



# Molecular subtypes and prognostic factors of lung large cell neuroendocrine carcinoma

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**Background:** Lung large cell neuroendocrine carcinoma (LCNEC) is an aggressive disease with poor prognosis and short-term survival, which lacks effective prognostic indicators. The study aims to investigate the molecular subtypes and prognostic markers of lung LCNEC.

**Methods:** Patients diagnosed with lung LCNEC at Sun Yat-sen University Cancer Center (SYSUCC) between November 2007 and January 2021 were screened. Baseline clinical data were collected and routine blood indexes including lymphocyte-to-monocyte ratio (LMR), neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR) and systemic immune-inflammation index (SII) were calculated. Immunohistochemistry (IHC) of ASCL1, NEUROD1, POU2F3, YAP1 were done to perform molecular subtyping, while CD56, Syn, CgA, CD3, CD8, CD20, CD68, and CD163 were also stained on tissue samples. Then prognostic factors of lung LCNEC were explored.

**Results:** One hundred and fifty-one lung LCNEC patients were identified, 103 of whom had complete clinical information, available routine blood and biochemical indexes were eventually included in the present study. Tumor tissue specimens were available from 64 patients. Positive expression rates of ASCL1, NEUROD1, and YAP1 were 82.8%, 50.0%, and 28.1%, respectively. No POU2F3<sup>+</sup> cases were detected. Forty (62.5%) patients co-expressed with two or three markers. High LMR (>3.3) was an independent predictor of favorable prognosis of disease-free survival (DFS) [hazard ratio (HR), 0.391; 95% confidence interval (CI): 0.161–0.948; P=0.04] and overall survival (OS) (HR, 0.201; 95% CI: 0.071–0.574; P=0.003). Notably, high LMR was correlated with higher intra-tumoral CD3<sup>+</sup> (P=0.004), CD8<sup>+</sup> (P=0.01), and CD68<sup>+</sup> (P<0.001) immune cell infiltration compared to low LMR in lung LCNEC.

**Conclusions:** Our study validated molecular subtypes by IHC in lung LCNEC, and co-expression was found among different subtypes, with no prognostic effect. High blood LMR level was associated with a favorable prognosis in lung LCNEC, which might partly reflect a hot tumor tissue immune microenvironment. Our findings may benefit clinical practice, and further studies are warranted.

**Keywords:** Lung large cell neuroendocrine carcinoma (lung LCNEC); molecular subtypes; lymphocyte-to-monocyte ratio (LMR); tumor-infiltrating lymphocytes and macrophages (TILs and TIMs); prognosis

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## Introduction

Lung large cell neuroendocrine carcinoma (LCNEC) is a rare and aggressive malignant tumor that accounts for ~3% of lung cancer. Additionally, it is classified as high-grade neuroendocrine (NE) carcinoma and characterized by a close association with smoking and with high metastasis potential (1-3). The diagnosis of lung LCNEC is based on NE morphology, cytological features of non-small cell lung cancer (NSCLC), high proliferation rate, and NE marker expression (1,4). The prognosis of lung LCNEC is poor, and therapeutic means are currently limited (3,5-8).

Therefore, identifying new promising biomarkers involved in the prognosis of lung LCNEC is necessary, as they may contribute to identifying high-risk populations and therapeutic interventions.

Lung LCNEC patients were categorized into two types: type I showing overlapping key genes with lung adenocarcinoma or squamous cell carcinoma in genome analysis but with a high NE expression in transcriptome analysis (9). In contrast, type II showed overlapping key genes with small cell lung cancer (SCLC) but with a low NE expression in transcriptome analysis (9). This reminds us that genome-based typing of lung LCNEC may not be consistent with its histological features of NE differentiation. Another study suggested that different histological types of lung cancer (including lung LCNEC) are associated with specific transcriptome characteristics rather than genome characteristics, highlighting the importance of exploring the molecular biological features at the gene expression level in lung cancer (10).

SCLC subtypes could be defined by the expression of ASCL1, NEUROD1, POU2F3, and YAP1 transcription factors using transcriptome data (11), which has been verified in clinical specimens at the protein level (12-14). The expression of ASCL1, NEUROD1, POU2F3, and YAP1 are likely associated with the prognosis of SCLC (15-18). In lung LCNEC, relatively few studies have systematically analyzed the four transcription factors (19,20), the prognostic differences among different molecular subtypes were unclear. Thus, we comprehensively evaluated the expression patterns and prognosis of ASCL1, NEUROD1, POU2F3, and YAP1 in lung LCNEC in the present study.

Systemic inflammation has been shown to correlate with pathogenesis, disease progression, and prognosis across various tumors (21,22). Peripheral blood lymphocyte-to-monocyte ratio (LMR), neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR) and systemic immune-inflammation index (SII) were widely used as systemic inflammatory indicators (23,24). Tumor-infiltrating lymphocytes (TILs) and macrophages

### Highlight box

#### Key findings

- Molecular subtypes by immunohistochemistry (IHC) staining were validated, co-expression was found among different subtypes, with no prognostic value.
- Blood lymphocyte-to-monocyte ratio (LMR) was an independent prognostic predictor of disease-free survival (DFS) and overall survival (OS), high blood LMR might partly reflect a hot tumor tissue immune microenvironment (high intra-tumoral CD3<sup>+</sup>, CD8<sup>+</sup>, and CD68<sup>+</sup> immune cell infiltration).

#### What is known and what is new?

- Previously studies have reported the molecular subtypes defined by IHC, co-expression was found among different subtypes.
- It was the first time to confirm that there is no prognosis effect of molecular subtypes in lung large cell neuroendocrine carcinoma (LCNEC). However, high LMR was associated with a favorable prognosis in lung LCNEC patients, which was correlated with higher intra-tumoral CD3<sup>+</sup>, CD8<sup>+</sup>, and CD68<sup>+</sup> immune cell infiltration.

#### What is the implication and what should change now?

- No prognosis difference among different molecular subtypes was observed, it is necessary to explore other prognostic markers in lung LCNEC.
- High blood LMR was a favorable prognostic indicator for DFS and OS in lung LCNEC, which might partly reflect a hot tumor immune microenvironment, suggesting that LMR might be a new candidate to predict prognosis and indicate tumor immune status in lung LCNEC.

