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ORIGINAL ARTICLE

Programmed death-ligand 1 expression and tumor-infiltrating lymphocytes in non-small cell lung cancer: association with clinicopathologic parameters

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Background: Data on the prevalence of programmed death-ligand 1 (PD-L1) expression and tumor-infiltrating lymphocytes (TILs) in non–small cell lung cancer (NSCLC) and their clinical significance in Indian patients are limited. **Methods:** Newly diagnosed NSCLC cases (adenocarcinoma or squamous cell carcinoma [SqCC] histology) were included in the present study. The TILs were evaluated based on morphology on hematoxylin and eosin–stained slides. PD-L1 expression in tumors was assessed using immunohistochemistry with rabbit monoclonal antibody (SP263) on the Ventana automated immunostainer. Tumors with PD-L1 expression >50% on tumor cells were considered PD-L1–positive. Tumors in which TILs occupy >25% of stroma were considered to have high TILs. The association of PD-L1 expression and TILs with various clinical parameters including overall survival (OS) was investigated. **Results:** The present study included 128 cases of NSCLC (67 adenocarcinoma, 61 SqCC). PD-L1 positivity was observed in 17.2% of the patients with NSCLC. Baseline characteristics of PD-L1–positive subjects were similar to PD-L1–negative subjects except for a higher prevalence of liver metastasis (18.2% vs. 2.8%; p=.018) and a higher probability of diagnosis from extrapulmonary biopsies. High TILs were observed in 26.6% of the subjects. However, PD-L1 expression and high TIL did not affect OS. **Conclusions:** PD-L1 positivity and high TILs were observed in 20% and 25% of the patients with NSCLC, respectively, however, neither were predictors of survival in SqCC.

Key Words: Lung neoplasms; Non-small cell lung carcinoma; PD-L1; Immunotherapy; Tumor-infiltrating lymphocytes

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Interaction of tumor cells with their microenvironment may result in infiltration of the tumor by immune cells of the host. Although such immune cell infiltration does not universally occur in all tumors, when present, these tumor-infiltrating lymphocytes (TILs) may provide tumor-specific immune response to prevent further tumorigenesis. However, tumor cells may evade host immunity through the expression of several transmembrane proteins including programmed death-ligand 1 (PD-L1). PD-L1–targeted therapy is a form of immunotherapy which targets the components of the PD-L1 pathway to prevent downregulation of anti-tumoral immunity. Unfortunately, PD-L1–targeted therapy is effective in < 50% of non-small cell lung cancers (NSCLCs) despite the presence of PD-L1 expression. However, in general, better results are observed in patients with higher PD-L1 expression [1–4]. Therefore, testing for PD-L1 expression using immunohistochemistry is often performed before initiating PD-L1–targeted therapy. The prevalence of PD-L1 positivity in NSCLC varies widely across studies performed in different parts of the world [2–5]. A large part of this difference could be attributed to methodological differences which is supported by the results of a recent multinational study (EXPRESS) which showed similar PD-L1 expression in advanced NSCLCs across various geographical regions when a uniform testing strategy was used [6]. However, the reported prevalence of PD-L1 expression in Indian patients with NSCLC, albeit with different methods, is much lower [7,8]. Furthermore, the PD-L1 expression in NSCLC has been associated with certain clinicopathological characteristics and poor clinical outcomes in some but not all studies [5]. The intensity of tumor infiltration by TILs has been associated with survival [9,10]. In the present study, the prevalence and prog-

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nostic significance of PD-L1 expression in a cohort of NSCLC patients from North India were reported.

MATERIALS AND METHODS

Patients

This was a single institute, prospective, observational study conducted over 2 years. Consecutive patients with newly diagnosed NSCLC with adenocarcinoma or squamous cell carcinoma (SqCC) histology were considered for inclusion in this study. Subjects with a biopsy sample insufficient for the necessary histopathological analyses were excluded from the study.

