. On the basis of their relative expression, 69% of cases were ASCL1-dominant and 17% were NEUROD1-dominant. POU2F3 was expressed in 7% of SCLC and was mutually exclusive of ASCL1 and NEUROD1. YAP1 was expressed at low levels, primarily in combined SCLC, and was not exclusive of other subtypes.

Both ASCL1-dominant and NEUROD1-dominant subtypes were associated with neuroendocrine marker/high/TTF-1/high/DLL3/high profile, whereas POU2F3 and other ASCL1/NEUROD1 double-negative tumors were neuroendocrine marker/low/TTF-1/low/DLL3/low.

**Conclusions:** This is the first comprehensive immunohistochemical and histopathologic analysis of novel SCLC subtypes in patient samples. We confirm that ASCL1/NEUROD1 double-negative tumors represent a distinct neuroendocrine-low subtype of SCLC, which is either uniquely associated with POU2F3 or lacks a known dominant regulator. The expression profiles of these markers appear more heterogeneous in native samples than in experimental models, particularly with regard to the high prevalence of ASCL1/NEUROD1...
coexpression. These findings may have prognostic and therapeutic implications and warrant further clinical investigation.

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Keywords: ASCL1; NEUROD1; POU2F3; YAP1; Small cell lung carcinoma; Neuroendocrine

Introduction

SCLC is a highly aggressive malignancy with few available treatment options.1,2 Pathologically, SCLC is characterized by a distinct morphology, expression of neuroendocrine (NE) markers, and an exceptionally high proliferation rate.3 The hallmarks of SCLC and small cell carcinomas arising in other organs include an overall histologic homogeneity with only limited variability under light microscopy. Only two subtypes of SCLC are recognized in standard pathologic classification—one in its pure form and another combined with NSCLC or large cell NE carcinoma (LCNEC).5 Although the expression of NE markers is well known to be variable in SCLC,4 historically, the extent of NE marker expression has not been regarded as biologically relevant and was not known to be associated with any specific tumor characteristics. Genomically, SCLC is also a highly homogeneous disease, with most cases characterized by inactivating RB1 and TP53 alterations.5,6

The biological heterogeneity of SCLC has started to emerge only recently. First, recent studies (based primarily on preclinical models utilizing SCLC cell lines, genetically engineered mouse models, and patient-derived xenografts) have identified distinct subtypes of SCLC defined by divergent gene expression programs driven by neuronal transcription factors ASCL1 and NEUROD1.7 ASCL1-high tumors were suggested to be associated with a high expression of NE markers,5,8-11 whereas NEUROD1-high tumors—lower overall NE marker expression.8-12 However, the characteristics of NEUROD1-high SCLC are not firmly established, given that a closer similarity to ASCL1-high tumors was reported in several later studies.13,14 Subsequently, a subset of ASCL1/NEUROD1 double-negative, so-called non-NE SCLC, was found to express and exhibit dependence on POU2F3—a marker of chemosensory tuft cells, which, in the lung airways, are also known as brush cells.15-17 Finally, YAP1, a transcriptional regulator in the HIPPO growth signaling pathway, was found to be preferentially expressed in a subset of non-NE SCLC5,16; however, its role as a subtype-defining transcriptional driver is not well established. Preclinical studies suggested distinct therapeutic vulnerabilities in the novel marker-defined subtypes of SCLC.15,15,19,21 Furthermore, several molecules with suggested roles in the selection of emerging therapeutic agents, including DLL3—an inhibitor of NOTCH signaling pathway, which is regulated by ASCL1 and associated with NE phenotype,16,14,22,23 were found to be differentially distributed among SCLC subtypes.9,10,12,24 Overall, the emerging data on molecular heterogeneity of SCLC holds promise for biomarker-driven personalized therapeutic approaches for this aggressive disease.25,26