(TIMs) are essential components of the tumor immune microenvironment (TIME), which is formed by peripheral blood lymphocytes and monocytes infiltrating into tumor tissue through systemic circulation and exerting anti- and pro-tumor effects (25,26). In lung LCNEC, evidence on the relationship between these inflammatory indicators and prognosis was limited (27-29).

This study's objectives were: (I) to investigate the protein expression patterns of ASCL1, NEUROD1, POU2F3, and YAP1 and explore their prognosis; (II) to evaluate the prognostic value of inflammatory indicators and their relationship with tissue TIL/TIM status. We present this article in accordance with the STROBE reporting checklist (available at <https://tldr.amegroups.com/article/view/10.21037/tldr-24-292/rc>).

## Methods

### *Study population*

From November 2007 to January 2021, patients diagnosed with lung LCNEC at Sun Yat-sen University Cancer Center (SYSUCC) were all identified in this study. Mixed lung LCNEC was defined as lung LCNEC mixed with NSCLC (adenocarcinoma/squamous cell carcinoma) or SCLC (4). The patients' tissue specimens were collected if available. The inclusion criteria included age >18 years, no previous treatment for lung LCNEC, and complete clinical information. Exclusion criteria included multiple primary cancers ( $\geq 1$  primary cancer of other organs except for lung) within 5 years, incomplete clinical information, and lack of baseline blood routine and biochemical test. The study was approved by the Institutional Review Board of SYSUCC (ID: B2021-030-01), and written informed consent was obtained from each participant. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

### *Information extraction and follow-up*

Demographic and clinical information were collected from electronic medical records, incorporating age, sex, smoking status, Eastern Cooperative Oncology Group performance status (ECOG-PS), tumor stage, treatment means, and body mass index (BMI). Lymphocyte count, monocyte count, neutrophil count, platelet count, C-reactive protein (CRP), lactate dehydrogenase (LDH), and albumin (ALB) values were collected from blood samples and biochemical

reports within 3 days before initial therapy. LMR was calculated as the lymphocyte count divided by monocyte count. NLR was calculated as the neutrophil count divided by lymphocyte count. PLR was calculated as the platelet count divided by lymphocyte count. SII was calculated by multiplying the platelet count with neutrophil count and dividing it by lymphocyte count (30). Individuals who had smoked  $\geq 100$  cigarettes in their lifetime were classified as current or ever smokers. Furthermore, tumor staging followed the American Joint Committee on Cancer (AJCC) eighth edition staging manual (31).

Telephone follow-up was conducted every 6 to 12 months until death or loss of follow-up. Tumor response assessment was performed every 2 cycles of chemotherapy or at every 3 months interval following radical surgery by computed tomography (CT), according to Response Evaluation Criteria in Solid Tumor (RECIST) 1.1 (32,33).

### *Immunohistochemistry (IHC) staining*

Tissue sections were deparaffinized with xylene, hydrated with ethyl alcohol, and washed in phosphate-buffered saline (PBS), followed by antigen repair. Sections were treated with 3% H<sub>2</sub>O<sub>2</sub> for 10 minutes to quench endogenous peroxidase activity. Primary antibodies of ASCL1, NEUROD1, POU2F3, YAP1, CD3, CD8, CD20, CD68, CD163, CD56, Syn, and CgA (source, clone and working dilution detailed in [Table S1](#)) were incubated 1 h at 37 °C, washed in PBS twice for 3 minutes. Secondary antibodies were incubated for 45 minutes for ASCL1, NEUROD1, POU2F3, and YAP1, and were incubated for 20 minutes with CD3, CD8, CD20, CD68, CD163, CD56, Syn, and CgA at room temperature, washed in PBS and stained by 3,3'-diaminobenzidine (DAB).

Images of all stained slides were acquired using the digital pathology slide scanner (KFBIO, Ningbo Jiangfeng Biological Information Technology Co., Ltd., Ningbo, China) at 40 $\times$  resolution and analyzed by K-viewer software (version 1.5.3.1; KFBIO). Two pathologists completed an independent evaluation of IHC staining results. CD56, Syn, and CgA were considered positive when there was  $\geq 1\%$  membrane staining of tumor cells. Histological score (H-score) was measured to evaluate ASCL1, NEUROD1, POU2F3, and YAP1 expression. H-score was derived by multiplying nuclear staining intensity (0, negative; 1, weak; 2, moderate; 3, strong; 4, very strong) by percentage of positive nuclei (1–100%). Additionally, the density of immune cell infiltration was evaluated via QuPath (version

0.3.2) software. Intra-tumoral immune cell infiltration was considered immune cells directly contacting tumor cells. In contrast, infiltration of stromal immune cells was considered immune cells that did not directly contact tumor cells. Positive immune cell density was counted in the average value of five randomly chosen intra-tumoral or stromal regions (0.1 mm<sup>2</sup>/per region).

### Statistical analysis

Differences in clinicopathological features of different groups were compared using Chi-squared tests, continuity corrections, or Fisher's exact test for categorical variables. Two independent sample *t*-test/one-way analysis of variance (ANOVA) test/Mann-Whitney test/Kruskal-Wallis test were used for continuity variables. Disease-free survival (DFS) was defined as the time from lung resection until the first disease progression or death of any cause or loss to follow-up. Overall survival (OS) was defined as the time from pathological diagnosis until the death of any cause or loss to follow-up. The Kaplan-Meier method performed DFS and OS survival curves with the log-rank test. Univariate and multivariate analyses of DFS and OS were analyzed using the Cox proportional hazards model. A two-sided of  $P < 0.05$  was considered significant. All the above statistical analyses were conducted using GraphPad Prism (version 8.0).

