pagepress

Subtyping of advanced lung cancer based on PD-L1 expression, tumor histopathology and mutation burden (EGFR and KRAS): a study from North India

Ritu Kulshrestha,[1] Himanshi Saxena,[1] Raj Kumar,[2] Sonam Spalgais,[2] Parul Mrigpuri,[2] Nitin Goel,[2] Balakrishnan Menon,[2] Meenu Rani,[1] Mahor Pawan,[1] Ishita Buthani[1]

[1]Department of Pathology; [2]Department of Pulmonary Medicine, Vallabhbhai Patel Chest Institute, University of Delhi, India

Correspondence: Dr. Ritu Kulshrestha, Department of Pathology. Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India.
E-mail: ritukumar71@yahoo.com

Key words: subtyping-lung cancer; PD-L1; coexisting EGFR/KRAS mutations; tumor histopathology; North India.

Contributions: all authors have contributed significantly, read and approved the final version of the manuscript. Dr Ritu Kulshrestha, corresponding author, is the guarantor of the content of the manuscript, including the data and analysis (original research).

Conflict of interest: the authors declare that there is no conflict of interest.

Availability of data and materials: all data generated or analysed during this study are included in this published article.

Ethics approval and consent to participate: Institutional Review Board approval was not required for this study as only de-identified compliant data were used in the analysis.

Patient consent for publication: not applicable.

Informed consent: written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article. The manuscript does not contain any individual person's data in any form.

Funding: the intramural funding was provided by Vallabhbhai Patel Chest Institute, University of Delhi.

Received: 3 October 2022.
Accepted: 13 December 2022.
Early view: 1 February 2023.

Publisher's note: all claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article or claim that may be made by its manufacturer is not guaranteed or endorsed by the publisher.

Monaldi Archives for Chest Disease 2023; 93:2449
doi: 10.4081/monaldi.2023.2449

Abstract

Immune checkpoint inhibitor (PD-L1) therapy for advanced non-small-cell lung cancer (NSCLC) has variable outcomes. Tumor subtypes based on PD-L1 expression, histopathology, mutation burden is required for patient stratification and formulation of treatment guidelines. Lung cancers (n=57) diagnosed at Pathology department, VPCI (2018-2021) were retrospectively analyzed. PD-L1(SP263) expressed by tumor cells [low (<1%), medium (1-49%), high (≥50%)] was correlated with histopathology, microenvironment, EGFR, KRAS expression. Patients were categorized into high and low risk based on their: i) gender: males (n=47, 30-89 years), females (n=10, 45-80 years); ii) smoking history: males 26/47 (45.61%), females 1/10 (10%); iii) tumor subtyping: squamous cell carcinoma 15/57 (26.32%), adenocarcinoma 6/57 (17.54%), NSCLC-undifferentiated 24/57 (42.10%), adenosquamous carcinoma 5/57 (8.77 %), carcinosarcoma 4/57 (7.02%), small cell carcinoma 1/57 (1.75%); iv) inflammatory tumor microenvironment/TILs 44/57 (77.1%); iv) PD-L1 positivity-31/57 (54.3%); v) concomitant EGFR/KRAS positivity. PD-L1positive cases showed squamous/undifferentiated histopathology, concomitant EGFR+ (9/20, 45%) and KRAS+ (8/15, 53.3%), smoking+ (21/31,67.74%).PD-L1 negative cases (26/57, 45.6%), were EGFR+ (2/14, 14.28%) and KRAS+ (6/19, 31.5%). The high-risk lung cancer subtypes show squamous/undifferentiated histopathology, inflammatory microenvironment, male preponderance, smoking history, higher concomitant PD-L1, KRAS and EGFR positivity. Lung cancer subtyping can predict clinical response/resistance of patients prior to initiation of PD-L1 inhibitor therapies and can be used to guide therapy.

Introduction

Lung cancer is the most common cause of cancer death worldwide. The immune check point PDL1 (programmed death-1) inhibitors such as pembrolizumab, durvalumabare rapidly becoming integral in treatment of advanced stage non-small cell lung cancer [1]. The therapies are initiated after PD-L1 immunohistochemistry and visual scoring. The PD-L1 expression has been scored using manual and computer-based machine learning algorithms to drive clinical decision making [2]. However, in spite of this, less than 30% patients reportedly respond to anti-PD-L1 therapy [3]. Indicating that the assessment of PD-L1 expression by tumor cells alone is not enough to influence survival advanced

NSCLC patients. Making it important to evaluate these patients for additional biomarkers of clinical response [4].

In lung adenocarcinoma (LADC), two distinct patient subtypes (high- and low-risk) correlating with significantly different survival outcomes have been identified [5]. The molecular subtype of these patients was correlated with their potential therapeutic response [tumor immune dysfunction and exclusion (TIDE) algorithm]. The high-risk subtype had lower TIDE score, upregulated PD-L1 expression, and higher tumor mutation burden (TMB). This high-risk subtype showed higher response with PD-L1 therapy [5]. Recently, PD-L1 expression in squamous cell carcinoma lung has been assessed using the Dako PD-L1 [6] assay and a computer aided automated tumor proportion scoring system (TPS) [7]. The correlation of PD-L1 expression with other tumor subtypes however remains to be done. Other independent predictors of the anti PD-1 response and patient survival include i) tumor burden and neutrophil-lymphocyte ratio [4]; ii) immune cells infiltrating tumor/tumor immune microenvironment [3,8,9]; iii) coexistent oncogenic mutations- epidermal growth factor receptor (EGFR) or the Kirsten rat sarcoma viral oncogene (KRAS) [10,11]; iv) TP53, NAV3, COL11A1, KEAP1 and other 19 mutations [12]; v) vascular endothelial growth factor (VEGF-C) upregulation [13].

The present study aims to define the histopathological type of lung cancers and their microenvironment/tumor infiltrating lymphocytes and correlate them with their PD-L1 positivity, gender, smoking history, concomitant KRAS and EGFR positivity. This pan-cancer panel can subtype the lung cancers (high and low risk) and be used to predict clinical response prior to initiation of cancer immunotherapy.

Methodology

A retrospective analysis of the lung cancers (n=57 cases) diagnosed at Pathology Department, Vallabhbhai Patel Chest Institute, Delhi, India, over a four-year period (2018-2021) was done. PD-L1 expressed was quantified and correlated with tumor histology, tumor infiltrating lymphocytes, gender, smoking history, and presence of concomitant EGFR and KRAS mutations in advanced lung cancer.

In brief, lung biopsies were processed and 5 µm serial tissue sections were cut from formalin-fixed paraffin-embedded blocks. Two sections were stained with Hematoxylin & Eosin after deparaffinization in xylene and rehydration through graded ethanol concentrations. The cancers were morphologically categorized using the WHO classification of tumors of the lung (epithelial tumors) [14]. Two sections were taken on histobond®+S glass slides (Cat. No. 0810501; Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) for immunostaining with PD-L1 and KRAS. At Least 10 sections were cut from each block for DNA extraction for molecular studies.

