Am J Transl Res 2026;18(1):788-796
www.ajtr.org /ISSN:1943-8141/AJTR0169296

Original Article

Immunohistochemical detection of PD-L1 in small cell lung cancer and its prognostic values

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Received October 13, 2025; Accepted December 31, 2025; Epub January 15, 2026; Published January 30, 2026

Abstract: Objective: The molecule known as Programmed death-ligand 1 (PD-L1) exerts an inhibitory effect on immune system reactions and promotes cancer progression. Its prognostic role in small cell lung cancer (SCLC) remains less defined than in non-small cell lung cancer. This study aimed to evaluate PD-L1 expression and its prognostic value in SCLC, comparing detection by immunohistochemistry (IHC) and reverse transcription quantitative polymerase chain reaction (RT-qPCR). Methods: PD-L1 expression was assessed in paired tumor and non-tumor tissues from 66 SCLC patients using IHC and RT-qPCR. IHC positivity was defined as membrane staining in >5% of tumor cells. Associations with clinicopathological factors were examined by Fisher's exact test. Survival analysis employed Kaplan-Meier curves and log-rank tests. Univariate and multivariate Cox regression identified independent prognostic factors. Results: IHC analysis showed PD-L1 positivity in 34/66 patients. RT-qPCR revealed significantly higher PD-L1 mRNA levels in tumor versus non-tumor tissues ($P<0.01$). Both IHC positivity and high mRNA levels were associated with larger tumor size, metastasis, and advanced clinical stage (all $P<0.05$), but not with age, gender, or smoking/drinking history. Patients with PD-L1-positive IHC staining or high PD-L1 mRNA exhibited significantly worse 5-year overall survival ($P<0.05$), with IHC showing stronger prognostic discrimination. Multivariate analysis confirmed IHC positivity (HR=2.45, P=0.004) and high mRNA level (HR=2.12, P=0.012) as independent predictors of poor survival. Conclusion: PD-L1 expression is associated with aggressive clinicopathological features and independently predicts poor survival in SCLC. IHC appears to be a more sensitive detection method than RT-qPCR for prognostic assessment.

Keywords: PD-L1, small cell lung cancer, prognosis

Introduction

Lung cancer is a major threat to public health [1]. It is estimated that lung cancer is responsible for about 18.7% of all cancer-related deaths across the world [2]. More than 80% of lung cancer cases can be attributed to smoking, while the development of this malignancy in never-smokers is rare [3, 4]. As the largest tobacco consumer and producer, China accounts for 1/3 of all smokers in the world [5]. As a consequence, more than 600,000 people die of lung cancer each year in China, and the mortality rate shows an increasing trend [6, 7]. Compared to non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC) is a more aggressive form of malignancy in the lungs [8]. In general, no more than 10% of SCLC patients survive 5 years after diagnosis [9].

Lung cancer in most cases is diagnosed at advanced stages, mainly owing to the lack of classic symptoms at early stages. Therefore, resection of the primary tumor as the only cure is usually not appropriate [10, 11]. Although advanced lung cancer including SCLC can be treated with combined radiation therapy and chemotherapy, resistance will inevitably develop and prognosis is generally poor [12]. Accurate prognosis may help to enhance the longevity of SCLC patients by guiding the design of treatment and care approaches [13].

In effect, several biomarkers, such as circulating DNAs and miRNAs, have been developed to predict SCLC patients' survival [14, 15]. However, these biomarkers are limited by the low accuracy. Programmed death-1 ligand-1 (PD-L1) expression increases tumor aggressiveness by suppressing the immune system

https://doi.org/10.62347/OZEK2695

[16, 17]. Therefore, altered PD-L1 is frequently utilized as an indicator of cancer progression. In effect, the role of PD-L1 in forecasting the survival rates of NSCLC patients has been thoroughly investigated [18, 19]. On the contrast, the role of PD-L1 as a predictive marker for SCLC remains unclear. Most existing studies on PD-L1 in SCLC have relied on a single detection methodology, either immunohistochemistry (IHC) or mRNA quantification. There is a lack of direct comparative data on the concordance and relative prognostic performance of these two commonly used techniques within the same SCLC cohort. Therefore, this study was undertaken not only to elucidate the relationship between PD-L1 expression and clinicopathological features but also to directly compare the efficacy of IHC and reverse transcription quantitative polymerase chain reaction (RT-qPCR) in evaluating the prognostic significance of PD-L1 for SCLC. Such a comparison is crucial for informing future biomarker testing strategies in both clinical and research settings.

