Original Article

Correlation of programmed death-ligand 1 expression with gene expression and clinicopathological parameters in Indian patients with non-small cell lung cancer

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ABSTRACT

Objectives: The aim of this study is to evaluate the incidence of programmed cell death-ligand 1 (PD-L1) expression in non-small cell lung cancer (NSCLC) cases and its correlation with gene mutation and clinicopathological parameters. **Methods:** Samples from NSCLCs patients were studied for PD-L1 expression through immunohistochemistry (IHC) using Rabbit anti-human PDL-1/CD274 Monoclonal Antibody. Genetic mutations were studied using IHC/fluorescence *in situ* hybridization (FISH) methods (for anaplastic lymphoma kinase [ALK]) or polymerase chain reaction/gene sequencing analysis (for epidermal growth factor receptor [EGFR]). Pearson's correlation coefficient (*r*) was used for correlation analysis. PD-L1 expression was analyzed for association with clinicopathological features. **Results:** Of the 101 NSCLC cases, PD-L1 expression was observed in 33.66% (34/101) cases; tumor proportion score of <50%: 67.65% (23/34) and ≥50%: 32.35% (11/34) cases. PD-L1 positivity was seen in; males: 35.5%, females: 28%, smokers: 37.7%, cases with brain metastasis: 20%, cases with pleural effusion: 20.8%, and histopathological evaluation (well-differentiated: 21.42%, moderately-differentiated: 13.79%, poorly-differentiated: 36.11%, and adenosquamous disease: 40.9%). Genetic mutation studies revealed PD-L1 positivity in 18.1% cases with EGFR mutation, 50% of ALK-IHC positive cases, and 33.3% ALK-FISH positive cases. No or very weak correlation ($r < 0.3$) in PD-L1 expression with gene mutations or clinicopathological parameters was observed. **Conclusions:** The study demonstrated PD-L1 expression in ~ 1/3rd cases of NSCLC patients. No or very weak correlation was observed for PD-L1 expression with genetic mutations and other parameters studied. The presence of gene mutations in PD-L1 expressed samples suggests further investigation on PD-L1 inhibitors in such patients for decisive treatments.

KEY WORDS: Anaplastic lymphoma kinase, clinicopathological, epidermal growth factor receptor, non-small cell lung cancer, programmed cell death-ligand 1

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INTRODUCTION

Non-small cell lung cancer (NSCLC) constitutes most (~85%) of the lung cancer cases, and at the time of diagnosis, majority of these patients present with an inoperable advanced disease.[1,2] The treatment armamentarium of advanced NSCLC has an increasingly important role for molecular-targeted therapy.[3] A large

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proportion of NSCLC patients represent with driver gene mutation including epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK), which have been shown to affect chemotherapy treatment outcomes. Targeted agents to the genetic alterations have led to the development of newer agents with improved treatment outcomes.[4]

The genetic alterations in the cancer cells lead to the development of immune resistance mechanisms, of which, "immune-inhibitory pathways," termed "immune checkpoints" such as programmed cell death protein 1 (PD1) inhibits the T-cell proliferation and activation by combining with tumor cells.[5] The blockade of PD1/PD ligand 1 (PD-L1) has been established as a novel target for NSCLC immunotherapy,[6,7] and several anti-PD-1/PD-L1 agents including nivolumab, pembrolizumab, and atezolizumab are approved for the treatment of advanced NSCLCs.[7] PD-L1 is a predictive biomarker that provides information on the probability of response to the PD-1/PD-L1 inhibitors, and thus assists in optimizing NSCLC therapy decisions.[8] Furthermore, predictive biomarkers are the need of the hour for the management of cancer to identify patients which are more likely to benefit from immunotherapy in view of the high cost of treatment for an indefinite duration.

Preclinical studies have indicated the correlation of genetic mutations (EGFR, ALK, and KRAS) with PD-L1 expression, though the underlying mechanism is not known.[7] Clinical studies have also evaluated the correlation of PD-L1 expression with genetic mutations; however, the data on Indian patients with NSCLC are scarce. In the current report, we evaluated the incidence of PD-L1 expression and its correlation with gene mutation along with other clinicopathological parameters including gender, smoking status, presence of brain metastasis, pleural effusion, and histopathological subtypes in Indian patients with NSCLC.

