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Original contribution

Clinicopathological features and prognostic analysis of 247 small cell lung cancer with limited-stage after surgery☆,☆☆

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Summary The objective of this study was to analyze the clinical and pathological characteristics of patients with small cell lung cancer (SCLC) after curative surgery and to explore prognostic factors for disease-free survival (DFS) and overall survival (OS). Clinical data of 247 patients were collected, and clinicopathological features were retrieved, including gender, age, smoking history, tumor location, and distant metastasis. Histopathological features were also reviewed by three pathologists, including primary tumor (T), lymph node metastasis (N), pleural invasion, bronchial invasion, nerve invasion, spread through air spaces (STAS), tumor thrombosis, major cell shape (round Vs. spindle), tumor necrosis, stromal fibrosis, and tumor-infiltrating lymphocytes (TILs). Immunohistochemical staining of neuroendocrine markers (CD56, synapsin, chromogranin A) was also reviewed. All patients were followed up for recurrence, distant metastasis, and survival. Kaplan-Meier curves and log-rank tests were applied for survival analysis. The median DFS was 98 months, and the 1-year, 3-year, and 5-year DFS rates were 70.9%, 54.4%, and 52.2%, respectively. The median OS was not reached, and the 1-year, 3-

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year, and 5-year survival rates were 94.2%, 72.3%, and 65.4%, respectively. Univariate analysis revealed clinicopathological features with DFS (gender, smoking history, primary tumor, regional lymph node metastasis, major cell shape, and TILs) and OS (age, primary tumor, regional lymph node metastasis, distant metastasis, nerve invasion, major cell shape, and TILs). Multivariate analysis revealed DFS-related factors (smoking history, regional lymph node metastasis and major cell shape) and OS-related factors (age, primary tumor, distant metastasis in the brain, liver, bone, nerve invasion, and TILs). Age more than 65 years, smoking, advanced stage (T and N), distant metastasis, nerve invasion, major cell shape as spindle and TILs >30% were negatively correlated with survival. Neuroendocrine immunostaining markers showed no correlation with survival. Of interest, spindle cell type and TILs >30% are revealed as independent negative prognostic factors, and further molecular mechanisms need to be explored.

1. Introduction

Small cell lung cancer (SCLC) is the most aggressive and lethal form of lung cancer. SCLC grows fast, metastasizes early, and acquires resistance shorty after any current chemotherapies and/or radiotherapies [1,2]. Over the last several decades, there have been only modest improvements in patient survival and no molecular targeted therapy has proven beneficial for patients with SCLC [3]. However, in terms of molecular mechanisms, the study of SCLC has made great progress in recent decades. Early in 1985, Gazdar et al. proposed classification of SCLC into "classic" and "variant" subtypes, as a subset of "variant" SCLC cell lines were observed to exhibit altered morphology, culture condition and loss of neuroendocrine properties [4], More recently, Rudin et al. [5] proposed four subtypes defined by differential expression of key transcription regulators, namely as ASCL1, NeuroD1, YAP1, and POU2F3, and listed unique therapeutic vulnerabilities of these subtypes, such as selective activity of LSD1 inhibitors for patients with SCLC-ASCL1. Preclinical studies using cell lines and animal models strongly suggest that SCLC tumors have different morphological types and molecular subtypes, which will further guide the generation of targeted therapies similar to lung adenocarcinoma [6]. Further validation of the morphological and molecular subtypes using surgical specimens from patients with SCLC may enhance progress in SCLC diagnosis and treatment. However, until the release of the 2002 National Comprehensive Cancer Network guidelines, radical surgery was not the recommended treatment option for limited-stage SCLC [7], which has limited the analysis of these tumors. Most of the existing literature reports that the prognostic study of postoperative SCLC is based on small biopsies or cytological samples of not more than 100 cases which only focused on description of clinical parameters instead of analysis of pathological features [2,8,9]. By analyzing clinicopathological variables of 297 SCLC samples after radical resection, our group found that pure and combined SCLC have different prognostic factors [10], including some specific pathological and morphological characteristics. Based on these data, it is likely that within pure SCLC, certain pathological features may also be correlated to prognosis. Here, we report an analysis of limited-stage pure SCLC specimens after surgical resection in our hospital for more than a decade, in which we retrospectively reviewed the clinicopathological characteristics and prognostic factors. This study represents the largest cohort of surgically resected pure SCLC in limited-stage focusing on prognostic analysis related to pathological features.

