

# Molecular subtypes, predictive markers and prognosis in small-cell lung carcinoma

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## ABSTRACT

**Aims** A new molecular subtype classification was proposed for small-cell lung carcinoma (SCLC). We aimed to further validate the classification in various SCLC patient samples using immunohistochemistry (IHC) to highlight its clinical significance.

**Methods** We analysed the protein expression of four subtype (achaete-scute family BHLH transcription factor 1 (ASCL1), neuronal differentiation 1 (NEUROD1), POU class 2 homeobox 3 (POU2F3) and Yes1-associated transcriptional regulator (YAP1)) and two predictive markers (delta-like ligand 3 (DLL3) and MYC) using IHC in 216 specimens from 195 SCLC patients, including 21 pairs of resected biopsy tumours. Associations among molecular subtypes, clinicopathological features and prognostic implications were also explored.

**Results** The ASCL1, NEUROD1, POU2F3, YAP1, DLL3 and MYC-positive expression rates were 70.3%, 56.9%, 14.9%, 19.0%, 75.4% and 22.6%, respectively. DLL3 expression had positive and negative associations with that of ASCL1 and POU2F3/YAP1, respectively, whereas MYC had the opposite effect. Strong associations of ASCL1 ( $P=0.8603$ ,  $p<0.0001$ ), NEUROD1 ( $P=0.8326$ ,  $p<0.0001$ ), POU2F3 ( $P=0.6950$ ,  $p<0.0001$ ) and YAP1 ( $P=0.7466$ ,  $p<0.0001$ ) expressions were detected between paired resected biopsy tumours. In addition to SCLC-A (ASCL1-dominant), SCLC-N (NEUROD1-dominant) and SCLC-P (POU2F3-dominant), unsupervised hierarchical cluster analyses identified a fourth, quadruple-negative SCLC subtype (SCLC-QN) characterised by the low expression of all four subtype-specific proteins, and 55.4% ( $n=108$ ), 27.2% ( $n=53$ ), 11.8% ( $n=23$ ) and 5.6% ( $n=11$ ) were categorised as SCLC-A, SCLC-N, SCLC-P and SCLC-QN, respectively. Significant enrichment of SCLC-P in the combined SCLC cohort was observed, and adenocarcinoma was more prevalent in SCLC-A, while large-cell neuroendocrine carcinoma was more commonly seen in SCLC-P. No survival difference was found among molecular subtypes.

**Conclusions** Our results provide clinical insights into the diagnostic, prognostic and predictive significance of SCLC molecular subtype classifications.

## INTRODUCTION

Small-cell lung carcinoma (SCLC) accounts for approximately 15% of newly diagnosed lung cancers.<sup>1</sup> Current therapeutic approaches for SCLC are generally based on disease stage, with no consideration of distinct biomarkers that might have predictive or prognostic significance. Recently, SCLC profiling studies have suggested molecular subtypes defined by the relative expression profiles

## WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Although small-cell lung carcinoma (SCLC) molecular subtype classification was observed in human tumours and preclinical models, it requires further validation in a large cohort of SCLC patients at various disease stages and variable specimens to highlight its clinical significance.

## WHAT THIS STUDY ADDS

⇒ Our results validated the feasibility of lung biopsy samples for identifying SCLC molecular subtypes, and revealed that combined SCLCs were significantly enriched in the subtype of POU2F3-dominant SCLC (SCLC-P) and for the non-SCLC components of combined SCLCs, adenocarcinoma was more prevalent in ASCL1-dominant SCLC, while large-cell neuroendocrine carcinoma was more commonly seen in SCLC-P.

## HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study describes the diagnosis, prognosis and predictive significance of SCLC molecular subtype classifications, which would aid in the development of personalised treatments and subtype-specific treatment of SCLC.

of four key transcriptional regulators: achaete-scute family Basic Helix-Loop-Helix (BHLH) transcription factor 1 (ASCL1; SCLC-A), neuronal differentiation 1 (NEUROD; SCLC-N), POU class 2 homeobox 3 (POU2F3; SCLC-P) and Yes1-associated transcriptional regulator (YAP; SCLC-Y).<sup>2</sup> These four subtypes are associated with neuroendocrine (NE) differentiation, showing that the first two groups have significant NE differentiation, namely, the NE phenotype, and the latter are non-NE phenotypes.<sup>2</sup> This molecular subtyping concept has inspired many researchers to stratify patients into subsets with distinct therapeutic vulnerabilities.<sup>3–5</sup> Notably, delta-like ligand 3 (DLL3), an inhibitory NOTCH ligand upregulated in ASCL1-dominant SCLC, is overexpressed in 80% of SCLC tumours but is not expressed in normal tissues.<sup>6,7</sup> It is currently under investigation as a potential therapeutic target.<sup>8</sup> The NEUROD1 and POU2F3 subtypes are characterised by MYC gene overexpression,<sup>4</sup> which is also a novel therapeutic target under clinical trial.<sup>8</sup>

Despite a substantial volume of data on SCLC molecular subtypes in preclinical models, there is currently minimal information on the expression



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of a full panel of these markers in SCLC clinical samples.<sup>9–18</sup> Some of these studies lack information on previous treatments and outcomes.<sup>9–11 17</sup> Additionally, most specimens were biopsies/tumour microarrays (TMAs) or surgically resected SCLC samples from limited stage patients.<sup>9–18</sup> However, emerging evidence suggests that different subtypes are dynamically changing, emphasising the intratumoral heterogeneity and strong plasticity of SCLC.<sup>19 20</sup> Thus, biopsies/TMAs may not represent the molecular subtype of the whole tumour, and surgically resected tumours might not completely mirror the clinicopathological characteristics of the entire disease spectrum.

Therefore, we collected specimens from SCLC patient with various stages and investigated the expression of molecular subtypes and predictive markers by immunohistochemistry (IHC) in whole SCLC tissue/cell blocks of formalin-fixed paraffin-embedded (FFPE), to explore the clinicopathological features and prognostic implications of SCLC molecular subtype. To the best of our knowledge, this is the first study to investigate the expression of subtype markers in paired resected-biopsy SCLC specimens.

MATERIALS AND METHODS

Patient selection and data collection

This retrospective study was performed using archived samples from the Peking University Cancer Hospital database between January 2010 and April 2023. We collected surgically resected SCLC primary tumours, resected biopsy of SCLC metastases and pleural effusions diagnosed as SCLC by cytology. In addition, treatment-naïve patients with paired lung core-needle biopsies of surgically resected SCLC were included. Patients with unavailable FFPE and poor specimen quality from IHC analysis were excluded. The approval of the Ethics Committee of Beijing Cancer Hospital (2023KT23) was obtained and waived the need for individual informed consent for this study.