Histopathological examination

All specimens were processed for paraffin sections for routine hematoxylin and eosin (H&E) staining and immunohistochemistry. The specimens included lung biopsies (bronchoscopic biopsies and computed tomography [CT]/positron emission tomography (PET)–guided biopsies) and biopsies from metastatic sites (lymph nodes, pleura, bone, and brain). Cytology samples were not included in the study. Based on H&E, the tumors were categorized as adenocarcinoma or SqCC as defined by the *2015 World Health Organization classification of lung tumors* [11]. Tumors in which histological subtyping on the basis of routine H&E staining was difficult, immunohistochemistry with p63, thyroid transcription factor 1, cytokeratin (CK) 5/6, CK7, neuron-specific enolase, and CD56 was used.

Tumor-infiltrating lymphocytes

The TILs were evaluated based on morphology on H&E-stained slides. Only lymphocytes and plasma cells were included in the scoring. Necrotic areas within the tumor and alveolar macrophages were not included in the scoring [12]. TILs were assessed in five areas of the tumor to determine the mean TIL score. The TIL score was assigned based on the proportion of tumor stroma occupied by TILs (TIL0, 0 to ≤5%; TIL1, >5% to ≤25%; TIL2, >25 to ≤50%; TIL3, >50%). Subjects with a score of TIL0 were considered to have low TILs and subjects with a score of TIL2 or TIL3 were considered to have high TILs.

PD-L1 expression

PD-L1 expression in tumors was assessed using immunohistochemistry with rabbit monoclonal antibody (SP263) on the Ventana automated immunostainer. Detection was optimized with the OptiView DAB IHC Detection Kit (Ventana Medical Systems, Tucson, AZ, USA). For positive controls, sections of the human placenta, as recommended in the data sheet of SP263 antibody (Fig. 1A) as well as human tonsil, were included in each batch. A negative control was run for each case. At least 100 viable tumor cells were scored. Tumor cells were considered to express PD-L1 when they showed complete or partial membranous staining with or without cytoplasmic staining of any intensity. PD-L1 expression on tumor cells was assigned scores based on the proportion of tumor cells (TC0, 0 to <1%; TC1, ≥1 to ≤50%; TC2, >50%) expressing PD-L1. Tumors with a PD-L1 expression on tumor cells >50% (TC2) were considered PD-L1–positive.

Mutation analysis

Epidermal growth factor receptor (*EGFR*) mutations and anaplastic lymphoma kinase (ALK) rearrangements were evaluated in subjects with adenocarcinoma histology. *EGFR* mutation analysis was performed using real-time PCR (EnteroGen, Agility Biotech, Los Angeles, CA, USA). ALK rearrangements were identified on immunohistochemistry performed on a Ventana BenchMark XT automated slide-processing system using the D5F3 clone [13].

Clinical details

The following clinical parameters were recorded at baseline: age, sex, smoking status, body mass index, performance status, and TNM. Performance status was evaluated using the Eastern Cooperative Oncology Group scale [14]. Contrast-enhanced CT scan of the thorax and upper abdomen (including the liver and adrenals) or whole-body PET was obtained for baseline staging evaluation in all patients. Tumor staging was performed using the seventh edition of the American Joint Committee on Cancer (AJCC) TNM classification [15]. Tumor staging was also performed using the eighth edition of the AJCC TNM classification in 106 patients (Supplementary Table S1). Because the proportion of patients between the seventh and eighth edition of the AJCC TNM classification was not significantly different, and to ensure completeness of data, the staging in the seventh edition of the AJCC TNM classification was used in the present study.

Subjects were treated with chemotherapy, targeted therapy, immunotherapy, radiotherapy, or surgery as indicated by tumor histopathology, mutation status, and clinical status, as previously described [16]. Briefly, subjects with adenocarcinoma without any driver mutation were treated with pemetrexed-based platinum doublet followed by maintenance pemetrexed therapy until disease progression [17]. Subjects with squamous histology were treated with docetaxel or gemcitabine-based

platinum doublet. All patients receiving chemotherapy were administered at least four cycles of chemotherapy before response assessment using the Response Evaluation Criteria in Solid Tumors ver. 1.0 [18]. Subjects with sensitizing *EGFR* mutation or *ALK* rearrangement were treated with appropriate EGFR tyrosine kinase or ALK inhibitors, respectively [19,20].