As a result of the aforementioned studies, a recent consensus proposal suggested grouping SCLC into four subtypes defined by RNA expression of ASCL1, NEUROD1, POU2F3, and YAP1, referring to these as SCLC-A, SCLC-N, SCLC-P, and SCLC-Y, respectively.7 Although there is a substantial volume of data on SCLC subtypes in preclinical models, there is currently very limited information on the expression of a full set of these markers in the native clinical samples of SCLC and the pathologic characteristics associated with these subtypes. In addition, although SCLC subtypes have been defined on the basis of transcriptional profiling, the expression of the subtype-defining markers has not been characterized in detail at the protein level. In this study, we analyzed the protein expression of ASCL1, NEUROD1, POU2F3, and YAP1 as measured by immunohistochemistry (IHC) in a large cohort of clinical samples of SCLC (N = 174). We assessed the distribution of these markers and comprehensively analyzed associated pathologic characteristics. This consisted of the analysis of morphologic features and standard diagnostic markers of SCLC, including conventional NE markers (synaptophysin, chromogranin A, CD56, and INSM1) and other classic markers of SCLC (TTF-1/NKX2-1 and Ki-67 proliferation marker). We also evaluated the expression of DLL3 as both an additional marker of NE differentiation and as a therapeutic target.

Materials and Methods

Sample Selection

The study was performed with the approval of the institutional review board of Memorial Sloan Kettering Cancer Center (MSKCC), New York. The specimens included in the analysis comprised 122 clinical samples of SCLC diagnosed at MSKCC primarily during the period of January 2017 and January 2020, with a minority of samples diagnosed before these dates. In addition, 52 SCLC were analyzed in tissue microarrays (TMAs).

Immunohistochemistry

All cases were stained for novel markers of SCLC subtypes (ASCL1, NEUROD1, POU2F3, and YAP1) and conventional markers of SCLC (synaptophysin, chromogranin A, CD56, and INSM1) and other classic markers of SCLC.
chromogranin A, CD56/NCAM, INSM1, Ki-67, and TTF-1. A subset of cases (TMA only) was also analyzed for DLL3. Samples were included in the analysis if an evaluable result was available for at least one of the subtype defining markers (ASCL1, NEUROD1, POU2F3, or YAP1). Non-evaluable stains (such as those with insufficient tissue, technical artifacts or other factors) were excluded. The detailed IHC protocols and the total number of cases with evaluable results for each marker are summarized in Supplementary Tables 1 and 2, respectively.

With the exception of POU2F3, all IHC protocols were performed using previously established methods. The POU2F3 protocol was developed using a commercial antibody (clone 6D1, Santa Cruz) and confirmed to exhibit specific strong nuclear labeling in scattered intramucosal cells of the gastrointestinal tract and pulmonary bronchial epithelium.

**IHC Scoring Criteria**

The detailed scoring criteria for each marker are summarized in Supplementary Table 1. The expression of markers was recorded by two parameters: percent of positive cells (1%–100%) and intensity of labeling (1 = weak, 2 = moderate, 3 = strong). For all markers except Ki-67 and TTF-1, a histoscore (H-score) was derived by multiplying the percent positivity by intensity score, yielding a range of possible H-scores of 0 to 300. The Ki-67 index was recorded as a percentage of positive cells, and TTF-1 was scored as either positive (any extent and intensity of labeling) or negative. To determine the combined NE score, the average of H-scores for chromogranin A, synaptophysin, CD56, and INSM1 was derived. Only cases with all four NE markers available were included in the combined NE score analysis. For descriptive purposes, for individual markers, H-scores of less than or equal to 50 were regarded as low, and those greater than 50 as high. For combined NE score, scores of less than or equal to 150 were regarded as NE-low and those of greater than 150 as NE-high. To define the dominant phenotype for cases with expression of both ASCL1 and NEUROD1, those with higher ASCL1 than NEUROD1 H-scores were regarded as ASCL1-dominant and vice versa. In SCLC with combined SCLC and NSCLC components, IHC scores reflect expression exclusively in the SCLC component.

