

# PD-L1 Protein Expression and Gene Amplification Correlate with the Clinicopathological Characteristics and Prognosis of Lung Squamous Cell Carcinoma

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**Purpose:** To investigate PD-L1 protein expression and gene amplification in lung squamous cell carcinoma (LUSC) and analyse their correlation with the clinicopathological characteristics and prognosis of LUSC patients.

**Patients and Methods:** Tissue samples from 164 LUSC patients were collected. PD-L1 protein was detected by immunochemistry (IHC), and PD-L1 gene amplification was investigated by fluorescence in situ hybridization in LUSC patients.

**Results:** The positive expression rate of PD-L1 in LUSC was 47.6% (78/164), and the amplification rate of PD-L1 was 6.7% (11/164); both rates were higher than those of paratumor tissue. Both PD-L1 positive expression and gene amplification were correlated with clinical stage and lymph node metastasis ( $P < 0.05$ ). PD-L1 protein expression, PD-L1 gene amplification, late stage, lymph node metastasis and distant metastasis were significantly correlated with the prognosis of patients. Among these factors, late stage, lymph node metastasis, PD-L1 protein expression and PD-L1 gene amplification were independent prognostic factors for LUSC.

**Conclusion:** Positive PD-L1 protein expression and gene amplification are involved in the malignant progression and metastasis of LUSC. Both PD-L1 protein expression and gene amplification are associated with poor prognosis.

**Keywords:** PD-L1 protein expression, PD-L1 gene amplification, lung squamous cell carcinoma, prognosis

## Introduction

Lung cancer is a malignant tumor with the highest morbidity and mortality worldwide.<sup>1</sup> Histological types of lung cancer include non-small-cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC is a malignant tumor with high rates of recurrence and metastasis.<sup>2,3</sup> NSCLC has several histological subtypes, including adenocarcinoma, squamous cell carcinoma (LUSC), and large cell carcinoma. LUSC and adenocarcinoma account for the majority of NSCLC. Various oncogene mutations mainly occur in lung adenocarcinoma,<sup>4</sup> and driver oncogene mutations, such as EGFR and KRAS mutations, can be found in more than three-quarters of Chinese lung adenocarcinoma patients. Therefore, a variety of gene-targeting drugs for lung adenocarcinoma, such as erlotinib, have been widely used in clinical practice. However, driver gene mutation incidence in

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LUSC is low, and molecular targeted therapy in LUSC patients is ineffective.<sup>5-7</sup> Fortunately, PD-1/PD-L1 immune checkpoint inhibitors have been observed to play increasingly important roles in LUSC. At present, screening patients who benefit from PD-1/PD-L1 inhibitors mainly depends on PD-L1 protein expression detection by IHC. However, due to the use of different reagent manufacturers and platforms, detection criteria have not been standardised until recently. Therefore, it is urgent to find new biomarkers to provide more evidence for precisely screening beneficiaries.

Lee KS found that in CRC patients, PD-L1 gene amplification definitely contributes to the upregulation of the PD 1/PD-L1 axis.<sup>8</sup> However, only a few studies with small samples have reported detectable PD-L1 amplification in head and neck squamous cell carcinoma, cervical squamous cell carcinoma and triple-negative breast cancer. Generally, according to genetic central dogma, gene amplification leads to high expression of the protein, but it is not necessarily the only cause, because the protein overexpression may be caused by complex molecular mechanism. Therefore, it is one of the purposes of the study to analyse whether PD-L1 protein overexpression and PD-L1 gene amplification are consistent. If they are consistent, the two methods, that is, the protein expression detected by IHC and gene amplification detected by FISH, can be mutually verified in clinical practice.

Currently, the prognosis significance of PD-L1 gene amplification has been rarely studied and the findings of PD-L1 protein expression in malignant tumours remain controversial.<sup>9-16</sup> Therefore, we analysed the prognosis significance of PD-L1 protein overexpression and PD-L1 gene amplification in LUSC to determine whether the biomarker is a predictor of the clinical prognosis in LUSC patients.

## Materials and Methods

### Patients and Samples

A total of 164 LUSC patients in Shanxi Tumor Hospital between December 2012 and December 2013 were enrolled. All patients underwent radical surgical treatment for lung cancer. Patients with radiation and chemotherapy were excluded. Complete clinicopathologic and follow-up data of the patients were collected. Clinical stage was determined according to the ninth edition of the TNM staging system (Union for International Cancer Control, UICC). Among 164 patients, 146 were male and 18 were

female. All patients were followed-up, and the deadline for follow-up was March 2020. All collected specimens were handled and made anonymous according to accepted ethical and legal standards. This study was approved by the Institute Research Medical Ethics Committee of Shanxi Tumor Hospital and was performed in accordance with the Declaration of Helsinki. All patients signed written informed consent.