All archival slides stained with H&E and conventional NE diagnostic markers, CD56, chromogranin A (CgA), and

Table 1 Expression of these subtyping and predictive markers of SCLC

Marker	H-score range N (% samples)			H-score mean (Full range)
	1–50	>50–150	>150	
ASCL1	58 (29.7)	54 (27.7)	83 (42.6)	114 (0–265)
NEUROD1	84 (43.1)	77 (39.5)	34 (17.4)	77 (0–220)
POU2F3	166 (85.1)	7 (3.6)	22 (11.3)	28 (0–265)
YAP1	158 (81.0)	37 (19.0)	0 (0)	22 (0–130)
DLL3	48 (24.6)	68 (34.9)	79 (40.5)	118 (0–265)
MYC	151 (77.4)	39 (20.0)	5 (2.6)	24 (0–200)

ASCL1, achaete-scute family BHLH transcription factor 1; DLL3, delta-like ligand 3; H-score, histoscore; NEUROD1, neuronal differentiation 1; POU2F3, POU class 2 homeobox 3; SCLC, small-cell lung carcinoma; YAP1, Yes1-associated transcriptional regulator.

synaptophysin (Syn) were retrieved and re-evaluated by two senior pathologists (YZ and HW) independently to confirm the diagnosis of SCLC. tumor-node-metastasis (TNM) stage was determined according to the eighth edition of the TNM classification system proposed by the American Joint Committee on Cancer. Detailed patient demographic and clinical information were collected from the electronic medical records. Follow-up data were obtained from the clinical records and telephone interviews. Overall survival (OS) was calculated as the date from pathological diagnosis to the time of death from any cause. Progression-free survival (PFS) was calculated from the date of pathological diagnosis to the time of the last clinical evidence of recurrence, progression, or death.

IHC staining and scoring criteria

FFPE specimens were prepared, and consecutive 4 µm-thick tissue sections were cut from the specimens for IHC analysis. All samples were stained for the six markers (ASCL1, NEUROD1,

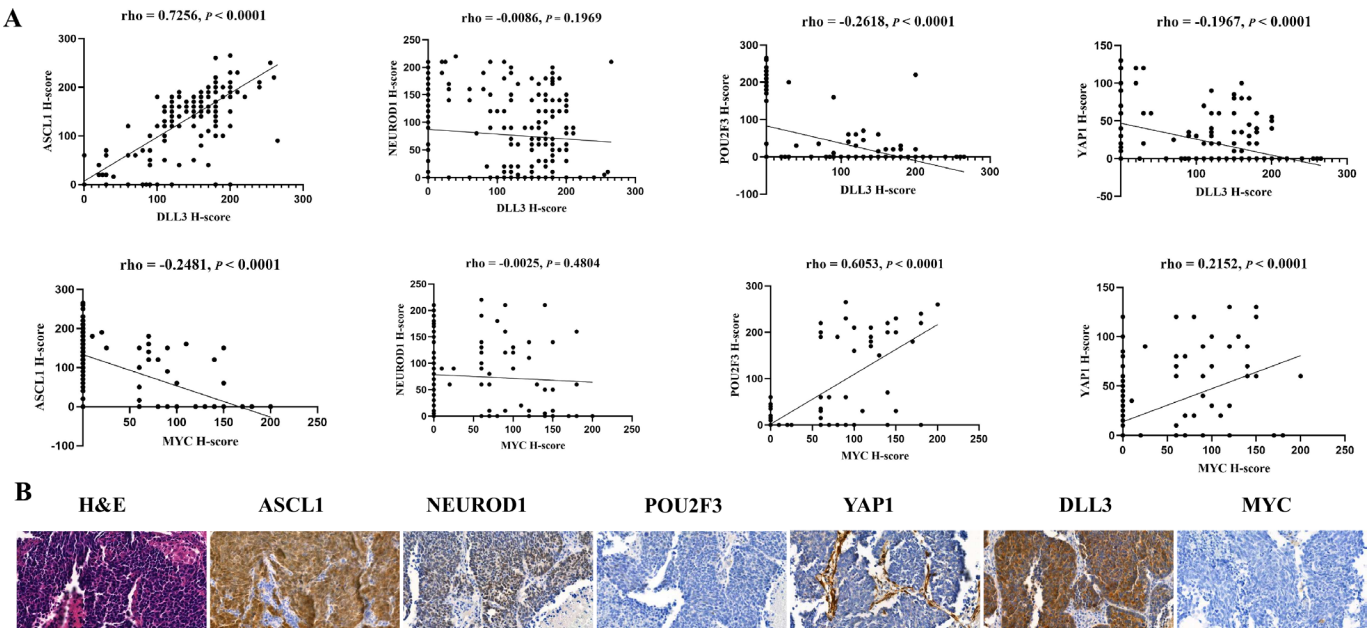


Figure 1 Molecular subtypes and predictive markers' expression correlations. (A) DLL3 positively correlated with ASCL1, whereas negatively correlated with POU2F3/YAP1. MYC positively correlated with POU2F3/YAP1, whereas negatively correlated with ASCL1. (B) Representative histopathological images revealing the relationship between subtype-specific protein expression and DLL3/MYC expression. ASCL1, achaete-scute family BHLH transcription factor 1; DLL3, delta-like ligand 3; POU2F3, POU class 2 homeobox 3; YAP1, Yes1-associated transcriptional regulator.

POU2F3, YAP1, DLL3 and MYC). Detailed IHC protocols are summarised in online supplemental table S1.

We evaluated the entire tumour area for all specimens, and the expression of markers was recorded by a histoscore (H-score), which was derived by multiplying the percentage positivity by the intensity score (1, 2 and 3), yielding a range of possible H-scores of 0–300.<sup>21</sup> The WHO classification of lung tumours defines combined SCLC as tumours containing both SCLC components plus any type of non-SCLC (NSCLC) component, including adenocarcinoma (ADC), squamous cell carcinoma (SCC), large-cell neuroendocrine carcinoma (LCNEC), large-cell carcinoma or rarely other,<sup>22</sup> and in combined SCLCs, we selected FFPE blocks in which at least half of the tumours were SCLC component, and IHC scores reflect expression exclusively in the SCLC component. For descriptive purposes, H scores  $\leq 50$  were regarded as negative, and those  $> 50$  were regarded as positive. To classify SCLC molecular subtypes, an unsupervised hierarchical clustering based on expression of the four subtype-defining transcription factors (ASCL1, NEUROD1, POU2F3, YAP1) was performed.