2. Material and methods

2.1. Selection of patients

A total of 300 patients with histologically confirmed SCLC who underwent radical resection of lung cancer and systematic lymph node dissection from July 2005 to April 2016 were collected from the Cancer Hospital of the Chinese Academy of Medical Sciences. Among these, eight were extensive-stage according to the Veterans Administration Lung Study Group (VALSG) staging system [11] and 45 were combined SCLC according to WHO (World Health Organization) lung cancer classification [12]. By excluding extensive-stage and combined SCLC cases, 247 remained for this study. All the enrolled patients underwent postoperative chemotherapy and/or radiotherapy, and the chemotherapy regimen was mainly paclitaxel and platinum drugs.

2.2. Slide reviewing and immunohistochemical staining

All archived formalin-fixed, paraffin-embedded sections were reviewed by two junior pathologists (L.L. and J.W.) and checked by one senior clinical pathologist (L.Y.) specializing in chest tumor pathology. All slides were reviewed, and no pneumonia disease was recorded. For cases with atypical morphology, neuroendocrine markers, namely as CD56, synaptophysin, and chromogranin A, as well as the proliferation

marker Ki-67, were applied to differentiate poorly differentiated squamous cell carcinoma and typical or atypical carcinoid according to 2015 WHO classification [12].

All surgical specimens were routinely fixed in 10% formalin for about 24 h at 10 times the volume of the tissue liquid and then embedded in paraffin. Consecutive 4-μm-thick sections were prepared for immunohistochemical staining. CD56, synaptophysin, chromogranin A, and Ki-67 protein expression was determined by immunohistochemistry. All staining steps were completed on the fully automatic Roche immunohistochemical instruments (Roche Diagnostics, Shanghai, China) according to the recommended standard protocols. CD56 protein was localized primarily in the cytomembrane, and synaptophysin and chromogranin A protein were localized primarily in the cytoplasm. Ki-67 protein was localized primarily in the nucleus. According to the manufacture's scoring algorithm, intensity was scored according to a four-tier systems: including negative (0), no staining or less than 5% dying; weakly positive (1+), 5%～25% tumor cells stained; moderately positive (2+), 25%～50% tumor cells stained; strongly positive (3+), >50% tumor cells stained. Before evaluation of results from the patient samples, we examined negative quality control sections and found no false positive signal from immunostaining. For statistical analysis, negative or low expression was defined as 0 and 1+, and high expression was defined as 2+ and 3+.et al.

2.3. Observation of variables

The clinical characteristics of patients were retrieved from archived database, including gender, age, smoking history, tumor location (left/right), distant metastasis (e.g. pleural metastasis and brain, liver, and bone metastasis). In addition to the identification of SCLC histology, pathological review focused on the observation and recording of features including primary tumor (T), lymph node metastasis (N), and other aspects of tumor invasion (pleural invasion, bronchial invasion, nerve invasion, spread though air spaces (STAS) and tumor thrombosis), shape of tumor cells, tumor microenvironment—related features including tumor cell necrosis, interstitial fibrosis, tumor-infiltrating lymphocytes, and so on. Tumor cell shape is based on the preliminary classic/variant morphological feature proposed by Gazdar et al. [4] and redefined according to the ratio between cell length and cell diameter (≥2 for spindle cells, <2 for round cells, Fig. 1). The degree of necrosis, fibrosis, and lymphocyte infiltration were semiquantitated according to the percentage of the aforementioned components in the tumor area of the slice. Tumor-infiltrating lymphocytes (TILs) is defined as lymphocytes in tumor area, including stromal and intratumoral lymphocytes [13]. For example, 10% TILs means that 10% of the tumor area shows a dense mononuclear infiltrate. The degree of necrosis and lymphocyte infiltration were divided by X-tile software into two groups by a threshold of 30% and the degree of fibrosis by 10%.