Methods

Study design and rationale

This retrospective cohort study was designed with a dual-detection approach. To comprehensively assess PD-L1 status, we performed both IHC staining for protein localization and RT-qPCR for mRNA quantification on paired tumor and non-tumor tissues from the same patients. This design allows for an internal comparison of the two methods regarding their association with clinicopathological parameters and survival outcomes.

Patients

A cohort of 66 patients diagnosed with SCLC at the Affiliated Hospital of Nantong University between May 2015 and January 2017 was retrospectively enrolled. Approval for this study was granted by the Affiliated Hospital of Nantong University's Ethics Committee. Prior to the initiation of this study, all participants provided informed consent. All SCLC patients were diagnosed by CT and confirmed by histopathological analysis. Paired SCLC and non-tumor samples were obtained from these patients by dissecting resected tumors in cases where primary tumor resection was performed (n=42) and dissecting biopsies in cases where tumor resection was not applicable (n=24). Clinical and pathological data, including age, gender, smoking history, drinking history, tumor size, American Joint Committee on Cancer (AJCC) stage, and metastasis status, were collected retrospectively.

Follow-up

To investigate the prognostic implications of PD-L1 immunohistochemical staining signals and the levels of PD-L1 expression associated with SCLC, a 5-year follow-up was conducted with the 66 participants. Monthly contacts were made via telephone or outpatient visits to monitor and document their survival status. The collected survival data were then compiled and utilized to generate survival curves.

RNA isolation

Preparation of fresh RNA from fresh tissues samples was carried out using MagMAX™-96 Total RNA Isolation Kit (ThermoFisher Scientific). Briefly, beads were mixed with each sample, followed by shaking for 5 min. RNA binding beads were then captured magnetically and washed with Wash Solution 1 for min by shaking. Then the beads were washed with Wash Solution 2. Removal of gDNA was performed by adding 50 ul TURBO DNase, followed by shaking for 15 min. RNA rebinding solution was then added, followed by incubation for 3 min. Then beads were captured magnetically, followed by washing with Wash Solution 2 twice. Finally, the beads were dried by shaking for 2 min, followed by RNA elution using 50 ul elution buffer.

RT-qPCR

Super Script III system (Invitrogen) was applied to prepare cDNA samples using RNA samples as template. In each reaction, 2,000 ng RNA was used after RNA concentration was determined using NanoDrop ND-1000. Primer Express 3.0 was used to design primers. After that, qPCR was carried out to determine the expression of PD-L1. Finally, Ct values were normalized using the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemical analysis

Tumor tissue specimens were fixed using formalin, and paraffin-embedded blocks were subsequently prepared. Then tissue sections