METHODS

Study design, population, and methodology

This was an observational study, and all patients who gave consent for the study were included in the study. The data of 101 adult patients (≥18 years) with proven NSCLC (adenocarcinoma) are presented. The patients were registered at Malignant Diseases Treatment Center, Army Hospital Research and Referral, New Delhi, India. The study duration was between November 2016 and November 2018. Detailed patient history was recorded for age, gender, smoking status, symptoms on presentation, duration of symptoms, cancer stage, pleural effusion, presence of brain metastasis, and histopathological evaluation (well-, poorly-, moderately-differentiated, or adenosquamous status). A simultaneous evaluation of patient's samples was done for EGFR by polymerase chain reaction (PCR) or gene sequencing analysis, ALK and ROS1 by immunohistochemistry (IHC)/fluorescence *in situ* hybridization (FISH) methods and PD-L1 by IHC.

Immunohistochemistry for programmed cell death-ligand 1 expression

PD-L1 by IHC was carried out on 5 μm sections, using Rabbit anti-human PDL-1/CD274 Monoclonal Antibody (Ventana SP263 clone [MedGenome diagnostics]), OptiView DAB IHC Detection Kit on Ventana Benchmark GX equipment (MedGenome diagnostics). Hematoxylin was used as counterstaining. The PD-L1 tumor proportion score (TPS) was calculated as the percentage of ≥100 viable tumor cells with partial or complete staining. PDL-1 staining/expression is defined as complete or partial circumferential linear plasma membrane staining at any intensity that can be differentiated from background and diffuse cytoplasmic staining; cytoplasmic only staining is not considered significant.

Fluorescence *in situ* hybridization for anaplastic lymphoma kinase rearrangement

ALK rearrangement using FISH technique was done using a dual color break apart probe (ZytoLight SPEC) in formalin-fixed, paraffin-embedded (FFPE) tissue with ALK Probe (ZytoVision Cat # Z-2124-200) and was studied on a BX-61 Olympus Fluorescence microscope equipped with Cytovision software. One hundred nuclei were counted (50 each by 2 readers) and a sample was considered FISH-negative if <5 cells of 50 were positive, whereas it was considered positive if >25 cells out of 50 were positive. A sample was considered equivocal if 5–25 cells of 50 were found positive. In such cases, a second reader also evaluated the slide. In the final analysis, if <15% cells of 100 were positive, then the sample was considered "negative" while if >15% had translocations, it was considered "positive."

Immunohistochemistry for anaplastic lymphoma kinase rearrangement

ANL rearrangement by IHC was performed using anti-ALK (D5F3) rabbit monoclonal primary antibody along with Optiview DAB IHC detection and Optiview Amplification kits by automated method on Ventana Benchmark XT. The presence of strong granular cytoplasmic staining in tumor cells (any percentage of positive tumor cells) was indicative of positive scoring criteria for the determination of ALK status in NSCLC.

Epidermal growth factor receptor mutation analysis

The EGFR assay was based on PCR and gene sequencing analysis developed and performance evaluated at Oncquest Laboratories Ltd., New Delhi, India. The tumor samples were fixed under appropriate conditions (10% neutral buffered formalin for 6–48 h; 12 h for small biopsies) to ensure preservation of amplifiable quality DNA. The mutations were screened in exons 18–21 of the *EGFR* gene in the FFPE tissue blocks.

Study assessments

The study endpoints included the incidence of PD-L1 expression and frequency of patients with high PD-L1 expression (defined as PD-L1 TPS ≥50%). Correlation of PD-L1 was evaluated with EGFR and ALK (by IHC and

FISH) mutations, and clinicopathological parameters including gender, smoking, cancer stage, brain metastasis, pleural effusion, and histopathological examination.