## Results

### Clinicopathological characteristics

A total of 151 lung LCNEC patients at SYSUCC were enrolled, of whom 41 had incomplete clinical information, 3 had multiple primary cancers within 5 years, and 4 did not have available blood routine and biochemistry test within 3 days before initial treatment. Finally, 103 lung LCNEC patients were included in this study (Figure S1). Tumor tissue specimens were available from 64 patients, and all specimens were surgical resected primary tumor lesions. The median age of the whole cohort was 60 years (range, 37–80 years). Sixty-eight patients (66.0%) were diagnosed as pure LCNEC, and 35 patients (34.0%) were diagnosed with mixed LCNEC, including 25 (24.3%) mixed with NSCLC and 10 (9.7%) mixed with SCLC. The study population was 87.4% (90/103) male, 74.8% (77/103) current or ever smokers, and all had a favorable ECOG-PS of 0–1. The stage at initial diagnosis was stage

I in 35, stage II in 26, stage III in 32, and stage IV in 10 cases. Of the entire cohort, 92 patients (89.3%) underwent lung cancer resection, and 52 patients (50.4%) were treated with platinum-based chemotherapy, including paclitaxel/etoposide/pemetrexed plus platinum regimens (Table S2).

### Expression of ASCL1, NEUROD1, YAP1 and POU2F3

Using a cutoff H-score of 0, positivity for ASCL1, NEUROD1, and YAP1 were detected in 53 (82.8%), 32 (50.0%) and 18 (28.1%) patients, respectively (Figure 1A,1B). No POU2F3 positive staining was detected in our study. The average H-score for ASCL1 was 145 (range, 10–360), NEUROD1 was 113 (range, 10–320), and YAP1 was 74 (range, 5–180) (Figure 1B).

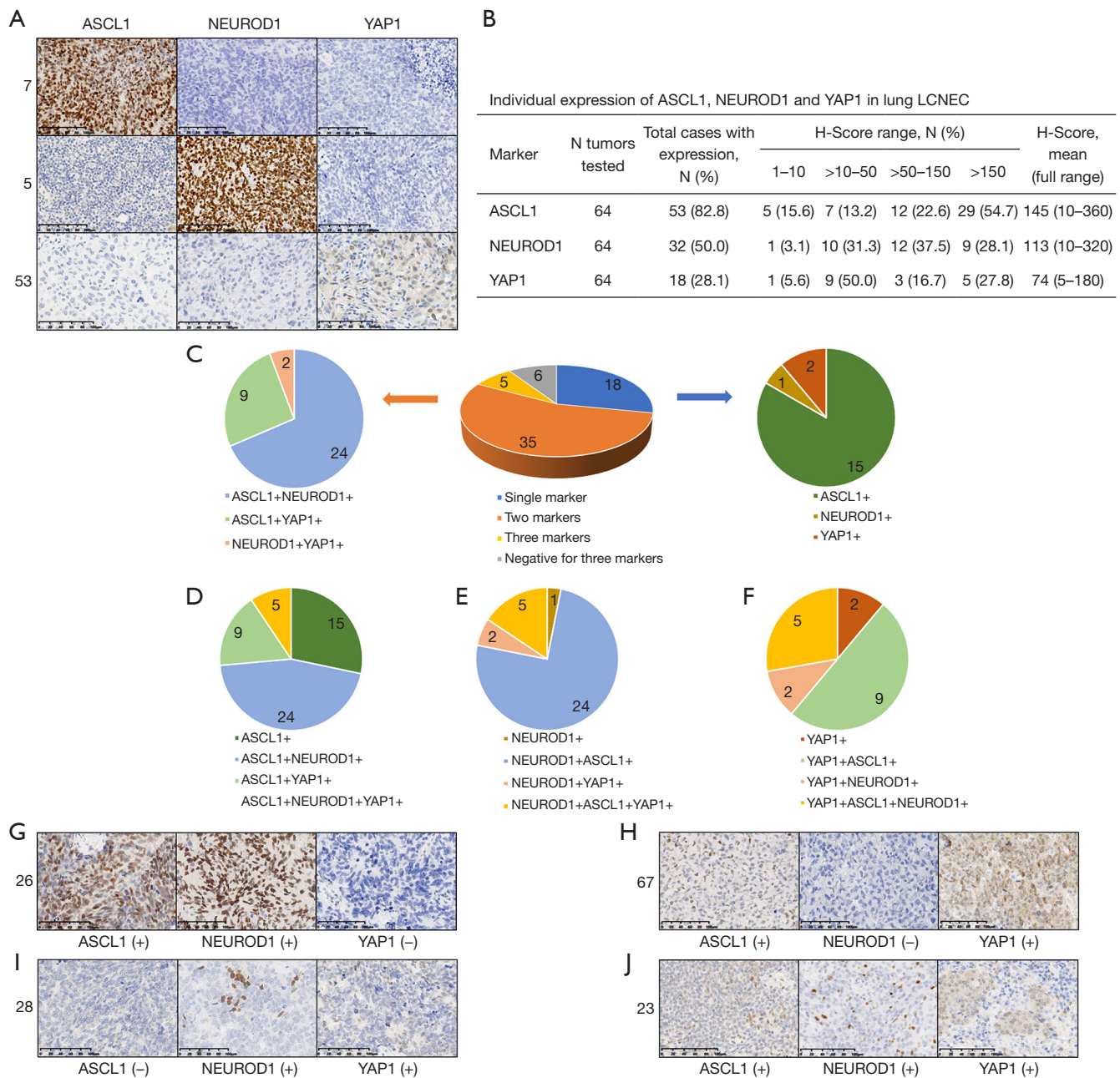
The co-expression pattern of ASCL1, NEUROD1, and YAP1 is shown in Figure 1C-1F. Six out of 64 patients (9.4%) detected negative expression of all four molecules. Additionally, 18/64 (28.1%) patients expressed single molecules, and 5/64 (7.8%) patients co-expressed three molecules. Thirty-five out of 64 patients (54.7%) co-expressed two molecules, including 24 co-expressed ASCL1 and NEUROD1, 9 co-expressed ASCL1 and YAP1, and 2 co-expressed NEUROD1 and YAP1. Figure 1G-1J shows the representative IHC images for ASCL1 and NEUROD1 co-expression, ASCL1 and YAP1 co-expression, NEUROD1 and YAP1 co-expression, and ASCL1, NEUROD1 and YAP1 co-expression, respectively.