Statistical analysis

Continuous variables were expressed as the mean and standard deviation and categorical values were expressed as the numbers and percentages. The differences between continuous and categorical variables were analyzed using the Mann-Whitney U test and the chi-square test (or Fisher exact test), respectively. Overall survival (OS) was defined as the time between initial diagnosis and date of death or last follow-up. The cutoff date for survival analysis was November 30, 2018. Survival curves were generated using the Kaplan-Meir method and were compared using the log-rank test. Multivariate analysis to identify predictors of survival was performed using the Cox proportional hazard model. Statistical analyses were performed using the commercial statistical package SPSS (IBM SPSS Statistics, ver. 22, IBM Corp., Armonk, NY, USA). A p-value < 0.05 was considered statistically significant.

RESULTS

A total of 128 cases of NSCLC were included in the present study (Table 1); 103 (80.5%) were males and the median age of the study population was 61 ± 15 years. The majority of patients had advanced disease (84.1% were stage IIIB/IV) and 44.8% of the patients had extrathoracic disease at diagnosis. Tissue samples (small biopsies: endobronchial biopsies, transbronchial biopsies, and imaging-guided core biopsies) were predominantly obtained from the lung (82.4%) and metastatic lymph nodes (8%), and 9.6% were resection samples. Adenocarcinoma and SqCC were observed in 52.3% and 47.7% of the subjects, respectively. A larger proportion of patients who were younger, female, non-smokers, and presented with advanced disease had adenocarcinoma. *EGFR* and *ALK* alterations were observed in 6.2% and 5.4% of the subjects, respectively. Chemotherapy (74.2%) was the most commonly used first-line therapy. Due to economic constraints, PD-L1–targeted therapy despite PD-L1 expression could not be provided to any patient in this study.

Based on immunohistochemistry, the majority of patients had PD-L1 expression on < 1% of tumor cells (TC0, 61.7%) (Table 2, Fig. 1). PD-L1 expression on ≥ 1% of tumor cells (TC1 or TC2) was observed in 38.3% of the subjects. However, this did not differ between subjects with adenocarcinoma or SqCC (p = .335). TIL1 (31.3%) and TIL0 (23.4%) were the most commonly observed TIL scores (Table 2, Fig. 2). High TILs (TIL occupying > 25% of tumor stroma, TIL2 or TIL3) were observed in 26.6% of patients. Infiltration > 50% of tumor stroma by TILs (TIL3) occurred in the least number of cases (7.8%). Difference in the TIL scores between adenocarcinoma and SqCC was not observed (p = .126). The baseline characteristics were not different between subjects with high and low TILs (data not shown). A higher proportion of patients with PD-L1 expression

Table 1. Baseline clinical characteristics

Characteristic	Total (n = 128)	Adenocarcinoma (n = 67)	SqCC (n = 61)	p-value
Age (yr)	59.5 ± 11.1	57.5±11.4	61.8 ± 10.5	.047
Male sex	103 (80.5)	45 (67.2)	58 (95.1)	< .001
Smokers[a]	95 (74.2)	39 (58.2)	56 (94.9)	< .001
Body mass index (kg/m^2)	20.3 ± 4.0	20.9 ± 3.9	19.7 (4.0)	.093
ECOG PS score ≥ 2	19 (14.8)	9 (13.4)	10 (16.4)	.638
TNM stage at diagnosis[a]				.024[b]
I	2 (1.6)	2 (3.0)	0	
II	5 (4.0)	2 (3.0)	3 (5.1)	
IIIA	13 (10.3)	2 (3.0)	11 (18.6)	
IIIB	27 (21.4)	9 (13.4)	18 (30.5)	
IV	79 (62.7)	52 (77.6)	27 (45.8)	
Extrathoracic disease[c]	56 (44.8)	41 (61.2)	15 (25.9)	< .001
Biopsy site[c]				.087[d]
Lung	103 (82.4)	50 (75.8)	53 (89.8)	
Lymph node	10 (8.0)	6 (9.1)	4 (6.8)	
Pleura	5 (4.0)	5 (7.6)	0	
Other	7 (5.6)	5 (7.6)	2 (3.4)	
EGFR-positive	8 (6.2)	8 (11.9)	0	.005
ALK-positive	7 (5.4)	7 (10.4)	0	.009
First-line treatment				.011
Chemotherapy	95 (74.2)	49 (73.1)	46 (75.4)	
Targeted therapy[e]	16 (12.5)	13 (19.4)	3 (4.9)	
Other	17 (13.3)	5 (7.5)	12 (19.7)	

