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Programmed cell death-ligand 1 expression and CD8 positive tumor-infiltrating lymphocyte density in non-small cell lung carcinoma and its association with histopathological grading

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Abstract

Non-small cell lung carcinoma (NSCLC), comprising 85% of lung cancers, remains a leading cause of cancer mortality despite advances in treatment. Immunotherapy, particularly immune checkpoint inhibitors targeting the PD-1/PD-L1 axis, has revolutionized therapy, though outcomes vary. This study aimed to explore the association between PD-L1 expression, CD8 tumor-infiltrating lymphocyte (TIL) density, and histopathological grading in NSCLC. Our retrospective, single-center cohort comprised 64 biopsy samples of NSCLC. PD-L1 and CD8 TIL density were assessed through immunohistochemistry. We also classified the tumors into four groups based on the PD-L1 and CD8-positive TIL statuses and evaluated their association with clinicopathological parameters. Male subjects were the predominant population in the study group (86%), with a mean age of 60 years. Most of the cases were smokers/ex-smokers (70.3%). Among 64 cases, PD-L1 positivity was observed in 62.5%, correlating with poorly differentiated tumors (grade 3) (p=0.03), suggesting its association with poor prognosis. Among PD-L1-positive cases, 55% had high expression and 45% had low expression. CD8 TIL density was low in 62.5% of cases and showed no significant correlation with clinical variables. Combined analysis revealed that 42.19% of cases were PD-L1+/CD8 low, a phenotype indicative of immune evasion and aggressive tumor behavior. Overall, our results emphasize that while PD-L1 immunohistochemistry remains a critical tool for identifying candidates for immunotherapy, it is not a standalone predictor of treatment response. Integrating CD8 TIL density provides additional prognostic information, potentially guiding more personalized treatment strategies.

Key words: tumor microenvironment, immunohistochemistry, immune checkpoints, tumor differentiation, lung cancer.

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Introduction

Lung carcinoma is the most diagnosed cancer and the leading cause of cancer-related deaths worldwide. As reported by GLOBOCAN 2022, approximately 2.5 million new lung cancer cases were diagnosed globally, representing 12.4% of all cancer diagnoses with 18.7% of all cancer-related fatalities [1]. In India, lung cancer constitutes 5.9% of new cancer cases and 8.1% of cancer-related deaths, with the highest incidence in Mizoram [2-5].

Lung cancer is broadly divided into non-small cell lung cancer (NSCLC) and small cell lung cancer, with NSCLC making up approximately 85% of cases. Most NSCLC cases are diagnosed at an advanced stage, where surgical options are limited, and survival rates are poor. In this context, the treatment landscape for advanced-stage NSCLC has evolved dramatically, particularly with the advent of targeted therapies and immune checkpoint inhibitors (ICI). Targeted therapies, such as tyrosine kinase inhibitors, specifically address genetic mutations like *EGFR*, *ALK*, and *ROS1*, offering significant improvements in survival and quality of life for subsets of patients with these molecular alterations [6-8]. Immunotherapy, particularly ICIs targeting the PD-1/PD-L1 pathway, has emerged as a promising treatment avenue, showing durable responses and extended survival in advanced NSCLC, with specific populations benefiting regardless of the subtype [9-11]. According to the World Health Organization, in addition to molecular testing, NSCLC analysis includes evaluating PD-L1 expression to direct the use of ICIs [12].

PD-L1, also referred to as CD274 or B7-H1, belongs to the B7 family and functions as an immune checkpoint, promoting antitumor suppression of the immune pathway. It is a 33-kDa type 1 transmembrane glycoprotein composed of 290 amino acids, featuring Ig- and IgC domains in its extracellular region [13]. PD-L1 is normally expressed by macrophages, activated T and B cells, dendritic cells, and epithelial, muscle, and endothelial cells, particularly during inflammatory conditions. Additionally, tumor cells exploit PD-L1 expression as an “adaptive immune mechanism” to evade immune-mediated anti-tumor responses. PD-1 (also called CD279) is a receptor for PD-L1 and is expressed predominantly on activated cytotoxic T-cells and natural killer cells.

PD-L1 ligand binds to the PD-1 receptor on activated T-cells, suppressing the immune system [14,15].

PD-L1 expression within tumors has been linked to unfavorable prognostic outcomes across various solid tumors, including lung cancer. Monoclonal antibodies targeting PD-1 and PDL-1 are currently being used successfully to inhibit the interaction between the PD-1 receptor and the PD-L1 protein. Despite studies indicating interassay and biological heterogeneity in PD-L1 expression, IHC testing has quickly become a key stratifying biomarker for patients receiving PD-1/PD-L1 inhibitors [16-18].

Clinical trial evidence indicates that not all patients with elevated PD-L1 expression respond to PD-1/PD-L1 inhibitors, while some individuals with low or absent PD-L1 expression do respond. Moreover, these agents are expensive and have side effects that may lead to life-threatening toxicity [19]. In the era of precision medicine and combination immunotherapy, additional biomarkers are essential to maximize clinical benefits, spare unnecessary costs and toxicity, and stratify patients to select the most appropriate treatment options. Indeed, PD-L1 expression alone remains an unreliable predictor of the effectiveness of ICIs in lung cancer, as only a limited subset of NSCLC patients with high PD-L1 expression exhibit a favorable response to ICI therapy [8]. Factors such as PD-L1 expression status, mutational load, driver gene mutation, and tumor-infiltrating lymphocyte (TIL) presence impact immunotherapy response with anti-PD-L1 therapy. Tumor mutation burden, when combined with elevated PD-L1 expression, proves to be a more effective predictor of responses to ICIs than PD-L1 expression alone [10].