### Construction of Tissue Microarrays (TMAs)

Three TMAs were constructed containing 164 LUSC and 16 normal control tissue cores. First, each H&E-stained section was reviewed retrospectively. Two pathologists selected representative formalin-fixed and paraffin-embedded blocks. Then, a hollow needle was used to punch and extract cores (0.9-mm diameter) from each tumor sample. Two cores were extracted from typical cancer cell nest zones in each sample for TMAs construction, and the protein expression rates of the two cores were averaged. Next, the cores were embedded in paraffin blocks of more than 10 lines across 6 rows for a total of three tissue chips with 180 samples.

### Immunohistochemistry (IHC)

PD-L1 protein expression was detected by immunohistochemistry. Immunohistochemistry was conducted using a Dako PD-L1 IHC 22C3 Pharm DX Assay Kit (Agilent Technologies Co. Ltd., USA) on the Dako Autostainer Link 48 (ASL48) platform. The operation was performed according to manufacturer recommendations. Briefly, the tissue sections underwent deparaffinization, rehydration and antigen retrieval. Following FLEX peroxidase blocking for 5 minutes, specimens were incubated with 22C3 clone anti-PD-L1 antibody (mouse, 1:50, Dako, Carpinteria, CA, USA) for 60 minutes at room temperature. Specimens were then incubated with the EnVision™ FLEX+ Mouse LINKER and the EnVision™ FLEX HRP visualization reagent for 30 minutes at room temperature. Finally, the specimens were developed with DAB and counterstained with haematoxylin and covered with a cover-slip. Each IHC run contained a positive control (on-slide tonsil tissue) and a negative antibody control (buffer, no primary antibody). PD-L1 expression was determined using TPS, and staining of the tumor cell membrane or basal membrane and lateral membrane with brown-yellow particles was regarded as positive

expression. Cytoplasmic staining was considered nonspecific and was excluded in the assessment of staining intensity. Normal cells and tumor-associated immune cells, such as infiltrating lymphocytes or macrophages, were not included in the scoring for determining PD-L1 expression level. Using  $TPS \geq 1\%$  and  $TPS \geq 50\%$  as cut-offs, the expression of PD-L1 was classified into three levels (Table 1): PD-L1  $TPS < 1\%$  (negative); PD-L1  $1\% \leq TPS \leq 49\%$  (Figure 1A); PD-L1  $TPS \geq 50\%$  (Figure 1B). Two experienced pathologists each independently read the sections.

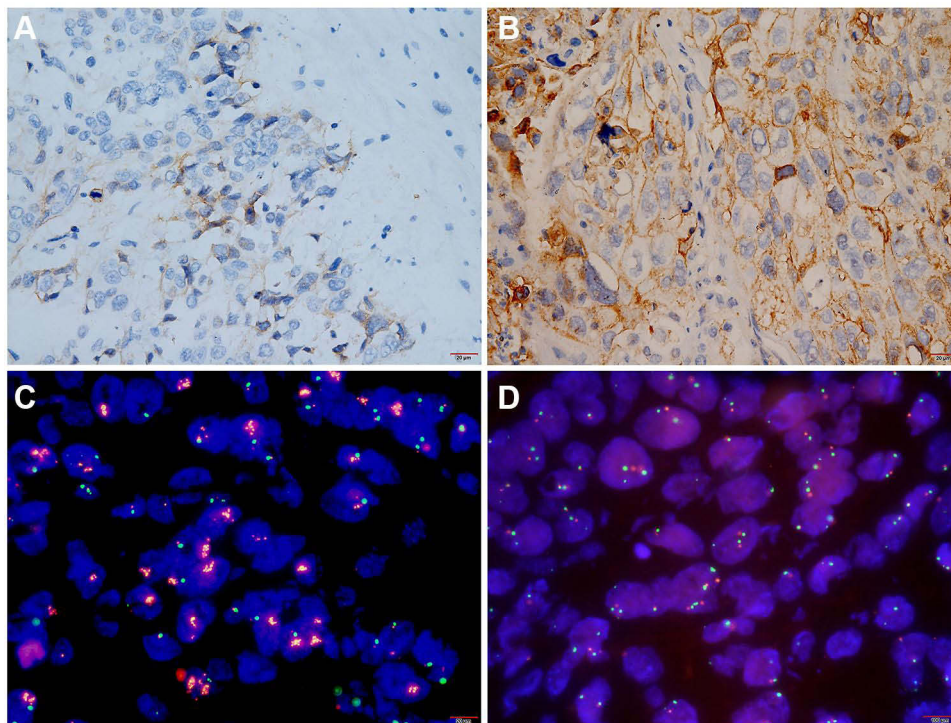
### Fluorescence in situ Hybridization (FISH)