The PD-L1 expression was estimated by immunohistochemistry using the BenchMark GX IHC/ISH automated analyser, Roche Diagnostics and VentanaPD-L1-SP263 assay. The PD-L1 expressed by tumor cells was evaluated and images were quantified by using Nikon 90i microscope and NIS-AR image analysis system. PD-L1 expression was measured by estimating the tumor proportion score (TPS), similar to the Dako (Glostrup, Denmark) PD-L1 IHC 22C3 and 28-8 pharmaDx assays. The TPS was determined as the percentage of PD-L1 positive stained tumor cells (TCs) with at least partial membrane staining relative to the total number of TCs, using the following equation: TPS (%)=# of PD-L1 positive TCs/ Total # of viable TCs ×100. In tumor sections having a single PD-L1 positive tumor area, the TPS was determined as the product of the % of positive staining area and the % of positive TCs in the area. Whereas in heterogeneous tumor areas, TPS is calculated by averaging the stained tumor cell percentages of several divided tumor areas [15,16]. The tumor-associated interstitial cells (ICs), necrotic, normal or non-neoplastic cells were excluded. The PD-L1 positive cases were categorized into low (<1%), medium (1-49%), high (≥50%). This has been used to recommend anti-PD-L1 inhibitors as first or second line therapy [17].

EGFR mutations were detected using the allele-specific Scorpion Amplified Refractory Mutation (ARMS)-PCR detection system (EGFR RGQ PCR Kit; Cat No. 870121). Seven somatic mutations in exons 19, 20 and 21 were qualitatively detected using the Rotor-Gene® Q 5plex (HRM®, Qiagen, Hilden, Germany). The data was analysed using Rotor-Gene Q software, version 2.0.2. In this two-step procedure, first the control assay was performed to assess the total amplifiable DNA in the sample. Then the mutation assay was done to detect the presence or absence of *EGFR* mutations. If the mutated DNA fully matched and annealed with the ARMS primers, the Taq DNA polymerase initiated full selective amplification of specific mutated sequences. If the 3' base mismatched, only low-level background amplification occurred.

KRAS gene mutations in codon 12, 13 were assessed using the allele-specific ARMS PCR (therascreen KRAS RGQ PCR Kit, Cat No. 874011; Qiagen). Briefly, the DNA was extracted using the QIAamp DNA Mini Kit, (Cat No. 51304; Qiagen). Real-time PCR assays was done using therascreen KRAS RGQ PCR Kit which qualitatively detects 7 mutations in codons 12 and 13 of the human *KRAS* gene (G12A, G12D, G12R, G12C, G12S, G12V, G13D) [18]. The Scorpion primer was used to detect the amplification product. Scorpions are bi-functional molecules that contain a PCR primer covalently linked to a probe. The Scorpion primer hybridizes with a DNA sequence upstream of the target region. This primer is also extended by Taq DNA polymerase, and the newly copied region is complementary to the probe region of the Scorpion. When the solution cools, the Scorpions probe self hybridizes. The fluorophore is separated from the quencher and a fluorescence signal is generated.

KRAS oncoprotein estimation was done by immunohistochemistry (SAB- WH0003845M1, clone 3B10-2F2). KRAS expression was semi quantitatively classified into Grade 1-4 as compared to Control. KRAS expressed was estimated by Image analysis-AR Software using the Nikon 90i microscope and reciprocal intensity (a.u.) was calculated.

Results

Fifty-seven (57) cases of lung cancers diagnosed at Pathology Department, Vallabhbhai Patel Chest Institute, over a four-year period (20182021) were assessed. 47 were males (30-89 years) and 10 were females (45-80 years). History of smoking was present in 26/47 (55.3%) males and 1/10 (10%) females. The tumors were categorized on basis of morphology (Table 1), into: Squamous cell carcinoma 15/57 (26.32%, Figures 1 and 2), adenocarcinoma 6/57 (10.52%, Figure 3), NSCLC (Undifferentiated, 24/57 (42.10%, Figures 4 and 5), metastasis to pleura, (1/57 (1.75%), Figure 6), adenosquamous carcinoma 5/57 (8.77 %, Figure 7), carcinosarcoma 4/57 (7.02%, Figure 8), small cell carcinoma 1/57 (1.75%) and lymphangitis carcinomatosis 1/57 (1.75%, Figure 9). An inflammatory background with tumor infiltrating lymphocytes was seen in 44/57 (77.19%, Figure 10) cases. The lung cancer cells showed variable PD-L1 expression (Table 1, Figures 1 to 9); 31/57 cases (54.3%) were PD-L1 positive while 26/57 (45.6%) were PD-L1 negative. These patients underwent analysis for coexisting KRAS oncoprotein expression (12/20, 60%, Figure 11) and EGFR and KRAS mutations (Graph 1 and 2 respec-

tively). In EGFR L858R was the commonest type of mutation seen 4/12(33.4%) followed by G719X 2/12 (16.67%) and Deletion 2/12 (16.67%) (Graph 1). The spectrum of KRAS mutations in lung cancer were mainly under Codons-12, 13 and occurred as transversions (6/9 ~66.7%) - G12C, G12V, G12R. Transitions were less common G12D, G13D (Gly to Aspartate), and accounted for 3/9 ~33.4% cases (Graphs 1 and 2). KRAS gene and oncoprotein expression inversely correlated with EGFR expression. Positive correlation between

Table 1. Correlation of tumor histopathology with PD-L1, EGFR/KRAS mutations and clinical features.

	PD-L1 (SP263) positive	PD-L1 (SP263) negative	Age (years)	Sex	Smokers (n of cases)	TILs (n of cases)	EGFR positive	KRAS positive
Squamous cell carcinoma (n=15)	10	5	45-73	M-11 F-4	17	13	2	4
Adenocarcinoma (n=6)	2	4	47-75	M-4 F-2	0	2	2	1
NSCLC- Undifferentiated (n=24)	15	9	40-87	M-21 F-3	7	19	4	5
Adenosquamous carcinoma (n=5)	0	5	40-71	M-5 F-0	0	5	0	2
Carcinosarcoma (n=4)	3	1	37-71	M-3 F-1	0	2	2	2
Lymphangitis carcinomatosis (n=1)	0	1	58	M-1 F-0	1	1	1	0
Ca metastasis to pleura (n=1)	0	1	48	M-1 F-0	1	0	0	0
Small cell (n=1)	1	0	60	M-1	1	1	0	0

TILs, tumor infiltrating lymphocytes.

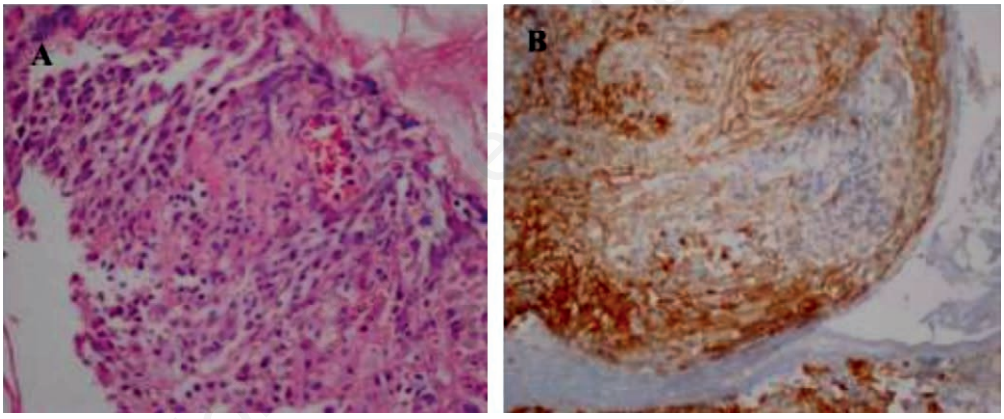

Figure 1. Moderately differentiated squamous cell carcinoma showing high PD-L1 (>50%) expression in tumor cells. H&E (A) and PD-L1 stain (B). Magnification: 400 x.