### Statistical analysis

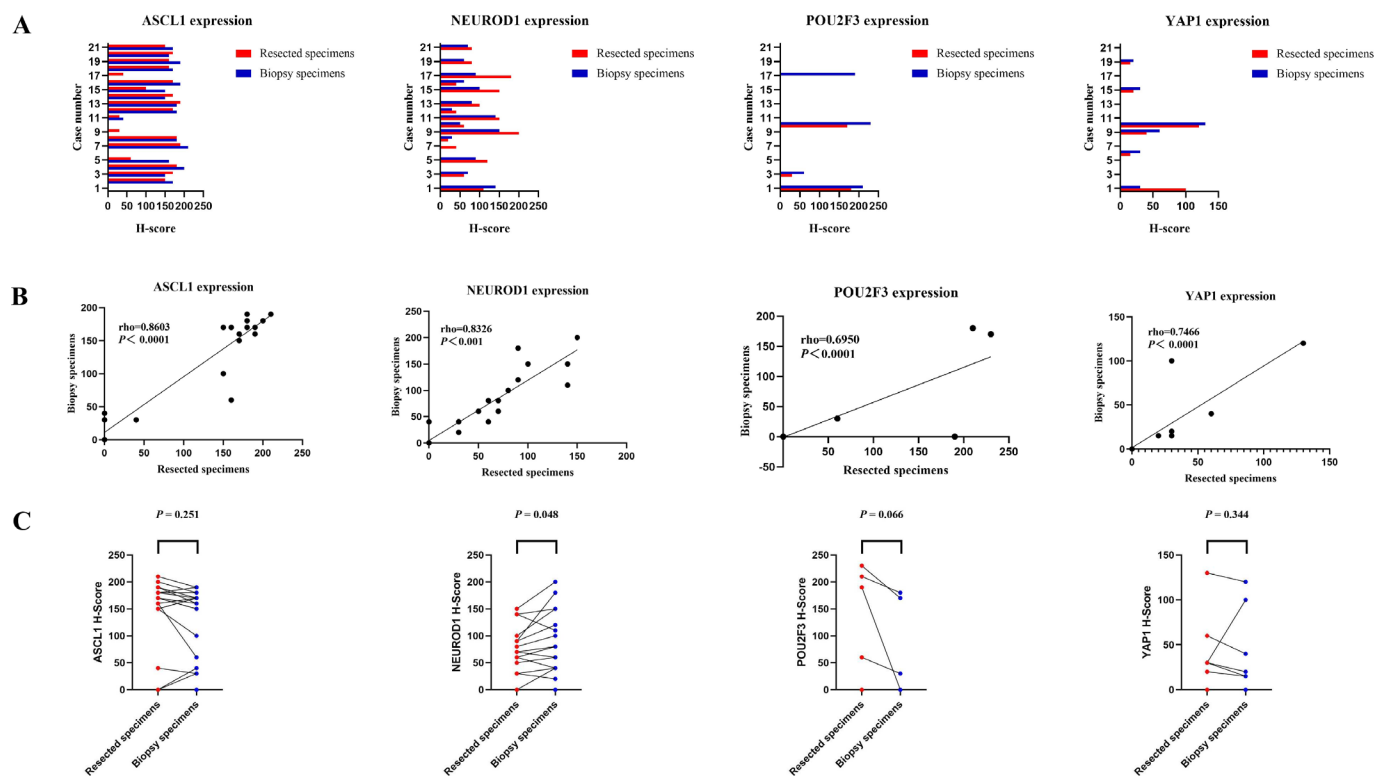
SPSS software (V.21.0; SPSS) and Prism V.8.0 (GraphPad Software, San Diego, California) were used for data visualisation and statistical analysis. The correlation between continuous variables was calculated using the Spearman's rank correlation. Correlations of subtype-defining markers' expression between the resected specimens and paired biopsy specimens were examined using the Spearman's rank correlation and Wilcoxon

matched-pairs signed-rank test. Hierarchical clustering of samples based on the measured expression levels was performed with the Complex Heatmap R package (R V.3.6.3). Clinicopathological characteristics of each molecular subtype were analysed by the  $\chi^2$  test,  $\chi^2$  test of independence with Yate's continuity correction or Fisher's exact test. A Cox proportional hazards regression model was used to evaluate factors independently associated with OS and PFS. Variables with  $p < 0.20$  in the univariate analyses were considered in the multivariate Cox proportional hazards regression model. Survival curves were delineated using the Kaplan-Meier method, and differences between curves were estimated using the log-rank test. Statistical significance was set at  $p < 0.05$ .

## RESULTS

### Patient and sample characteristics

A total of 195 SCLC patients were included in this study. The mean patient age was 61.5 years (range, 33–87 years), 106 (54.4%) patients were older than 60 years, 145 (74.4%) were men and 43 (22.1%) were never-smokers with de novo SCLC; none was histologic transformation of tyrosine kinase inhibitors-treated patients. The number of patients with pure SCLC and combined SCLC was 146 (74.9%) and 49 (25.1%), respectively. The NSCLC component for combined SCLCs was lung ADC (n=23), lung SCC (n=8) and LCNEC (n=18). 78 (40.0%), 38 (19.5%), 42 (21.5%) and 37 (19.0%) patients had stage I, II, III and stage IV disease, respectively. Most patients were treatment-naïve before surgery or biopsy, with only 34 (17.4%) patients undergoing neoadjuvant chemotherapy (ChT). Beside surgical



**Figure 2** Comparative analysis of paired resected-biopsy tumours. (A) The expression of subtype-defining markers in resected tumours and paired biopsies. (B) Spearman's rank correlation tests were used to investigate the expression correlation between resected tumours and paired biopsies, and showed strong associations of ASCL1, NEUROD1, POU2F3 and YAP1 expressions in the paired two groups. (C) By Wilcoxon matched-pairs signed-rank tests, we found that the heterogeneity of NEUROD1 expression significantly differed between the resected and biopsy specimens ( $p = 0.048$ ), revealing that NEUROD1 expression in resected tumours was stronger than in biopsies. ASCL1, achaete-scute family BHLH transcription factor 1; NEUROD1, neuronal differentiation 1; POU2F3, POU class 2 homeobox 3; YAP1, Yes1-associated transcriptional regulator.



resection, SCLC patients also received adjuvant ChT with or without adjuvant thoracic radiotherapy according to the lung cancer treatment guidelines of our hospital; thus, one hundred and thirty-one (67.2%) patients received ChT, 60 (30.8%) patients received radiotherapy, and 81 (41.5%) patients received prophylactic cranial irradiation (PCI) treatment.

We collected 216 specimens from 195 patients with SCLC, including 150 surgically resected primary tumours, 17 metastatic samples by biopsy, 28 pleural effusions and 21 lung biopsies paired with surgically resected SCLC.

### Expression of molecular subtypes and predictive markers and their correlations

We examined the expression of molecular subtypes and predictive markers for the samples ( $n=195$ ) besides the paired biopsies ( $n=21$ ). When dichotomised into positive versus negative using an H-score threshold of  $>50$ , the ASCL1, NEUROD1, POU2F3, YAP1, DLL3 and MYC-positive expression rates were 70.3%, 56.9%, 14.9%, 19.0%, 75.4% and 22.6%, respectively. The expression distribution of these markers is listed in [table 1](#).