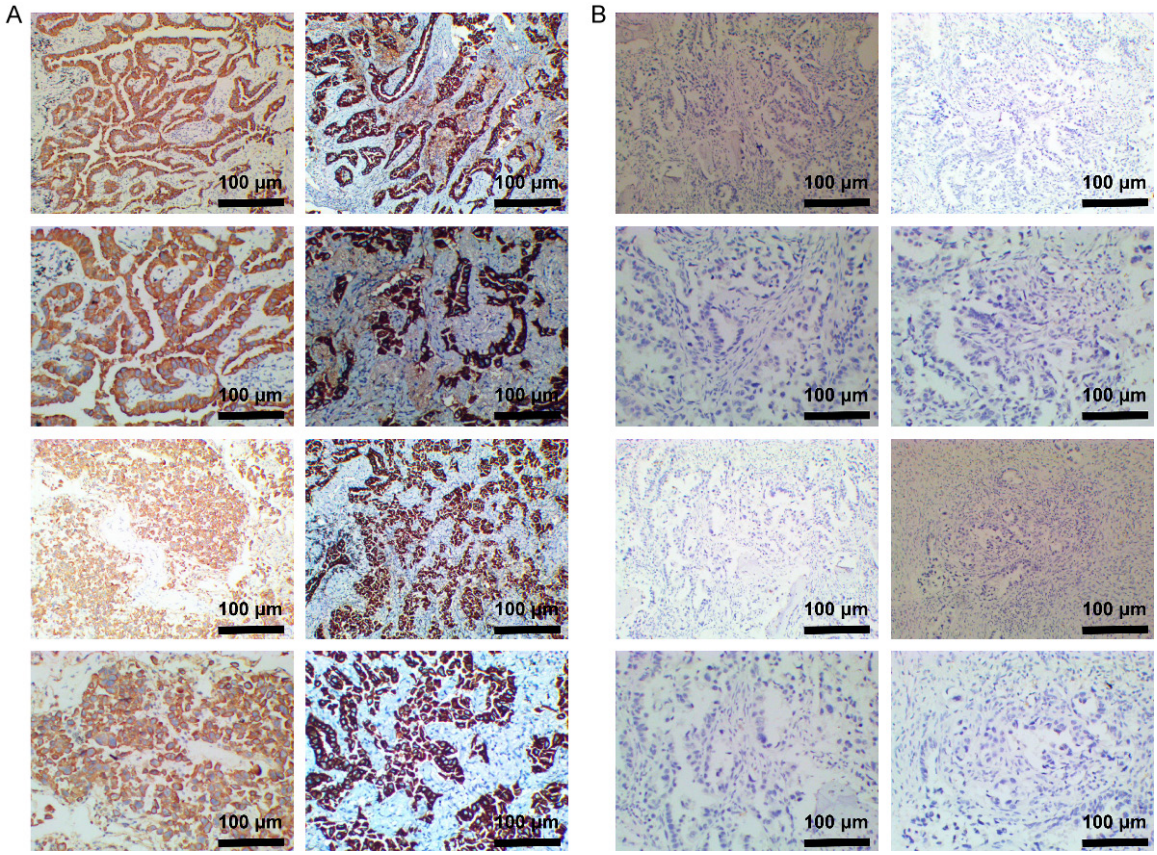

Figure 1. Immunohistochemical analysis of PD-L1 using anti-PD-L1 antibody. Expression of PD-L1 protein in SCLC tumor cells was detected through staining with anti-PD-L1 antibody. A. SCLC samples with PD-L1 signal in tumor cell membrane. B. No signal of PD-L1 was detected after negative staining. PD-L1: Programmed Death-Ligand 1; SCLC: Small Cell Lung Cancer.

(4 μm) were prepared and deparaffinization was performed with 100% xylene (Sigma-Aldrich). Rehydration was then performed using graded ethyl alcohol (from 100% to 30%). After washing with PBS, pre-heated Tris-EDTA buffer solution (pH 9.0) was used to wash each section. Following incubation with REAL™ Peroxidase-Blocking solution (Agilent Technologies), tissue sections were further incubated with rabbit polyclonal anti-PD-L1 antibody (1:2000, Abcam) overnight in a cold room. Finally, signals were developed using Dako REAL™ EnVision™ Detection system (Agilent Technologies). Negative control experiments were performed without adding anti-PD-L1 antibody.

PD-L1 signal quantification in membranes of SCLC cells

PD-L1 expression signal was analyzed and quantified by researchers who were blind to patient grouping. Under a light microscope (×400), 25 visual fields were randomly selected for each sample. About 40 cells were included for each visual field. The sample was defined as positive for PD-L1 if ≥5% of tumor cells membranes exhibited PD-L1 positivity (i.e., at least 50 out of 1000 cells), with each individual cell being individually analyzed. PD-L1 mainly accumulated in the cytoplasm and its signal in the nuclear membrane was rarely detected (**Figure 1A**). No signal of PD-L1 was detected in the negative control group (**Figure 1B**).

Statistical analysis

PD-L1 expression levels were compared between SCLC and paired with non-tumor tissue samples using paired samples Wilcoxon test. In order to investigate the prognostic significance of PD-L1 immunohistochemical staining signal and expression level for SCLC, the 66 patients were divided into PD-L1-positive (n=34) and PD-L1-negative (n=32) as well as high and low PD-L1 level groups (n=33, cutoff value = median PD-L1 level in SCLC tissue samples). Survival

Table 1. Baseline characteristics of SCLC patients (n=66)

Characteristics	Category	Number (%)
Age (years)	≥60	34 (51.5%)
	<60	32 (48.5%)
Gender	Male	44 (66.7%)
	Female	22 (33.3%)
Smoking	Yes	50 (75.8%)
	No	16 (24.2%)
Drinking	Yes	42 (63.6%)
	No	24 (36.4%)
Tumor size (cm)	≤5	16 (24.2%)
	>5	50 (75.8%)
AJCC stage	I/II	28 (42.4%)
	III/IV	38 (57.6%)
Distant metastasis	Yes	18 (27.3%)
	No	48 (72.7%)

SCLC: Small Cell Lung Cancer; AJCC: American Joint Committee on Cancer.