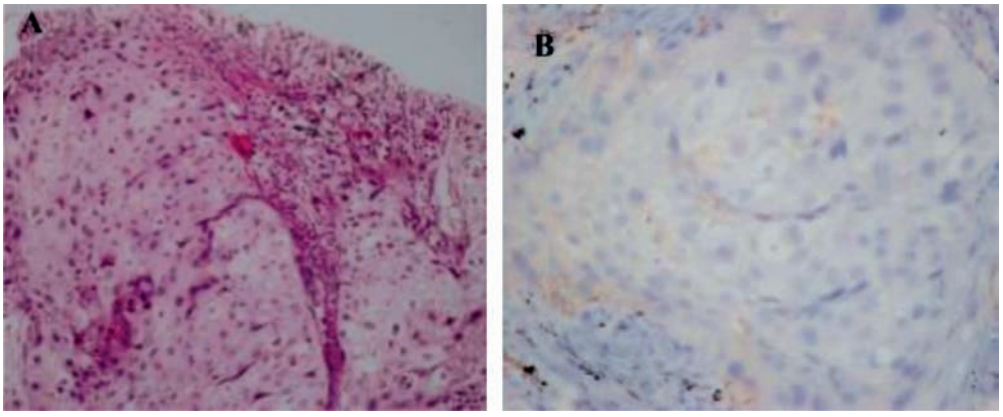

Figure 2. Squamous cell carcinoma, clear cell variant, PD-L1 negative. H&E (A) and PD-L1 stain (B). Magnification: 400 x.

KRAS oncogene and oncoprotein expression was seen in 3/5 (60%) cases. On examining each histological type (Table 1), the squamous cell carcinomas were PDL1+(66.67% cases)/KRAS+ (26.67%); adenocarcinomas were PD-L1+/EGFR+ (33.33%), adenosquamous were PD-L1-ve (100%) /EGFR-ve (100%)/KRAS +(40%), carcinosarcomas were PD-L1+(75%). The carcinosarcomas were EGFR+(75%)/KRAS+(75%). Undifferentiated NSCLC were PD-L1+/TILs+ (62.5%). Small cell lung cancer (n=1) was PD-L1+/EGFR-Ve. Lymphangitic Carcinomatosis (n=1), was PDL1-ve/KRAS-ve/EGFR+ve. The cases were subtyped into high risk (Table 1), based on their PD-L1 positivity-31/57 cases (54.3%), concomitant EGFR positivity (9/20cases, 45%), KRAS positivity (8/15,53.3%). This group showed male preponderance with more patients presenting at younger age with non-small cell lung carcinoma and tumor infiltrating lymphocytes (Table 1). History of smoking was present in 21/31 (67.74 %) and correlated mainly with squamous cell and undifferentiated type of lung cancers. In low-risk subtype (Table 2), the patients were PD-L1 negative-26/57 (45.6%), with con-

Table 2. High-risk subtype of lung cancer patients PD-L1 positive with concomitant EGFR/KRAS positivity, male preponderance, younger age of presentation, squamous /undifferentiated histopathology and tumor invasion in microenvironment of tumor infiltrating lymphocytes indicative of immune escape.

	Total PD-L1+ve	PD-L1+/ E GFR +ve	PD-L1+/ E GFR -ve	PD-L1+ / KRAS+	PD-L1+ / KRAS-	Age in yrs	Sex	TILs
Squamous cell carcinoma	10	1	4	3	1	45-70	M-7F-3	9
Adenocarcinoma	2	2	0	0	1	47-70	M-0F-2	1
NSCLC- undifferentiated	15	4	6	3	5	50-82	M-13F-2	15
Carcinosarcoma	3	2	0	2	0	37-68	M-2F-1	2
Small cell carcinoma	1	0	1	0	0	60	M-1F-0	0

TILs, tumor infiltrating lymphocytes.

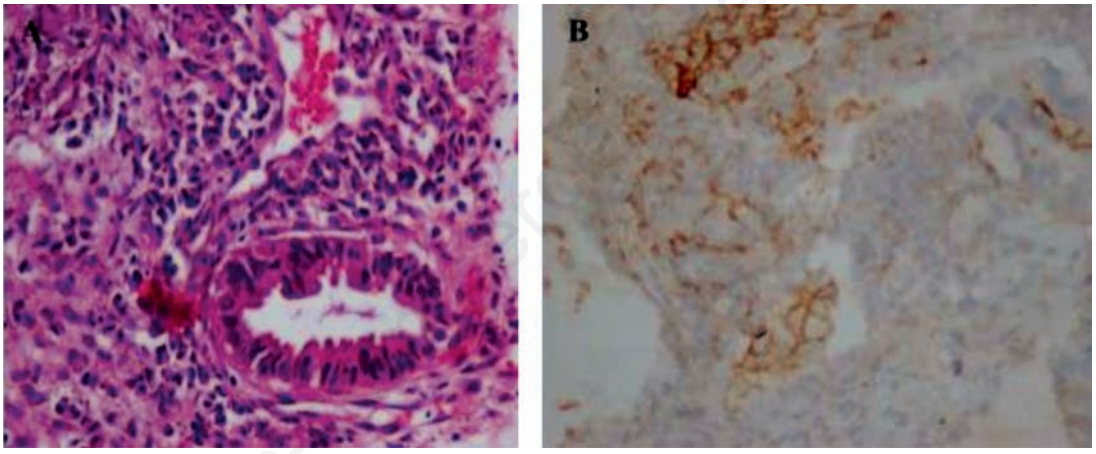

Figure 3. Well differentiated adenocarcinoma showing medium PD-L1 expression (~10%) in tumor cells. H&E (A) and PD-L1 stain (B). Magnification: 400 x.