Furthermore, CD8+ TILs have emerged as a critical determinant in predicting ICI efficacy due to their direct role in tumor cell destruction. TIL s, especially CD8+ TILs, represent a critical component of the tumor immune microenvironment, indicating active immune responses and correlating with better outcomes in NSCLC patients undergoing ICI treatment [20-23].

Combining PD-L1 expression and CD8+ TIL density could enhance predictive accuracy for immunotherapy response, given CD8+ TILs' direct role in tumor cell destruction. The tumor immune microenvironment can be classified into four types: type I (PD-L1 positive with TILs positive), type II (PD-L1 negative with TILs negative), type III (PD-L1 positive with TILs negative), and type IV (PD-L1 negative with TILs positive). This classification may help predict the response to immunotherapy and personalize treatment strategies [24].

This study aims to evaluate the expression of PD-L1 and CD8-positive TIL density in NSCLC and their association with histopathological grading, thereby contributing to precision medicine strategies for NSCLC patients.

Materials and Methods

This cross-sectional study was conducted over 18 months in the Department of Pathology in collaboration with the Department of Pulmonary Medicine at a tertiary care hospital in New Delhi, India. After approval from the Institutional Ethics Committee, the study included all histopathologically diagnosed cases of non-small cell lung carcinoma in the Department of Pathology from March 2023 to September 2024.

Study population

A total of 64 histopathologically confirmed cases of NSCLC were included in the study. Exclusion criteria included patients with metastatic carcinoma to the lungs or co-existing malignancies.

Collection and preparation of materials

Clinical examination and staging were conducted per standard protocols in the Department of Pulmonary Medicine, incorporating routine clinical and radiological details. For each suspected lung carcinoma case, biopsies were taken from representative lesions, fixed in 10% formalin, embedded in paraffin, and sectioned at 3-5 µm thickness. Histopathological evaluation was carried out on hematoxylin and eosin-stained slides to determine the histological type and grade.

Immunohistochemical analysis

Two representative paraffin blocks per case were selected for immunohistochemistry (IHC). Sections were prepared at 3 µm thickness and processed with antibodies for PD-L1 and CD8 markers, using monoclonal antibodies from BIOCARE. For PD-L1, a primary rabbit monoclonal antibody (clone CAL10) was used and incubated overnight at 4°C. For CD8, a primary rabbit monoclonal antibody (clonal SP16) was used and incubated for 30 minutes at 36°C. Positive and negative controls were included in each IHC run. All IHC results were evaluated by two independent pathologists.

Assessment of immunohistochemical expression of PD-L1

PD-L1 expression was assessed by Tumor Proportion Score (TPS), defined as the percentage of viable tumor cells showing partial or complete membrane staining at any intensity [25]. PD-L1 immunoreactive tumor cells were counted per high-power field in five different fields for each case. Positivity in tumor cells was taken as follows: i) only membrane staining was evaluated; ii) partial or complete membrane staining was included; iii) cytoplasmic staining was not included [Eq. 1].

$$TPS = \frac{\text{(Number of PDL1 positive tumor cells)}}{\text{(Total number of PDL1 positive + PDL1 negative tumor cells)}} \times 100 \quad \text{[Eq. 1]}$$

Lesions exhibiting ≥1% of tumor cells were regarded as positive. Lesions exhibiting no or <1% of tumor cells were considered negative. PD-L1 positivity was further divided into high (≥50%) and low PD-L1 (1-49%) expression.

CD8 TIL evaluation [26,27]: CD8+ TIL s were evaluated in the stromal compartment within tumor borders and were expressed as a percentage of stromal TILs, which refers to the area occupied by mononuclear inflammatory cells over the total intratumoral stromal area. TILs outside of the tumor border and in tumor zones with crushing artifacts and necrosis were excluded. In each slide, 100 cells in 5 high-power fields were counted. CD8 staining was evaluated as membranous staining of TILs for staining frequency (based on percentage of positively stained lymphocytes: 0 if no cells, 1 if 1-25%, 2 if 26-50% 3 if 51-75% and 4 if 76-100%) and staining intensity (0 if negative, 1 if weak, 2 if moderate and 3 if strong). The final score, derived by multiplying the intensity and frequency scores, categorized cases into low (≤3) and high (>3) TIL density.

PD-L1/CD8 tumor-infiltrating lymphocyte density groups

According to PD-L1 expression and CD8 TIL density, we further categorized the cases into four groups: PD-L1+/CD8high group included cases positive for PD-L1 expression and showed high CD8 TIL density, PD-L1+/CD8low group included cases positive for PD-L1 expression with low CD8 TIL density, PD-L1−/CD8high group included cases negative for PD-L1 staining with high CD8 TIL density, and PD-L1−/CD8low group included cases negative for PD-L1 expression and showed low CD8 TIL density.

Statistical analysis

The categorical variables were presented as numbers and percentages (%); quantitative data as the means ± standard deviation and as median with 25th and 75th percentiles (interquartile range). The data normality was checked by the Shapiro-Wilk test. The association of variables that were quantitative was analyzed using an independent t-test (for two groups) and analysis of variance (for more than two groups). The association of the qualitative variables was analyzed using Fisher's exact test as at least one cell had an expected value of less than 5. The data entry was done in the Microsoft EXCEL spreadsheet, and the final analysis was done with the use of Statistical Package for Social Sciences (SPSS) software (IBM, Chicago, IL, USA) version 25.0. For statistical significance, a p-value of less than 0.05 was considered statistically significant.