Statistical analysis

The data collected were entered into Microsoft Excel 2010 (Microsoft Corporation, USA) and analyzed with SAS® Version 9.4 (SAS Institute Inc., USA). The demographic variables were expressed in numbers and percentages. The incidence of PD-L1 was evaluated descriptively. The relationship between PD-L1 and clinicopathological parameters was established using Pearson's correlation coefficient (*r*) method.

RESULTS

This study evaluated a total of 101 proven cases with NSCLC (adenocarcinoma). Majority of the cases were male (75.25%), had Stage IV cancer (86.14%) and had presented with hemoptysis and cough (23.76%) symptoms. The mean age of the patients was 57.9 (range: 27–83) years and duration of symptoms was 3.7 (2.65) months (range: 1–12 months). Histopathological evaluation revealed poorly-differentiated adenocarcinoma in majority (35.64%) of the patients. Patients with Stage III disease had received concurrent chemo-radiotherapy, whereas patients with Stage IV disease had received first- or second-line chemotherapy and were reported for further management. Table 1 presents the baseline demographic and characteristics of the patients.

The IHC testing revealed positive PD-L1 expression in 33.66% (34/101) cases; of these, majority (67.65%, 23/34) of the patients had PD-L1 TPS of <50% and 32.35% (11/34) patients had TPS of ≥50% [Figure 1]; none of the patients showed <1% TPS. PD-L1 expression was present in 18.1% (2/11) patients with EGFR mutation (TPS: <50% in both patients), 50% (4/8) ALK-IHC positive cases (TPS <50%: 2, TPS ≥50%: 2), and 33.3% (3/10) ALK-FISH positive cases (TPS <50%: 2, TPS ≥50%: 1) [Figure 1]. In patients with PD-L1 expression, EGFR mutation was present in 5.9% (2/34) patients, ALK-IHC rearrangement in 11.8% (4/34), and ALK-FISH rearrangement in 8.8% (3/34) patients.

Table 1: Patient disposition and baseline characteristics (*n*=101)

Parameters	Value
Age (years), mean±SD (range)	57.9±11.26 (27-83)
Sex, *n* (%)	
Men	76 (75.25)
Women	25 (24.75)
Smoking present, *n* (%)	61 (60.40)
Cancer stage, *n* (%)	
II	1 (0.99)
III	13 (12.87)
IV	87 (86.14)
Brain metastasis present, *n* (%)	15 (14.85)
Histopathology evaluation, *n* (%)	
Well differentiated	14 (13.86)
Moderately differentiated	29 (28.71)
Poorly differentiated	36 (35.64)
Adenosquamous disease	22 (21.78)
Previous treatment received, *n* (%)	13 (12.87)
EGFR mutation present	11 (10.89)
L858R	7 (6.93)
Del19	3 (2.97)
Other	1 (0.9)
ALK rearrangement positive by IHC	8 (7.92)
ALK rearrangement positive by FISH	10 (9.90)

n: Number of patients, SD: Standard deviation, EGFR: Epidermal growth factor receptor, IHC: Immunohistochemistry, FISH: Fluorescence *in situ* hybridization, ALK: Anaplastic lymphoma kinase

The clinicopathological evaluation revealed PD-L1 positivity in 35.5% (27/76) male and 28% (7/25) female samples. PD-L1 positivity was seen in 37.7% of smokers (23/61; TPS <50%: 17, TPS ≥50%: 6), 20% cases of brain metastasis (3/15; TPS <50%: 2, TPS ≥50%: 1), 20.8% cases of pleural effusion (5/24; TPS <50%: 2, TPS ≥50%: 3), 46.1% cases with Stage III disease (6/13, TPS <50%: 5, TPS ≥50%: 1), and 32.2% cases with Stage IV disease (28/87, TPS <50%: 18, TPS ≥50%: 10). Histopathological evaluation showed PD-L1 positivity in 21.4% of patients with well-differentiated histology (3/14, TPS <50%: 2, TPS ≥50%: 1), 31% with moderately differentiated (9/29, TPS <50%: 6, TPS ≥50%: 3), 36.1% with poorly differentiated (13/36, TPS <50%: 7, TPS ≥50%: 6), and 40.9% with adenosquamous histology (9/22; TPS <50%: 8, TPS ≥50%: 1).