A PD-L1 (9p24) two-color probe was used (Guangzhou Ambiping Pharmaceutical Technology Co., Ltd., China) to perform FISH. The sections were placed in an oven at  $65^\circ\text{C}$  for 30 minutes and successively immersed in xylene, 100% ethanol and distilled water 2–3 times. Afterwards, proteolysis was performed with a pepsin working liquid at  $37^\circ\text{C}$  for 10 minutes. Then, each section was immersed in  $2 \times \text{SSC}$  for 5 minutes, dehydrated in gradient ethanol for 2 minutes, and dried naturally. Probe liquid was poured on the tissue area and covered with a cover glass. The slices

**Table 1** Classification of PD-L1 Protein Expression Level in LUSC

PD-L1 Expression Status	PD-L1 Expression Levels	Staining Type
No PD-L1 Expression	$TPS < 1\%$	Tumor cells showing partial or complete cell membrane staining ( $\geq 1+$ ) accounted for less than 1% of viable tumor cells.
PD-L1 Expression (Low to medium)	$1\% \leq TPS \leq 49\%$	Tumor cells with partial or complete cell membrane staining ( $\geq 1+$ ) accounted for 1–49% of viable tumor cells.
High PD-L1 Expression	$TPS \geq 50\%$	Tumor cells with partial or complete cell membrane staining ( $\geq 1+$ ) accounted for more than 50% of viable tumor cells.

were placed in a hybridization instrument (Abbott Laboratories, USA). DNA was denatured at  $85^\circ\text{C}$  for 5 minutes and hybridized at  $37^\circ\text{C}$  for 10–16 hours. Finally, cells were counterstained with DAPI, and the number of double-color signals indicative of tumor cells was counted



**Figure 1** PD-L1 protein expression and gene amplification in lung squamous cell carcinoma. (A and B) Representative images of PD-L1 protein expression. (A) Low to moderate expression of PD-L1 ( $1\% \leq TPS \leq 49\%$ )  $\times 400$ . (B) High expression of PD-L1 protein ( $TPS > 50\%$ )  $\times 400$ . (C and D) Representative images of PD-L1 gene amplification analysed by FISH. (C) PD-L1 gene amplified Ratio  $> 2$ , parts of PD-L1 gene (red fluorescence) were expressed in clusters  $\times 1000$ . (D) PD-L1 gene without amplification  $\times 1000$ .

in at least 30 cells in a high-power field. Red signals signified the PD-L1 gene, and green signals were indicative of the chromosome 9 centromere. PD-L1 gene amplification was defined as follows: (1) ratio value  $\geq 2.0$ ; ratio value was average gene/average centromere (or average value of the red signal/average value of the green signal  $\geq 2.0$ ) (Figure 1C); (2) ratio value  $< 2.0$ , but average copy number of PD-L1  $\geq 6.0$  (or average red signal number  $\geq 6.0$  or many red signals were connected in clusters) (Figure 1D). No PD-L1 gene amplification was indicated if the ratio value was less than 2.0 and the average red signal number was less than 4.0.

## Statistical Analysis

SPSS 26.0 statistical software (IBM Corp, Armonk, NY, USA) was used to analyse the data. The relationship of PD-L1 protein expression and PD-L1 gene amplification with clinicopathological factors was analysed by the  $\chi^2$  test. Correlation between PD-L1 protein expression and PD-L1 gene amplification was analysed by Spearman correlation analysis. Overall survival (OS) was defined from the date of surgery to the last follow-up or death. Disease-free survival (DFS) was defined from the date of surgery to recurrence. Univariate analysis of prognostic factors was done by Kaplan-Meier survival analysis and Log rank test. Multivariate analysis was performed by Cox regression analysis.  $P < 0.05$  was considered significant for all the above analyses.