Results

Patient characteristics

The socio-demographic characteristics are summarized in Table 1. The median age was 61 years (range 34-89 years), and 55 (86%) patients were men. At diagnosis, 45 (70.3%) patients were current smokers/ ex-smokers, while 19 (29.7%) were never smokers. The most predominant histological type was squamous cell carcinoma (38 patients), followed by adenocarcinoma (24 patients), with one case each of adenosquamous and large cell carcinoma. Tumor differentiation/ histopathological grade was classified as either poor (50%), moderate (37.5%), or well-differentiated (12.5%) (Figure 1).

Relation between PD-L1 expression and clinicopathologic parameters

PD-L1 positive expression was detected in 40 (62.5%) of NSCLC cases, while 24 (37.5%) were PD-L1 negative. PD-L1 expression was demonstrated as brownish staining in the cell membranes of tumor cells. The relation between PD-L1 expression and clinicopathological parameters is summarized in Table 2. There was a significant association between PD-L1 expression and high tumor grade (p=0.03). Among grade 3 cases, 32 (50%) of cases were positive for PD-L1 expression, whereas 24 (37.5%) of grade 2 cases showed PD-L1 positivity. No significant association was observed between PD-L1 expression and age (p=0.8), gender (p=0.7), smoking status (p=0.9), and histologic type (p=1). Among the positive PD-L1 cases, Low expression (1-49%) was seen in 18/64 (45%) cases and high expression (≥50%) in 22/64 (55%). Except for gender (female *vs*. male), none of the clinicopathological parameters was associated with higher PD-L1 expression. Higher PD-L1 expression was significantly associated with the male gender (p=0.05) (Figure 2).

Relation between CD8+ tumor-infiltrating lymphocytes and clinicopathologic parameters

A total of 24 patients had high CD8+TIL density, and 40 had low CD8+TIL density. The relation between CD8+ TILs and clinicopathologic parameters is summarized in Table 2. No significant association was seen between CD8 TIL density and clinicopathological parameters (Figure 3).

Relation between PD-L1/CD8 tumor-infiltrating lymphocyte density groups and clinicopathologic parameters

The combined PD-L1 and CD8 TIL status of the cases was as follows, maximum number of cases 27 (42.2%) were PD-L1 positive and CD8 low, followed by 13 cases (20.3%) of PD-L1 negative and CD8 low, 13 cases (20.3%) of PD-L1 positive and CD8 high and 11 cases (17.2%) were PD-L1 negative and CD8 high. Table 3 summarizes the relation between PD-L1/CD8 TIL density groups and clinicopathologic parameters. The mean age across groups showed no significant difference (p=0.853), with a male predominance (85.94%), especially in the PD-L1+/CD8 high group (100%). Poorly differentiated tumors (grade 3) were most associated with PD-L1+/CD8 high (61.54%) and PD-L1+/CD8 low (59.26%) groups, while well-differentiated tumors (grade 1) were predominant in the PD-L1−/CD8 high group (45.45%, p=0.05).

Table 1. Clinicopathological characteristics of patients with non-small cell lung carcinoma.

Clinicopathological parameters	Cases (%)
Age, mean ± standard deviation	61.97±10.8
Sex, n (%)	
Male	55 (86)
Female	9 (14)
Smoking status, n (%)	
Current/ex-smoker	45 (70.3)
Never smoker	19 (29.7)
Histologic type, n (%)	
Squamous cell carcinoma	38 (59.5)
Adenocarcinoma	24 (37.5)
Large cell carcinoma	1 (1.5)
Adenosquamous carcinoma	1 (1.5)
Histopathological grade, n (%)	
Poorly differentiated (grade 3)	32 (50)
Moderately differentiated (grade 2)	24 (37.5)
Well-differentiated (grade 1)	8 (12.5)
PD-L1 expression, n (%)	
Positive	40 (62.5)
Negative	24 (37.5)
PD-L1 high/low, n (%)	
Low	18 (45)
High	22 (55)
CD8+ TIL density, n (%)	
High	24 (37.5)
Low	40 (62.5)

TILs, tumor-infiltrating lymphocytes.

Table 2. Relation between PD-L1 expression and CD8+ tumor-infiltrating lymphocytes with clinicopathologic parameters.