**TMA Construction**

SCLC TMA was constructed in the Pathology Core Laboratory, Precision Pathology Biobanking Center (PPBC), MSKCC using the automated TMA Grand Master (3DHISTECH, Budapest, Hungary) and TMA Control software (version 2.4). The hematoxylin and eosin slides were reviewed to select the most viable tumor areas, and corresponding areas in the paraffin block were punched as 1.0 mm cores (two cores per case). A custom TMA layout was designed using the TMA Control software.

**Statistical Analysis**

JMP version 14.0 software (SAS Institute) was used for statistical evaluation of the data. The two-tailed t-test was used for the analysis of continuous variables (H-scores, Ki-67 proliferative index, etc.), whereas the likelihood-ratio chi-square test was used for the analysis of categorical data.

**Results**

**Patient and Sample Characteristics**

The detailed patient and sample characteristics for 174 SCLC cases in this study are summarized in Supplementary Table 3. The patients included 96 women (55%), had an average age of 67 years, and an average smoking history of 45 pack-years. Specimens comprised 43 primary tumor resections (25%), 105 biopsies (60%), and 26 fine-needle aspirates (15%). Of biopsy/cytology samples, 57 (44%) were from the primary lung tumors and 74 (56%) from metastatic sites, including intrathoracic lymph nodes (N = 40) and distant metastases (N = 34). Histologically, 82% of cases were pure SCLC, whereas 18% were combined, including combinations of SCLC with adenocarcinoma (7%), squamous cell carcinoma (3%), and LCNEC (9%).

**Individual Expression of ASCL1, NEUROD1, POU2F3, and YAP1**

As summarized in Table 1, the expression of ASCL1, NEUROD1, POU2F3, and YAP1 at any level was detected in 80%, 56%, 8%, and 33% of tumors, respectively. The mean H-score in positive cases was 173 (range: 4–300) for ASCL1, 95 (range: 1–300) for NEUROD1, 137 (range: 6–280) for POU2F3, and 22 (range: 1–100) for YAP1. Among positive cases, the expression of either ASCL1 or POU2F3 was typically high, with an H-score greater than 50 seen in 92% of cases with each marker. NEUROD1 exhibited a broader range of expression, with a similar distribution of high expressors (H-score >50 in 52% of cases) and low expressors (48% of cases). In contrast, YAP1 expression was generally low, with only four cases (7%) above an H-scores of 50, and none above an H-score of 100.

**SCLC Subtypes Defined by Relative Expression of ASCL1 and NEUROD1**

Results for both ASCL1 and NEUROD1 were available for a total of 159 SCLC, yielding the following distribution: (1) 41% ASCL1-only; (2) 8% NEUROD1-only; (3)
YAP1 was enriched in ASCL1/NEUROD1-negative distributed widely in all subtypes (Fig. 2 for 151 SCLC. Unlike POU2F3, YAP1 expression was in all cases. 

strictly mutually exclusive of both ASCL1 and NEUROD1 at an individual cell level, the expression of POU2F3 was and NEUROD1 expression (Supplementary Fig. 1). Thus, an area with POU2F3 expression in the absence of ASCL1 mentioned unique case that contained distinct double-negative tumors. Notably, this was the afore—was only a single case with POU2F3 expression in non double-negative tumors (Fig. 1). Despite the frequent coexpression of ASCL1 and NEUROD1, in most cases (120 of 137; 88%), one of the markers was strongly dominant over the other (H-score differential of >50 points) (Fig. 1C). Cases with near-equivalent expression mostly occurred in the low end of the expression of both markers. 

Microscopic examination of tumors with dual-high ASCL1 and NEUROD1 expression revealed that all but one case had both markers coexpressed in the same tumor cell populations (Fig. 1D). Only a single case exhibited ASCL1 and NEUROD1 expression in spatially distinct subpopulations (Supplementary Fig. 1).