A significant positive association was observed between ASCL1 and DLL3 ( $P=0.7256$ ,  $p<0.0001$ ), whereas a significant negative association was observed between POU2F3/YAP1 and DLL3 ( $P=-0.2618$ ,  $p<0.0001$  and  $P=-0.1967$ ,  $p<0.0001$ ). In contrast to DLL3, expression levels of MYC had a strong positive association with those of POU2F3/YAP1 ( $P=0.6053$ ,  $p<0.0001$  and  $P=0.2152$ ,  $p<0.0001$ ) and a negative association with those of ASCL1 ( $P=-0.2481$ ,  $p<0.0001$ ) ([figure 1A](#)). Additionally, we observed no significant correlation between NEUROD1 and DLL3/MYC ([figure 1A](#)). Representative

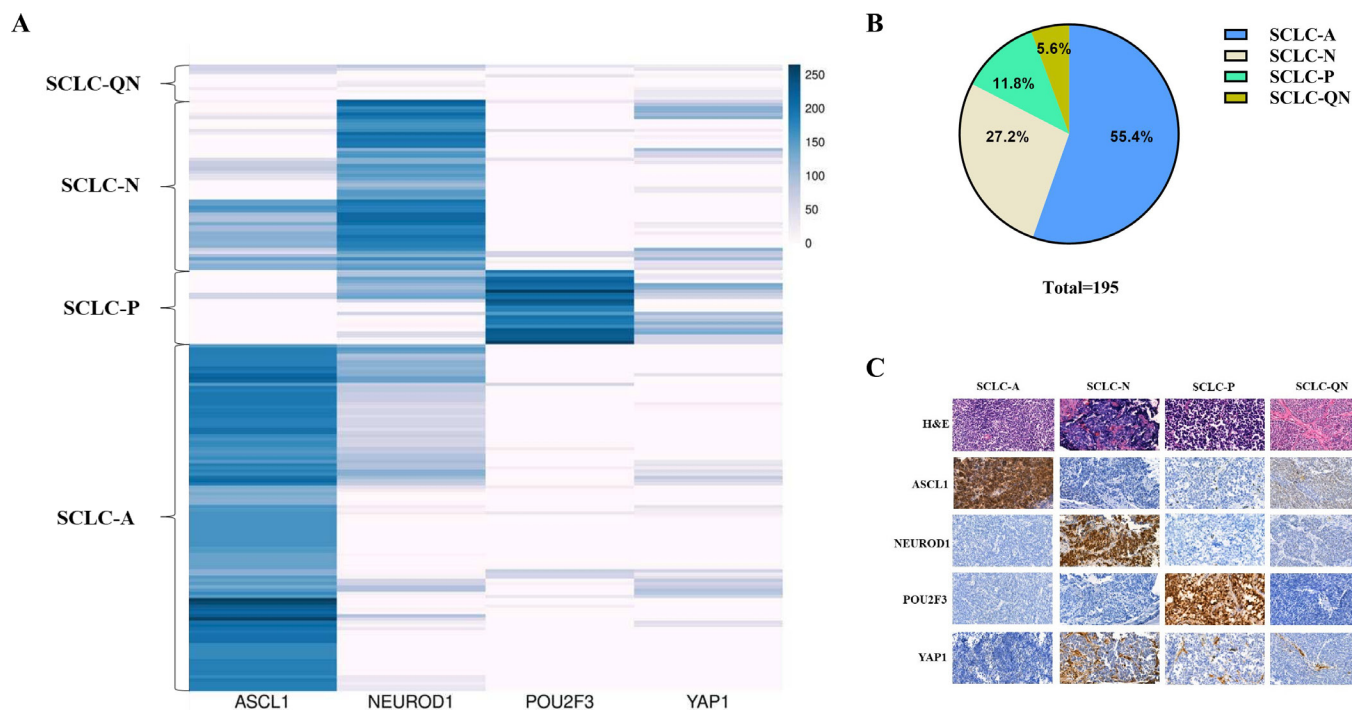
histopathological images revealing the relationship between subtype-specific protein expression and DLL3/MYC expression are shown in [figure 1B](#).

### Subtype-specific protein expressions in SCLC-paired resected tumours and biopsies

The expression of subtype-defining markers in resected tumours and paired biopsies is shown in [figure 2A](#). To determine the relationship between subtype-defining markers' expressions in resected tumours and biopsies, we examined the correlation between these markers' expressions in the matched specimens using Spearman's rank correlation test. Strong associations of ASCL1 ( $P=0.8603$ ,  $p<0.0001$ ), NEUROD1 ( $P=0.8326$ ,  $p<0.0001$ ), POU2F3 ( $P=0.6950$ ,  $p<0.0001$ ) and YAP1 ( $P=0.7466$ ,  $p<0.0001$ ) expressions were detected in the paired two groups ([figure 2B](#)). Next, to investigate subtype-specific markers heterogeneity, we assessed the differences in these marker expressions between the resected and biopsy tissues using the Wilcoxon matched-pairs signed-rank test. The heterogeneity of NEUROD1 expression significantly differed between the resected and biopsy specimens ( $p=0.048$ ), and these data suggested that NEUROD1 expression in resected tumours was stronger than in biopsies ([figure 2C](#)).

### Molecular subtypes and clinicopathologic correlation

Unsupervised hierarchical cluster analyses were performed to identify the dominant marker in 195 samples besides the paired biopsies ( $n=21$ ) based on the IHC expressional levels of the four molecular subtype markers (ASCL1, NEUROD1,



**Figure 3** Molecular subtypes distribution. (A) Unsupervised hierarchical clustering analyses of ASCL1, NEUROD1, POU2F3 and YAP1 immunohistochemistry expression were performed in 195 samples besides the paired biopsies. Differential expression of the four markers clearly distinguished four major SCLC subtypes. In addition to SCLC-A (ASCL1-dominant), SCLC-N (NEUROD1-dominant) and SCLC-P (POU2F3-dominant), cluster analyses identified a fourth, quadruple-negative SCLC subtype (SCLC-QN) characterised by the low expression of all four subtype-specific proteins. (B) The pie chart illustrating the percentage of each dominant molecular subtype tumour in specimens besides the paired biopsy ( $n=195$ ). (C) Representative histopathological images of SCLC by molecular subtypes. ASCL1, achaete-scute family BHLH transcription factor 1; NEUROD1, neuronal differentiation 1; POU2F3, POU class 2 homeobox 3; SCLC, small-cell lung carcinoma; YAP1, Yes1-associated transcriptional regulator.