Clinicopathological parameters	Total	PD-L1 positive (n=40)	PD-L1 negative (n=24)	p
Age, mean ± standard deviation	61.97±10.83	61.7±11.52	62.42±9.79	0.8‡
Sex, n (%)				
Male	55 (85.94)	35 (87.50)	20 (83.33)	0.7*
Female	9 (14.06)	5 (12.50)	4 (16.67)	
Smoking status, n (%)				
Current/ex-smoker	45 (70.31)	28 (70)	17 (70.83)	0.9†
Never smoker	19 (29.69)	12 (30)	7 (29.17)	
Histologic type, n (%)				
Squamous cell carcinoma	38 (59.38)	23 (57.50)	15 (62.50)	1*
Adenocarcinoma	24 (37.50)	15 (37.50)	9 (37.50)	
Large cell carcinoma	1 (1.56)	1 (2.50)	0 (0)	
Adenosquamous carcinoma	1 (1.56)	1 (2.50)	0 (0)	
Histopathological grade, n (%)				
Poorly differentiated (grade 3)	32 (50)	24 (60)	8 (33.33)	0.03*
Moderately differentiated (grade 2)	24 (37.50)	14 (35)	10 (41.67)	
Well-differentiated (grade 1)	8 (12.50)	2 (5)	6 (25)	
CD8 TIL density, n (%)				
Low CD8 TIL density	40 (62.50)	27 (67.50)	13 (54.17)	0.286†
High CD8 TIL density	24 (37.50)	13 (32.50)	11 (45.83)	
Clinicopathological parameters	**Total**	**Low CD8+ TILs (n=40)**	**High CD8+ TILs (n=24)**	**p**
Age, mean ± standard deviation	61.97±10.83	62.82±10.7	60.54±11.12	0.418‡
Sex, n (%)				
Male	55 (85.94)	32 (80)	23 (95.83)	0.136*
Female	9 (14.06)	8 (20)	1 (4.17)	
Smoking status, n (%)				
Current/Ex smoker	45 (70.31)	30 (75)	15 (62.50)	0.289†
Never smoker	19 (29.69)	10 (25)	9 (37.50)	
Histologic type, n (%)				
Squamous cell carcinoma	38 (59.38)	27 (67.50)	11 (45.83)	0.116*
Adenocarcinoma	24 (37.50)	27 (67.50)	11 (45.83)	
Adenosquamous carcinoma	1 (1.56)	0 (0)	1 (4.17)	
Large cell carcinoma	1 (1.56)	1 (2.50)	0 (0)	
Histopathological grade, n (%)				
Poorly differentiated (grade 3)	32 (50)	21 (52.50)	11 (45.83)	0.367*
Moderately differentiated (grade 2)	24 (37.50)	16 (40)	8 (33.33)	
Well-differentiated (grade 1)	8 (12.50)	3 (7.50)	5 (20.83)	

TILs, tumor-infiltrating lymphocytes. ‡Independent t-test; *Fisher's exact test; †Chi-square test.

Table 3. Relation between PD-L1/CD8 tumor-infiltrating lymphocyte density groups and clinicopathologic parameters.

Clinicopathological parameters	Total	PD-L1+/CD8^{high} (n=13)	PD-L1+/CD8^{low} (n=27)	PD-L1−/CD8^{high} (n=11)	PD-L1-/CD8^{low} (n=13)	p
Age, mean ± standard deviation	61.97±10.83	59.92±12.77	62.56±11.02	61.27±9.36	63.38±10.42	0.853†
Sex, n (%)						
Male	55 (85.94)	13 (100)	22 (81.48)	10 (90.91)	10 (76.92)	0.325*
Female	9 (14.06)	0 (0)	5 (18.52)	1 (9.09)	3 (23.08)	
Smoking status, n (%)						
Current/ex smoker	45 (70.31)	9 (69.23)	19 (70.37)	6 (54.55)	11 (84.62)	0.465*
Never smoker	19 (29.69)	4 (30.77)	8 (29.63)	5 (45.45)	2 (15.38)	
Histologic type, n (%)						
Squamous cell carcinoma	38 (59.38)	7 (53.85)	16 (59.26)	4 (36.36)	11 (84.62)	0.155*
Adenocarcinoma	24 (37.5)	5 (38.46)	10 (37.04)	7 (63.64)	2 (15.38)	
Large cell carcinoma	1 (1.56)	0 (0)	1 (3.70)	0 (0)	0 (0)	
Adenosquamous carcinoma	1 (1.56)	1 (7.69)	0 (0)	0 (0)	0 (0)	
Histopathological grade, n (%)						
Poorly differentiated (grade 3)	32 (50)	8 (61.54)	16 (59.26)	3 (27.27)	5 (38.46)	0.05*
Moderately differentiated (grade 2)	24 (37.50)	5 (38.46)	9 (33.33)	3 (27.27)	7 (53.85)	
Well-differentiated (grade 1)	8 (12.50)	0 (0)	2 (7.41)	5 (45.45)	1 (7.69)	

†Independent t test; *Fisher's exact test.