Values are presented as mean ± SD or number (%).
SqCC, squamous cell carcinoma; ECOG PS, Eastern Cooperative Oncology Group performance status; EGFR, epidermal growth factor receptor; ALK, anaplastic lymphoma kinase; SD, standard deviation.
[a]Data were not available in two patients; [b]For comparison of stage IIIB or IV between adenocarcinomas and SqCCs; [c]Data were not available in three patients; [d]For comparison of lung biopsy between adenocarcinomas and SqCCs; [e]Patients who had a high probability of underlying *EGFR* mutation (e.g., non-smoking females) in whom molecular testing could not be performed (inadequate tissue for molecular analysis in the initial sample with the patient unwilling or unfit for a repeat invasive procedure) and whose performance status did not permit the use of chemotherapy were provided targeted therapy on compassionate grounds. Therefore, the number of patients with driver mutations and the number of patients who received targeted therapy was not equal.

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Table 2. Histopathological characteristics of the overall population

Variable	Total (n=128)	Adenocarcinoma (n=67)	SqCC (n=61)	p-value
PD-L1 TC score				.335[a]
TC0	79 (61.7)	44 (65.7)	35 (57.4)	
TC1	27 (21.1)	8 (11.9)	19 (31.1)	
TC2	22 (17.2)	15 (22.4)	7 (11.5)	
TIL score[b]				.126[c]
TIL0	30 (23.4)	18 (26.9)	12 (19.7)	
TIL1	40 (31.3)	22 (32.8)	18 (29.5)	
TIL2	24 (18.8)	10 (14.9)	14 (23.0)	
TIL3	10 (7.8)	4 (6.0)	6 (9.8)	

Values are presented as number (%).
The TC score was assigned based on the proportion of tumor cells expressing PD-L1 (TC0: <1%, TC1: ≥1 but ≤50%, TC2: >50%). The TIL score was assigned based on the proportion of tumor stroma occupied by TILs (TIL0: ≤5%, TIL1: ≤25%, TIL2: >25 but ≤50%, TIL3: >50%).
SqCC, squamous cell carcinoma; PD-L1, programmed death-ligand 1; TC, tumor cell; TILs, tumor-infiltrating lymphocytes.
[a]For comparison of the proportion of subjects with PD-L1 expression on ≥ 1% of tumor cells (TC1 or TC2) between adenocarcinomas and SqCCs; [b]TIL score was not available for 26 patients; [c]For comparison of the proportion of subjects with ≥25% of tumor stroma infiltrated by TILs (TIL2 or TIL3) between adenocarcinomas and SqCCs.

≥1% on tumor cells (TC1 or TC2: 58.8% vs. 35.7%, p=.026) had high TILs than low TILs.

A total of 22 subjects (17.2%) were classified as PD-L1–positive (PD-L1 expression on tumor cells >50%, TC2). Most of the clinical characteristics of PD-L1–positive subjects were not different from PD-L1–negative subjects (Table 3). However, subjects who were PD-L1–positive had a higher prevalence of liver metastasis at baseline (18.2% vs. 2.8%; p=.018). PD-L1–positive subjects were also more often diagnosed based on extrapulmonary biopsies (Table 3). The median OS (95% confidence interval [CI]) was 7.6 (6.5–8.8) months in subjects who were PD-L1–negative compared with 8.5 (1.1–16.0) months in subjects who were PD-L1–positive (log-rank p=.584) (Fig. 3). On multivariate analysis, sex was the only factor associated with survival (hazard ratio, 0.39; 95% CI, 0.16 to 0.98; p=.046) (Table 4). The survival of patients with tumors showing high TILs was not different from subjects with low TILs (data not shown).