Table 1. Expression of Individual Subtype-Defining Markers in SCLC

<table>
<thead>
<tr>
<th>Marker</th>
<th>N Tumors Tested</th>
<th>Total Cases With Expression, N (%)</th>
<th>H-Score Range, N (% Cases With Expression)</th>
<th>H-Score Mean (Full Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASCL1</td>
<td>160</td>
<td>128 (80)</td>
<td>2 (2) &gt;10-50 &gt;50-150 &gt;150</td>
<td>173 (4-300)</td>
</tr>
<tr>
<td>NEUROD1</td>
<td>163</td>
<td>91 (56)</td>
<td>18 (20) 25 (28) 24 (26) 24 (26)</td>
<td>95 (1-300)</td>
</tr>
<tr>
<td>POU2F3</td>
<td>159</td>
<td>12 (8)</td>
<td>1 (8) 0 (0) 7 (58) 4 (34)</td>
<td>137 (6-280)</td>
</tr>
<tr>
<td>YAP1</td>
<td>164</td>
<td>54 (33)</td>
<td>22 (41) 28 (52) 4 (7) 0 (0)</td>
<td>22 (1-100)</td>
</tr>
</tbody>
</table>

H-score, histoscore.

37% ASCL1/NEUROD1 double-positive; and (4) 14% ASCL1/NEUROD1 double-negative (Fig. 1A). Dual-high expression (both ASCL1 and NEUROD1 H-score of >50) was seen in 35 tumors (22%). 

The distribution of SCLC defined by the relative dominance of ASCL1 and NEUROD1 was as follows: (1) 69% ASCL1-dominant, (2) 17% NEUROD1-dominant, and (3) 14% double-negative (Fig. 1B). Despite the frequent coexpression of ASCL1 and NEUROD1, in most cases (120 of 137; 88%), one of the markers was strongly dominant over the other (H-score differential of >50 points) (Fig. 1C). Cases with near-equivalent expression mostly occurred in the low end of the expression of both markers. 

ASCL1/NEUROD1-Defined Groups and Expression of POU2F3 and YAP1

Results for ASCL1+NEUROD1+POU2F3 were available for 152 SCLC. Strikingly, POU2F3 expression was nearly exclusively restricted to ASCL1/NEUROD1 double-negative tumors (p < 0.0001, Fig. 2A), of which 45% (10 of 22) were POU2F3-negative. Conversely, there was only a single case with POU2F3 expression in non—double-negative tumors. Notably, this was the aforementioned unique case that contained distinct geographic regions with divergent ASCL1/NEUROD1 immunoprofiles; this case also contained a third distinct area with POU2F3 expression in the absence of ASCL1 and NEUROD1 expression (Supplementary Fig. 1). Thus, at an individual cell level, the expression of POU2F3 was strictly mutually exclusive of both ASCL1 and NEUROD1 in all cases. 

Results for ASCL1+NEUROD1+YAP1 were available for 151 SCLC. Unlike POU2F3, YAP1 expression was distributed widely in all subtypes (Fig. 2B). Although YAP1 was enriched in ASCL1/NEUROD1-negative tumors, within this subgroup, its expression was similar in cases with POU2F3 versus without POU2F3. This, together with its wide distribution in other subtypes, precluded delineation of a distinct SCLC subtype defined by YAP1.

On the basis of the above findings, we grouped the tumors as follows: (1) ASCL1-dominant; (2) NEUROD1-dominant; (3) ASCL1/NEUROD1 double-negative with POU2F3 expression (POU2F3); and (4) ASCL1/NEUROD1 double-negative not otherwise specified (NOS). In the following sections, we compared the immunophenotypic and histologic characteristics of these subtypes. A separate analysis for ASCL1-only versus NEUROD1-only versus double-positive tumors is illustrated in Supplementary Figure 2.