2.4. Clinical outcome

In this study, regional recurrence was defined at the observation of lymph node metastasis in the surgical stump, hilum, and mediastinum; distant metastasis was distant lymph node metastasis, pleural metastasis, and brain, liver, and bone metastasis. Disease-free survival

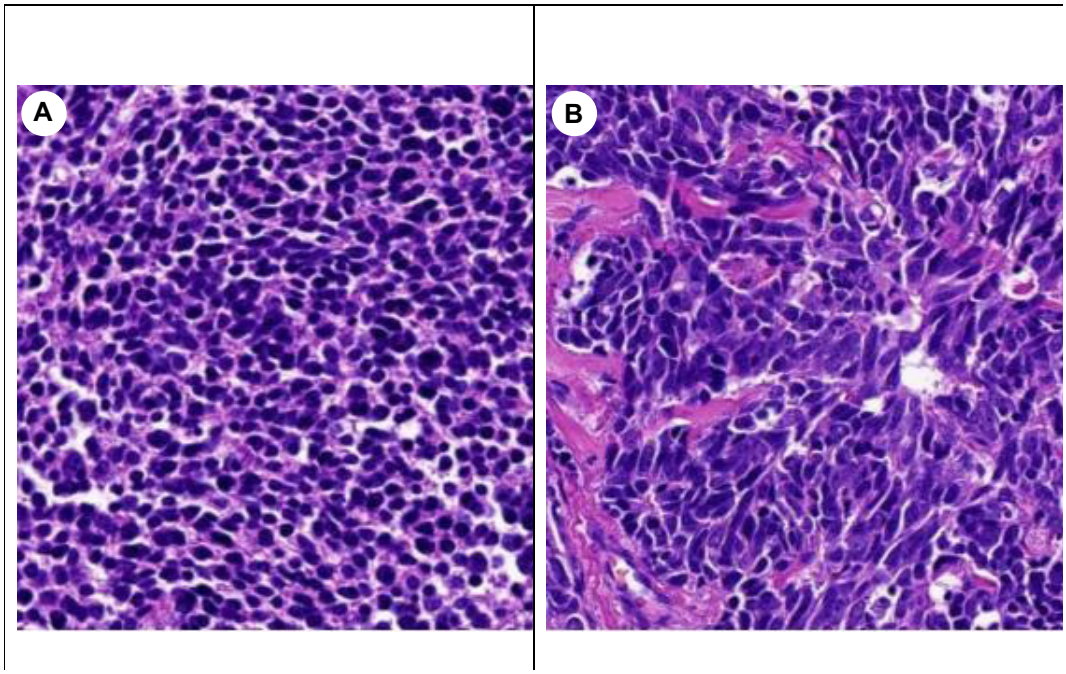

Fig. 1 The shape of tumor cells (HE ×400). 1A: Round, the ratio of long diameter to short diameter<2.1B: Spindle, the ratio of long diameter to short diameter≥2.

Table 1 Univariate analysis of DFS and OS in patients' clinical factors.

Factors	N (%)	DFS		OS	
		5-yr DFS %	*p* value	5-yr OS %	*p* value
Baseline					
Gender					
Male	175 (70.85)	46.0	0.005[a]	61.4	0.082
Female	72 (29.15)	66.6		75.3	
Age					
≤65	202 (81.78)	52.0	0.995	68.9	0.038[a]
> 65	45 (18.22)	53.1		48.7	
Smoking history					
No	89 (36.03)	61.2	0.039[a]	69.7	0.370
Yes	158 (63.97)	46.9		63.0	
Tumor location					
Left lung	122 (49.39)	52.9	0.612	65.7	0.990
Right lung	125 (50.61)	51.5		65.1	
Follow-up					
Brain metastasis					
No	204 (82.59)	–	–	71.8	3.892E-07[a]
Yes	43 (17.41)	–	–	35.8	
Liver metastasis					
No	227 (91.90)	–	–	69.5	1.858E-08[a]
Yes	20 (8.10)	–	–	21.3	
Bone metastasis					
No	227 (91.90)	–	–	69.3	5.541E-07[a]
Yes	20 (8.10)	–	–	25.4	
Pleural metastasis					
No	241 (97.57)	–	–	66.8	0.018[a]
Yes	6 (2.42)	–	–	16.7	

DFS, disease-free survival; OS, overall survival.
[a] statistically significant.

(DFS) was defined as the time durance from the start of the follow-up to observation of relapse or metastasis; overall survival (OS) was defined as the time durance from the start of the follow-up to the censor or the end of the follow-up.