### ASCL1/NEUROD1-defined groups and NE markers

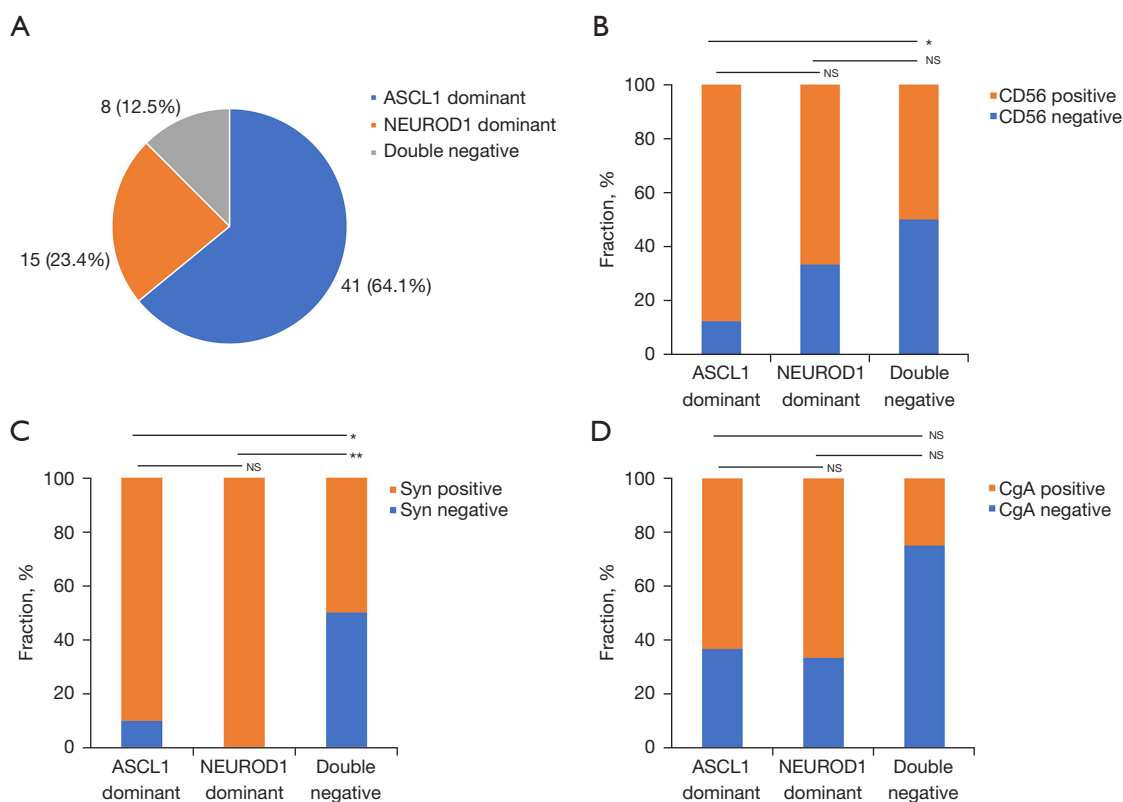
Due to no POU2F3 positive cases being detected in our study, and since YAP1 was widely co-expressed with ASCL1/NEUROD1 (Figure 1F), we applied the molecular subtyping approach as reported previously (14). Thus, a subsequent analysis was carried out based on this.

The molecular subtyping was based on the relative dominant expression of ASCL1 (H-score of ASCL1-NEUROD1 >0) or NEUROD1 (H-score of NEUROD1-ASCL1 >0), negative expression (H-score =0) of both ASCL1 and NEUROD1 was classified as a double negative group (14). According to the approach, the distribution of groups was as follows: ASCL1 dominant group, N=41 (64.1%); NEUROD1 dominant group, N=15 (23.4%); and double negative group, N=8 (12.5%) (Figure 2A).

We performed IHC staining for three NE markers typically used in clinical practice: CD56, Syn, and CgA. The positive expression rates of CD56 in the ASCL1



**Figure 1** Protein expression patterns of ASCL1, NEUROD1 and YAP1. (A) Represent IHC images for ASCL1 (patient No. 7), NEUROD1 (patient No. 5) and YAP1 (patient No. 53). (B) The table showing the individual expression of ASCL1, NEUROD1 and YAP1 protein. (C) Pie chart showing the patient number of positive for none, single, double and triple markers of ASCL1, NEUROD1 and YAP1. (D) Pie chart showing the patient number of positive for ASCL1 alone and co-expression of ASCL1 with NEUROD1/YAP1. (E) Pie chart showing the patient number of positive for NEUROD1 alone and co-expression of NEUROD1 with ASCL1/YAP1. (F) Pie chart showing the patient number of positive for YAP1 alone and co-expression of YAP1 with ASCL1/NEUROD1. (G) Representative IHC image for co-expression of ASCL1 and NEUROD1 (patient No. 26). (H) Representative IHC image for co-expression of ASCL1 and YAP1 (patient No. 67). (I) Representative IHC image for co-expression of NEUROD1 and YAP1 (patient No. 28). (J) Representative IHC image for co-expression of ASCL1, NEUROD1 and YAP1 (patient No. 23). (+) indicates the positive expression (H-score >0); (-) indicates the negative expression (H-score =0). LCNEC, large cell neuroendocrine carcinoma; IHC, immunohistochemistry.



**Figure 2** ASCL1/NEUROD1 defined groups and their association with CD56, Syn and CgA. Pie chart (A) illustrating the distribution of ASCL1/NEUROD1 defined groups: ASCL1 dominant group (H-score of ASCL1-NEUROD1 >0), NEUROD1 dominant group (H-score of NEUROD1-ASCL1 >0) and double negative group (H-score =0 for both NEUROD1 and ASCL1). Bar charts illustrating comparison of CD56 (B), Syn (C) and CgA (D) expression among ASCL1 dominant, NEUROD1 dominant and double negative groups. Statistical significance was calculated by Fisher exact test in (B-D). P values are indicated as NS, not significant; \*, P<0.05 and \*\*, P<0.01.