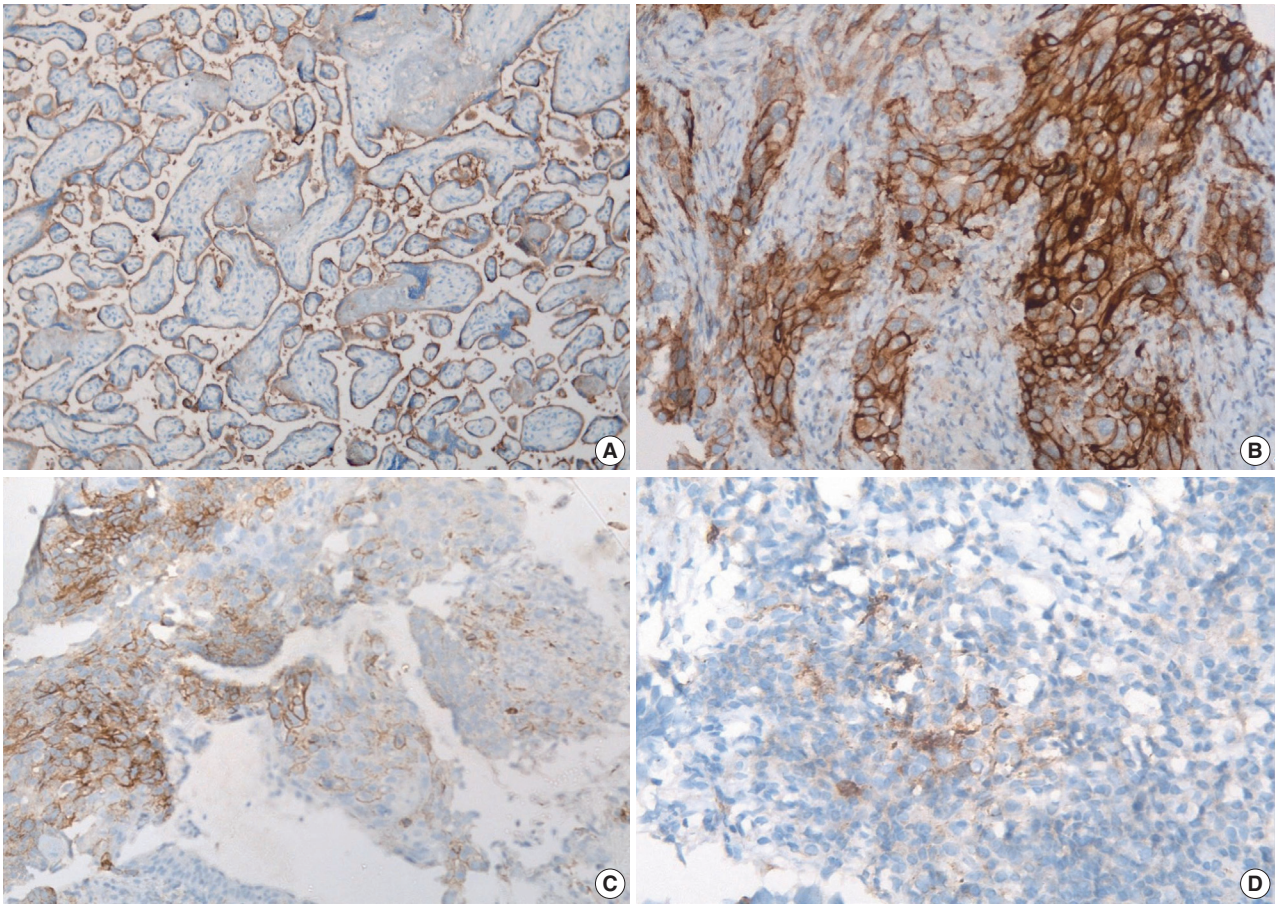

Fig. 1. Photomicrographs (programmed death-ligand 1 [PD-L1] staining using SP263 clone) showing positive control (PD-L1 staining in placenta) (A), TC2 (PD-L1 expression on >50% of tumor cells [TCs]) (B), TC1 (PD-L1 expression on 1%–50% TCs) (C), and TC0 (PD-L1 expression on <1% of TCs) (D).