2.5. Statistics

Survival distributions were made by the Kaplan-Meier method for each variable, with the log-rank test performed to determine significance of differences between different groups. Variables with $p < 0.2$ were included for multi-factor analysis by the Cox proportional hazards model. Statistical significance was set at two-sided $p < 0.05$. All statistical analyses were performed using SPSS software (version 23.0; IBM-SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Clinicopathological characteristics

Among all enrolled 247 histologically confirmed pure SCLC cases, 78 cases (31.6%) were in stage I, 68 cases (27.5%) in stage II, and 101 cases (40.9%) in stage III according to the 7th AJCC/UICC (American Joint Committee on Cancer/Union for International Cancer Control) stage, with an age range from 19 to 82yrs (average age: 56.5 yrs And median age: 56.0 yrs), of which 202 cases (81.8%) were older than 65 years. The ratio of male to female was 2.4 (175/72). Smokers counted for 64.0% (158), of which male smokers were significantly more than female smokers by chi square tests with $p < 0.0001$.

3.2. Survival analysis

The follow-up deadline for all cases was February 28, 2019. The follow-up duration for the entire cohort was 0–166 months, with a median follow-up time of 48 months. One hundred twenty (48.6%) patients had recurrence or metastasis and 89 (36.0%) patients deceased at the end of the follow-up. During the follow-up, 35 (14.2%) patients were lost. The overall 1-year, 3-year, and 5-year DFS rates were 70.9%, 54.4%, and 52.2%, respectively; the 1-year, 3-year, and 5-year OS rates were 94.2%, 72.3%, and 65.4%, respectively. The median DFS was 98 months (95%

Table 2 Univariate analysis of DFS and OS in patients' pathological factors.

Factors	N (%)	DFS		OS	
		5-yr DFS %	*p* value	5-yr OS %	*p* value
Primary tumor					
T1	78 (31.58)	60.2	4.264E-07[a]	73.6	4.235E-10[a]
T2	126 (51.01)	56.5		72.3	
T3	34 (13.77)	27.3		41.0	
T4	9 (3.64)	12.7			
Regional lymph node metastasis					
N0	104 (42.11)	67.9	2.000E-06[a]	74.7	0.001[a]
N1	65 (26.32)	49.9		69.6	
N2	76 (30.77)	34.7		50.2	
N3	2 (0.81)	0.0		50.0	
Pleural invasion					
No	170 (68.83)	54.5	0.477	69.6	0.104
Yes	77 (31.17)	46.9		56.3	
Bronchus invasion					
No	37 (14.98)	59.7	0.384	72.1	0.186
Yes	210 (85.02)	51.0		64.1	
Vascular invasion					
No	41 (16.60)	50.9	0.713	64.3	0.851
Yes	206 (83.40)	52.3		65.4	
Nerve invasion					
No	163 (65.99)	55.7	0.063	71.6	0.004[a]
Yes	84 (34.01)	45.5		52.0	
STAS					
No	65 (26.32)	56.2	0.517	67.2	0.584
Yes	182 (73.68)	50.7		64.7	
Tumor thrombosis					
No	122 (49.39)	56.8	0.098	68.3	0.082
Yes	125 (50.61)	47.7		62.9	
Major cell shape					
Round	193 (78.14)	58	4.250E-04[a]	68.3	0.018[a]
Spindle	54 (21.86)	33.9		55.7	
Necrosis proportion					
≤30%	172 (69.64)	52.1	0.922	64.4	0.836
>30%	75 (30.36)	52.4		67.8	
Fibrosis proportion					
≤10%	116 (46.96)	50.6	0.931	66.6	0.910
>10%	131 (53.04)	53.6		64.4	
TILs					
≤30%	212 (85.83)	54.2	0.028[a]	68.0	0.026[a]
>30%	35 (14.17)	40.0		50.6	

DFS, disease-free survival; OS, overall survival; STAS, spread through air spaces; TILs, tumor-infiltrating lymphocytes.
[a] statistically significant.

confidence interval: 49−147 months), and the median OS was not reached.

For DFS, univariate analysis suggested that gender, smoking history, primary tumor, regional lymph node metastasis, major cell shape, and TILs were influencing factors for postoperative tumor recurrence or metastasis ($p < 0.05$, Table 1 and Table 2, Fig. 2). Multivariate analysis showed that smoking, spindle cell shape, and regional lymph node metastasis were independent risk factors ($p < 0.05$, Table 3, Fig. 2).

For OS, univariate analysis showed age, primary tumor, distant metastasis to the brain, liver, bone, and pleura, regional lymph node metastasis, nerve invasion, major cell shape, and TILs are influencing factors for postoperative survival ($p < 0.05$, Tables 1 and 2). Multivariate analysis showed that an age of more than 65 years, T3 and T4, distant metastasis to the brain, liver, and bone, nerve invasion, and TILs >30% were independent factors for prognosis ($p < 0.05$, Table 3).