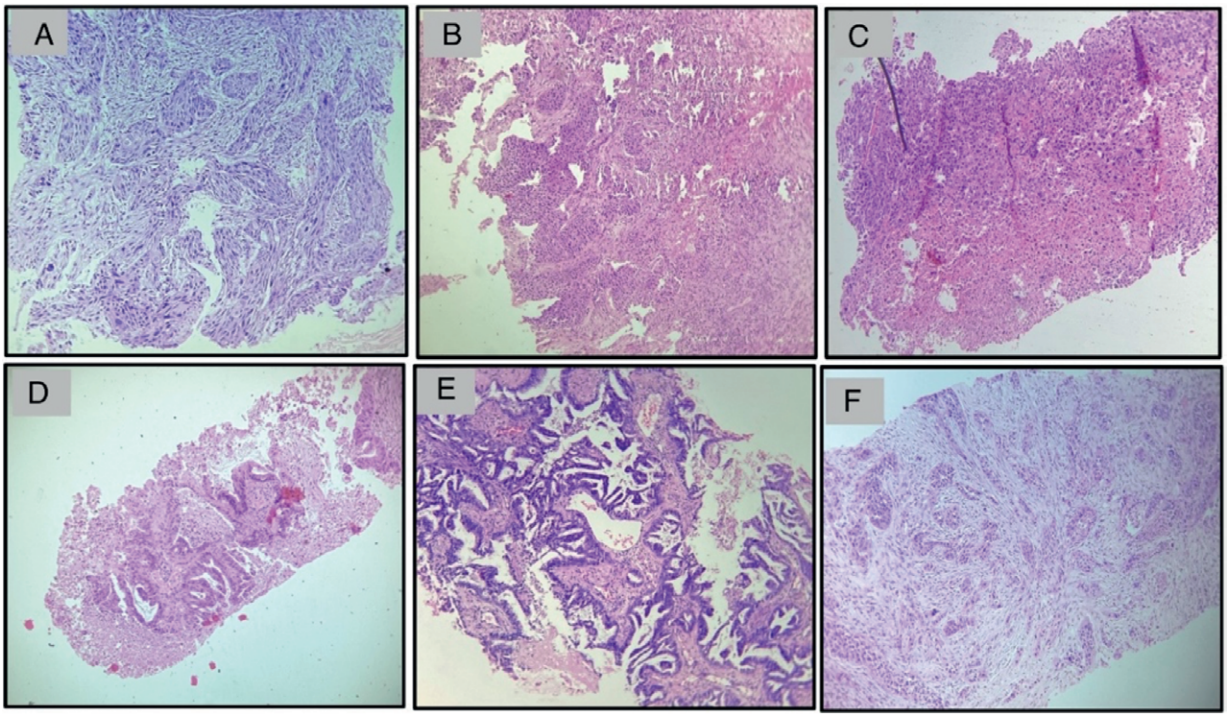

Figure 1. A) Hematoxylin and eosin-stained section showing grade 1 (well differentiated) squamous cell carcinoma (100×); B) grade 2 (moderately differentiated) squamous cell carcinoma (100×); C) grade 3 (poorly differentiated) Squamous cell carcinoma (100×); D) grade 1 (well differentiated) adenocarcinoma (100×); E) grade 2 (moderately differentiated) adenocarcinoma (100×) F) grade 3 (poorly differentiated) adenocarcinoma (100×).

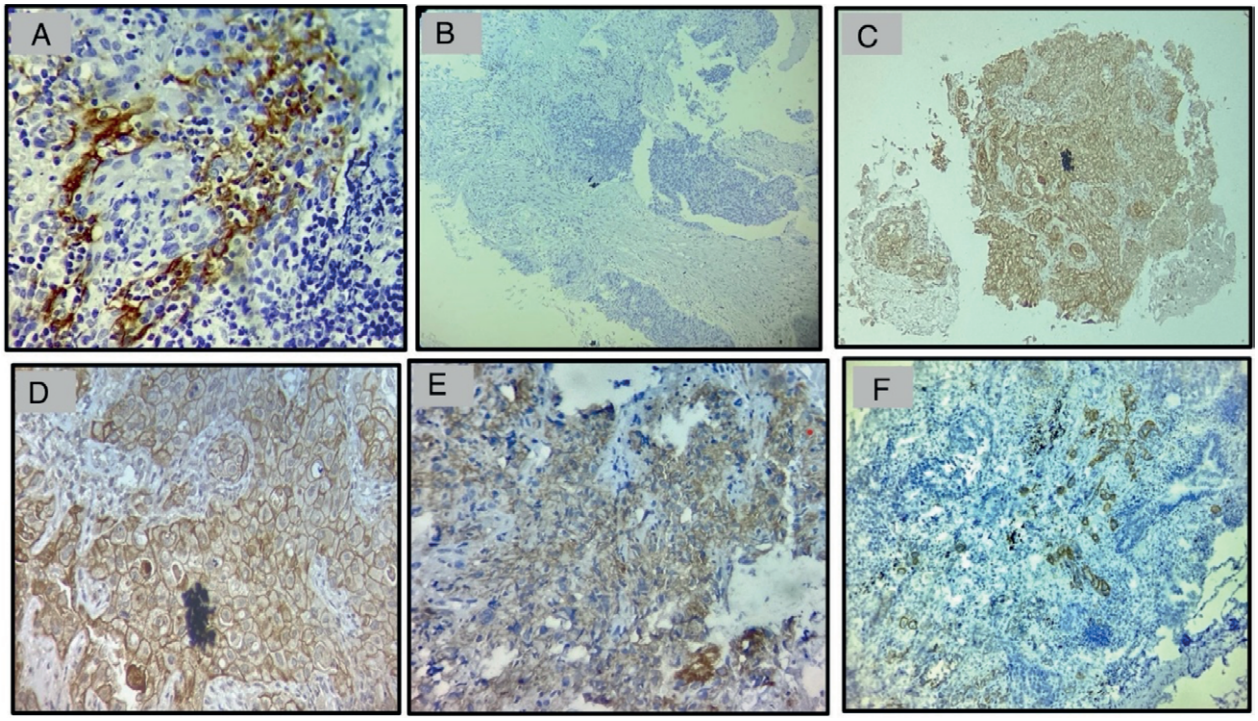

Figure 2. A) Lymphocytes (external control) showing membranous positivity for PD-L1 antibody (400×); B) immunohistochemistry (IHC) showing membranous staining for negative PD-L1 in squamous cell carcinoma (400×); C) IHC showing membranous staining for high PD-L1 in squamous cell carcinoma (100×); D) IHC showing membranous staining for high PD-L1 in squamous cell carcinoma (400×); E) IHC showing membranous staining for high PD-L1 in adenocarcinoma in (400×); F) IHC showing membranous staining for low PD-L1 in adenocarcinoma in 400× magnification.