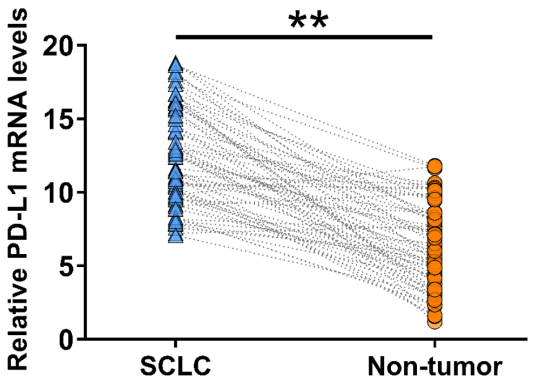

Figure 2. RT-qPCR analysis of PD-L1 in SCLC tumor cells. Expression of PD-L1 in SCLC and paired non-tumor samples collected from 66 SCLC patients was determined by performing RNA isolation, followed by RT-qPCR. PD-L1 expression levels were compared between SCLC and paired non-tumor tissue samples using paired samples Wilcoxon test. **, P<0.01. RT-qPCR: Reverse Transcription Quantitative Polymerase Chain Reaction; PD-L1: Programmed Death-Ligand 1; SCLC: Small Cell Lung Cancer.

curves were plotted from each group, and comparisons between the curves were conducted using the log-rank test. To analyze the relationships between patients' clinical data and PD-L1 immunohistochemical staining signals or PD-L1 expression levels, Fisher's exact test was employed. Cox proportional hazards models were used to determine independent prognostic factors. Multivariate Cox proportional hazards models were adjusted for age, gender, smoking history, drinking history, tumor size, AJCC stage, and metastasis status to control for potential confounders. A *p*-value less than 0.05 was deemed to indicate statistical significance.

Results

Patient characteristics

The study included 66 SCLC patients (44 males, 22 females) with a mean age of 59.8± 8.9 years. Clinical stages I or II were identified in 28 patients, whereas stages III or IV were found in 38 patients. Distant metastasis was observed in 18 patients. Detailed baseline information is summarized in **Table 1**.

Comparative analysis of PD-L1 expression by immunohistochemistry and RT-qPCR

Expression of PD-L1 in paired SCLC and non-tumor samples from 66 SCLC patients were detected by performing both immunohistochemical staining and RT-qPCR. Immunohistochemical staining analysis revealed that PD-L1 was positive in 34 out of 66 patients. RT-qPCR analysis revealed that PD-L1 was markedly overexpressed for SCLC tissues relative to non-tumor tissues (**Figure 2**, P<0.01).

Associations between PD-L1 and SCLC patients' clinical data

Associations between patients' clinical data and PD-L1 immunohistochemical staining signal or PD-L1 expression level were evaluated using Fisher's exact test. Positive PD-L1 signal (immunohistochemical staining) and elevated PD-L1 expression levels (RT-qPCR) showed a strong association with tumor size, tumor metastasis and clinical stage, but not age, gender, habits of smoking and drinking (**Table 2**). In addition, correlation of PD-L1 immunohistochemical staining signal with patients' clinical data is more significant than that between high PD-L1 RNA expression level and patients' clinical data (**Table 3**). Therefore, immunohistochemical staining is more sensitive than RT-qPCR to predict the contribution of PD-L1 in SCLC progression.

Prognostic value of PD-L1 for SCLC

The 66 subjects were divided into PD-L1-positive (n=34) and PD-L1-negative (n=32) as

Table 2. Associations between PD-L1 signal and SCLC patients' clinical data

Characteristics	Cases (n)	PD-L1 signal		*P* value
		Positive (n=34)	Negative (n=32)	
AJCC stage				
I/II	28	7	21	0.0004
III/IV	38	27	11	
Distant metastasis				
Yes	18	16	2	0.0002
No	48	18	30	
Tumor size (cm)				
≤5	16	2	14	0.0004
>5	50	32	18	
Age (years)				
≥60	32	15	17	>0.05
<60	34	19	15	
Gender				
Male	44	20	24	>0.05
Female	22	14	8	
Smoking				
Yes	50	27	23	>0.05
No	16	7	9	
Drinking				
Yes	42	24	18	>0.05
No	24	10	14	