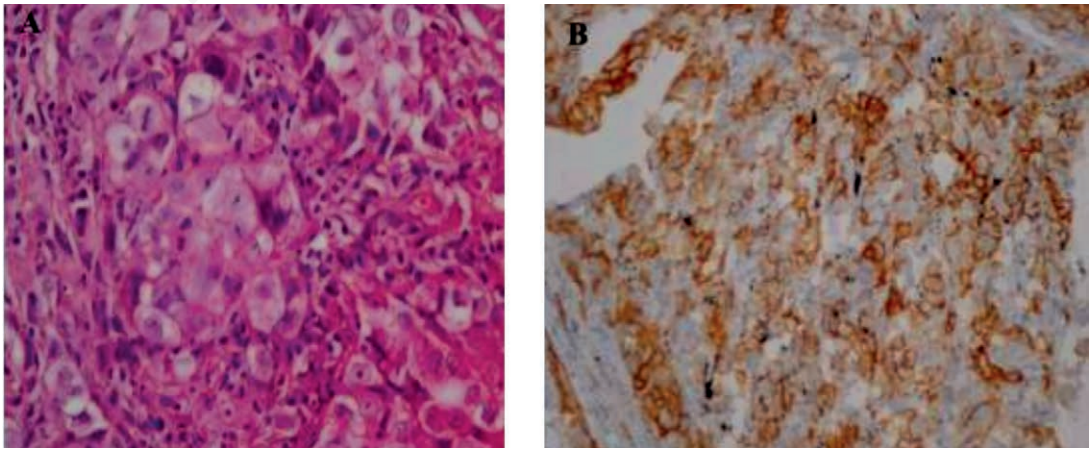

Figure 4. Poorly differentiated carcinoma showing medium PD-L1 expression (1-50%) in tumor cells. H&E (A) and PD-L1 stain (B). Magnification: 400 x.

comitant EGFR positivity seen in 2/14(14.28%) and KRAS positivity seen in 6/19 (31.5%) cases. This group showed male preponderance with more patients presenting in older age as compared to high-risk group. History of smoking was present in 6/26 (23.08%) and correlated with adenocarcinoma, adenosquamous cell and undifferentiated type of lung cancers. Therefore, we propose that the cases of lung cancer can be categorized into subtypes based on histopathology, age/gender, PD-L1 positivity and concomitant EGFR/KRAS/TILs profile: i) the high-risk subset which displays: PD-L1+/EGFR +/-/KRAS+/- /TIL+/- (Table 1); ii) the low-risk subset 2, which displays: PD-L1-/EGFR +/-/KRAS+/- / TIL+/- (Table 2).

Discussion

The Programmed cell death ligand-1 (PD-L1) expression status is critical to effectively treat lung cancer patients. It not only reflects the occurrence of tumor growth in spite of triggering the immune checkpoint pathway. It is also predictive of their postoperative tumor recurrence [20]. However, in spite of rapid progress many challenges of anti-PD-1/PD-L1 immunotherapy in treating NSCLC remain [16]. The association between PD-L1 expression and various clinicopathological factors remains limited to date [20]. Also, there is an unmet need to subtype lung cancers and assess the predictors of clinical response/resistance prior to initiation of tyrosine kinase (TKI) and PD-L1 inhibitor therapies [21].

The PD-1 and its ligands PD-L1 and PD-L2 are expressed on tumor cells and immune cells. These immune check point proteins inhibit the T cells, suppress the host immune response, and help tumor cells evade anti-cancer immunity [16,22,23]. Therefore, monoclonal antibodies (such as pembrolizumab) that block the PD-1 and PD-L1 interaction and restore the host anti-cancer immune response are being increasingly used [17,24]. These effectively treat lung cancer as first line treatment when PD-L1 positivity is ≥50% [25]. Even when PD-L1-positive tumor cells are sparse (≥1%), second-line treatment with PD-L1 inhibitors has been shown to be effective [26]. High expression of PD-L1 is seen in males, smokers, undifferentiated lung cancers with pleomorphism [27], advanced pathologic stages, positive capillary and lymphatic invasion [20]. However, some of these patients with high PD-L1 levels fail to show clinical response to PD1/PD-L1 therapy [2]. Thus, indicating the need for subtyping the lung cancer patients at time of initial diagnosis, based on clinical and molecular features.

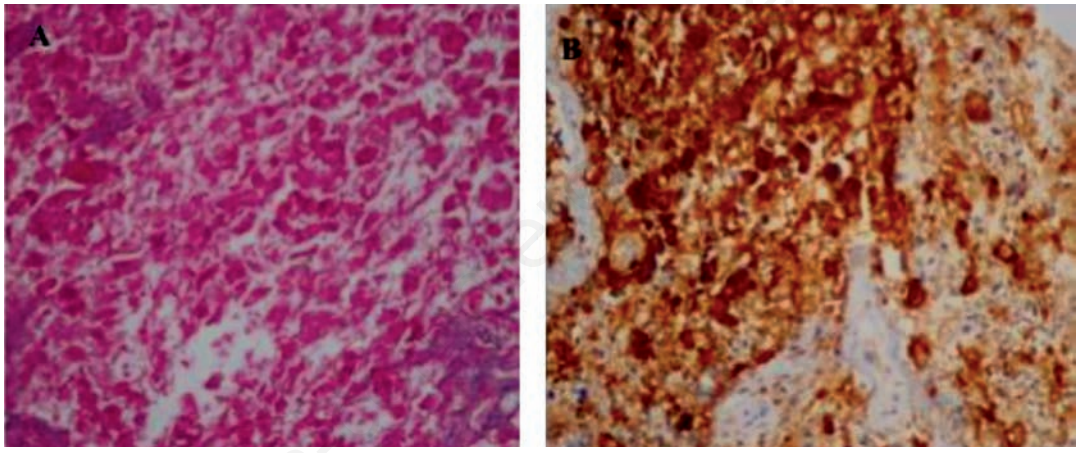

Figure 5. Undifferentiated non-small cell lung cancer showing high PD-L1 expression (>80%) in tumor cells. H&E (A) and PD-L1 stain (B). Magnification: 400 x.

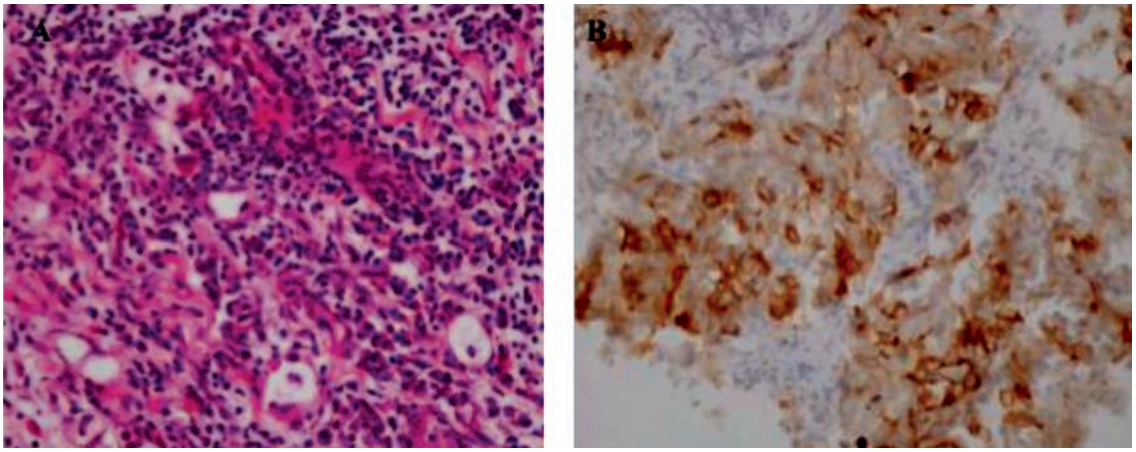

Figure 6. Non-small cell lung cancer metastasis to pleura showing high PD-L1 expression (>50%) in tumor cells, infiltrating pleura. H&E (A) and PD-L1 stain (B). Magnification: 400 x.