ASM1/NEUROD1/POU2F3-Defined Subtypes and Expression of Conventional Markers of SCLC

We next evaluated the relationship between SCLC subtypes (defined by dominance of ASCL1 versus NEUROD1 and expression of POU2F3) and the expression of standard diagnostic markers of SCLC, including conventional markers of NE differentiation (synaptophysin, chromogranin A, CD56, and INSM1), Ki-67 proliferation marker, and TTF-1. Strikingly, the expression of conventional NE markers—analyzed as a combined NE score for the four markers (see Methods section)—was significantly lower in ASCL1/NEUROD1 double-negative compared with ASCL1- or NEUROD1-dominant subtypes (p < 0.0001) (Fig. 3A). The mean combined NE score in ASCL1- and NEUROD1-dominant subtypes was 196 and 191, respectively, whereas in POU2F3 and NOS cases, it was 76 and 89, respectively. Overall, 100% of POU2F3 and 83% of NOS cases had a combined NE H-score of ≤150 (NE-low) compared with only 20% of ASCL1- and NEUROD1-dominant cases. When analyzed individually, each of the four conventional NE markers was expressed at lower levels in POU2F3 and NOS subtypes (data not shown). Furthermore, the number of expressed NE markers was significantly higher in the ASCL1- and NEUROD1-dominant subtypes compared with POU2F3 and NOS.
subtypes ($p < 0.0001$), with all four markers typically expressed in the former versus an average of approximately two in the latter subtypes (Fig. 3B). Notably, none of the ASCL1/NEUROD1 double-negative tumors were entirely negative for conventional NE markers, because at least one NE marker was expressed at least weakly or focally in all POU2F3 and NOS cases. For ASCL1- versus NEUROD1-dominant subtypes, the extent and number of expressed NE markers was equivalent. Similarly, analysis of ASCL1-only versus NEUROD1-only subtypes exhibited an equivalent extent and number of expressed NE markers (Supplementary Fig. 2D and E, respectively).

Analysis of Ki-67 revealed equivalent distribution in all groups, with mean proliferation indices of 88%, 90%, 89%, and 86% in ASCL1-dominant, NEUROD1-dominant, POU2F3, and NOS subtypes, respectively (Fig. 3C). Conversely, TTF-1 expression was strongly linked with ASCL1: most SCLC with ASCL1 expression were TTF-1-positive, whereas tumors with NEUROD1-only expression were entirely TTF-1-negative (Fig. 3D and Supplementary Fig. 2F). Accordingly, the NEUROD1-dominant subtype had a substantially lower rate of TTF-1 expression compared with the ASCL1-dominant subtype (Fig. 3D). Nearly all POU2F3 and most of NOS tumors were TTF-1-negative.

Figure 1. ASCL1 and NEUROD1 expression profiles. (A) Pie chart illustrating the expression patterns of ASCL1 [A] and NEUROD1 [N] in SCLC. (B) Pie chart illustrating the distribution of marker-dominant subtypes: (1) ASCL1-dominant (ASCL1 H-score > NEUROD1 H-score); (2) NEUROD1-dominant (NEUROD1 H-score > ASCL1 H-score); and (3) double-negative (negative for both markers). (C) Dot plot depicting the $\Delta$H-score, with each dot representing an individual case with its corresponding $\Delta$H-score on the y-axis. All positive values represent ASCL1-dominant tumors, and all negative values represent NEUROD1-dominant ones. Bracket indicates a minority of cases with close scores for both markers ($\Delta$H-score $\leq 50$). (D) A case with dual-high ASCL1 and NEUROD1, illustrating that both markers are coexpressed in the same cell population. H&E, hematoxylin and eosin; H-score, histoscore; $\Delta$H-score, H-score difference between ASCL1 and NEUROD1.
ASCL1/NEUROD1/POU2F3-Defined Subtypes and Expression of DLL3

Given the previously reported association of DLL3 with ASCL1-high SCLC and its role as a marker associated with NE-high phenotype, in an exploratory analysis, we evaluated DLL3 expression in a subgroup of SCLC typed for other markers using a TMA (N = 41 with evaluable results). Strikingly, DLL3 was entirely negative in ASCL1/NEUROD1 double-negative tumors but had a consistently high expression in both ASCL1-dominant and NEUROD1-dominant groups (p < 0.0001 for each comparison, Fig. 3E). The expression of DLL3 was equivalent in ASCL1-dominant versus NEUROD1-dominant subtypes (see Supplementary Figure 3 for an illustrative example).