**Table 2** Clinicopathological characteristics of each molecular subtype in small cell lung carcinoma

	SCLC-A	SCLC-N	SCLC-P	SCLC-QN	P value
Sex					0.128
Male	83 (76.9%)	36 (67.9%)	20 (87.0%)	6 (54.5%)	
Female	25 (23.1%)	17 (32.1%)	3 (13.0%)	5 (45.5%)	
Age					0.133
≤60	53 (49.1%)	23 (43.4%)	6 (26.1%)	7 (63.6%)	
>60	55 (50.9%)	30 (56.6%)	17 (73.9%)	4 (36.4%)	
Smoking history					<b>0.017</b>
Yes	87 (80.6%)	37 (69.8%)	22 (95.7%)	6 (54.5%)	
No	21 (19.4%)	16 (30.2%)	1 (4.3%)	5 (45.5%)	
TNM stage					0.261
I	42 (38.9%)	18 (34.0%)	11 (47.8%)	7 (63.6%)	
II-IV	66 (61.1%)	35 (66.0%)	12 (52.2%)	4 (36.4%)	
Histology					<b>0.005</b>
Pure	89 (82.4%)	39 (73.6%)	11 (47.8%)	7 (63.6%)	
Combined	19 (17.6%)	14 (26.4%)	12 (52.2%)	4 (36.4%)	
Combined SCLC					<b>0.020</b>
ADC	15 (78.9%)	7 (50.0%)	0 (0)	1 (25.0%)	
SCC	1 (5.3%)	2 (14.3%)	3 (25.0%)	2 (50.0%)	
LCNEC	3 (15.8%)	5 (35.7%)	9 (75.0%)	1 (25.0%)	
Neoadjuvant ChT					0.065
Yes	17 (15.7%)	6 (11.3%)	7 (30.4%)	4 (36.4%)	
No	91 (84.3%)	47 (88.7%)	16 (69.6%)	7 (63.6%)	
PCI					0.583
Yes	46 (42.6%)	19 (35.8%)	12 (52.2%)	4 (36.4%)	
No	62 (57.4%)	34 (64.2%)	11 (47.8%)	7 (63.6%)	
DLL3 expression					<b>&lt;0.001</b>
Yes	108 (100%)	35 (66.0%)	2 (8.7%)	3 (27.3%)	
No	0 (0)	18 (34.0%)	21 (91.3%)	8 (72.7%)	
MYC expression					<b>&lt;0.001</b>
Yes	9 (8.3%)	11 (20.8%)	23 (100%)	1 (9.1%)	
No	99 (91.7%)	42 (79.2%)	0 (0)	10 (90.9%)	

Note: (1) Neoadjuvant chemotherapy means formalin-fixed paraffin-embedded tissue samples from primary tumours or metastases were obtained after chemotherapy. (2) Significant p values are highlighted in bold.

ADC, adenocarcinoma; ChT, chemotherapy; LCNEC, large-cell neuroendocrine carcinoma; PCI, prophylactic cranial irradiation; SCC, squamous cell carcinoma; SCLC, small-cell lung carcinoma; SCLC-A, small-cell lung carcinomas with ASCL1 dominant expression; SCLC-N, small-cell lung carcinomas with NEUROD1 dominant expression; SCLC-P, small-cell lung carcinomas with POU2F3 dominant expression; SCLC-QN, small-cell lung carcinomas characterised by the low expression of all four investigated transcription factors.

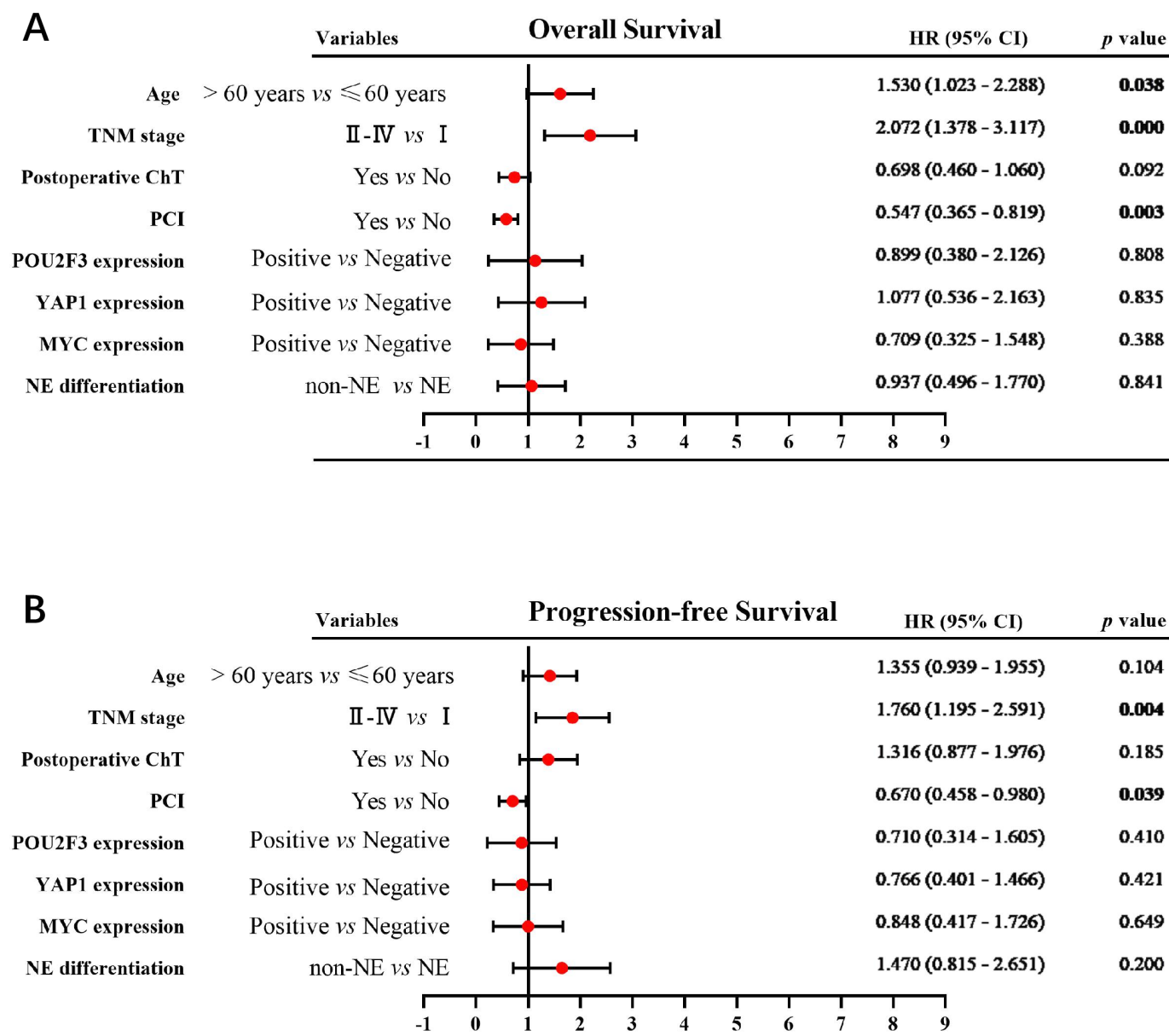
POU2F3 and YAP1). Differential expression of the four markers clearly distinguished four major SCLC subtypes. In addition to SCLC-A (ASCL1-dominant), SCLC-N (NEUROD1-dominant) and SCLC-P (POU2F3-dominant), cluster analyses identified a fourth, quadruple-negative SCLC subtype (SCLC-QN) characterised by the low expression of all four subtype-specific proteins (figure 3A). As shown in figure 3B, 55.4% (n=108), 27.2% (n=53), 11.8% (n=23) and 5.6% (n=11) were categorised as SCLC-A, SCLC-N, SCLC-P and SCLC-QN, respectively. Representative histopathological images of the marker-defined SCLC molecular subtypes are shown in figure 3C.