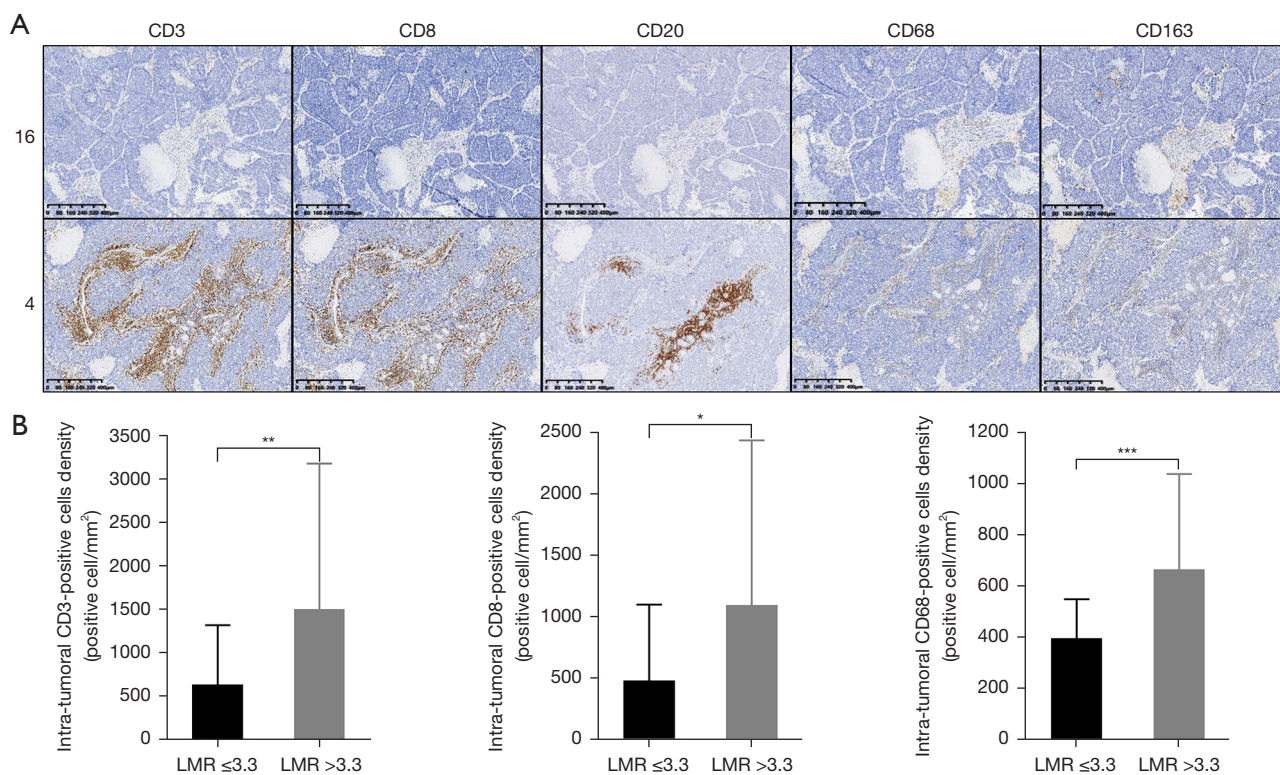
dominant group were higher than those in the double negative group (87.8% vs. 50.0%, P=0.03, *Figure 2B*). Syn positive rates were also higher in ASCL1 dominant (90.2% vs. 50.0%, P=0.02) or NEUROD1 dominant (100.0% vs. 50.0%, P=0.008) groups versus those in the double negative group (*Figure 2C*). No significant difference in CgA positive rate was seen among different groups (P=0.12, *Figure 2D*). Furthermore, age, sex, smoking status, tumor stage, pathological classification, and chemotherapy regimens were balanced among different groups (*Table S3*).

**Peripheral blood inflammation indicators and clinicopathological features**

Baseline LMR, NLR, PLR, and SII values were available for all patients in the cohort. The optimal cutoff value was 3.3 for LMR, 2.9 for NLR, 95.4 for PLR, and 530.8 for SII, which was determined according to OS by X-tile

software (34). Compared with patients with low LMR ( $\leq 3.3$ ), patients with high LMR ( $> 3.3$ ) had lower CRP levels [mean  $\pm$  standard deviation (SD),  $8.1 \pm 14.6$  vs.  $25.4 \pm 40.3$ , P=0.004] and higher ALB levels (mean  $\pm$  SD,  $43.0 \pm 3.1$  vs.  $40.0 \pm 5.2$ , P=0.005). Patients with SII  $> 530.8$  had higher CRP level (mean  $\pm$  SD,  $18.2 \pm 32.1$  vs.  $5.4 \pm 8.8$ , P=0.02) than those with SII  $\leq 530.8$ . Other clinicopathological parameters did not differ between groups stratified by LMR, NLR, PLR or SII (*Table S4*).

*Figure 3A* shows the representative IHC staining image for intra-tumoral and stromal TIL (marked by CD3, CD8, or CD20) and TIM (marked by CD68 or CD163). See *Table S5* for detailed expression levels (mean  $\pm$  SD and range). Compared to patients with low LMR (N=21), patients with high LMR (N=43) had significantly more intra-tumoral CD3<sup>+</sup> ( $641 \pm 685$  vs.  $1,508 \pm 1,672$  cells/mm<sup>2</sup>, P=0.004), CD8<sup>+</sup> ( $484 \pm 618$  vs.  $1,096 \pm 1,342$  cells/mm<sup>2</sup>, P=0.01), and CD68<sup>+</sup> ( $398 \pm 153$  vs.  $667 \pm 373$  cells/mm<sup>2</sup>,



**Figure 3** Association of peripheral blood LMR level and tumor TILs and TIM status. (A) Representative IHC images for low density (patient No. 16) and high density (patient No. 4) TIL (marked by CD3, CD8 and CD20) and TIM (marked by CD68 and CD163) in tumor tissue. (B) Comparison of intra-tumoral CD3<sup>+</sup>, CD8<sup>+</sup> and CD68<sup>+</sup> infiltrating immune cells between low ( $\leq 3.3$ ) LMR and high ( $> 3.3$ ) LMR groups. Statistical significance was calculated by two independent sample *t*-test in difference on intra-tumoral CD68<sup>+</sup> TIM between two groups, by Mann-Whitney test in difference on intra-tumoral CD3<sup>+</sup>/CD8<sup>+</sup> TIL, between two groups. P values are indicated as \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  and \*\*\*,  $P < 0.001$ . LMR, lymphocyte and monocyte ratio; TIL, tissue infiltrating lymphocyte; TIM, tumor-infiltrating macrophage; IHC, immunohistochemistry.