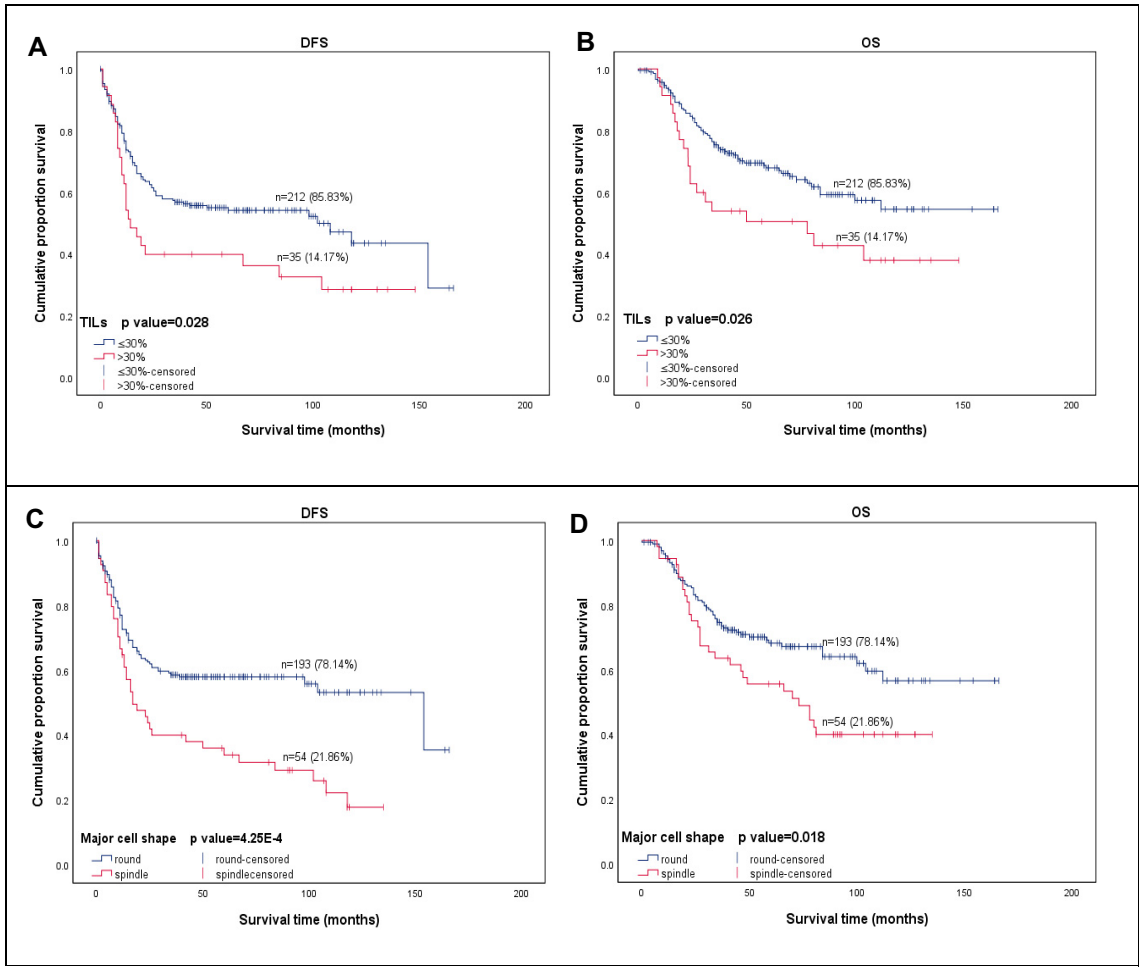

Fig. 2 2A and 2B, TILs≤30% Vs. TILs>30% showed a significance difference both for DFS (p = 0.028) and OS (p = 0.00.026); 2C and 2D, Major cell shape of round Vs. spindle showed a significance difference both for DFS(p = 4.25E-04) and OS(p = 0.00.018). TILs, tumor-infiltrating lymphocytes; DFS, disease-free survival; OS, overall survival.