We then analysed the relationship between molecular subtypes and clinicopathologic parameters. Notably, combined SCLCs were significantly enriched in the subtype of SCLC-P, and patients with the tumour of SCLC-P tended to have a history of smoking. For the NSCLC components of combined SCLCs, ADC was more prevalent in SCLC-A, while LCNEC was more commonly seen in SCLC-P. All SCLC-A cases showed DLL3 expression, similarly, we observed MYC expression in all SCLC-P cases (table 2).

### Survival analysis in relation to clinicopathologic parameters and molecular subtypes

All patients were followed-up routinely. By the follow-up deadline of 30 April 2023, 123 cases (63.1%) had tumour recurrence or metastasis, 50 (25.6%) were disease-free, 108 (55.4%) died, 65 (33.3%) were alive and 22 (11.3%) were lost during follow-up. The median OS and PFS were 40 and 28 months, respectively. One-year, 3-year and 5-year OS and PFS rates were 87.3% and 60.1%, 47.4% and 29.5%, and 23.7% and 15.6%, respectively.

To assess the prognostic value of ASCL1, NEUROD1, POU2F3, YAP1, DLL3 and MYC expression and other clinicopathologic variables, we performed univariate Cox's regression analyses, and the results are summarised in online supplemental table S2. Next, clinicopathologic parameters with p value less than 0.2 in univariate analyses were included in a multivariate Cox proportional hazards regression model (figure 4). Furthermore, Kaplan-Meier analysis was used to compare the risk stratification of subgroups according to these prognostic factors, which were included in multivariate analysis (figure 5 and online



**Figure 4** Multivariate analyses for prognostic significance of clinicopathologic parameters in the cohort. Clinicopathologic parameters with p value <0.2 in univariate analyses were included in a multivariate Cox proportional-hazards regression model. (A) An older age and median and advanced TNM stage (II and IV) were independently correlated with worse clinical survival outcomes, while performing PCI was associated with favourable OS. (B) Advanced TNM stage (II and IV) was independently correlated with short PFS, conversely, performing PCI was associated with prolong PFS. ASCL1, achaete-scute family BHLH transcription factor 1; DLL3, delta-like ligand 3; OS, overall survival; PCI, prophylactic cranial irradiation; PFS, progression-free survival; POU2F3, POU class 2 homeobox 3; YAP1, Yes1-associated transcriptional regulator; TNM, tumor-node-metastasis.

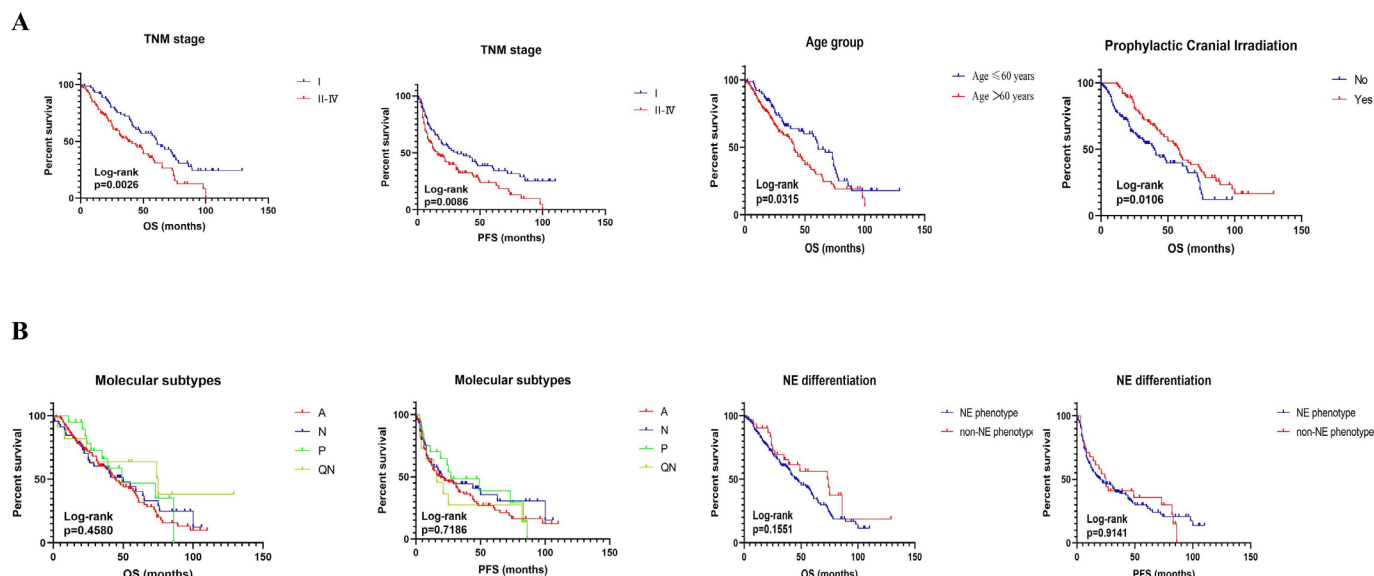
supplemental figure S1). Together, these results revealed median and advanced TNM stage (II–IV) was associated with short OS and PFS compared with early TNM stage (I), and TNM stage was an independent prognostic factor in the cohort (figure 5A). Additionally, an older age (HR=1.530, 95% CI 1.023 to 2.288; p=0.038) was independently correlated with worse clinical survival outcomes, while performing PCI was an independent predictive factor (HR=0.547, 95% CI 0.365 to 0.819; p=0.003) (figure 4A and figure 5A).

We then conducted a subgroup analysis according to molecular subtypes. The median OS and PFS values in patients with the SCLC-A, SCLC-N, SCLC-P and SCLC-QN subtypes were 38.7 and 27.1 months, 39.7 and 28.1 months, 40.1 and 32.9 months and 55.3 and 28.6 months, respectively. The Kaplan-Meier curves

for OS and PFS according to molecular subtypes were examined, and no difference was found among molecular subtypes (SCLC-A, SCLC-N, SCLC-P and SCLC-QN) (figure 5B). In addition, based on the NE differentiation of their tumours, patients were further dichotomised into the two groups of NE phenotype (SCLC-A and SCLC-N) and non-NE phenotype (SCLC-P and SCLC-QN); there were no significant differences in OS and PFS between the two groups (p=0.1551 and p=0.9141) (figure 5B). However, a consistent trend was noted towards a better SCLC outcome for patients in the non-NE-phenotype group.