## Results

### PD-L1 Protein Expression and Gene Amplification in LUSC

PD-L1 was negatively expressed in all paratumor tissues and weakly expressed in inflammatory cells. PD-L1 protein was mainly observed on cancer cell membranes (Figure 1); the results are shown in Table 2. The positive rate of PD-L1 was 47.6% (78/164). Among the 164 LUSC patients, 86 cases (52.4%) had negative expression (TPS  $< 1\%$ ), 47 cases (28.7%) had moderate expression ( $1\% \leq \text{TPS} \leq 49\%$ ), and 31 cases (18.9%) had high expression of PD-L1 (TPS  $\geq 50\%$ ). The amplification rate of PD-L1 in LUSC was 6.7% (11/164). Among these 11 patients (Table 3), the positive expression rate of PD-L1 protein was 100% (11/11), including 7 patients with high expression (7/11, 63.6%) and 4 patients with moderate expression of PD-L1 (4/11, 36.4%). Correlation analysis showed that PD-L1 gene amplification was positively correlated

with protein expression intensity ( $r=0.786$ ,  $P < 0.001$ , Table 3), which revealed that PD-L1 protein expression and PD-L1 gene amplification were consistent in LUSC.

### Relationship Between PD-L1 Protein Expression, Gene Amplification and Clinicopathological Characteristics of Patients with LUSC (See Table 2)

Positive PD-L1 protein expression was significantly correlated with age, tumor size, clinical stage and lymph node metastasis in LUSC patients ( $P < 0.05$ ) but not correlated with gender, smoking, histological grade, distant metastasis and recurrence ( $P > 0.05$ ). PD-L1 gene amplification was significantly correlated with lymph node metastasis and distant metastasis in LUSC patients ( $P < 0.05$ ) but was not related to gender, age, smoking, histological grade, tumor size, or recurrence ( $P > 0.05$ ). The PD-L1 protein positive expression rate in patients under 60 years old was significantly higher than that in patients over 60 years old ( $P = 0.026$ ). Gene amplification and protein expression rates of patients with advanced stage cancer ( $P < 0.001$ ) were significantly higher than those of patients in early stage cancer ( $P = 0.017$ ), and in those with lymph node metastasis, they were significantly higher than in those without lymph node metastasis ( $P = 0.001$ ;  $P < 0.001$ ). The amplification rate of PD-L1 gene in patients with distant metastasis was significantly higher than that in patients without distant metastasis ( $P < 0.001$ ).

### PD-L1 Positive Expression and Gene Amplification Predict Poor Prognosis in LUSC Patients

Kaplan-Meier survival curves (Figure 2) showed that the DFS and OS of patients with moderate and high PD-L1 expression were significantly shorter than those of patients with negative PD-L1 expression. The median survival ( $64.29 \pm 1.99$ ) of patients with low PD-L1 expression was significantly longer than those with moderate ( $52.89 \pm 2.847$ ) and high PD-L1 expression ( $36.359 \pm 2.297$ ) ( $P < 0.001$ , see Table 4). Compared with patients without PD-L1 amplification, the DFS and OS of patients with PD-L1 amplification were significantly shorter, and the median survival ( $27.909 \pm 3.942$ ) was significantly shorter than that of patients without PD-L1 amplification ( $58.184 \pm 1.636$ ) ( $P < 0.001$ , see Table 4). Patients with PD-L1 expression  $> 50\%$  and gene amplification positive had shorter survival