Clinicopathologic and Histologic Characteristics of ASCL1/NEUROD1/POU2F3-Defined Subtypes

Comparison of patient and tumor characteristics, including age, sex, pack-year smoking history, tumor site (primary versus metastatic), site of metastasis (lymph node versus other organs), and specimen type yielded similar distribution in SCLC subtypes (Supplementary Table 4).

We also confirmed that histologically, all tumors in ASCL1-dominant, NEUROD1-dominant, POU2F3, and NOS subtypes represented small cell carcinoma of either pure or combined subtype. However, we found that POU2F3 and NOS subtypes were significantly enriched in combined histology (Fig. 3F). Whereas combined histology comprised only 14% and 11% of ASCL1-dominant and NEUROD1-dominant subtypes, respectively, POU2F3 and NOS subtypes were combined in 50% and 45% of cases, respectively. In all cases, SCLC components of combined tumors had classic histologic features of SCLC, and expression of ASCL1, NEUROD1, and POU2F3 was present exclusively or primarily in SCLC components. The histologic subtype of NSCLC components (LCNEC versus adenocarcinoma versus squamous cell carcinoma) was distributed similarly in different subtypes (Supplementary Table 4).

Finally, given the previous suggestion of genomic similarity of some ASCL1/NEUROD1 double-negative SCLC to squamous cell carcinoma,29 we confirmed that these tumors lacked evidence of squamous differentiation morphologically and consistently lacked the expression of squamous master regulator p40 (ΔNp63).

A histologic illustration of marker-defined SCLC subtypes is provided in Figure 4.

Correlates of YAP1 Expression

Finally, we analyzed the correlates of YAP1 expression in SCLC. As illustrated in Figure 5A, YAP1 expression was inversely associated with NE marker...
expression as reflected by lower combined NE score, especially in tumors with higher YAP1 H-scores (>50). Furthermore, YAP1 expression was associated with combined SCLC histology. In particular, all tumors with a higher level of YAP1 expression (H-score >50) had combined histology compared with 18% of YAP-low or

![Figure 3](image-url)
Figure 4. Histology illustration. Examples of SCLC subtypes as defined by ASCL1, NEUROD1, and POU2F3 expression. H&E illustrates the classic histologic features of SCLC in all subtypes. Low-level coexpression of NEUROD1 is seen in the ASCL1-dominant case, and low-level ASCL1 coexpression is seen in the NEUROD1-dominant case. YAP1 is negative in tumor cells, but labeling is seen in benign stromal and endothelial cells. The expression of Chromo illustrates high expression of neuroendocrine markers in ASCL1 and NEUROD1 subtypes, and low expression in the double-negative subtypes (for illustrated POU2F3-positive case, INSM1 and CD56 were expressed) (not provided). Chromo, chromogranin; H&E, hematoxylin and eosin; neg, negative.
negative tumors \((p < 0.001)\). In the combined SCLC, the expression of YAP1 was consistently high in the NSCLC components, and its expression in associated SCLC components ranged from absent to variably retained in a focal and/or weak pattern (Fig. 5B).

**Discussion**

In this study, using a large cohort \((N = 174)\) of SCLC clinical samples, we analyzed the protein expression of ASCL1, NEUROD1, POU2F3, and YAP1, and assessed their association with tumor histology and expression of standard markers of SCLC. This included the following: (1) conventional markers of NE differentiation (synaptophysin, chromogranin A, CD56, and INSM1), which are expressed at varying levels and combinations in virtually all SCLC; (2) transcriptional regulator TTF-1 \((NKK2-1)\), which is expressed in approximately 90% of SCLC; and (3) Ki-67 \((MIB1)\) proliferation index marker. In a smaller exploratory subset, we also evaluated the expression of DLL3, given the previous data on its distinctive distribution in ASCL1/NEUROD1-defined subtypes and its link with markers of NE differentiation. Our findings confirm some of the observations from experimental models regarding the predicted features of marker-defined subtypes but also reveal a higher level of marker expression heterogeneity and associated phenotypes in patient samples.