Correlation of PD-L1 with age/ sex and smoking

The efficacy of PD-1/PD-L1 inhibitors may correlate with age and sex [28,29]. Age-related differences in patients with advanced lung cancer receiving immunotherapy have been compared using patient reported outcomes [30]. No large differences at baseline or in the distributions of change from baseline functional domains were identified between younger and older patients of lung cancer in PRO undergoing anti-PD -1/PD-L 1 therapy [30]. Recently however, anti-PD-1/PDL1 inhibitors have been identified as a preferable treatment option for advanced/ metastatic lung cancer patients who are male, aged<65 years, current or former smokers [31]. PD-1/PD-L1 inhibitors are more effective in smoking NSCLC patients compared with non-smokers [32,33]. However, no direct evidence for the reasons behind the effectiveness of PD-1/PD-L1 inhibitors in smokers as compared to non-smokers has been identified so far. Smoking influences the; number of regulatory T cells (Treg) [34], natural killer cells function, maturation and function of dendritic cells [35] and may partly explain the immunological response. Patients of high-risk subtype were more likely to be current smokers [33].

These patients typically also have high mutation burden generated by the mutagenic effects of cigarette smoke [36]. These two factors underlie the response to immune checkpoint blockade therapy for patients of high-risk subtype.

Correlation of PD-L1 with tumor histology

Significant differences of PD-L1 expression in lung cancers are seen in relation to their histological patterns. In lung adenocarcinomas, PD-L1-positivity frequently occurs in acinar and solid variants as compared to other subtypes [20]. PD-L1 Levels are low/negative in invasive mucinous adenocarcinomas and lepidic non-mucinous adenocarcinomas. Based on their immune-related signatures, Wang *et al*. have identified two distinct subtypes of lung adenocarcinoma (high and low risk subtypes) that are characterized by significantly different survival outcomes [31]. The Findings of this study, suggest the efficacy of immune checkpoint blockade therapy for high-risk subtypes. In the present study, 33.33% were high risk acinar type of adenocarcinoma with PD-L1 positive while 66.67% LADC were low risk subtype (Table 2, Figure 3). Advanced squamous cell carcinoma patients who have disease progression during or after first-line chemotherapy have limited treatment options. Little therapeutic

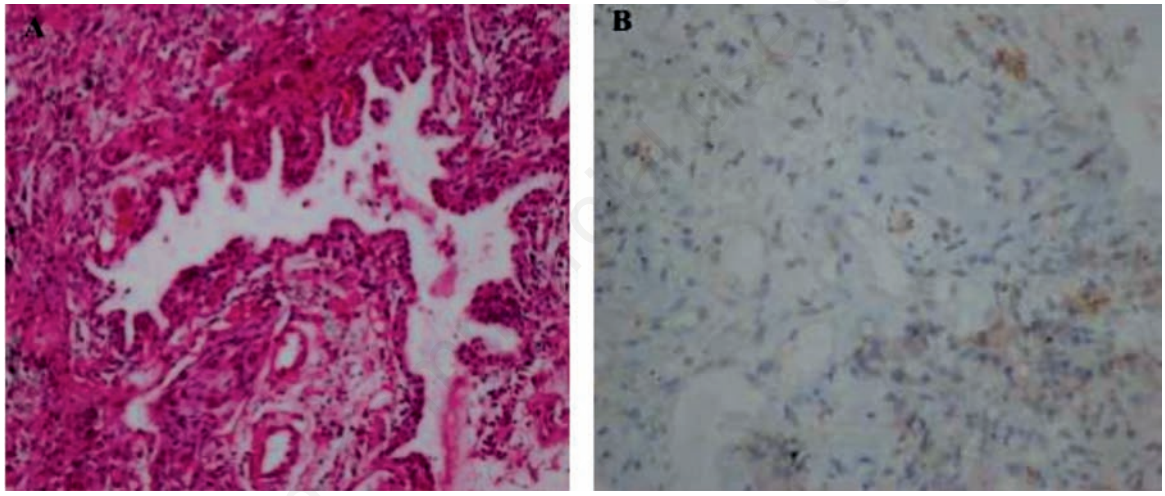

Figure 7. Adenosquamous carcinoma with PD-L1 negative tumor cells. H&E (A) and PD-L1 stain (B). Magnification: 400 x.

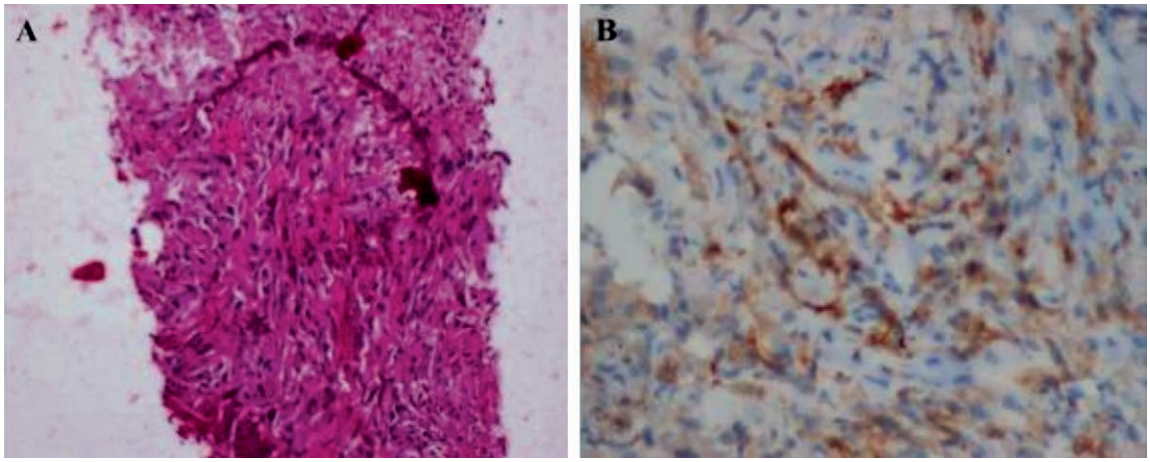

Figure 8. Carcinosarcoma showing medium PD-L1 expression (>10%) in tumor cells. H&E (A) and PD-L1 stain (B). Magnification: 400 x.

progress has been made since the approval of docetaxel for their second-line treatment in 1999 [37]. They have shown significantly better overall survival, response rate, and progression-free survival if treated with PD-1 inhibitor (nivolumab), as compared with docetaxel [38]. Treatment-related serious adverse events leading to treatment discontinuation occurred less frequently with PD-1 inhibitor than with docetaxel.