4. Discussion

Clinically, SCLC is characterized by a series of malignant manifestations including rapid growth, early metastases, and high recurrence rate; thus, the majority of patients with SCLC have dismal long-term survival outcomes [14]. Histologically, SCLC classification underwent a series of changes, which underscores the diversity and complexity of morphology and difficulty for differential diagnosis. Originally, in the first edition of the WHO classification system for lung cancer, published in 1967, SCLC was classified as lymphocyte-like, polygonal, spindle, containing squamous and glandular foci, according to cell shape [15]. Afterward, in the 2nd WHO classification system, published in 1982, it was classified into oat cell, intermediate, and combined types [16]. Later, in the 3rd edition in 1999, depending on whether a non-small cell lung cancer (NSCLC) component was mixed, SCLC was simplified as SCLC and combined SCLC, the former of which can be regarded as pure SCLC [17]. The classification remained the same in the latest version of 2015 WHO classification [12]. Biologically, Rudin et al. [5], in 2019, proposed the molecular classification of SCLC based on the dominant expression of transcription regulators that modulate different signal pathways in SCLC, some of which are may be targetable such as NOTCH and MYC signaling. Considering the remarkable progress in the treatment of lung adenocarcinoma oriented by molecular targets, similar developments in the treatment of SCLC can be predicted. Accordingly, specific morphological SCLC types associated with certain molecular subtypes such as ASCL1 and NeuroD1 are susceptible to targeted therapies that inhibit the transcription regulator modulated pathways [18]. Current studies on the prognostic factors of SCLC lack comparability due to insufficient number of cases, inclusion of different stages, and combination of both pure SCLC and combined SCLC which have different molecular and clinicopathological features [1,2,8,9,19]. Thus, in this study, we zoomed into

Table 3 Multivariate Analysis of DFS and OS in Patients' Factors.

Variables	DFS			OS		
	HR	95%CI	*p* value	HR	95%CI	*p* value
Clincal Factors						
Gender (Female)	0.801	0.450-1.426	0.451	0.844	0.502-1.420	0.524
Age (>65y)	-	-	-	1.797	1.054-3.066	0.031[a]
Smoking history (Yes)	1.569	1.045-2.356	0.030[a]	-	-	-
Brain metastasis (Yes)	-	-	-	2.368	1.454-3.857	0.001[a]
Liver metastasis (Yes)	-	-	-	2.370	1.302-4.314	0.005[a]
Bone metastasis (Yes)	-	-	-	2.568	1.485-4.441	0.001[a]
Pleural metastasis (Yes)	-	-	-	1.985	0.740-5.326	0.173
Pathological Factors						
Primary tumor (T1)			0.056			9.300E-05[a]
T2	0.979	0.624-1.536	0.928	1.188	0.692-2.040	0.532
T3	1.693	0.964-2.974	0.067	3.206	1.729-5.945	2.170E-05[a]
T4	2.251	0.923-5.490	0.075	3.584	1.431-8.981	0.006[a]
Regional lymph node metastasis(N0)	(ref)		0.003[a]	(ref)		0.421
Regional lymph node metastasis(N1)	1.640	1.007-2.672	0.047[a]	1.093	0.589-2.028	0.778
Regional lymph node metastasis(N2)	2.424	1.494-3.932	3.380E-04[a]	1.560	0.851-2.860	0.151
Regional lymph node metastasis(N3)	4.034	0.888-18.33	0.071	0.651	0.078-5.463	0.692
Pleural invasion (Yes)	-	-	-	1.430	0.839-2.437	0.188
Bronchus invasion (Yes)	-	-	-	1.097	0.545-2.211	0.795
Nerve invasion (Yes)	1.409	0.955-2.079	0.084	1.632	1.036-2.570	0.035[a]
Tumor thrombosis (Yes)	1.039	0.698-1.547	0.851	0.999	0.616-1.621	0.998
Major cell shape (spindle)	2.104	1.403-3.157	3.240E-04[a]	1.564	0.937-2.611	0.087
TILs (>30%)	1.279	0.788-2.077	0.320	1.797	1.073-3.011	0.026[a]

DFS, disease-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; ref, reference category; TIL, tumor-infiltrating lymphocyte.
[a] statistically significant.

the limited-stage, pure SCLC to investigate the clinical and pathological factors related to prognosis and clinical outcome prediction. Compared with the current studies, our cohort is more robust based on cohort size and detailed record of clinical stage and pathological characteristics.

From the perspective of clinical prognosis, the median DFS in our cohort is 98 months, and the 5-year OS rate is 65.4%, which is close to the upper limit reported in the literature (27.6%−68.0%) [1,20]. According to our analysis, the difference in survival in different studies is caused by different proportions of pure and combined SCLC, stages, treatment regimens, and cohort size.