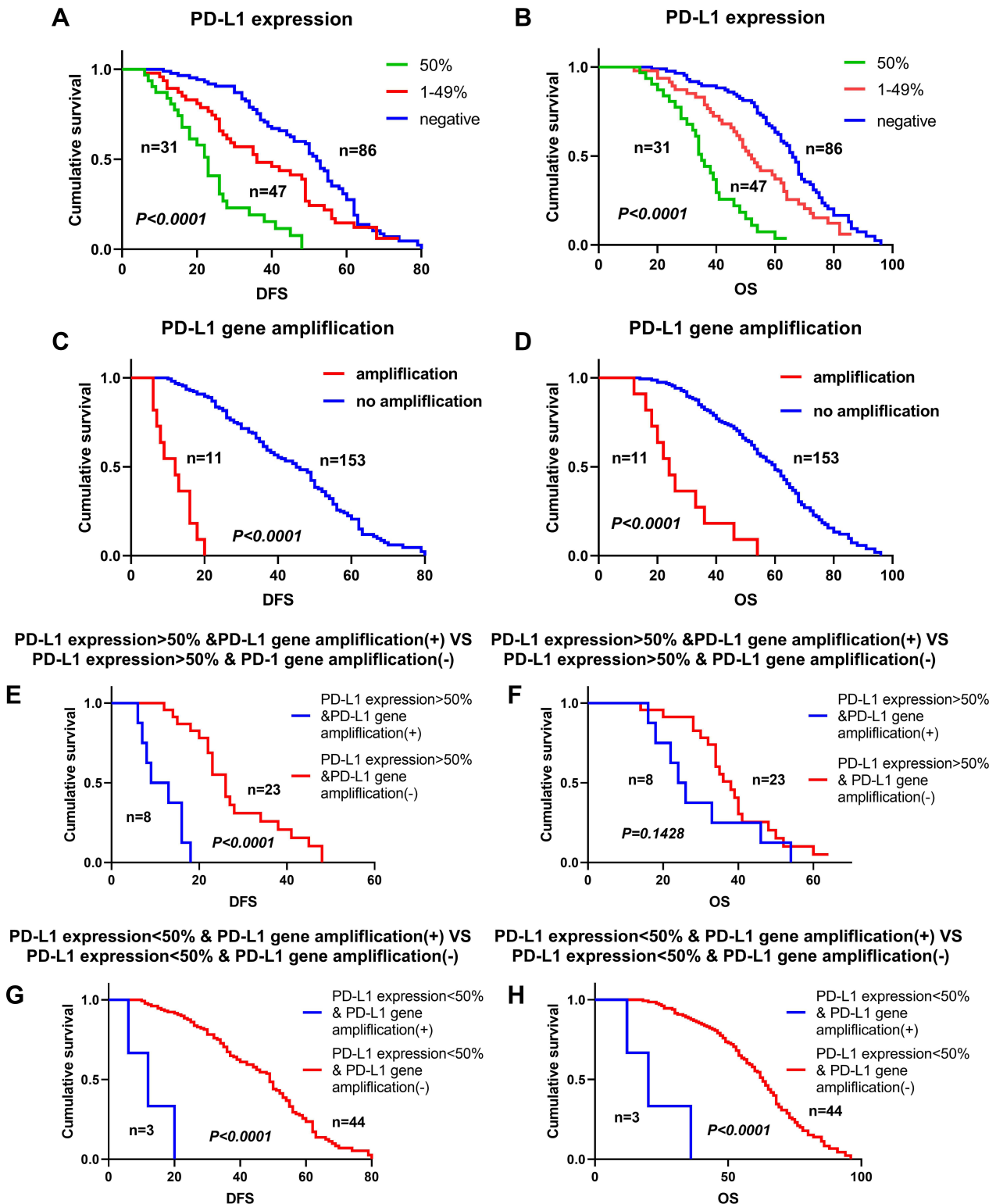
**Table 2** Relationship Between PD-L1 Gene Amplification and Clinicopathological Features of Lung Squamous Cell Carcinoma

Variable	Total	PD-L1 Amplification				PD-L1 Expression				
		No	Yes	$\chi^2$	p-value	<1%	1-49%	≥50%	$\chi^2$	p-value
Total	164	153	11			86	47	31		
Gender										
Female	18	15	3	3.495	0.073	6	8	4	3.283	0.194
Male	146	138	8							
Age (years)										
≤60	87	79	8	6.462	0.176	37	30	20	7.300	0.026
>60	77	74	3							
Smoking										
Yes	101	94	7	0.728	0.885	60	26	15	5.496	0.064
No	63	59	4							
Tumor size										
T1	4	4	0	9.847	0.340	1	2	1	20.450	<0.001
T2	75	72	3							
T3	85	77	8							
Histological grade										
I	44	40	4	1.802	0.531	26	10	8	1.347	0.853
2	49	45	4							
3	71	68	3							
Stage										
I	39	39	0	52.897	<0.001	27	9	3	15.391	0.017
II	84	81	3							
III	37	32	5							
IV	4	1	3							
Lymph node metastasis										
Yes	71	61	10	49.639	0.001	14	34	23	53.774	<0.001
No	93	92	1							
Metastasis										
Yes	4	1	3	30.973	<0.001	1	3	0	4.436	0.109
No	160	152	8							
Relapse										
Yes	7	7	0	1.501	0.468	3	3	1	0.725	0.696
No	157	146	11							