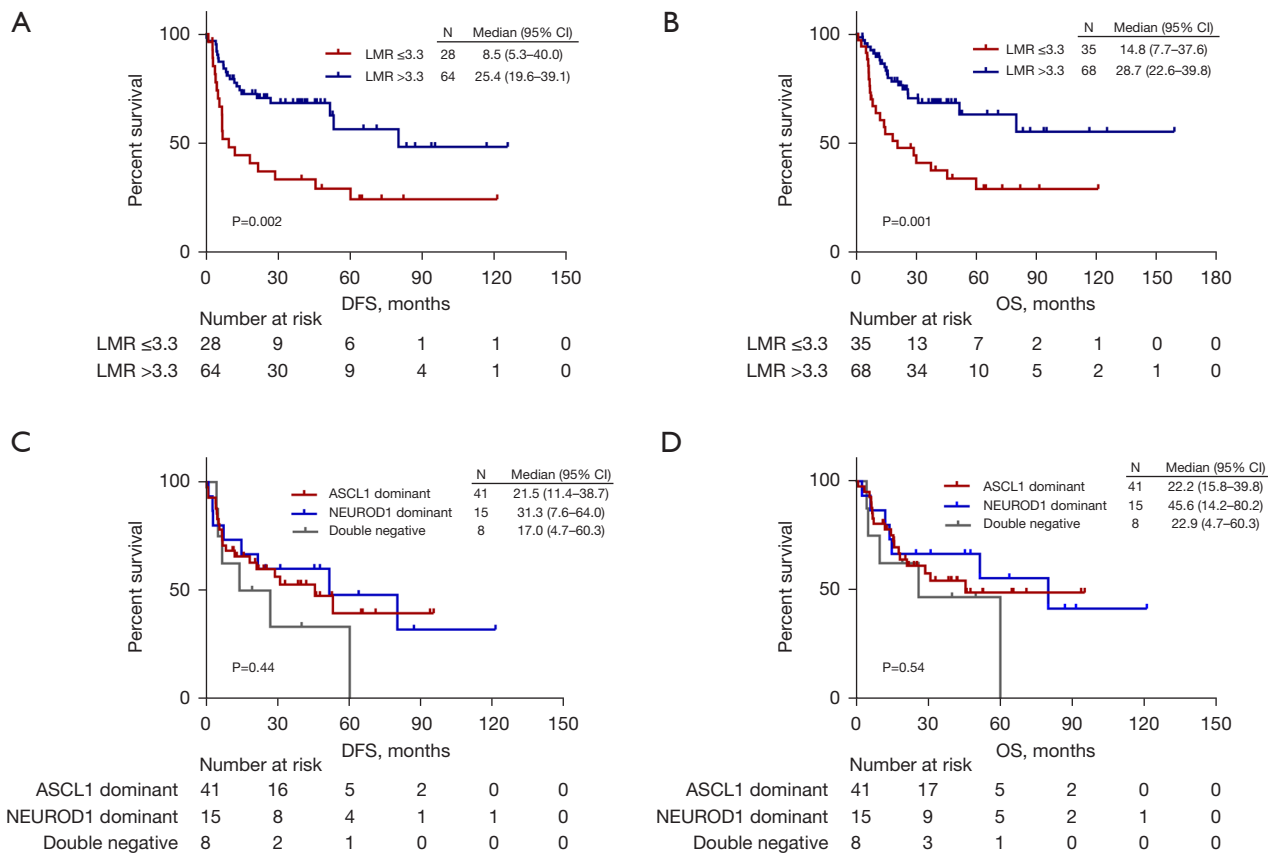
$P < 0.001$ ) immune cell infiltration (Figure 3B, Table S6). The intra-tumoral and stromal TIL/TIM status were balanced between groups stratified by NLR, PLR or SII (Table S6). The LMR level was not differ among ASCL1 dominant, NEUROD1 dominant, and double negative groups ( $P = 0.30$ ), as well as NLR, PLR, SII level and intra-tumoral or stromal TIL/TIM status (Table S7).

#### Prognostic indicators for DFS and OS

At the last follow-up date of January 30, 2023, the median follow-up time of the whole cohort was 37.4 months (range, 3.0–159.1 months). The median DFS and OS were 23.5 months (range, 0.6–125.5 months) and 25.6 months (range, 1.1–159.1 months), respectively. Kaplan-Meier survival curves showed that the median DFS ( $P = 0.002$ , Figure 4A) and OS ( $P = 0.001$ , Figure 4B) were longer in

patients with high LMR than those with low LMR. No significant differences were observed in DFS ( $P = 0.44$ , Figure 4C) and OS ( $P = 0.54$ , Figure 4D) among ASCL1 dominant, NEUROD1 dominant, and double negative groups. No statistically substantial differences in DFS or OS were observed between groups stratified by NLR, PLR or SII (Figure S2).