PD-L1: Programmed Death-Ligand 1; SCLC: Small Cell Lung Cancer; AJCC: American Joint Committee on Cancer.

well as high and low PD-L1 level groups (n=33) to investigate the prognostic significance of PD-L1 immunohistochemical staining signal and expression level for SCLC. Survival curves were generated for each group and comparisons among these curves were conducted by log-rank test. Subjects with positive PD-L1 signal (immunohistochemical staining, **Figure 3A**) and high PD-L1 expression levels (RT-qPCR, **Figure 3B**) showed higher mortality rate during a 5-year follow-up. However, immunohistochemical staining signal is much more sensitive.

Univariate and multivariate Cox regression analysis

Univariate analysis identified AJCC stage, metastasis, tumor size, PD-L1 signal positivity, and high PD-L1 level as significant indicators of OS (**Table 4**). Multivariate analysis demonstrated that PD-L1 signal positivity (HR=2.45, P=0.004) and high PD-L1 level (HR=2.12, P=0.012) were independent prognostic factors (**Table 5**).

Discussion

The current study evaluated the expression and prognostic significance of PD-L1 in SCLC using two complementary approaches: immunohistochemical staining and RT-qPCR. A key distinctive aspect of our work is the head-to-head comparison of these methods within a well-defined SCLC cohort. Our findings demonstrate that PD-L1 is overexpressed in SCLC tissues relative to non-tumor tissues by both measures. More importantly, our analysis reveals differential sensitivities and associations, providing evidence that IHC may offer superior clinical utility as a prognostic tool in this context.

Our findings demonstrate that PD-L1 is overexpressed in SCLC tissues relative to non-tumor tissues. More importantly, both PD-L1 protein expression (detected by IHC) and PD-L1 mRNA levels (detected by RT-qPCR) were closely associated with aggressive clinicopathological features, including advanced AJCC stage, larger tumor size, and the presence of distant metastasis. Moreover, both high PD-L1 expression and IHC positivity were independent predictors of poor overall survival in SCLC patients, as confirmed by multivariate Cox regression analysis. These results indicate that PD-L1 may have a significant impact on SCLC progression and could serve as an important prognostic biomarker.

Compared to non-tumor tissues, SCLC samples exhibited significantly elevated PD-L1 expression in both protein and mRNA. This finding matches the known function of PD-L1 in immune evasion, where its interaction with PD-1 on T cells inhibits antitumor immunity, thereby facilitating tumor growth and metastasis. The strong correlation of PD-L1 expression with advanced disease characteristics (tumor size, stage, and metastasis) supports the notion that PD-L1 upregulation is involved in SCLC aggressiveness [20, 21]. This finding

Table 3. Associations between PD-L1 level and SCLC patients' clinical data

Characteristics	Cases (n)	PD-L1 level		*P* value
		High (n=33)	Low (n=33)	
AJCC stage				
I/II	28	9	19	0.024
III/IV	38	24	14	
Distant metastasis				
Yes	18	14	4	0.0116
No	48	19	29	
Tumor size (cm)				
≤5	16	4	12	0.0424
>5	50	29	21	
Age (years)				
≥60	32	14	18	>0.05
<60	34	15	15	
Gender				
Male	44	21	23	>0.05
Female	22	12	10	
Smoking				
Yes	50	26	24	>0.05
No	16	7	9	
Drinking				
Yes	42	22	20	>0.05
No	24	11	13	

PD-L1: Programmed Death-Ligand 1; SCLC: Small Cell Lung Cancer; AJCC: American Joint Committee on Cancer.

aligns with previous studies in NSCLC, where PD-L1 expression has been linked to poorer patient prognoses [22]. However, this is unlike other cancers like breast carcinoma, where PD-L1 mRNA expression has been associated with favorable outcomes; our data in SCLC indicate its expression has a clear association with poor prognosis [23].