**Table 3** Correlation Between PD-L1 Gene Amplification and Protein Expression

PD-L1 Amplification	PD-L1 Expression			r=0.786	P<0.001
	<1%	1-49%	≥50%		
Yes	0	4	7		
No	86	44	23		

time than those patients with PD-L1 expression >50% and negative gene amplification (P<0.001, see Table 4). Even in patients with PD-L1 protein expression below 50%, that accompanied by positive gene amplification had significantly shorter survival time than patients with negative gene amplification (P<0.001, see Table 4). Multivariate analysis showed that clinical stage, lymph node metastasis, PD-L1 protein expression and PD-L1 gene amplification



**Figure 2** Kaplan-Meier survival curves of PD-L1 amplification and expression in patients with lung squamous cell carcinoma. **(A and C)** DFS curve of patients with lung squamous cell carcinoma. **(B and D)** OS curve of patients with lung squamous cell carcinoma. **(E)** DFS curve of PD-L1 expression >50% and gene amplification. **(F)** OS curve of PD-L1 expression >50% and gene amplification. **(G)** DFS curve of PD-L1 expression <50% and gene amplification. **(H)** OS curve of PD-L1 expression <50% and gene amplification.

**Table 4** Univariate Prognostic Factor Analysis by Log Rank Test

Variable	Total	Disease-Free Survival			Overall Survival		
		Mean $\pm$ SE (Months)	95% CI	P	Mean $\pm$ SE (Months)	95% CI	P
Gender							
Female	18	38.726 $\pm$ 4.682	29.550–47.903	0.458	52.434 $\pm$ 5.124	42.391–62.478	0.358
Male	146	42.153 $\pm$ 1.641	38.936–45.370		56.618 $\pm$ 1.757	53.173–60.062	
Age (years)							
$\leq$ 60	87	38.142 $\pm$ 2.114	33.999–42.284	0.046	51.473 $\pm$ 2.026	47.149–55.707	0.006
>60	77	45.851 $\pm$ 2.168	41.601–50.101		61.394 $\pm$ 2.351	56.787–66.001	
Smoking							
Yes	101	43.377 $\pm$ 2.018	39.422–47.333	0.221	57.998 $\pm$ 2.181	48.347–58.373	0.153
No	63	39.372 $\pm$ 2.406	34.656–44.089		53.360 $\pm$ 2.558	53.723 $\pm$ 2.273	
Tumor status							
T1	4	46.500 $\pm$ 10.618	25.688–67.312	0.155	60.250 $\pm$ 9.630	41.376–79.124	0.169
T2	75	45.797 $\pm$ 2.127	41.627–49.967		60.478 $\pm$ 2.302	55.967–64.989	
T3	85	38.053 $\pm$ 2.216	33.709–42.379		52.235 $\pm$ 2.388	47.555–56.915	
Histological grade							
I	44	42.243 $\pm$ 3.224	35.924–48.562	0.689	57.26 $\pm$ 03.602	50.200–64.319	0.555
2	49	40.361 $\pm$ 2.653	35.161–45.561		54.681 $\pm$ 2.814	49.165–60.198	
3	71	42.350 $\pm$ 2.311	37.820–46.880		56.449 $\pm$ 2.461	51.626–61.272	
Stage							
I	39	49.736 $\pm$ 2.760	44.327–55.145	<0.001	66.524 $\pm$ 2.816	61.004–72.043	<0.001
II	84	43.933 $\pm$ 1.976	40.059–47.807		57.701 $\pm$ 2.037	53.708–61.695	
III	37	30.872 $\pm$ 3.271	24.461–37.283		44.554 $\pm$ 3.819	37.068–52.039	
IV	4	14.500 $\pm$ 3.610	7.425–21.575		26.000 $\pm$ 6.364	13.527–38.473	
Lymph node							
Yes	71	36.594 $\pm$ 2.414	31.863–41.325	0.026	49.510 $\pm$ 2.635	44.344–54.675	0.015
No	93	45.531 $\pm$ 1.853	41.898–49.163		60.844 $\pm$ 1.920	57.081–64.606	
Metastasis							
Yes	4	14.500 $\pm$ 3.610	7.425–21.575	<0.001	26.000 $\pm$ 6.364	13.527–38.473	<0.001
No	160	42.309 $\pm$ 1.540	39.291–45.327		56.753 $\pm$ 1.646	53.527–59.979	
Relapse							
Yes	7	42.143 $\pm$ 8.221	26.029–58.257	0.901	55.000 $\pm$ 8.608	38.129–71.871	0.926
No	157	41.771 $\pm$ 1.579	38.677–44.865		56.199 $\pm$ 1.696	52.875–59.522	
PD-L1 expression							
<1%	86	49.281 $\pm$ 1.801	45.751–52.811	<0.001	64.291 $\pm$ 1.992	60.388–68.195	<0.001
1–49%	47	38.652 $\pm$ 2.801	33.161–44.143		52.892 $\pm$ 2.847	47.311–58.473	
$\geq$ 50%	31	23.925 $\pm$ 2.221	19.572–44.143		36.359 $\pm$ 2.297	31.858–40.860	
PD-L1 amplification							
No	153	43.934 $\pm$ 1.507	40.980–46.889	<0.001	58.184 $\pm$ 1.636	54.978–61.391	<0.001
Yes	11	11.909 $\pm$ 1.522	8.925–14.893		27.909 $\pm$ 3.942	20.184–35.634	
PD-L1 >50% (+)							
And gene amplification	8	11.625 $\pm$ 1.658	8.376–14.874	<0.001	29.875 $\pm$ 4.812	20.443–39.307	0.130
And gene amplification (-)	22	28.461 $\pm$ 2.454	23.650–33.271		38.885 $\pm$ 2.608	33.774–43.996	