Small cell carcinomas of lung (SCLC) are an exceptionally lethal subtype of lung cancer. Majority of these patients present with extensive -stage disease and are treated by platinum-doublet chemotherapy. However, SCLC is relatively refractory to treatment upon relapse and many approaches have been used to delay resistance and improve survival [7] chemo-immunotherapy combinations are being explored in SCLC. In a Phase III randomized trial, the addition of ipilimumab (anti-Cytotoxic T-lymphocyte-associated protein 4 (CTLA4) antibodies) to first-line chemotherapy did not improve survival, in contrast to atezolizumab (anti-PD-L1 antibodies) in SCLC [26]. The atezolizumab plus carboplatin plus etoposide regimen improved survival for patients with treatment-naïve ES-SCLC and was approved as first-line therapy for ES-SCLC by the US FDA in March 2019 [39]. In 2019 and 2020, the U.S. FDA granted approval to atezolizumab and durvalumab, respectively, for use in combination with chemotherapy as first line treatment of patients with extensive stage SCLC [12].

NSCLC (undifferentiated) accounted for 42.1 % cases in present study; 62.5% of these expressed PD-L1 and were categorized as high-risk subtype. A biphasic prognostic significance of PD-L1 expression status in early- and locally advanced-stage NSCLC patients has been observed. In early-stage NSCLC, high tumor PD-L1 is reflective of induction of an antitumor immune response and a biomarker of good prognosis postoperatively. While, in locally advanced stage NSCLC, tumor PD-L1 indicates tumor progression in spite of triggering of the immune checkpoint pathway and is predictive of tumor recurrence after surgery [19]. Adenosquamous carcinoma accounted for 5/57 (8.77 %) cases in present study. All the cases were PD-L1 negative/EGFR negative and categorized as low risk subtype (Table 3). The mixed adenocarcinomas and adenosquamous carcinomas of lung show spatial and temporal heterogeneity of PD-L1 Expression [40-42]. This is found to be significantly related to the presence of micropapillary and solid adeno patterns in the tumor. This heterogeneity can also partly explain the discrepant clinical effectiveness of PD-L1-positive or negative LADC diagnosed on cytology/small biopsy [41]. The carcinosarcoma lung, is a rare subset of NSCLC, associated with worse prognosis and resistant to platinum-based regimens [6]. Lung carcinosarcomas were identified in 4/57 (7.02%) cases with 75% of these showing positive PD-L1 expression, in present study. This is similar to recent studies which have shown high levels of PD-L1 expression in pulmonary sarcomatoid carcinoma of lung providing a rationale for the potential use of immunotherapy. ThePD-L1 overexpression is also strongly associated with tumors aggressiveness and occurrence of both local and distant metastasis [43]. Overall, the PD-L1 expression appears to

Table 3. Low-risk subtype of lung cancer patients. PD-L1 negative with concomitant EGFR/KRAS negativity, male preponderance, non-small cell histopathology and tumor microenvironment/TILs not conducive for immune escape.

	Total PD-L1 negative	PD-L1 -/ EGF R +	PD-L1-/ EGFR-ve	PD-L1- / KRAS+	PD-L1-/ KRAS -	Age in yrs	Sex	TILs
Squamous cell carcinoma	5	1	2	1	3	50-73	M-4F-1	4
Adenocarcinoma	4	0	1	1	1	52-75	M-4F-0	1
NSCLC-undifferentiated	9	0	4	2	6	50-82	M-8F-1	4
Adenosquamous carcinoma	5	0	5	2	2	40-71	M-5F-0	5
Carcinosarcoma	1	0	0	0	1	71	M-1F-0	0
Lymphangitis carcinomatosis	1	1	0	0	0	58	M-1F-0	1
Carcinoma metastasis to pleura	1	0	0	0	0	48	M-1F-0	0

TILs, tumor infiltrating lymphocytes.

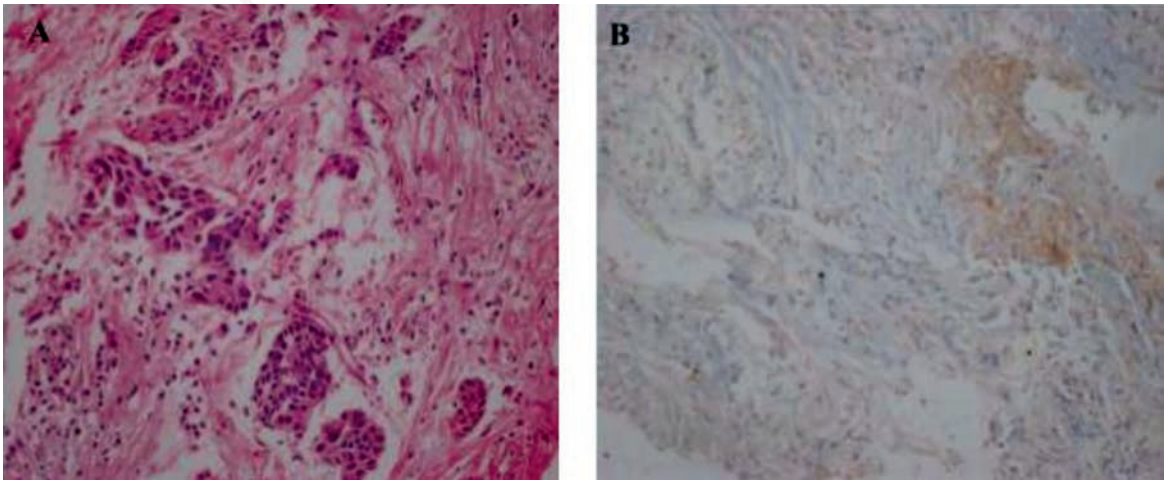

Figure 9. Lymphangitic carcinomatosis with PD-L1 negative tumor cells. H&E (A) and PD-L1 stain (B). Magnification: 400 x.

increase with pathologic NSCLC progression [20] and is suggestive of a worse prognosis.

Correlation of PD-L1 with coexisting mutational burden

Previous investigators have suggested that NSCLC with high tumor mutational burden (TMB) or combinations of immune markers are more likely to benefit from PD-1 inhibitor (pembrolizumab) therapy [9]. These include coexisting KRAS and EGFR mutations. The KRAS mutation affect the immune microenvironment and tumor response in a heterogenous way. A correlation of KRAS mutations with an inflammatory tumor microenvironment and increased tumor immunogenicity is seen. The anti-PD-1/PD-L1 immunotherapy show superior efficacy in KRAS-mutant NSCLC patients. Therefore, the KRAS status should be considered as a predictive biomarker prior to initiation of PD-L1 inhibitor therapy in lung cancer patients. Also suggesting that PD-1/PD-L1 inhibitors may be the optimal therapeutic schedule in NSCLC patients harbouring KRAS mutations [7]. The efficacy of immunotherapy in EGFR-activated NSCLC is limited [32]. However, in a recent study of patients with EGFR alterations (G719X, L861Q, S768I, and Ex20 ins) clinical benefit from PD-1 inhibitor therapy has been reported. These patients had a high frequency (36.7%) of concurrent PD-L1 expression and abundant CD8 + TILs infiltration. Thereby suggesting the existence of a subgroup of EGFR mutant patients with therapeutic sensitivity to PD-1 inhibitors [32].