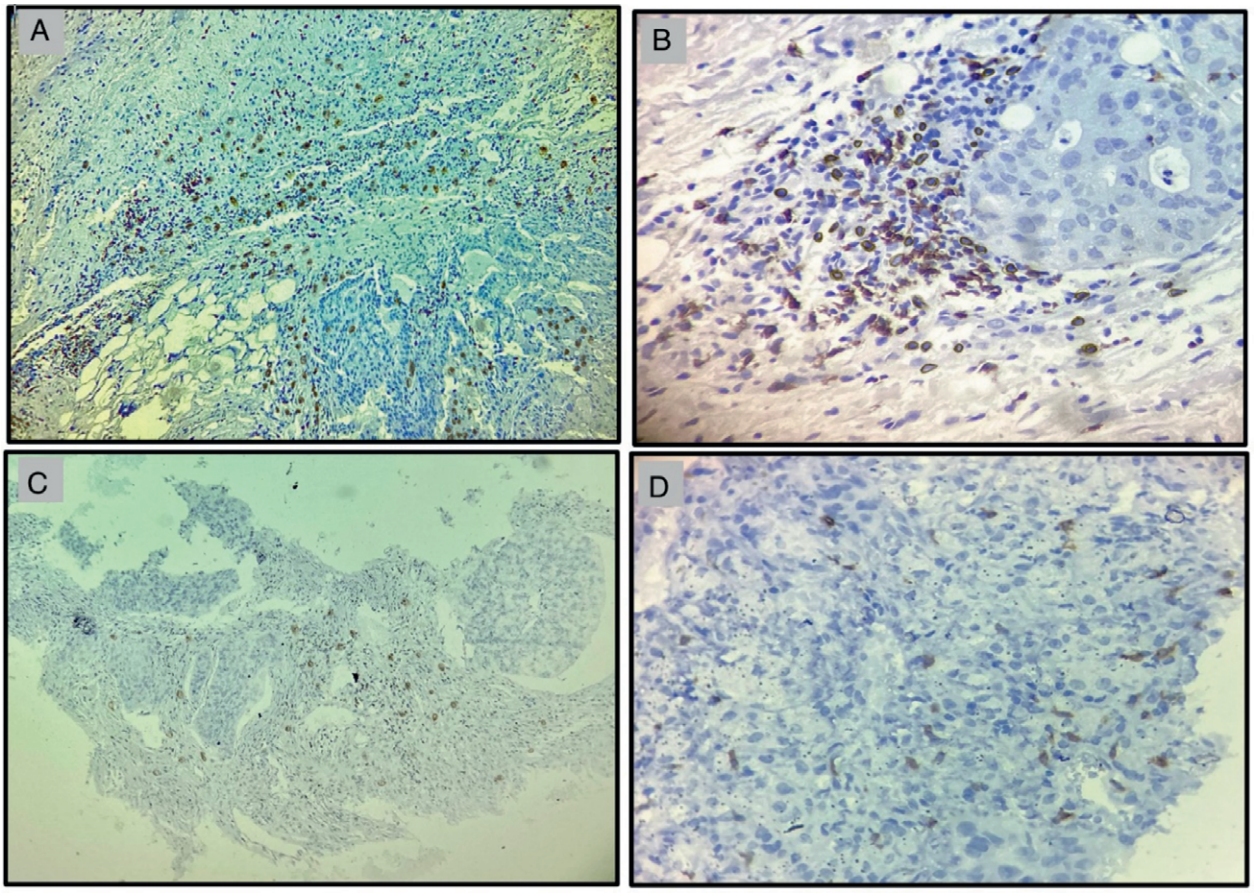

Figure 3. A) Immunohistochemistry (IHC) showing membranous staining for high CD8 tumor-infiltrating lymphocytes (TILs) in squamous cell carcinoma in 100× magnification; B) IHC showing membranous staining for high CD8 TILs in squamous cell carcinoma in 400× magnification; C) IHC showing membranous staining for low CD8 TILs in squamous cell carcinoma in 100X magnification; D) IHC showing membranous staining for low CD8 TILs in squamous cell carcinoma in 400× magnification.

Discussion and Conclusions

The study population included 64 cases, including 38 cases of squamous cell carcinoma, 24 cases of adenocarcinoma, and 1 case each of adenosquamous and large cell carcinoma. Out of 64 cases, grades 1, 2, and 3 were 2, 24 and 32, respectively. The mean age in our study population was 62 years (range 34-89 years). This aligns with the findings of Guindy *et al*., who reported a mean age of 65 years in their study [28]. The observed age distribution reflects global epidemiological trends, where NSCLC primarily affects older adults, with diagnosis typically occurring in the sixth to seventh decade of life [29]. In our study, out of 64 patients, 23 belonged to the age group of 51-60 years, followed by 22 patients aged 61-70 years, and 9 were in the age group of <50 years. Most of the cases in our study were males, 55 cases (86%), followed by females, 9 cases (14%). The male-to-female ratio was 6.1:1. Similar to our study, the study by Elaska *et al*. also had predominantly male cases [27]. In our study, 70.3% of patients were smokers, a finding that is consistent with most previous studies [27]. The high proportion of male smokers in this study mirrors global patterns, where smoking is more prevalent among males and is strongly associated with the development of lung carcinoma [1].

The most common histological type in our study was squamous cell carcinoma (59.5%), followed by adenocarcinoma (37.5%), with only a small proportion of cases diagnosed as adenosquamous carcinoma and large cell carcinoma (1.5% each). However, in a study by Jin *et al.*, the most common histological type was adenocarcinoma [30]. The rationale behind the difference can be attributed to the high prevalence of smoking in this study, as SCC is strongly associated with tobacco exposure. In a similar study done by Pawelcyzk *et al*., SCC was the most common histological type, followed by adenocarcinoma [31]. In our study, the majority of cases (50%) were poorly differentiated (grade 3), followed by moderately differentiated (37.5%) and well-differentiated tumors (12.5%). This distribution is consistent with other studies, such as those by Rashed *et al*., which similarly reported a predominance of poorly differentiated tumors in NSCLC [32].