(Continued)

**Table 4** (Continued).

Variable	Total	Disease-Free Survival			Overall Survival		
		Mean $\pm$ SE (Months)	95% CI	P	Mean $\pm$ SE (Months)	95% CI	P
PD-L1 <50% And gene amplification (+)	3	12.667 $\pm$ 4.055	4.719–20.615	<0.001	22.667 $\pm$ 7.055	57.756–64.380	<0.001
And gene amplification (-)	131	46.269–1.591	43.149–49.388		61.068 $\pm$ 1.690	59.275–66.725	

were independent adverse prognostic factors for LUSC (see Table 5).

## Discussion

Programmed cell death ligand 1 (PD-L1, also known as B7-H1, CD274) is a transmembrane protein that belongs to the B7 family.<sup>17</sup> Human PD-L1 gene is located on chromosome 9p24.1. Programmed death receptor-1 (PD-1) is mainly expressed on T lymphocytes.<sup>18</sup> High PD-L1 expression is found in a variety of solid tumors, such as lung cancer, liver cancer, breast cancer, and ovarian cancer. PD-L1 on tumor cells binds PD-1 receptors on T cells, resulting in the dephosphorylation of Sh2p-driven T cell receptors and its coreceptor CD28, which inhibits T cell activation,<sup>19</sup> weakening the ability of T cells to kill tumor cells. Under this mechanism, tumor cells can evade immune surveillance and promote tumor migration.<sup>20,21</sup> The results of this study showed that there were 47.6% patients with PD-L1 positive expression in LUSC, and the expression of PD-L1 was related to lymph node metastasis

and advanced stage. The results indicated that high PD-L1 expression was involved in progression and metastasis of lung squamous cell carcinoma.

The significance of PD-L1 expression in the prognosis of lung cancer remains controversial.

A few studies have shown that PD-L1 expression was associated with better prognosis or longer overall survival,<sup>22–26</sup> only Ameratunga et al have suggested that the expression of PD-L1 was not associated with prognosis;<sup>27</sup> most studies have suggested that the expression of PD-L1 in lung cancer cells was associated with poor prognoses,<sup>11,28–35</sup> which is consistent with the results of this study. Both univariate analysis and multivariate analysis showed PD-L1 protein expression was significantly correlated with poor prognosis and was an independent risk factor for poor prognosis in clinical practice.

Generally, gene amplification leads to high expression of the protein, but it is not necessarily the only cause, because the protein overexpression may be caused by complex molecular mechanism, such as gene enhancer