In the present study, PD-L1 positivity and high TMB (EGFR and KRAS) correlated with squamous/undifferentiated histopathology and high-risk subtype. Identifying targetable alterations and developing cost-effective cancer panel assays is getting more and more critical for predicting responses to immunotherapy [43]. The high-risk patients showed PD-L1 positivity, male preponderance, history

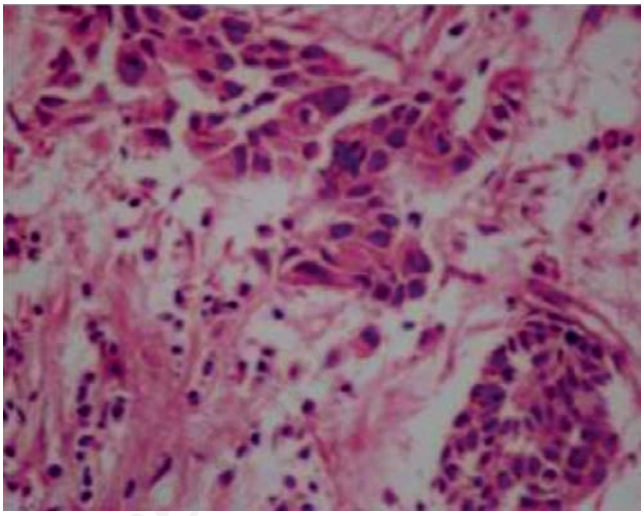

Figure 10. Tumor microenvironment comprising clusters of tumor cells arranged in an inflammatory background. H&E stain. Magnification: 400 x.

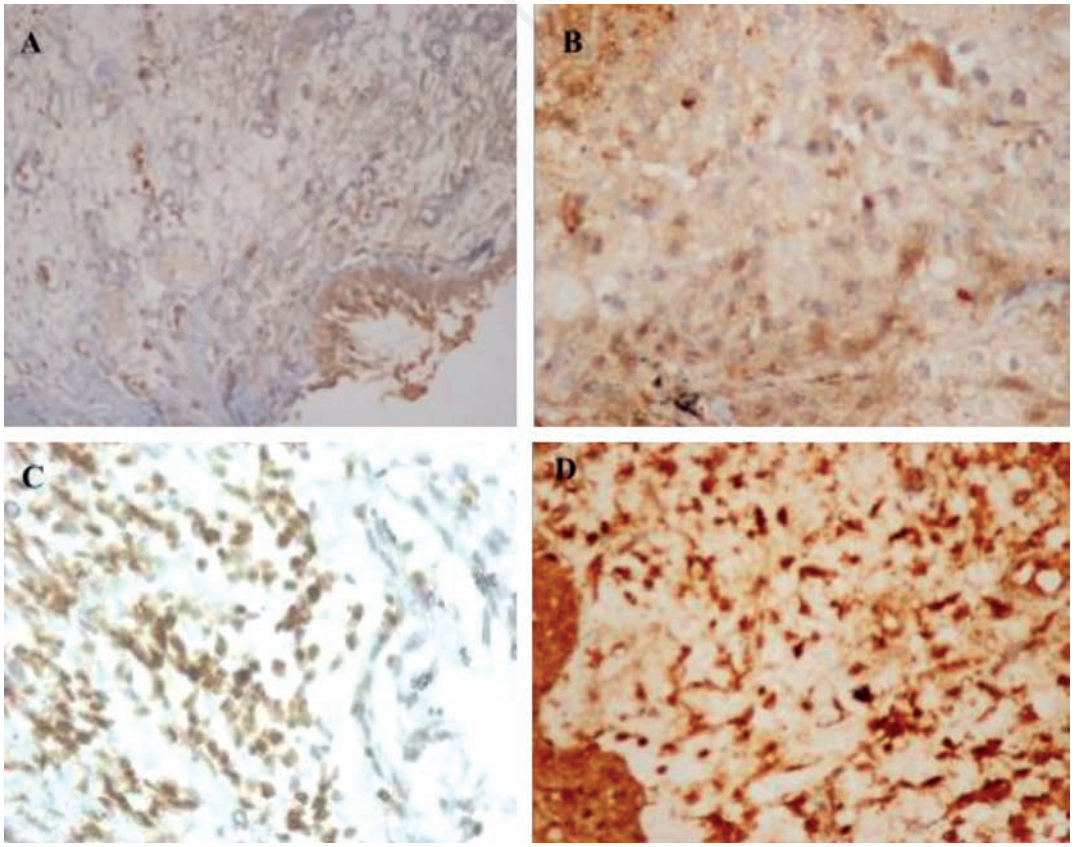

Figure 11. KRAS oncoprotein expressed by tumor cells. A-D) Grades 1 to 4 of KRAS oncoprotein expression. Magnification: 100 x.

of smoking, higher KRAS positivity, EGFR positivity in nearly 50%, in the present study. High PD-L1 positivity correlated with squamous /undifferentiated histopathology and tumor invasion in the microenvironment of tumor infiltrating lymphocytes (TILs) indicative of immune escape. These subtypes can be used as predictors of clinical response/resistance prior to initiation of TKI and PD-L1 inhibitor therapies. To the best of our knowledge, this is the first such study from North India.