Among 64 cases included in our study, PD-L1 positivity was observed in 62.5% of cases, with 55% of these cases showing high PD-L1 expression. These findings are in line with existing literature, where the prevalence of PD-L1 positivity in NSCLC ranges from 19% to 100%, depending on the cutoffs used for PD-L1 expression and the patient population studied [33-35]. Studies such as those by Rashed *et al*. and Guindy *et al*. have reported similar rates of PD-L1 positivity, especially in the advanced stages of

NSCLC [28,32]. However, in a few studies, PD-L1-positive cancer cells were found in a much smaller percentage [31,36]. Cooper *et al.* conducted a study on a group of 678 patients, and IHC was performed using tissue microarrays [36]. They found membranous PD-L1 expression in 32.8% of cases, with high expression observed in only 7.4% of NSCLC cases. This difference and the wide range of immunohistochemical expression may be related to different antibodies used, genetic and environmental factors, and sample size. Another explanation for the diverse expression of PD-L1 in NSCLC may be related to the use of different scoring methods and cut-off levels for evaluation. A study detected PD-L1 expression in 65.3% of NSCLC cases using the IRS scale, with a positive score above 3 [37]. Cooper *et al*. found PD-L1 expression in 32% of cases, considering positivity when over 50% of cells were stained, while Tang *et al.* reported 65.9% positivity with a threshold of 5% [36,37]. Our study used the TPS, a standard method in diagnostics, to assess PD-L1 expression and immunotherapy response, classifying expression as <1%, ≥1% to 49%, or ≥50%. Recent studies suggest that small biopsy specimens, such as cell blocks or core needle biopsies, may not accurately represent PD-L1 expression in NSCLC due to tumor heterogeneity and the dynamic dispersion of PD-L1 distribution [38]. Variability in PD-L1 expression may also result from sampling small areas or necrotic regions, leading to inconsistent evaluations. In addition, in our study, PDL1 expression was higher with the squamous cell carcinoma histology variant, but without a statistically significant difference. On the other hand, one study stated that the PD-L1 expression was significantly associated with adenocarcinoma, and another reported it to be associated with the squamous cell carcinoma variant [35,39].

The association between PD-L1 expression and clinicopathologic features remains controversial. In this study, PD-L1 expression was significantly associated with grade 3, thus pointing out that positive PD-L1 expression is associated with poor prognosis. High PD-L1 expression is often associated with aggressive tumor behavior and poor prognosis, as highlighted by the studies of Velcheti *et al*. and Ilie *et al*. [39,40] The overexpression of PD-L1 in poorly differentiated tumors, which was also observed in this study, reinforces the fact that poorly differentiated tumors may be more immunologically "cold", making them more resistant to immune surveillance. On the other hand, no significant relationship was detected between PD-L1 expression and clinical features, including age, gender, and smoking status. Jiang *et al*. showed that high PD-L1 expression was associated with male gender, smoking, and higher histologic grade [41]. On the other hand, some studies demonstrated no association of PD-L1 expression with age, gender, and smoking status [40]. These discrepancies among studies could be attributed to different sizes, baseline characteristics, PD-L1 antibodies used, and evaluation methods applied.

CD8-positive TILs have long been regarded as prognostic indicators in solid organ malignancies [21]. This has been especially evident in colorectal cancer, leading to efforts to establish a validated tool called the immunoscore to complement standard prognostic markers [42]. Some studies have indicated that increased CD8+ TIL infiltration correlates with improved survival outcomes, but other research has not found such associations [43-47]. Donnem *et al.* published the most consistent findings to date, proposing a straightforward scoring system for stromal CD8+ counts that demonstrates strong prognostic value [45]. Our study employed a similar methodology, reinforcing its reliability and reproducibility.

CD8 TIL density, a marker for immune infiltration and response, was found to be low in 62.5% of the cases, with only 37.5% exhibiting high CD8 TIL density. This is consistent with findings from studies such as Teng *et al*., which describe tumors with low immune infiltration as having an immunosuppressive microenvironment [24]. The low density of CD8 TILs observed in this study may explain these tumors' limited natural immune response, which correlates with more aggressive tumor behavior and potential resistance to immune-based therapies [29]. In this study, CD8 TIL density showed no significant association with age, sex, smoking status, histological type, or tumor grade. This aligns with a study done by Schalper *et al*., where CD8 TIL levels were not consistently linked to these clinical variables but were independently associated with improved survival, highlighting their predictive value [43].

Malignant cells expressing PD-L1 will interact with the negative signal-generating immune receptor on the surface of CD8+T cells and PD-1, thereby blocking anti-tumor activity. Therapeutic suppression of this interaction will show promise in treating many cancers by restoring functional antitumor T-cell activity [43,48]. Several studies have demonstrated an association between PD-L1 expression and CD8 TIL density in NSCLC. [37,44] However, our result revealed no significant correlation between PD-L1 and CD8+ TIL density. Consistent with our findings, Ameratunga *et al*. also observed no significant correlation between PD-L1 and CD8 TIL density in NSCLC patients [49].