Overall, we confirmed that the vast majority of SCLC is characterized by dominant expression of ASCL1 \((69%)\) and/or NEUROD1 \((17%)\), although a minor subset of SCLC \((14%)\) lacks both of these regulators. We found that YAP1 had a distinctly low expression and did not define a distinct subtype of SCLC. Conversely, POU2F3 was uniquely associated with the ASCL1/NEUROD1 double-negative subtype, comprising 7% of SCLC, whereas the rest of double-negative tumors \((7% \text{ of SCLC})\) remained with no identified dominant transcriptional regulator (i.e., not otherwise specified). We found highly distinctive pathologic and immunophenotypic characteristics of SCLC defined by the presence versus...
absence of ASCL1 and/or NEUROD1. In particular, previous studies have suggested that the expression of markers of NE differentiation, DLL3, and TTF-1 represents a coordinated NE program in SCLC. As such, we found that SCLC with ASCL1 and/or NEUROD1 expression was associated with a high NE program (NE marker\textsuperscript{high}/DLL3\textsuperscript{high}/TTF-1\textsuperscript{high}), whereas POU2F3 and NOS subtypes were associated with a low NE program (NE marker\textsuperscript{low}/DLL3\textsuperscript{low}/TTF-1\textsuperscript{low}).

An important clarification provided by our data relates to the degree of ASCL1 and NEUROD1 coexpression in the clinical samples of SCLC. In several experimental models, ASCL1 and NEUROD1 were found to have predominantly exclusive expression, with only a low degree of coexpression. Conversely, we found a significant level of coexpression, with 37% of SCLC expressing both ASCL1 and NEUROD1, and 22% being dual-high expressors (H-score > 50 for both markers). Notably, our data are closely in line with a study by Zhang et al. in which using a smaller set of human SCLC formalin-fixed paraffin-embedded (FFPE) samples (N = 81), the authors also found frequent (19.8%) ASCL1 and NEUROD1 dual-high expression. It is possible that in experimental models, there is a clonal selection for a dominant transcription factor and inhibition of the minor factor under cell culture or patient-derived xenograft conditions, as supported by experimental evidence of SCLC subtype plasticity.

Notably, in the series by Zhang et al., the question of whether ASCL1 and NEUROD1 were coexpressed in the same cell populations or exhibited subclonal expression in distinct tumor cell populations was not addressed. In a study of circulating tumor cell–derived xenografts, it was suggested that dual expression was present in distinct cell subpopulations. Conversely, to our knowledge, our study is the first to document that in native patient samples, nearly all cases of dual-high expression represent coexpression within the same cell populations, as illustrated in Figure 1D, whereas only a single case exhibited subclonal areas with divergent ASCL1 and NEUROD1 (and POU2F3) expression.

Several distinctive characteristics of SCLC-N subtype have been suggested in previous studies, including highly divergent global gene expression, with lower expression of NE genes and DLL3 compared to SCLC-A tumors. Conversely, we found that in patient samples, NEUROD1-dominant SCLC expressed high levels of NE markers and DLL3, equivalent to those of ASCL1-dominant tumors. The analysis of NEUROD1-associated characteristics was complicated by the fact that most (83%) of NEUROD1-dominant tumors coexpressed ASCL1, and only a small subset of cases had exclusive NEUROD1 expression. However, our findings in the NEUROD1-only tumors confirmed that they also had high expression of NE markers and DLL3. This discrepancy could reflect RNA versus protein level analysis in the current versus previous studies, respectively, in part owing to the wider dynamic range of gene expression measurements when compared with IHC. Nonetheless, we note that, similar to the results of this study, other recent RNA-based investigations also suggested that the SCLC-N subtype has greater transcriptional overlap with SCLC-A tumors than previously proposed. Interestingly, we did find that TTF-1 was consistently negative in NEUROD1-only tumors but positive in nearly all tumors with ASCL1 expression at any level, which is in line with the known codependence of TTF-1 and ASCL1 expression. This might suggest that distinct transcriptional programs may correspond to SCLC with exclusive NEUROD1 expression, as measured by IHC. However, further studies using parallel mRNA and protein analysis will be required to clarify IHC-based criteria for SCLC-N versus SCLC-A subtypes associated with globally distinct transcriptional programs as defined in mRNA-based studies.