## DISCUSSION

The positive rate of ASCL1 (70.3%) in this study was consistent with previously reported 59.5%–79.2%,<sup>9 11 13 16 17 23 24</sup> whereas



**Figure 5** Kaplan-Meier estimates for OS and PFS. (A) Kaplan-Meier analysis was used to compare the risk stratification of subgroups according to the prognostic factors in multivariate analysis. (B) The Kaplan-Meier curves for OS and PFS according to molecular subtypes and NE-phenotype or non-NE-phenotype, showing that there was no survival difference among molecular subtypes (SCLC-A, SCLC-N, SCLC-P and SCLC-QN) and there were no significant differences in OS and PFS between NE-phenotype and non-NE-phenotype cohorts. However, SCLC patients with a non-NE phenotype tended to have a better outcome. OS, overall survival; PFS, progression-free survival; SCLC, small-cell lung carcinoma; SCLC-A, SCLC-ASCL1-dominant; SCLC-N, SCLC-NEUROD1-dominant; SCLC-P, SCLC-POU2F3-dominant; SCLC-QN, SCLC characterised by the low expression of all four investigated transcription factors.

that of NEUROD1 (56.9%) was higher.<sup>9 11 13 16 17</sup> However, SCLC-A was the predominant subtype, which was also in line with prior studies.<sup>9 10 13 14 16–18</sup> The high NEUROD1-positive rate might be attributed to antibodies used and the different clinical stages of patients included. Notably, we observed a significant coexpression of ASCL1 and NEUROD1, which is consistent with those of other reports in the literature.<sup>9 12 17 18</sup> In support of this, other recent RNA-based investigations have also suggested that the SCLC-N subtype has greater transcriptional overlap with SCLC-A tumours,<sup>2 25</sup> and recent genetically engineered mouse SCLC models have demonstrated temporal evolution from one molecular subtype to another (such as the transition from SCLC-A to SCLC-N). These findings indicate transcriptional plasticity in SCLC, which may explain the discordant expression of ASCL1 and NEUROD1 in various studies. However, consistent with prior studies,<sup>9–11 16–18</sup> most SCLCs (82.6%) were characterised by dominant expression of ASCL1 and/or NEUROD1 (SCLC-A and SCLC-N) in our study population, and the ASCL1/NEUROD1 double-negative subtype (SCLC-P and SCLC-QN) comprised only a minor subset of SCLC (17.4%).

No YAP subtype was found in our study, which is in line with other tissue-based studies that failed to identify a distinct subtype with high YAP1 expression.<sup>4 9 12 18</sup> Conversely, a small subset of SCLC with dominant YAP1 expression was reported in primary SCLC tumours,<sup>10 14 26</sup> and it also has been pointed out that some samples may be sourced from YAP1-expressing subclones of SCLC in cell line and circulating tumour cell-derived explant data.<sup>27 28</sup> Collectively, the role of YAP1 as a subtype-defining marker in SCLC warrants further investigation for clarification. Notably, given the previously reported association of YAP1-abundance and ChT-resistance in preclinical studies,<sup>29 30</sup> the YAP1 lineage might indeed be more prominent in patients already treated with ChT and could emerge during disease evolution.<sup>31</sup> In addition, caution should be taken with the results concerning YAP1 expression by using bulk RNA sequencing as

YAP1 could be positive in stromal cells and the NSCLC component of combined SCLC.<sup>9 10</sup>

Consistent with the previous studies,<sup>9 11 23 32 33</sup> a positive correlation was observed between ASCL1 and DLL3 expression. Furthermore, we detected significantly negative associations between POU2F3/YAP1 and DLL3, indicating that POU2F3/YAP1-dominant SCLCs might be unsuitable for targeted therapy for DLL3. Unlike the *in vitro* findings that MYC is often overexpressed in the NEUROD1-subtype SCLC lines,<sup>27</sup> we found no significant correlation between NEUROD1 and MYC expression, and expression levels of MYC had a strongly positive association with those of ASCL1. These findings are in agreement with those of a recent study by Qu *et al.*<sup>10</sup> Collectively, our results show the significance of guiding targeted therapy for patients with SCLC and the need to be validated in prospective clinical trials.

In the present study, 49 cases of combined SCLC were enrolled, and this is the largest cohort of combined SCLC samples to investigate the molecular subtype distribution in terms of histological subtypes (pure and combined SCLC).<sup>9 16–18</sup> In the first comprehensive IHC-based study on SCLC molecular subtypes in patient samples,<sup>9</sup> combined SCLCs were significantly enriched in the non-NE phenotype, and in line with this, we observed significant enrichment of SCLC-P in the combined SCLC cohort. Conversely, Hwang *et al* found that SCLC-N was more commonly seen in the combined SCLCs.<sup>18</sup> SCLC molecular subtypes were not associated with histology in the other two studies,<sup>16 17</sup> while the number of combined SCLC cases included was relatively small. In addition, we collected information on the NSCLC components of combined SCLCs to explore whether they differed according to the molecular subtype, and the results revealed that ADC was more prevalent in SCLC-A, while LCNEC was more commonly seen in SCLC-P, which requires further prospective validation.



Although no difference was found among molecular subtypes, we noted that SCLC with a non-NE phenotype tended to have a better outcome, and this finding is generally consistent with other reports in the literature.<sup>12,13</sup> Conversely, SCLC-P ( $p=0.023$ ) was an independent poor prognostic factor for resected SCLC in the Cox multivariate analysis by Ding *et al*,<sup>16</sup> and in the most recent study, it was pointed out that YAP1 expression was a poor prognostic factor for patients undergoing surgical resection.<sup>18</sup> The difference in prognosis prediction is probably due to the constitution of different TNM stages and therapeutic regimens, relatively small cohort size or different antibodies used, and studies in larger cohorts will be needed to further evaluate the prognostic and treatment implications of different SCLC molecular subtypes.

There were some limitations in our study. First, it was a retrospective and single-centre study, which inevitably caused selection bias. Second, due to the limited number of paired lung biopsies of surgically resected SCLC primary tumours, the number of studied populations was limited reducing the statistical power. Third, targeted therapy of DLL3 and MYC is not yet applied in the clinic, together with the nature of the retrospective study, we could not identify the exact correlation of molecular subtypes and predictive markers' expression with treatment response.

## CONCLUSIONS

In summary, our study validated the proposed molecular subtype classification of SCLC in a large cohort of various patient samples using whole-section FFPE-based IHC. No YAP1 subtype was found. The expression of DLL3 was positively associated with that of ASCL1 and negatively associated with that of POU2F3/YAP1, whereas MYC had the opposite effect. Strong associations of subtype-defining markers in the paired resected-biopsy specimens were detected, proving the feasibility of biopsy samples for identifying SCLC molecular subtypes. We observed significant enrichment of SCLC-P in the combined SCLC cohort, and ADC was more prevalent in SCLC-A, while LCNEC was more commonly seen in SCLC-P. No survival difference was found among molecular subtypes, while SCLC with a non-NE phenotype tended to have a better outcome. Collectively, our results provide clinical insights into the diagnostic and prognostic significance of SCLC subtype classification and contribute to the selection of individualised treatments and subtype-specific management.

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Supplementary Table S1. Antibodies used for immunohistochemistry

Marker	Clone	Catalog number	Company	Host	Dilution
ASCL1	24B72D11.1	#556604	BD Biosciences, San José, CA, USA	mouse	1:50
NEUROD1	EPR17084	#ab205300	Abcam, Cambridge, U.K.	rabbit	1:200
POU2F3	E5N2D	#36135	Cell Signaling Technology, Leiden, The Netherlands	rabbit	1:300
YAP1	EP1674Y	#ab52771	Abcam, Cambridge, U.K.	rabbit	1:300
DLL3	EPR22592-18	#ab229902	Abcam, Cambridge, UK	rabbit	1:200
MYC	Y69	#ab32072	Abcam, Cambridge, UK	rabbit	1:200

Abbreviations: ASCL1, achaete-scute family BHLH transcription factor 1; NEUROD1, neuronal differentiation 1; POU2F3, POU class 2 homeobox 3; YAP1, Yes1-associated transcriptional regulator; DLL3, Delta-like ligand 3.