References

1. Vrankar M, Kern I, Stanic K. Prognostic value of PD-L1 expression in patients with unresectable stage III non-small cell lung cancer treated with chemoradiotherapy. Radiat Oncol 2020; 15:247.
2. Inge LJ, Dennis E. Development and applications of computer image analysis algorithms for scoring of PD-L1 immunohistochemistry. Immunooncol Technol 2020;6:2-8.
3. Garon EB, Rizvi NA, Hui R, et al. Pembrolizumab for the treatment of non-small-cell lung cancer. N Engl J Med 2015;372: 2018–28.
4. Ahn BC, So JW, Synn CB, et al. Clinical decision support algorithm based on machine learning to assess the clinical response to anti-programmed death-1 therapy in patients with non-small cell lung cancer. Eur J Cancer 2021;153:179-89.
5. Liu C, Zheng S, Jin R, et al. The superior efficacy of anti-PD-1/PD-L1 immunotherapy in KRAS-mutant non-small cell lung cancer that correlates with an inflammatory phenotype and increased immunogenicity. Cancer Lett 2019;470: 95-105.
6. Lococo F, Torricelli F, Rossi G, et al. Inter-relationship between PD-L1 expression and clinic-pathological features and driver gene mutations in pulmonary sarcomatoid carcinomas. Lung Cancer 2017;113: 93-101.
7. Armstrong SA, Liu SV. Dashing decades of defeat: Long anticipated advances in the first-line treatment of extensive-stage small cell lung cancer. Curr Oncol Rep 2020;22:20.
8. Cronin-Fenton D, Dalvi T, Movva N, et al. PD-L1 expression, EGFR and KRAS mutations and survival among stage III unresected non-small cell lung cancer patients: a Danish cohort study. Sci Rep 2021;11:16892.
9. Rizvi NA, Hellmann MD, Snyder A, et al. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science 2015;348:124-8.
10. Pao W, Girard N. New driver mutations in non-small-cell lung cancer. Lancet Oncol 2011;12: 175–80.
11. Liu J, Zheng Q, Mu X, et al. Automated tumor proportion score analysis for PD-L1 (22C3) expression in lung squamous cell carcinoma. Sci Reps 2021;11:15907.
12. Qin T, Xia J, Liu S, et al. Clinical importance of VEGFC and PD-L1 co-expression in lung adenocarcinoma patients. Thorac Cancer 2020;11:1139-48.
13. Travis WD, Brambilla E, Burke AP, et al. Introduction to the 2015 World Health Organization classification of tumors of the lung, pleura, thymus, and heart. J Thorac Oncol 2015;10:1240-2.
14. Agilent Dako. Pd-L1 ihc 22c3 pharmdx interpretation manual. 2018. Available from: https://www.agilent.com/cs/library/usermanuals/public/29158_pd-l1-ihc-22C3-pharmdx-nsclc-interpretation-manual.pdf
15. Qu J, Mei Q, Liu L, et al. The progress and challenge of anti-PD-1/PD-L1 immunotherapy in treating non-small cell lung cancer. Ther Adv Med Oncol 2021;13:1758835921992968.
16. Tamiya M, Tamiya A, Hosoya K, et al. Efficacy and safety of pembrolizumab as first-line therapy in advanced non-small cell lung cancer with at least 50% PD-L1 positivity: a multicenter retrospective cohort study (HOPE-001). Invest New Drugs 2019;37:1266-73.
17. Sherwood JL, Brown H, Rettino A, et al. Key differences between 13 KRAS mutation detection technologies and their relevance for clinical practice. ESMO Open 2017;2:e000235.
18. Teramoto K, Igarashi T, Kataoka Y, et al. Biphasic prognostic significance of PD-L1 expression status in patients with early and locally advanced-stage non-small cell lung cancer. Cancer Immunol Immunother 2021;70:1063-74.
19. Miyazawa T, Marushima H, Saji H, et al. PD-L1 expression in non-small-cell lung cancer including various adenocarcinoma subtypes. Ann Thorac Cardiovasc Surg 2019;25:1-9.
20. Shien K, Papadimitrakopoulou VA, Wistuba II. Predictive biomarkers of response to PD-1/PD-L1 immune checkpoint inhibitors in non-small cell lung cancer. Lung Cancer 2016;99: 79–87.
21. Iwai Y, Hamanishi J, Chamoto K, Honjo T. Cancer immunotherapies targeting the PD-1 signaling pathway. J Biomed Sci 2017;24:26.
22. Zou W, Wolchok JD, Chen L. PD-L1 (B7-H1) and PD-1 pathway blockade for cancer therapy: Mechanisms, response biomarkers, and combinations. Sci Transl Med 2016;8:328rv4.
23. Iwai Y, Ishida M, Tanaka Y, et al. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. Proc Natl Acad Sci USA 2002;99:12293-7.
24. Reck M, Luft A, Szczesna A, et al. Phase III randomized trial of ipilimumab plus etoposide and platinum versus placebo plus etoposide and platinum in extensive-stage small-cell lung cancer. J Clin Oncol 2016;34:3740-8.
25. Herbst RS, Baas P, Kim DW, et al. Pembrolizumab versus docetaxel for previously treated, PDL1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial. Lancet 2016;387:1540–50.
26. Ng KeeKwong F, Laggner U, McKinney O, et al. Expression of PD-L1 correlates with pleomorphic morphology and histological patterns of non-small-cell lung carcinomas. Histopathology 2018;72:1024-32.
27. Wu Y, Ju Q, Jia K, et al. Correlation between sex and efficacy of immune checkpoint inhibitors (PD-1 and CTLA-4 inhibitors). Int J Cancer 2018;14:45-51.
28. Wu Y, Ju Q, Qian B, et al. The effectiveness of PD-1 inhibitors in non-small cell lung cancer (NSCLC) patients of different ages. Oncotarget 2017;9:7942–8.
29. King-Kallimanis BL, Kanapuru B, Blumenthal GM, et al. Age-related differences in patient-reported outcomes in patients with advanced lung cancer receiving anti-PD-1/PD-L1 therapy. Semin Oncol 2018;45:201-9.
30. Sun L, Zhang L, Yu J, et al. Clinical efficacy and safety of anti-PD-1/PD-L1 inhibitors for the treatment of advanced or metastatic cancer: a systematic review and metaanalysis. Sci Rep 2020;10:2083.
31. Wang Q, Li M, Yang M, et al. Analysis of immune-related signatures of lung adenocarcinoma identified two distinct subtypes: implications for immune checkpoint blockade therapy. Aging 2020;12:3312-39.
32. Brahmer JR, Tykodi SS, Chow LQ, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Eng J Med 2012;366:2455–65.
33. Herberth G, Bauer M, Gasch M, et al. Maternal and cord blood miR-223 expression associates with prenatal tobacco smoke

exposure and low regulatory T-cell numbers. J Allergy Clin Immunol 2014;133:543-50.
34. Givi ME, Folkerts G, Wagenaar GT, et al. Cigarette smoke differentially modulates dendritic cell maturation and function in time. Respir Res 2015;16:131.
35. Alexandrov LB, Nik-Zainal S, Wedge DC, et al. Signatures of mutational processes in human cancer. Nature 2013; 500;415-21.
36. Shepherd FA, Dancey J, Ramlau R, et al. Prospective randomized trial of docetaxel versus best supportive care in patients with non-small-cell lung cancer previously treated with platinum-based chemotherapy. J Clin Oncol 2000;18:2095-103.
37. Brahmer J, Reckamp KL, Baas P, et al. Nivolumab versus docetaxel in advanced squamous-cell non-small-cell lung cancer. N Engl J Med 2015;373:123-35.
38. Horn L, Mansfield AS, Szczęsna A, et al. First-line atezolizumab plus chemotherapy in extensive-stage small-cell lung cancer. N Engl J Med 2018;379:2220-9.
39. Zito Marino F, Rossi G, Montella M, et al. Heterogeneity of PD-L1 expression in lung mixed adenocarcinomas and adenosquamous carcinomas. Am J Surg Pathol 2020;44: 378-36.
40. Stanta G, Bonin S. Overview on clinical relevance of intratumor heterogeneity. Front Med (Lausanne) 2018;5:85.
41. Munari E, Zamboni G, Marconi M, et al. PD-L1 expression heterogeneity in non-small cell lung cancer: evaluation of small biopsies reliability. Oncotarget 2017;8:90123-31.
42. Vieira T, Antoine M, Hamard C, et al. Sarcomatoid lung carcinomas show high levels of programmed death ligand-1 (PD-L1) and strong immune-cell infiltration by TCD3 cells and macrophages. Lung Cancer 2016;98:51-8.
43. Kroeze LI, de Voer RM, Kamping EJ, et al. Evaluation of a hybrid capture-based pan-cancer panel for analysis of treatment stratifying oncogenic aberrations and processes. J Mol Diagn 2020;22:757-69.

Appendix authors

Divya Nair, Rajiv Shukla, Amit Kumar

Department of Pathology, Vallabhbhai Patel Chest Institute, University of Delhi, India

Contributions:
DN, RS, AS, contributed substantially to the study data acquiring and analysis.