One of the major findings in this study is the confirmation of the highly distinctive nature of POU2F3-positive SCLC, defined by POU2F3 expression and strict lack of ASCL1 and NEUROD1. POU2F3 is a transcription factor required for the generation and chemosensory and immune functions of specialized tuft cells in the skin, oropharyngeal, gastrointestinal, and respiratory tracts. It has been proposed that POU2F3-defines a distinct subset of SCLC arising from or recapitulating the differentiation of the tuft cells. However, the singular case with distinct ASCL1-positive, NEUROD1-positive, and POU2F3-positive components calls into question the suggestion that SCLC-P might derive from a different cell of origin from other SCLC subtypes. We confirmed that POU2F3-positive SCLCs are characterized by NE marker\textsuperscript{low}/TTF-1\textsuperscript{low}/DLL3\textsuperscript{low} phenotype. We also confirmed that, pathologically, POU2F3-positive tumors represent true SCLC as defined by morphology and extremely high proliferation rate, despite the low NE markers and near-universal lack of TTF-1 expression. The enrichment of POU2F3 expression in combined SCLC suggests either greater morphologic plasticity or closer ontogenetic relationship with NSCLC than ASCL1/NEUROD1 subtypes. Similar to conventional SCLC, we confirmed that POU2F3-expressing tumors lack RB expression (data not shown).

YAP1 has been proposed to represent one of the subtype-defining markers of SCLC within the non-NE ASCL1/NEUROD1 double-negative tumors, associated with decreased INSM1 expression and enrichment for an intact RB. However, the role of YAP1 as a major transcriptional driver in SCLC remains unclear given that it was also reported that YAP1 expression in SCLC is
consistently absent or minimal,\textsuperscript{21} and no distinct YAP1 subtype was identified in a recent study of SCLC circulating tumor cell–derived xenografts.\textsuperscript{13} Consistent with these latter observations, we also found that YAP1 expression in SCLC was either entirely absent or present only at a low level. Few cases that exhibited moderate expression of YAP1 were confined to SCLC with combined histology, in which YAP1 was strongly expressed in the NSCLC component, and focally retained YAP1 expression in the SCLC component could reflect transitional phenotype between NSCLC and SCLC in such regions. Furthermore, YAP1 did not define a distinct subgroup of SCLC, although its expression was mildly elevated in the ASCL1/NEUROD1 double-negative tumors and was associated with a low expression of NE markers. Overall, the role of YAP1 as a subtype-defining marker in SCLC will require clarification in future studies.

In our series, 7% of SCLC lacked a known transcriptional regulator as they were negative for ASCL1, NEUROD1, and POU2F3, and were generally either negative or low for YAP1. Overall, these tumors were similar to the POU2F3 subtype in that they were NE marker\textsuperscript{low}/or low for YAP1. Overall, these tumors were similar to the POU2F3 subtype in that they were NE marker\textsuperscript{low}/or low for YAP1. Overall, these tumors were similar to ROD1, and POU2F3, and were generally either negative or low for TTF-1/DLL3 expression in SCLC.\textsuperscript{53} Enrichment for combined histology in the double-negative tumors, which account for 7% of SCLC. Further studies are warranted to determine whether expression-based subtypes of SCLC are associated with distinct patient outcomes and/or predict distinct therapeutic vulnerabilities.

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Supplementary Data
Note: To access the supplementary material accompanying this article, visit the online version of the Journal of Thoracic Oncology at www.jto.org and at https://doi.org/10.1016/j.jtho.2020.09.009.

References


43. Mukhopadhyay S, Dermawan JK, Lanigan CP, Farver CF. Insulinoma-associated protein 1 (INSM1) is a sensitive and highly specific marker of neuroendocrine differentiation in primary lung neoplasms: an immunohistochemical study of 345 cases, including 292 whole-tissue sections. Mod Pathol. 2019;32:100-109.