We performed the univariate and multivariate Cox regression analysis to investigate the independent prognostic indicators of DFS and OS in lung LCNEC. For DFS, high LMR ( $P = 0.003$ ) and early tumor stage ( $P = 0.006$ ) were associated with increased DFS in univariate analyses. From multivariate analyses, patients with high LMR ( $P = 0.04$ ) and early tumor stage ( $P = 0.02$ ) were independent predictors of favorable prognosis of DFS in lung LCNEC patients (Table 1). The univariate favorable prognostic factors for OS were high LMR ( $P = 0.002$ ) and early tumor stage



**Figure 4** Kaplan-Meier survival curves of DFS and OS. DFS (A) and OS (B) in patients with low LMR ( $\leq 3.3$ ) versus patients with high LMR ( $> 3.3$ ). DFS (C) and OS (D) among ASCL1 dominant group, NEUROD1 dominant group and double negative group. Statistical significance was calculated by Kaplan-Meier method with log-rank test. A two-sides of  $P < 0.05$  was considered significant. The number and median survival time (with 95% CI) of patients in each group are indicated. CI, confidence interval; LMR, lymphocyte and monocyte ratio; DFS, disease-free survival; OS, overall survival.

( $P = 0.002$ ). Furthermore, multivariate analyses indicated that high LMR ( $P = 0.003$ ) and low tumor stage ( $P = 0.03$ ) were independent predictors of favorable prognosis of OS in lung LCNEC patients (Table 2).

**Discussion**

In this study, we explored molecular subtypes and immune microenvironment in lung LCNEC and assessed their correlation with clinical outcomes. Our study demonstrated the feasibility of defining molecular subtypes of lung LCNEC patients by IHC, and found ASCL1, NEUROD1, and YAP1 were co-expressed in different degrees, with no prognostic effect. Moreover, peripheral blood LMR was a strong independent predictor of outcomes for DFS and OS, and significantly correlated with tissue intra-tumoral  $CD3^+$ ,

$CD8^+$ , and  $CD68^+$  immune cell infiltration.

Molecular subtypes defined by ASCL1, NEUROD1, POU2F3, and YAP1 were initially proposed for SCLC, called SCLC-A, SCLC-N, SCLC-P and SCLC-Y, respectively (11). In general, SCLC-A and SCLC-N demonstrated higher proportions, while SCLC-P and SCLC-Y showed lower proportions (16,35). Our study found that ASCL1 had the highest positive expression rate, followed by NEUROD1, and YAP1 was the lowest, consistent with the SCLC study (14). POU2F3<sup>+</sup> tumor originated from a rare tuft cell lineage different from ASCL1<sup>+</sup> and NEUROD1<sup>+</sup>. Although POU2F3 was detectable in lung LCNEC by RNA sequencing (20) and IHC (19,36) methods, its positive expression rate was low (about 10% to 15.9%). No POU2F3<sup>+</sup> cases were detected in our study, which may be due to the sample size restriction.



**Table 1** Univariate and multivariate Cox analysis for DFS in lung LCNEC

Variables	Subtypes	Univariate analysis			Multivariate analysis		
		HR	95% CI	P value	HR	95% CI	P value
Age, years	≤60	0.923	0.510–1.670	0.79	0.719	0.310–1.666	0.44
	>60	1 (Referent)	–		1 (Referent)	–	
Sex	Male	2.315	0.715–7.501	0.16	1.458	0.302–7.035	0.64
	Female	1 (Referent)	–		1 (Referent)	–	
Smoking status	Current/ever smokers	1.017	0.509–2.031	0.96	0.910	0.304–2.727	0.87
	Never smokers	1 (Referent)	–		1 (Referent)	–	
Tumor stage	I	0.147	0.049–0.441	0.006*	0.131	0.031–0.561	0.02*
	II	0.374	0.134–1.042		0.417	0.115–1.514	
	III	0.366	0.130–1.029		0.452	0.114–1.794	
	IV	1 (Referent)	–		1 (Referent)	–	
Molecular subtypes	ASCL1 dominant	0.589	0.236–1.470	0.45	0.724	0.241–2.176	0.63
	NEUROD1 dominant	0.522	0.178–1.530		0.551	0.161–1.887	
	Double negative	1 (Referent)	–		1 (Referent)	–	
LMR	>3.3	0.409	0.225–0.744	0.003*	0.391	0.161–0.948	0.04*
	≤3.3	1 (Referent)	–		1 (Referent)	–	
NLR	>2.9	1.029	0.545–1.944	0.93	0.376	0.137–1.032	0.06
	≤2.9	1 (Referent)	–		1 (Referent)	–	
PLR	>95.4	1.439	0.640–3.234	0.38	1.453	0.373–5.661	0.59
	≤95.4	1 (Referent)	–		1 (Referent)	–	
SII	>530.8	1.260	0.675–2.352	0.47	2.136	0.746–6.115	0.16
	≤530.8	1 (Referent)	–		1 (Referent)	–	

\*, statistically significance ( $P < 0.05$ ). DFS, disease-free survival; LCNEC, large cell neuroendocrine carcinoma; HR, hazard ratio; CI, confidential interval; LMR, lymphocyte and monocyte ratio; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; SII, systemic immune-inflammation index.

ASCL1, NEUROD1, POU2F3, and YAP1 were disproportionately co-expressed in one sample in previous studies of SCLC (35,37) and lung LCNEC (19,20). A similar phenomenon was observed in our study, in which most lung LCNEC patients co-expressed ASCL1/NEUROD1/YAP1 to different degrees, suggesting inter- and intra-tumoral heterogeneities. Notably, preclinical studies demonstrated remarkable heterogeneity and plasticity within SCLC, with the capacity to subtypes switching: MYC drive the ASCL1<sup>+</sup> to NEUROD1<sup>+</sup> to YAP1<sup>+</sup> phenotypic transition *in vitro* and *in vivo*, and KDM6A inactivation induced plasticity from ASCL1<sup>+</sup> to NEUROD1<sup>+</sup> state (35,38-41). A possible explanation for tumor heterogeneity of lung LCNEC is

the time evolution process among ASCL1, NEUROD1, and YAP1. This dynamic subtype transformation may be the reason why there is no difference in prognosis among different subtypes observed in the present study.