A male preponderance (79.41%, 27/34) with PD-L1 expression was observed. Of 34 patients with PD-L1 expression, 23 (67.6%) were smokers, 3 patients had brain metastasis, and 5 had pleural effusion. Histopathological evaluation in these 34 PD-L1 expressed cases showed: well differentiated in 3, moderately differentiated in 9, poorly differentiated in 13, and adenosquamous disease in 9 cases [Table 2].

Pearson's correlation coefficient evaluated for the association of TPSs of PD-L1 (<50% and ≥50%) with clinicopathological factors showed no or very weak correlation ($r < 0.3$ for all) [Table 3].

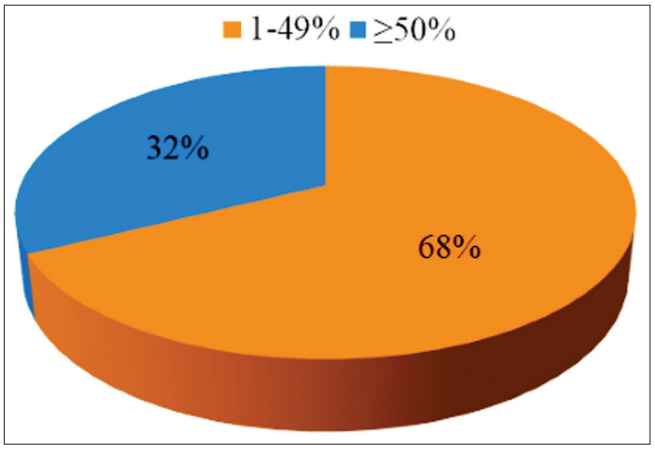

Figure 1: Programmed cell death-ligand 1 tumor proportion score (*n* = 34)

Table 2: Programmed death-ligand 1 staining by tumor proportion score

	PD-L1 staining		
	<50%, (*n*=23), *n* (%)	≥50%, (*n*=11), *n* (%)	Total, (*n*=34), *n* (%)
Sex			
Male	20 (86.96)	7 (63.64)	27 (79.41)
Female	3 (13.04)	4 (36.36)	7 (20.59)
Smoking present	17 (73.9)	6 (54.5)	23 (67.6)
Brain metastasis present	1 (4.3)	2 (18.1)	3 (8.8)
ALK-IHC positive	2 (8.70)	2 (18.18)	4 (11.76)
ALK-FISH positive	2 (8.70)	1 (9.09)	3 (8.82)
EGFR mutation status	2 (8.70)	-	2 (5.88)
Cancer stage			
III	5 (21.74)	1 (9.09)	6 (17.65)
IV	18 (78.26)	10 (90.91)	28 (82.35)
Diagnosis			
Well differentiated	2 (8.70)	1 (9.09)	3 (8.82)
Moderately differentiated	6 (26.09)	3 (27.27)	9 (26.47)
Poorly differentiated	7 (30.43)	6 (54.55)	13 (38.24)
Adenosquamous disease	8 (34.78)	1 (9.09)	9 (26.47)

PD-L1: Programmed death-ligand 1, EGFR: Epidermal growth factor receptor, IHC: Immunohistochemistry, FISH: Fluorescence *in situ* hybridization, ALK: Anaplastic lymphoma kinase

Table 3: Correlation analysis: Programmed death-ligand 1 expression with other parameters

Parameter	Pearson correlation coefficient	
	PD-L1 (1%-49%)	PD-L1 (≥50%)
Male	0.26981	−0.26981
Female	−0.26981	0.26981
Smoker	0.19368	−0.19368
Cancer Stage III	0.15522	−0.15522
Cancer Stage IV	−0.15522	0.15522
Brain metastasis present	−0.22817	0.22817
EGFR present	0.17289	−0.17289
ALK by IHC present	−0.13774	0.13774
ALK by FISH present	−0.00652	0.00652
Pleural effusion present	−0.24539	0.24539
Well-differentiated HPE	−0.00652	0.00652
Moderately-differentiated HPE	−0.01257	0.01257
Poorly-differentiated HPE	−0.23211	0.23211
Adenosquamous HPE	0.27243	−0.27243