A key finding of this study is that PD-L1 IHC staining showed greater sensitivity and stronger associations with clinical parameters compared to RT-qPCR. This was evident in the more significant *p*-values for associations with tumor size, metastasis, and clinical stage, as well as in the survival analyses. There are several possible explanations for this observation. First, IHC detects the functional PD-L1 protein localized in the cell membrane and cytoplasm, which directly participates in immune checkpoint signaling. In contrast, mRNA levels may not always correspond to protein expression because of post-transcriptional control [24, 25]. Second, IHC allows for visual assessment of staining distribution and intensity within tumor cells, providing spatial context that mRNA quantification lacks. This is particularly relevant given that PD-L1 expression can vary in tumors [26, 27]. Therefore, IHC may better reflect the biologically active state of PD-L1 and thus offer a more dependable prognostic indicator.

Focus on PD-L1 as a foundation for future research

We deliberately focused on PD-L1 as a primary biomarker in this initial study for several reasons. PD-L1 has the most direct and established link to currently available immune checkpoint inhibitor therapies, making its prognostic validation in SCLC of immediate translational relevance. Establishing a robust and clinically practical detection method for a single key marker is a necessary foundational step before embarking on more complex multiplex analyses. We acknowledge that the tumor immune microenvironment is multifaceted, involving other checkpoints and diverse immune cell populations. Our findings on PD-L1 establish a benchmark and a methodological framework. Future studies, ideally building upon this work, should indeed integrate multiplex IHC, RNA sequencing, or spatial transcriptomics to map the co-expression patterns of PD-L1 with other immune molecules and to define comprehensive immune phenotypes in SCLC, which will likely yield more powerful predictive models.

Prognostic value of PD-L1 was further validated through Cox regression analyses. Univariate analysis revealed that AJCC stage, metastasis, tumor size, PD-L1 IHC positivity, and high PD-L1 mRNA were significant predictors of overall survival. In the multivariate model, both PD-L1 IHC positivity and high PD-L1 mRNA level remained independent prognostic factors, even after

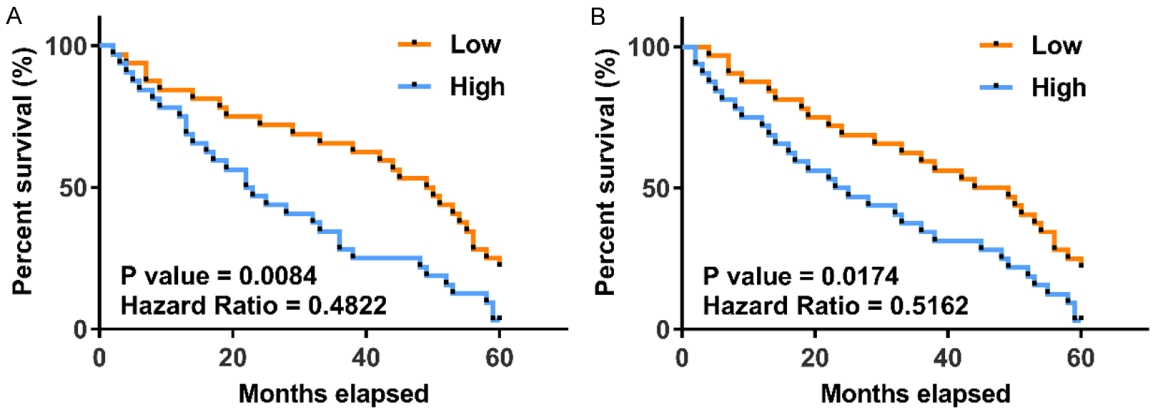

Figure 3. Prognostic value of PD-L1 for SCLC. To explore the prognostic value of PD-L1 immunohistochemical staining signal (A) and expression level (B) for SCLC, the 66 patients were divided into PD-L1-positive (n=34) and PD-L1-negative (n=32) as well as high and low PD-L1 level groups (n=33). Survival curves were generated for each group and comparisons between the curves were conducted using log-rank test. PD-L1: Programmed Death-Ligand 1; SCLC: Small Cell Lung Cancer.

Table 4. Univariate Cox regression analysis for overall survival

Variable	HR	95% CI	*P*-value
Age (≥60 vs <60)	1.12	0.65-1.93	0.681
Gender (Male vs Female)	1.08	0.59-1.98	0.804
Smoking (Yes vs No)	1.24	0.67-2.29	0.491
Drinking (Yes vs No)	1.15	0.66-2.01	0.620
Tumor size (>5 vs ≤5 cm)	2.01	1.10-3.68	0.024
AJCC stage (III/IV vs I/II)	2.56	1.45-4.52	0.001
Metastasis (Yes vs No)	2.89	1.62-5.16	<0.001
PD-L1 signal (Positive vs Negative)	2.78	1.58-4.89	<0.001
PD-L1 level (High vs Low)	2.32	1.32-4.08	0.003

PD-L1: Programmed Death-Ligand 1; AJCC: American Joint Committee on Cancer; HR: Hazard Ratio; CI: Confidence Interval.