Note: (1) Nuclear labeling is scored as positive reactivity for the marker of ASCL1, NEUROD1, POU2F3, and MYC.

(2) YAP1 expression is localized in the nucleus and cytoplasm.

(3) DLL3 expression is localized in the membrane of tumor cells.

**Supplementary Table S2. Univariate analyses for prognostic significance of clinicopathologic parameters in the cohort**

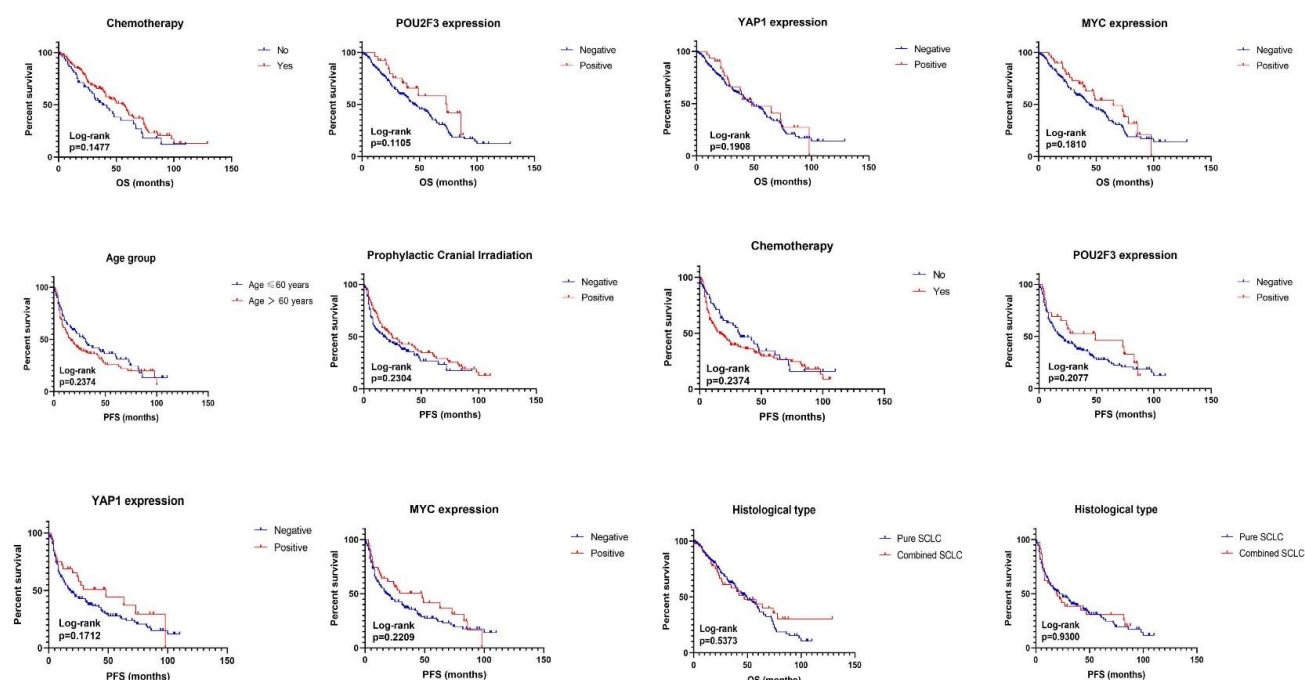
Clinicopathologic parameters	OS		PFS	
	<i>P</i> value	HR (95% CI)	<i>P</i> value	HR (95% CI)
Age, ≤60 v. > 60 years	<b>0.028</b>	0.648 (0.440-0.954)	0.209	0.796 (0.557-1.136)
Sex, Male v. Female	0.335	0.810 (0.527-1.243)	0.818	0.953 (0.634-1.434)
Smoking story, yes v. no	0.434	1.193 (0.767-1.856)	0.698	1.087 (0.713-1.658)
TNM stage, I v. II-IV	<b>0.003</b>	0.546 (0.369-0.809)	<b>0.006</b>	0.598 (0.414-0.865)
Histology, Pure v. Combined	0.505	1.168 (0.741-1.841)	0.921	1.021 (0.678-1.537)
Neoadjuvant ChT, yes v. no	0.271	1.348 (0.792-2.295)	0.927	0.979 (0.627-1.530)
Postoperative ChT, yes v. no	<b>0.115</b>	1.382 (0.924-2.066)	0.264	0.801 (0.542-1.182)
Postoperative radiotherapy, yes v. no	0.412	1.183 (0.792-1.767)	0.201	0.787 (0.546-1.136)
PCI, yes v. no	<b>0.010</b>	1.674 (1.134-2.473)	0.204	1.266 (0.880-1.820)
ASCL1 expression, Pos v. Neg	0.956	0.988 (0.650-1.502)	0.941	1.015 (0.690-1.491)
NEUROD1 expression, Pos v. Neg	0.526	1.131 (0.773-1.656)	0.286	1.213 (0.851-1.729)
POU2F3 expression, Pos v. Neg	<b>0.109</b>	1.635 (0.896-2.984)	<b>0.188</b>	1.413 (0.844-2.365)
YAP1 expression, Pos v. Neg	0.633	1.132 (0.681-1.880)	<b>0.151</b>	1.432 (0.877-2.336)
DLL3 expression, Pos v. Neg	0.410	1.200 (0.778-1.851)	0.476	1.158 (0.774-1.735)
MYC expression, Pos v. Neg	<b>0.172</b>	1.396 (0.865-2.251)	<b>0.189</b>	1.337 (0.866-2.065)
NE differentiation, NE v. non-NE	<b>0.150</b>	1.478 (0.868-2.517)	0.832	1.051 (0.667-1.655)

Abbreviations: OS, overall survival; PFS, progression-free survival; HR, hazard ratio; Pos, positive; Neg, negative; ChT, chemotherapy; PCI, prophylactic cranial irradiation; ASCL1, achaete-scute family BHLH transcription factor 1; NEUROD1, neuronal differentiation 1; POU2F3, POU class 2 homeobox 3; YAP1, Yes1-associated transcriptional regulator; DLL3, Delta-like ligand 3; NE, neuroendocrine.

Note: (1) Neoadjuvant chemotherapy means formalin-fixed paraffin-embedded tissue samples from primary tumors or metastases were obtained after chemotherapy.

(2) Significant *p* values (*p*<0.20) are highlighted in bold.





**Figure S1. The Kaplan–Meier curves for OS and PFS.**

Kaplan–Meier analysis was used to compare the risk stratification of subgroups according to other prognostic factors, which were included in multivariate analysis. The results showed that these prognostic factors were not associated with OS and PFS. OS, overall survival; PFS, progression-free survival.