ALK: Anaplastic lymphoma kinase, EGFR: Epidermal growth factor receptor, IHC: Immunohistochemistry, FISH: Fluorescence *in situ* hybridization HPE: Histopathologic evaluation, PD-L1: Programmed cell death-ligand 1

DISCUSSION

The identification of driver gene mutations/rearrangements has led to the development of newer targeted therapies for the treatment of NSCLC, and the availability of newer targeted agents has made the treatment of NSCLC increasingly complex.[9] Several predictive biomarkers have emerged for NSCLC treatment decisions including the most well-studied markers PD-L1, tumor mutation burden (TMB), and microsatellite instability (MSI).[10] Clinical studies have demonstrated that treatment outcomes in NSCLC correlate with high PD-L1 expression,[11] and the prognosis of NSCLC has improved with the use of immune checkpoint inhibitors targeting PD-1/PD-L1 receptors.[7] Furthermore, a higher mutational load is seen in patients who respond to anti-PD-1/PD-L1 therapies. TMB using hybrid capture-based next-generation sequencing (NGS) have shown superior response rate, progression free, and overall survivals in the high mutational burden group compared with intermediate and low mutational load groups.[12] However, TMB by hybrid capture based NGS is a time-consuming test requiring expertise and expensive as well, hence its applicability in routine clinical practice needs to be validated. MSI has emerged as another valuable marker with MSI-high (MSI-H) tumors showing good response to treatment with check point inhibitors. Based on the understanding and outcomes of five Keynote trials (016, 164, 012, 028, 158), pembrolizumab has been approved for MSI-H unresectable or metastatic colorectal cancers postprogression on all approved lines of treatment.[13]

The National Cancer Comprehensive Network guidelines recommend PD-L1 testing in patients diagnosed with advanced NSCLC.[14] IHC assay is one of the cost-effective and rapid tools for screening and the detection of PD-L1 expression. IHC using FFPE tissue has been widely used for detecting ALK ROS1 rearrangements and PD-L1 expression in NSCLC patients.[10]

Clinical studies have reported the prevalence of PD-L1 expression in NSCLC patients to be 24%–60%.[15] However, different antibodies have been used for PD-L1 evaluation in different studies and the ability of different PD-L1 antibodies to detect PD-L1 protein may vary. Among these, SP263 assay has been used with satisfactory results.[15] We report here the data of 101 proven cases of NSCLC in the Indian population, which showed that PD-L1 expression was present in approximately one-third of the cases (33.6%). PD-L1 expression was measured using IHC PD-L1 assay (SP263) with Rabbit anti-human PDL-1/CD274 Monoclonal Antibody in the current study. In another study in Indian patients (n = 134) with NSCLC, PD-L1 expression using Rabbit anti-human PD-L1 Monoclonal Antibody (clone SP263) was found to be 47%.[16] A meta-analysis of 61 studies involving 17 types of malignancies revealed PD-L1 expression rate of 44.5%; for NSCLC patients (13 studies), the rate of PD-L1 expression was 51.7%.[17]

Several clinical studies have reported the association of PD-L1 expression with EGFR and ALK mutations suggesting upregulation of PD-L1 expression by EGFR mutation through PI3K, MAPK, STAT3, and nuclear factor-κB signaling pathway or by ALK mutation through PI3K, MAPK, STAT3, and HIF-1α signaling pathways.[7,18] PD-L1 expression has been observed in up to 72% EGFR mutation cases and up to 78% ALK positive cases.[18] Our study showed PD-L1 positivity in 18.1% of the EGFR mutant cases and 33.3%–50% of ALK positive cases. Another study in Indian NSCLC patients reported PD-L1 expression in 35% of EGFR mutant cases and 1.5% ALK positive cases (1.5%).[16] A meta-analysis by Lan *et al*. from 24 studies with 4891 specimens revealed a lower PD-L1 expression with EGFR mutation. Furthermore, no

statistically significant correlation was observed between PD-L1 expression and ALK status from 11 studies with 3050 specimens.[7] However, in a review article, Ji *et al*. concluded based on the results of existing studies that the results of PD-L1 pathway blockade cannot be satisfactorily assessed through EGFR and ALK.[19]

Pawelczyk *et al*. evaluated the role of PD-L1 expression in NSCLC and their prognostic significance according to clinicopathological factors and diagnostic markers in 866 patients and reported that PD-L1 expression was seen in 42% patients with adenocarcinoma and 44% with squamous cell carcinoma.[20] Our study showed an abundance of PD-L1 positivity in poorly differentiated or adenosquamous disease.