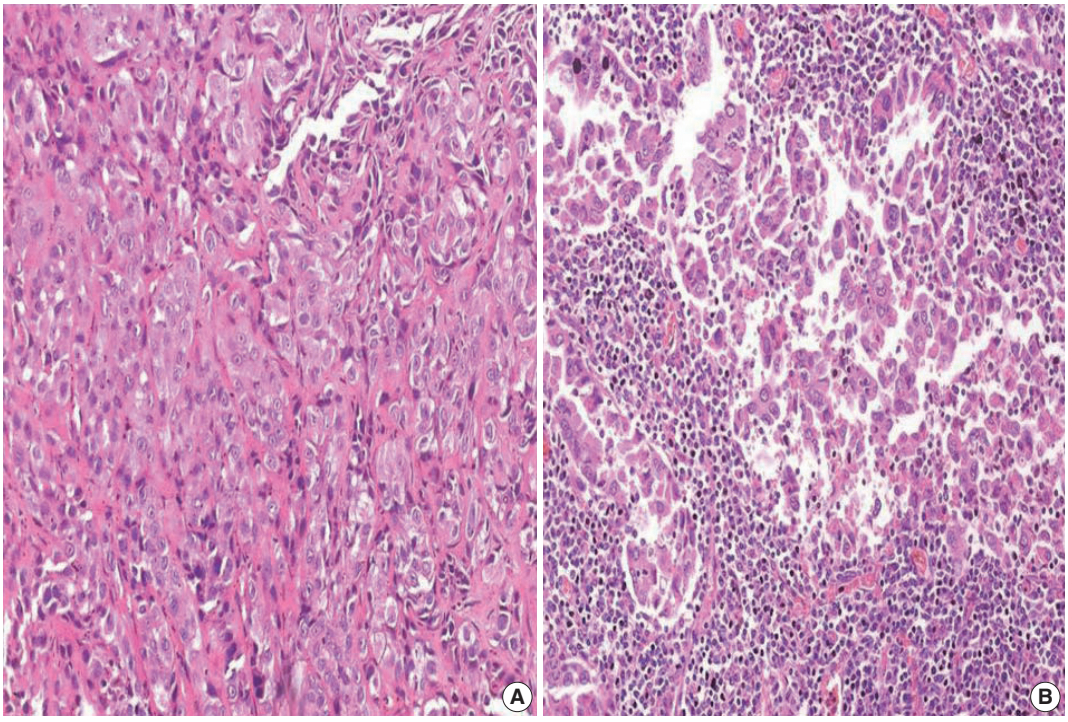

Fig. 2. Photomicrographs (×200) of hematoxylin and eosin staining showing TIL0 (0%–5% of tumor stroma occupied by tumor-infiltrating lymphocytes [TILs]) (A), TIL3 (>50% of tumor stroma occupied by TILs) (B).

DISCUSSION

In this study, 17.2% of the patients with NSCLC could be labelled as PD-L1–positive (PD-L1 expression on tumor cells >50%). PD-L1 expression ≥1% on tumor cells was observed in 38.3% of the subjects. PD-L1–positive subjects had a higher prevalence of liver metastasis and were more often diagnosed using biopsy samples obtained from extrapulmonary sites. The PD-L1 expression did not affect survival in the study population. High TILs (TILs occupying >25% of tumor stroma) were observed in 26.6% of the subjects, however, it did not affect survival. A larger proportion of patients with higher PD-L1 expression on tumor cells was more associated with subjects with high TILs than low TILs.

The assessment of PD-L1 expression based on immunohistochemistry is complicated by several factors including intra-tumor heterogeneity and inter-assay variation [21]. Therefore, the estimated prevalence of PD-L1–positive NSCLCs varies widely (7%–75%) [5,22,23]. In the multinational EXPRESS study, PD-L1 expression on ≥50% and ≥1% of tumor cells was observed in 22% and 52% of patients with advanced NSCLC, respectively, using the 22C3 pharmDx kit [6]. In the present study, PD-L1 positivity was observed in 17.2% of NSCLCs (38.3% had PD-L1 expression ≥1% on tumor cells). The SP263 antibody clone was used because its performance is comparable or better than the other available antibody clones [24-26]. In prior studies from India, a PD-L1 positivity rate of 27% (>5% PD-L1 expression on tumor cells) and 34% (≥1% PD-L1 expression on tumor cells) was reported using the SP142 and SP263 clones, respectively [7,8]. Overall, the reported rates of PD-L1 expression in lung cancer in India appears to be less than international estimates. Whether this difference is due to methodological differences or true geographic differences is unclear.