Table 5. Multivariate Cox regression analysis for overall survival

Variable	HR	95% CI	*P*-value
Tumor size (>5 cm)	1.78	0.95-3.33	0.072
AJCC stage (III/IV)	2.12	1.15-3.91	0.016
Metastasis (Yes)	2.34	1.27-4.31	0.006
PD-L1 signal (Positive)	2.45	1.32-4.55	0.004
PD-L1 level (High)	2.12	1.18-3.81	0.012

PD-L1: Programmed Death-Ligand 1; AJCC: American Joint Committee on Cancer; HR: Hazard Ratio; CI: Confidence Interval.

adjusting for other clinical variables. This underscores the robustness of PD-L1 as an indicator in SCLC and suggests that its evaluation could help stratify patients into different risk groups, potentially guiding personalized treatment strategies [28, 29].

From a clinical perspective, evaluating PD-L1 expression in resected tumors or biopsies could identify SCLC patients with a high risk of disease progression and poor survival. Such patients might benefit from more aggressive monitoring or adjuvant therapies, including immune checkpoint inhibitors like anti-PD-1/PD-L1 antibodies [29]. Several studies have already highlighted the efficacy of PD-1/PD-L1 blockers in NSCLC, and clinical trials are currently ongoing to assess their utility in SCLC [30, 31]. Our findings provide a rationale for including PD-L1 status for selecting patient in such trials. However, it is important to note that the relationship between PD-L1 expression and treatment response may be complex and influenced by additional factors like tumor mutational burden, immune cell infiltration, and co-expression of other immune checkpoints [32].

Our findings suggest that PD-L1 expression, particularly as measured by IHC, could function as a practical biomarker for risk stratification in SCLC. Patients with high PD-L1 expression may benefit from more intensive surveillance and adjuvant therapies, including ICIs. Although ICIs are not yet standard for SCLC, several trials are exploring anti-PD-1/PD-L1 agents in this setting. Our study supports inclusion of PD-L1 status as a potential enrichment bio-

marker in such trials. Moreover, combining PD-L1 with other biomarkers may improve patient selection and therapeutic outcomes.

This research has several limitations that should be considered when interpreting the results. Firstly, the sample size, while comparable to many single-institution studies on this aggressive malignancy, is modest (n=66). SCLC presents challenges for large-scale tissue collection due to its rapid progression and the frequent predominance of cytological over histological specimens at diagnosis. Our cohort, though not large, was carefully characterized with paired samples for both detection methods, ensuring internal validity for the primary aim of method comparison. Second, this was a single-center study. While this ensures consistency in pathological evaluation and laboratory protocols, it may limit the generalizability of the findings. Our results, particularly the proposed 5% IHC cutoff, require validation in independent, preferably multi-center, cohorts. Such validation is the essential next step to translate our observations into clinically applicable guidelines. Third, we used a single antibody and a fixed cutoff value for immunohistochemical positivity; different antibodies and thresholds may yield varying results. Additionally, although we employed multivariate analysis to control for key clinical variables, as with any observational study, residual confounding from unmeasured factors (e.g., detailed treatment regimens, performance status variations, comorbid conditions) cannot be entirely ruled out. The associations we report should therefore be interpreted as robust within the constraints of the available data. Future studies should also explore the fluctuating patterns of PD-L1 expression during disease progression and in reaction to therapy.

In summary, this research demonstrates that PD-L1 is overexpressed in SCLC and correlates with advanced disease and poor survival. Both IHC and RT-qPCR can be used to assess PD-L1 expression, but IHC appears to be more sensitive and clinically relevant. PD-L1 IHC positivity and high mRNA levels are independent prognostic factors, suggesting that PD-L1 may function as a useful biomarker for risk stratification in SCLC. Additional research is required to investigate the potential of PD-L1-directed therapies for improving outcomes for SCLC patients.

Disclosure of conflict of interest

None.

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