NE differentiation is an essential feature of lung LCNEC (5). It is reported that the biological function of ASCL1 and NEUROD1 is to promote NE differentiation (11,42-45). Compared with patients in the double negative group, the ASCL1 dominant group had significantly higher CD56 positive expression rates. The NEUROD1 dominant group patients had significantly higher CD56 and Syn positive expression rates. This indirectly demonstrates the role of ASCL1 and NEUROD1 in promoting NE

**Table 2** Univariate and multivariate Cox analysis for OS in lung LCNEC

Variables	Subtypes	Univariate analysis			Multivariate analysis		
		HR	95% CI	P value	HR	95% CI	P value
Age, years	≤60	0.904	0.496–1.646	0.74	0.704	0.282–1.754	0.45
	>60	1 (Referent)	–		1 (Referent)	–	
Sex	Male	1.856	0.572–6.017	0.30	0.667	0.129–3.448	0.63
	Female	1 (Referent)	–		1 (Referent)	–	
Smoking status	Current/ever smokers	1.175	0.578–2.389	0.66	1.468	0.469–4.588	0.51
	Never smokers	1 (Referent)	–		1 (Referent)	–	
Tumor stage	I	0.164	0.064–0.421	0.002*	0.122	0.027–0.554	0.03*
	II	0.368	0.156–0.870		0.439	0.121–1.593	
	III	0.318	0.132–0.765		0.467	0.118–1.852	
	IV	1 (Referent)	–		1 (Referent)	–	
Molecular subtypes	ASCL1 dominant	0.634	0.235–1.711	0.55	0.760	0.227–2.540	0.66
	NEUROD1 dominant	0.528	0.165–1.697		0.553	0.146–2.094	
	Double negative	1 (Referent)	–		1 (Referent)	–	
LMR	>3.3	0.386	0.212–0.703	0.002*	0.201	0.071–0.574	0.003*
	≤3.3	1 (Referent)	–		1 (Referent)	–	
NLR	>2.9	1.336	0.718–2.485	0.36	0.426	0.134–1.349	0.15
	≤2.9	1 (Referent)	–		1 (Referent)	–	
PLR	>95.4	2.070	0.813–5.271	0.13	1.465	0.334–6.420	0.61
	≤95.4	1 (Referent)	–		1 (Referent)	–	
SII	>530.8	1.519	0.779–2.960	0.19	1.208	0.400–3.645	0.74
	≤530.8	1 (Referent)	–		1 (Referent)	–	

\*, statistically significance (P<0.05). OS, overall survival; LCNEC, large cell neuroendocrine carcinoma; HR, hazard ratio; CI, confidential interval; LMR, lymphocyte and monocyte ratio; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; SII, systemic immune-inflammation index.

differentiation, which aligns with the observation in the SCLC study (14). Our findings validated the correlation between ASCL1/NEUROD1 expression and NE differentiation in lung LCNEC.

Our study was the first to explore the prognostic value of LMR in lung LCNEC and demonstrated that peripheral blood LMR was prognostic indicator for DFS and OS in lung LCNEC, and its prognostic value was independent of the tumor stage, suggesting that the blood LMR and tumor stage provide complementary prognostic information in lung LCNEC. This result was consistent with studies of lung cancer: high LMR level was significantly associated with improved OS and DFS (46,47).

We observed no prognostic effect of SII, NLR and PLR in our study. This is the first time to assess the prognostic value of SII in lung LCNEC. SII was reported to be a new prognostic systemic inflammation indicator, patients with high SII score had a worse prognosis in NSCLC (48,49). However, no correlation between SII and prognosis was found in lung LCNEC in our study. Previous two studies indicated that baseline high NLR or PLR was associated with poor prognosis in lung LCNEC patients (27-29), which is differ from our results. The differences may be related to different baseline characteristics of study population, small sample size and retrospective study design of these studies. Findings of the prognostic value of these

inflammation indicators were needed to be interpreted with caution and validated in larger prospective cohorts.

In tumor tissue, high blood LMR was significantly associated with higher intra-tumoral CD3<sup>+</sup> TIL, CD8<sup>+</sup> TIL, and CD68<sup>+</sup> TIM versus low LMR. CD3<sup>+</sup> TIL contains all T cells infiltrated in tumor tissue, including cytotoxic T lymphocytes (CTLs), T helper (Th) cells, and Treg cells, which kill tumor cells through the direct killing effect of CTL and pose an indirect effect via cytokines secretion (50,51). Moreover, CD8<sup>+</sup> cytotoxic T cells exert an antitumor effect by binding to MHC-I molecules on the surface of antigen-presenting cells (52). CD68 is a common marker of pan-macrophages in tumor tissue (53), which plays a dual role in antitumor immunity, tumor suppression, and promotion of tumor cell proliferation and invasion (54,55). Macrophages are classified into M1 and M2 macrophages, M1 macrophage can recruit an antitumor TIL response to prolong the survival of lung cancer patients (56,57). High CD3<sup>+</sup> TIL, CD8<sup>+</sup> TIL, and CD68<sup>+</sup> TIM were associated with good prognosis in cancers (58-62), and high immune cell infiltration with anti-tumor effect in tumor tissue has been regarded as a hot tumor microenvironment (63,64). Thus, we speculate that the good prognosis of high LMR may be closely related to the hot tumor microenvironment as reflected by high CD3<sup>+</sup>, CD8<sup>+</sup> TIL and CD68<sup>+</sup> macrophages infiltration, which needs to be explored in further studies.

Our study has several limitations. First, this is a single-center retrospective study with a small sample size. However, given the low incidence rate of lung LCNEC, the sample size is relatively large. Second, we explored the immune microenvironment of lung LCNEC using IHC which were not comprehensive enough. Thus, we are currently reaching out to conduct RNA sequencing for further investigation. Third, the result that peripheral blood LMR was an independent prognostic factor in lung LCNEC requires further validation in prospective studies with larger sample sizes.

## Conclusions

Our study validated the molecular subtypes by IHC in lung LCNEC, and co-expression was found between different subtypes with no prognostic effect. High blood LMR was a favorable prognostic indicator for DFS and OS in lung LCNEC, which may be partly related to a hot TIME. Our findings may benefit clinical practice while prospective studies with larger sample size are warranted.

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## Footnote

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*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Institutional Review Board of SYSUCC (ID: B2021-030-01), and written informed consent was obtained from each participant.

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