PD-L1 expression has been associated with male sex, smoking, advanced tumor stage, SqCC histology, and *EGFR* mutation [5,22,27,28]. In the present study, association between PD-L1 expression and sex, smoking, or TNM stage, was not found. Liver metastasis was more common in subjects who were PD-L1–positive. The PD-L1 positivity rate was not affected by histology.

The association between PD-L1 expression and survival is controversial [5,7,22]. In a meta-analysis of 41 studies, PD-L1 expression was associated with poor survival in NSCLCs, specifically in subgroups of patients with adenocarcinoma or early disease [5]. In contrast, another study showed that PD-L1 expression resulted in worse prognosis in SqCC but not adenocarcinoma [22]. Furthermore, in another study, PD-L1 positivity in immune cells was found associated with better prognosis in resected NSCLCs [7]. In the present study, PD-L1 expression was not as-

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Table 3. Difference in clinical characteristics between PD-L1–positive and PD-L1–negative subjects

Variable	PD-L1 negative (n = 106)	PD-L1 positive (n = 22)	p-value
Age (yr)	59.6 ± 10.6	59.5 ± 13.6	.934
Male sex	88 (83.0)	15 (68.2)	.240
Smokers[a]	82 (77.3)	13 (59.1)	.051
Body mass index (kg/m^2)	20.5 ± 4.0	19.3 ± 3.7	.308
ECOG score ≥ 2	17 (16.0)	2 (9.1)	.525
TNM stage at diagnosis[a]			.649
I	2 (1.9)	0	
II	4 (3.8)	1 (4.5)	
IIIA	11 (10.4)	2 (9.1)	
IIIB	25 (23.6)	2 (9.1)	
IV	62 (58.5)	17 (77.3)	
Extrathoracic disease at baseline[b]	44 (41.5)	12 (54.5)	.311
Liver metastasis	3 (2.8)	4 (18.2)	.018
Biopsy site[b]			.022
Lung	91 (85.8)	12 (54.5)	
Lymph node	6 (5.7)	4 (18.2)	
Pleura	3 (2.8)	2 (9.1)	
Other	4 (3.8)	3 (13.6)	
Histology			.102
Adenocarcinoma	52 (49.1)	15 (68.2)	
Squamous cell carcinoma	54 (50.9)	7 (31.8)	
EGFR-positive	5 (4.7)	3 (13.6)	.138
ALK-positive	5 (4.7)	2 (9.1)	.345
First-line treatment			.664
Chemotherapy	80 (75.5)	15 (68.2)	
Targeted therapy	12 (11.3)	4 (18.2)	
None	14 (13.2)	3 (13.6)	
TIL score (%)[c]			.890
0–5	23 (28.0)	7 (31.8)	
6–25	33 (40.2)	7 (31.8)	
26–50	18 (22.0)	6 (27.3)	
>50	8 (9.8)	2 (9.1)	

Values are presented as mean ± SD or number (%).
Tumors with PD-L1 expression > 50% in tumor cells (TC2) were considered PD-L1-positive.
PD-L1, programmed death-ligand 1; ECOG, Eastern Cooperative Oncology Group; EGFR, epidermal growth factor receptor; ALK, anaplastic lymphoma kinase; TIL, tumor-infiltrating lymphocyte; SD, standard deviation.
[a]Data were not available in two patients; [b]Data were not available in three patients; [c]TIL score was not available for 24 patients.

sociated with survival.

Infiltration ≥ 20% of tumor stroma with TILs has been observed in 50% of subjects with NSCLCs [26]. Higher TILs have been associated with higher PD-L1 expression and better survival in NSCLCs [9,10,29]. In the present study, approximately one-fourth of the tumors showed high TILs (infiltration > 25% of tumor stroma by lymphocytes TIL2 or TIL3). Although high TILs were associated with higher PD-L1 expression in both TILs and tumor cells, high TILs did not affect survival in our study cohort.