PD-L1 expression on tumor cells can be driven either as a response to T-cell activity or *via* oncogenic signaling pathways [45]. Given this, evaluating CD8 TIL density alongside PD-L1 expression is increasingly critical. This study assessed the combined impact of PD-L1 expression and CD8 TIL density on the clinicopathological features of NSCLC. The tumor immune microenvironment was divided into four groups based on the PD-L1 and CD8-positive TIL status as previously proposed by Teng's classification [24]. These included type I (PD-L1 positive and high CD8-positive TILs), type II (PD-L1 negative and low CD8-positive TILs), type III (PD-L1 positive and low CD8-positive TILs), and type IV (PD-L1 negative and high CD8-positive TILs). In our study, the PD-L1+/CD8 TIL low group was significantly associated with grade 3 of tumor, suggesting that low CD8 TIL infiltration in PD-L1 positive tumors may be linked to more aggressive tumor behavior. The finding was consistent with those of Guindy *et al*., who found that the PD-L1^{+}/CD8 low group was significantly associated with high tumor grade and advanced tumor stage as compared to the PD-L1^{-}/CD8 high group. They also found that the PD-L1^{-}/CD8 high group had the best OS and PFS, whereas the PD-L1^{+}/CD8^{low} group showed the worst OS and PFS [37].

The combined analysis of PD-L1 expression and CD8 TIL density showed that 42.19% of cases were PD-L1 positive with low CD8 TIL density, a subgroup that is clinically significant in the context of immunotherapy. This PD-L1-positive and CD8 low phenotype represents a subset of tumors that may evade immune detection despite the presence of immune checkpoint markers like PD-L1. Studies, such as that by Hwang *et al*., have suggested that these tumors may rely more on PD-L1-mediated immune escape, making them prime candidates for anti-PD-1/PD-L1 therapies [50]. However, the low CD8 TIL density could indicate a need for combination therapies that enhance immune infiltration, such as immune-modulating agents, to improve patient outcomes. In contrast, PD-L1-negative tumors with low CD8 TIL density (20.31% of cases) represent an immune desert phenotype. Such tumors are generally less responsive to ICIs due to the lack of both immune activation (low CD8) and immune suppression markers (PD-L1

negative). This is supported by studies such as that by Herbst *et al*., which emphasize that PD-L1-negative tumors with low immune infiltration often require alternative treatment strategies beyond immune checkpoint blockade, potentially including chemotherapy or targeted therapies [51].

Interestingly, 20.31% of cases were PD-L1 positive with high CD8 TIL density, a phenotype typically associated with better responses to ICIs. High CD8 TIL infiltration alongside PD-L1 positivity suggests a pre-existing immune response that is being suppressed by PD-L1. Taube *et al*. and Tumeh *et al*. have shown that this group of patients often benefits the most from PD-1/PD-L1 blockade therapies, as the immune system may be primed to attack the tumor once the PD-L1-mediated suppression is lost [52,53]. Lastly, 17.19% of cases were PD-L1 negative with high CD8 TIL density, a group that may represent tumors with a functional immune response that is independent of PD-L1 signaling. These tumors might respond to immune therapies targeting other pathways, such as CTLA-4, or through therapies that increase TIL activation. This phenotype has been less frequently studied, but emerging research, such as that by McGranahan *et al*., indicates that high TIL infiltration, even in the absence of PD-L1 expression, could still be a marker of favorable prognosis, especially when combined with other immune-activating treatments [54].

Various mechanisms may account for the relationship between cytotoxic CD8 T lymphocytes in the tumor microenvironment and PD-L1 expression on tumor cells. One explanation is that cytotoxic T-cells recognize and target tumor cells, producing interferon-γ, which in turn induces PD-L1 expression on tumor cells, allowing them to evade the immune response. This could explain the PD-L1-positive/CD8-high phenotype. In contrast, tumors lacking T-cell infiltration are typically PD-L1 negative unless PD-L1 expression is driven by oncogenic factors, as seen in the PD-L1-negative/CD8-low group [24,55].

Additionally, PD-L1 can be constitutively expressed in tumor cells *via* oncogenic signaling, independent of T-cell presence. This intrinsic production of PD-L1 by tumor cells is referred to as intrinsic induction, exemplified by the PD-L1-positive/CD8-low group. In certain cases, genetic alterations may prevent tumor cells from expressing PD-L1, even in the presence of T-cell infiltrates, which could account for the PD-L1-negative/CD8-high group [24,55].

PD-L1 immunohistochemistry is currently recommended as a first-line screening tool in the management of patients with NSCLC. It plays a crucial role in identifying candidates for immunotherapy, carrying both prognostic and therapeutic significance [12]. However, PD-L1 expression alone is not sufficient to predict response to ICIs, as it does not fully capture the complexity of tumor-immune interactions [8]. Additional biomarkers are needed to more accurately predict response and minimize the risk of adverse effects from both chemotherapy and immunotherapy. CD8 TIL s have emerged as a promising complementary biomarker, providing further insight into the likelihood of response to ICIs when combined with PD-L1 status [10].

Limitations

Our study has several limitations that warrant consideration. First, a relatively small sample size limits the generalizability of our findings and may reduce the power to detect subtle associations. Second, while we employed relatively standardized scoring systems for the immunohistochemistry analyses of PD-L1 expression and CD8+ TIL counts, it is important to note that the PD-L1 scoring protocols differ across the various ICIs currently available. The PD-L1 IHC assay used in this study was a laboratory-developed test using the CAL10 clone antibody and is not an FDA-approved companion or complementary diagnostic assay, which may affect the clinical applicability of our results. While there is a standardized stromal CD8+ TIL scoring system, a consensus scoring protocol for CD8+ TILs in non-small cell lung carcinoma is still lacking. Also, the use of core biopsies may have introduced sampling bias due to intratumoral heterogeneity, potentially underestimating or overestimating PD-L1 and CD8 TILs levels. Finally, the lack of long-term follow-up data restricts our ability to evaluate the prognostic impact of PD-L1 expression and CD8+ TIL density on patient outcomes, particularly in the context of PD-L1-targeted therapies.

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