**Table 5** Cox Multivariate Survival Analysis in LUSC Patients

Variable	Disease-Free Survival			Overall Survival		
	HR	95% CI	P	HR	95% CI	P
Age (years)	0.823	0.576–1.175	0.283	0.823	0.576–1.175	0.179
Stage			0.001			0.184
Stage (1)	0.219	0.043–1.101	0.065	0.315	0.063–1.577	0.160
Stage (2)	0.306	0.066–1.418	0.130	0.416	0.093–1.853	0.250
Stage (3)	0.838	0.180–3.897	0.822	0.691	0.157–3.042	0.625
Lymph node metastasis	2.696	1.521–4.779	0.001	0.494	0.284–0.861	0.013
PD-L1 expression			<0.001			<0.001
PD-L1 expression (1)	0.168	0.088–0.320	<0.001	0.050	0.010–0.249	<0.001
PD-L1 expression (2)	0.415	0.231–0.746	0.003	0.228	0.048–1.077	0.062
PD-L1 amplification	0.102	0.038–0.268	<0.001	2.552	1.547–4.210	<0.001



mutation, modification and regulation of transcription and translation levels, and protein stability etc. Hence, gene amplification and protein overexpression are not always consistent in malignant tumors. However, there were few researches into PD-L1 gene amplification and its consistency with protein expression in lung cancer. Inoue Yusuke et al evaluated the PD-L1 copy number of 194 cases of NSCLC and found that the PD-L1 amplification rate was only 2.6%,<sup>15</sup> Some studies in European, American and Japanese populations have shown that PD-L1 gene amplification in NSCLC patients the PD-L1 gene amplification rate was lower than 5.3%.<sup>10,36,37</sup> In this study, we found that the amplification rate of the PD-L1 gene in squamous cell carcinoma was 6.7%, which was slightly higher than the average amplification rate of all types of previously studied NSCLCs. Moreover, patients with PD-L1 gene amplification showed positive expression of PD-L1 protein, which supported that PD-L1 gene amplification contributes to PD-L1 protein expression in LUSC. Therefore, PD-L1 protein expression detected by IHC and PD-L1 gene amplification detected by FISH can be mutually verified in clinical practice.

As we all know, there are controversies surrounding the prognostic significance of PD-L1 gene amplification, and the prognostic implication of PD-L1 gene amplification has been rarely studied. Inoue Yusuke found that the DFS and OS of NSCLC patients with PD-L1 amplification showed good survival results.<sup>15</sup> Other studies have shown that PD-L1 gene amplification in NSCLC patients was associated with poor prognosis,<sup>36,37</sup> which corresponds with our findings. The results demonstrate that both PD-L1 protein expression and gene amplification can be poor prognosis predictors for LUSC patients.

As it is a retrospective investigation, this study has certain limitations. First of all, all the LUSC patients from this cohort underwent surgical therapy without being treated with PD-L1 inhibitor. So, we will expand the sample size to include patients who have undergone PD-L1 inhibitor therapy in the subsequent research so as to analyse the relationship between biomarkers and the efficacy of PD-L1 inhibitors. In addition, this study only observed the phenomenon that PD-L1 expression increased in patients in advanced clinical stages with high rates of LNs and distant metastasis, which suggested the accompanying relationship between PD-L1 and tumor progression. Further research needs to do be done to clarify whether high PD-L1 expression is a cause or

a result or an accompanied feature of highly aggressive tumors.

## Conclusion

PD-L1 protein overexpression and gene amplification are consistent in LUSC. PD-L1 protein and gene amplification related to the malignant progression and metastasis of lung squamous cell carcinoma and both can predict poor prognosis. Therefore, in addition to PD-L1 protein, PD-L1 gene amplification may be used as a new routine detection marker for the immunotherapy of lung squamous cell carcinoma.

## Abbreviations

LUSC, lung squamous cell carcinoma; IHC, immunohistochemistry; NSCLC, non-small-cell lung cancer; SCLC, small cell lung cancer; UICC, Union for International Cancer Control; TMAs, Construction of tissue microarrays; ASL48, Autostainer Link 48; FISH, Fluorescence in situ hybridization; DFS, Disease-free survival; OS, Overall survival; PD-1, Programmed death receptor-1; PD-L1, Programmed cell death ligand 1; HR, hazard ratio.

## Author Contributions

Zhenwen Chen, Ning Zhao and Yirong Xu designed the whole study, collected the tissue specimens and performed FISH methods, read the sections and taken the picture of FISH, collected and analyzed data, then wrote the draft paper and revised paper. Yanfeng Xi, Huiwen Wu, Xiaoi Tian and Qi Wang developed the idea for the clinical study, collected clinicopathological data, performed IHC and wrote the materials of draft paper and revised the paper. All authors have reviewed and approved the drafted and final paper. All authors have agreed on the journal to which the article will be submitted and agreed to take responsibility and be accountable for the contents of the article.

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## Disclosure

The authors report no conflicts of interest in this work.

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