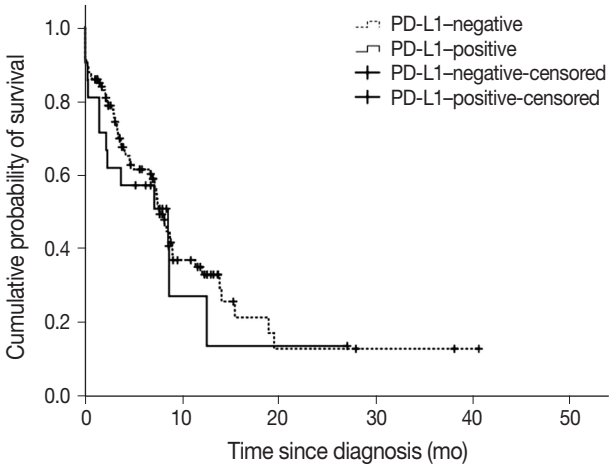

Fig. 3. Kaplan-Meir plots for overall survival (OS). OS of programmed death-ligand 1 (PD-L1)–positive subjects was not different from PD-L1–negative subjects. The median (95% confidence interval) OS was 7.6 months (6.5–8.8) in subjects who were PD-L1–negative compared with 8.5 months (1.1–16.0) in subjects who were PD-L1-positive (log-rank p = .584).

Table 4. Multivariate analysis for predictors of OS

Variable	HR (95% CI)	p-value
Age	1.00 (0.98–1.03)	.708
Female sex	0.39 (0.16–0.98)	.046
Smoking	0.84 (0.36–2.00)	.682
ECOG PS ≥ 2	0.66 (0.30–1.45)	.297
TNM stage IIIB or IV	1.68 (0.82–3.44)	.154
Adenocarcinoma histology	0.78 (0.46–1.33)	.358
PD-L1–positive	1.47 (0.78–2.77)	.231

Tumors with PD-L1 expression > 50% on tumor cells (TC2) or PD-L1 expression of 1%–50% on tumor cells (TC1) with PD-L1 expression > 10% in TILs (IC2) were considered PD-L1–positive.
OS, overall survival; HR, hazard ratio; CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance status; PD-L1, programmed death-ligand 1; TIL, tumor-infiltrating lymphocytes.

This was a single-center study with a relatively small sample size, thus, the results, especially subgroup analyses, should be interpreted cautiously. The TNM stage was not a prognostic factor in the present study for several reasons. The study had a relatively small sample size (n = 128). A disproportionately larger proportion of patients had stage IV disease (62.7% with stage IV disease in this study compared with 51.1% with stage IV disease in a previous analysis from our center with a much larger sample size (n = 1,501) [14]. The EGFR and ALK positivity rate observed in this study was considerably lower than what is generally observed at our center possibly due to selection bias [30]. Subjects who had undergone mutation testing could have been excluded from this study because they might have been left with inadequate tissue specimen for additional histopathological analyses.

Furthermore, a considerable proportion of lung cancer patients diagnosed based on cytology samples were not included in this study.

In conclusion, the present study results showed PD-L1 positivity and high TILs can be observed in approximately one-fifth and one-fourth of the patients with NSCLC, respectively. However, PD-L1 expression or high TILs did not affect the OS in our study cohort.

Supplementary Information

The Data Supplement is available with this article at https://doi.org/10.4132/jptm.2021.08.08.

Ethics Statement

Written informed consent was obtained from all the study participants. The study was approved by the Institutional Ethics Review Committee (Reference number: NK/4398/MD/381).

Availability of Data and Material

The datasets generated or analyzed during the study are available from the corresponding author on reasonable request.

Code Availability

Not applicable.

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Conceptualization of study: AB. Data curation: GG, KTP, NS, PG. Formal analysis: AB, AD, VM. Investigation: GG. Methodology: AB, AD, VM, GG, PG. Resources: AB, NS, KTP, GG, PG. Supervision: AB. Validation: AB, AD, KTP, NS. Writing—original draft: AB, GG. Writing—review & editing: AB, KTP, PG, AD, NS. Approval of final manuscript: all authors.

Conflicts of Interest

The authors declare that they have no potential conflicts of interest.

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