It is reasonable that age is one of the correlation factors with general health status [21] and that most studies set 65 years as age threshold. In our study, although gender was not an independent prognostic factor, women tended to have a better survival than men (Table 3). As for the effect of gender on the prognosis, Cai et al. [22] speculated that the biological behaviors of tumors might vary between different genders. We propose this may be related to the different smoking ratios of men and women. Our data showed that smoking patients have significantly lower DFS than non-smokers (5-year DFS 46.9% vs. 61.2%, $p < 0.05$), and OS also has a low trend in smokers compared with non-smokers (5-year OS: 63.0% vs. 69.7%), although not statistically significant ($p = 0.370$). When smokers were divided into two groups by gender, we found that male patients were significantly more smoker than female patients (147:11, $\chi 2 = 104.5$, $p < 0.001$). Considering the role of smoking in the carcinogenesis of SCLC [23], we speculate that the data in this group show that the correlation between gender and prognosis may be related to the higher smoking rate of male patients.

In terms of pathological morphology, primary tumor of T3 and T4, and regional lymph node metastasis were independent risk prognostic factors, which is in accord with the published studies [24,25] and clinical cognition. TNM (Tumor Node Metastasis) staging is inherent with prognosis [25]. Our data show that primary tumor is an independent factor for DFS and OS in limited-staged SCLC, which is consistent with the study of Zhang et al. [25]. Previously, SCLC staging has been mainly divided into limited and extensive-stage to guide radiotherapy by the VALSG staging system. As the role of surgery in SCLC had been put forward to the 7th edition of AJCC/UICC TNM staging [26] and emphasized in the 8th edition of TNM staging, prognostic significance of TNM staging in SCLC was confirmed [3]. With the increasing role of surgery in limited-staged SCLC, TNM staging may gradually replace the previous VALSG staging system and will gain more weight in prognosis prediction. Besides, nerve invasion is consistent with what we have learned from previous reports

[27,28]. However, there is little research on cell morphology, although SCLC tumors were basically classified according to cell morphology since the 1st WHO edition in 1967, in which four subtypes were suggested namely as lymphocyte-like, polygonal, spindle, containing squamous and glandular foci [15]. Later, in 1982, SCLC was classified into oat cell, intermediate, and combined types [15]. However, these classifications lack reproducibility between different pathologists and failed to demonstrate a consistent relationship between the histologic subtype and patient outcome [9,29−31]. In contrast, according to the *classic vs. variant* morphological classification defined by Gazdar et al. [4], we redefined the major cell types of SCLC into two categories, round and spindle, and found that the prognosis of SCLC with spindle cells was worse than that of the round cell group. Based on this preliminary observation, we speculate that different cell morphologies may have different biological behaviors or molecular biological mechanisms. Still, we admit that the cell morphology is determined by visual inspection of our group, which is somehow subjective. With the development of artificial intelligence in digital pathology, morphological categorization determined by analyzing the accurate cell measurement parameters using artificial intelligence has recently been applied in related field of pathology as exemplified in NSCLC [32]. In the future, we will validate the morphological observation via artificial intelligence.

Besides, our study also revealed that the content of TILs closely correlated with prognosis as an independent factor indicating a shorter OS, and there is a tendency to a shorter DFS. TILs represent aggregates of lymphocytes in the tumor microenvironment. It includes T/B lymphocytes, NK cells, macrophages, and myeloid-derived suppressor cells, and so on [33]. Recent study revealed that instead of TILs content, the specific lymphocyte type and its function in TILs is correlated with prognosis [13]. For example, medium to high levels of CD8+ T cells, CD4+ T cells, and CD3+ T cells have been associated with better OS in patients with lung cancer [34]. High ratio between neutrophils and lymphocytes correlate with shorter OS of patients with NSCLC [35]. As for SCLC, tumor immune phenotypes may be relevant for responses to immune checkpoint blockade combinations as Thomas et al. [36] reported in a phase II study of durvalumab in combination with olaparib in patients with relapsed SCLC. Prognostic role of tumor microenvironment and the function of different lymphocytes and related gene signatures in SCLC need further investigation.

In conclusion, by retrospectively analyzing 247 surgically resected stage I to III pure SCLC cases, we identified factors influencing prognosis such as age, smoking, and primary tumor, and so on. More importantly, we found spindle cell shape and TILs >30% were independent factor for worse prognosis, which need further biological validation.

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