A male preponderance for PD-L1 expression has been observed (73.9%),[20] similar to that seen in our study (79.4%). Male preponderance in PD-L1 expression was also observed by Domadia *et al*.,[16] (men: 51% vs. women: 33.4%). The clinical significance of PD-L1 expression in brain metastases from NSCLC was evaluated by Takamori *et al*., who reported that 21.9% (7/32) patients showed PD-L1 positivity using antibody against human PD-L1 (clone SP142).[21] PD-L1 positivity in brain metastasis was reported at 47.9% in a study by Domadia *et al*.,[16] versus 20% (3/15) in our study. Lower cancer stage was associated with lower prevalence of PD-L1 expression:[22] similarly in our study, none of the patients with cancer Stage I or II showed PD-L1 expression. The cells in pleural effusions exist in a different environment and there is a possibility of expression of various biomarkers. Hence, PD-L1 expression in pleural effusion can be a predictor for treatment. Xu *et al*. reported PD-L1 expression in 11.6% of 51 patients with pleural effusion[23] which was 20.8% in our study.

Pawelczyk *et al*. reported ≥50% PD-L1 expression in only 10.3% cases versus 32.4% in our study (1%–49% TPS: 22.3% vs. 67.6% in our study; 67.4% in their study showed <1% TPS, whereas none of the patient in our study showed <1% TPS). The difference in the TPS results could be attributed to the lower sample size in our study. The different clinicopathological parameters reported in the study by Pawelczyk *et al*. compared to that reported in our study are, smokers: 83.4% versus 67.6%; males: 73.9% versus 79.4%; cancer Stage III/IV: 30.3% versus 100%.[20]

An Asian study of 500 lung cancer (including 63 NSCLC) cases showed no significant difference in most parameters or biomarkers (including gender, smoking status, body weight, and genetic mutations [EGFR and ALK]) for PD-L1 expression. No significant difference in the brain metastasis, ALK and EGFR mutations with respect to TPS (<50% or ≥50%) was seen in this study, similar to that reported in our study.[24]

No or very week correlation of PD-L1 expression was reported with genetic mutations (ALK or EGFR) or other clinicopathologic parameters including gender, smoking, presence of brain metastasis, pleural effusion, and disease histopathology in our study. Similarly, no significant difference in PD-L1 expression with respect to age, gender, smoking, histology, sites of metastasis, and molecular profile was reported in another study in Indian patients with NSCLC.[16] Skov *et al*. reported that PD-L1 positivity (using IHC 22C3 pharmDx kit) was not affected by age, sex, smoking history, or performance status in 819 patients with NSCLC.[22] Dix Junqueira Pinto *et al*. highlighted the differences in PD-L1 positivity when measure by different platforms (DAKO and VENTANA) and different antibodies (28–8, 22C3, SP263, SP142, and MIH1, etc.).[25]

CONCLUSION

We report that PD-L1 expression was positive in approximately one-third of the cases of NSCLC studied. A high incidence of PD-L1 positivity was reported in males, smokers, and cases with advanced cancer Stages (III or IV). However, the correlation analysis established no or very weak correlation of PD-L1 expression with driver gene status and clinicopathological parameters including gender, smoking history, presence of brain metastasis, cancer stage, pleural effusion, and histopathology of the disease. As the sample size was low in this study, these correlations need to be confirmed in a large population to draw any conclusion. Furthermore, the method of checking PD-L1 expression by IHC is not yet standardized and also there is no uniform cutoff level beyond which the response is certainty.

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Conflicts of interest
There are no conflicts of interest.

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