

# PD-L1, Vimentin and Ki-67 Acting as Immunotherapy Predictive Biomarkers in Pulmonary Carcinomas Transthoracic and Bronchial Biopsies

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## Research Article

**Keywords:** Pulmonary Carcinoma, PD-L1, Immunotherapy, Epithelial-Mesenchymal Transition

**Posted Date:** December 9th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-1135697/v1>

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# Abstract

## Introduction:

Programmed death-ligand 1 (PD-L1) expression became a routine biomarker to preview response to programmed death-1 (PD-1)/PD-L1 inhibitors, with diverging parameters concerning PD-L1 scoring and variable response to immunotherapy agents. The aim of this study was to evaluate association between PD-L1 expression and immunohistochemistry panel applied in Pathology practice, defining any of those antibodies as biomarkers concurrent in patients selection for PD-1/PD-L1 blockade therapy.

## Methods

A total of 97 cTNM IIIb/IV staged pulmonary carcinoma biopsies were randomly selected between 2018/2020, after adequate representativeness and PD-L1 expression scored through Dako 22C3 antibody. The panel with cytokeratin 7, thyroid transcription factor 1 (TTF1), cytokeratin 5.6, cluster of differentiation 56 (CD56), periodic acid-Schiff (PAS-D), vimentin expression, and ki-67 labeling index (LI) was considered for retrieving reports and respective archival slides.

## Results

PD-L1 expression in tumor cells (TCs) was identified in 56 samples and significantly associated with male gender ( $p=0.028$ ), vimentin expression ( $p=0.018$ ) and ki-67 LI $>30\%$  ( $p=0.029$ ). A tendency to PD-L1 positivity came up in lymphocytic-predominant/immune-inflamed stroma (9/10), adenocarcinoma solid subtype (21/23) and CK7-negative squamous cell carcinomas (8/13). When more than 50% TCs expressed PD-L1, the risk of vimentin expression was 3.85 times higher (OR=3.85;  $p=0.013$ ), and for ki-67 LI $>30\%$  the risk was 9.90 times higher (OR=9.90;  $p=0.033$ ), compared with PD-L1-negative samples.

## Conclusion

High proliferation status defined by ki-67 LI $>30\%$  and epithelial-mesenchymal transition phenotype verified by vimentin staining analysis might complement PD-L1-positive TCs percentage determination for immunotherapy prescription. These patients will more likely benefit from PD-1/PD-L1 blockade therapy, overcoming the limitations of selection based on PD-L1 immunohistochemistry status.

## Introduction

Lung cancer remains clinically asymptomatic in early stages and 75% of cases are diagnosed at advanced stages, out of surgical staging resection and with 5-year survival rate of approximately 15% [1–3]. Targeted therapies with tyrosine kinase inhibitors (TKIs) have become the standard of care to approximately 20% of patients with pulmonary carcinomas, for the last two decades [3, 4].

Programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1) inhibitors are actually applied in combination with pemetrexed and carboplatin as first-line therapy in adenocarcinomas (ADCs), regardless of PD-L1 expression [5]. For pembrolizumab, PD-L1 expression determined by 22C3 Dako stain is mandatory for first-line therapy upon over 50% expression in tumor cells (TCs).

As a continuous biomarker within tumoral heterogenous compartments, without standardization among different assays, PD-L1 antibodies have been referred for the available drugs, after different detection antibodies and scoring systems [3, 6]. Blueprint Comparison Project demonstrated equivalency among 3 of the 4 currently used assays, with the limitation of including 39 tumor surgical samples [7], and Blueprint Phase 2 corroborated these results in 81 surgical samples [8].

Tumor mutation burden (TMB), evaluated by next-generation sequencing (NGS) [6], may emerge with predictive score for response to immunotherapy, aiding to overcome the limitations of PD-L1 immunohistochemistry (IHC) expression [9–11]. Rizvi et *al.* demonstrated that progression free survival (PFS) and clinical response to PD-L1 inhibitors were higher in patients with high TMB tumors, irrespective of PD-L1 status [12]. Also, higher TMB had been previously associated with clinical resistance to epidermal growth factor receptor (EGFR)-TKis [13], and Singal et *al.* found mutated EGFR, anaplastic lymphoma kinase (ALK), c-ros oncogene 1 (ROS1) and rearranged during transfection (RET) proto-oncogene correlated with significantly lower TMB [14]. TMB evaluation is not yet routinely used in clinical practice owing to elevated costs and interpretation complexity due to low *versus* high levels scores variability [9, 10].

Routine biopsies morphology and IHC panels support diagnosis, classification and screening for therapeutical targets in pulmonary carcinomas [15]. Defining tumoral histopathology subtyping with final diagnosis based on routine IHC panels allows: basic classification among incomplete represented tumors in biopsy samples, minimization of diagnostic mistakes, exclusion of metastatic origin and selection of samples for molecular testing and therapy guidance [16]. Recognition of ADCs patterns, namely solid, papillary, micropapillary, acinar and mucinous differentiation, and keratinizing *versus* non-keratinizing squamous cell carcinomas (SQCs), became feasible in Pathology practice [16].

A consistent panel of IHC antibodies, such as thyroid transcription factor 1 (TTF1) and NapsinA (both expressed in more than 85% of lung ADCs), cytokeratin (CK) 5/6 and p40 (to establish squamous cell differentiation), vimentin (mesenchymal marker) and proliferation marker ki-67 labeling index (LI), will change over 90% of biopsies sampling to correctly classify ADCs and SQCs, including other mixed subtypes [15]. IHC is definitely considered fast and cost-effective in routine Pathology practice, aiding the identification of predictive biomarkers of response to lung cancer therapies [15].

While high PD-L1 expression levels correlated with increased response to PD axis blockade therapy in several studies [3], some carcinomas harboring PD-L1-positive cells did not respond to immunotherapy, and 10-20% of responses to anti-PD therapy occurred in PD-L1-negative tumor biopsies [9, 17, 18]. Hence, since PD-L1 expression may not be an efficient predictive biomarker of response, but rather a risk factor used to select patients more likely to benefit from immunotherapy, additional predictive cost-effective

biomarkers are needed to identify potential responders to immunotherapy and be considered in follow-up [19].

The aim of this study was to evaluate the association between routine IHC panel applied to bronchial and pulmonary biopsies for pulmonary carcinomas classification, according to World Health Organization (WHO) 2015/2020 definitions, and PD-L1 status (22C3 Dako antibody). Proliferation marker ki-67 LI, dedifferentiation marker vimentin and tumoral stroma characteristics in association with PD-L1 expression were also considered in this study, to search patient selection for PD-1/PD-L1 blockade therapy in pulmonary carcinomas based in archival biopsy tissue, integrating the rationale for selecting the cases.

## Materials And Methods

### Tumor samples

Based on biopsy diagnosis of non-surgical stages bronchopulmonary carcinomas, cT3b and cT4 by 2017 TNM system, a series of 97 cases concerning 16 SQCs, 64 ADCs, 7 adenosquamous carcinomas (ADSQCs), 3 carcinomas not otherwise specified (NOS) and 7 pleomorphic carcinomas were included in this study. The cases were selected according to representative tumor tissue: at least three fragments in bronchial biopsies and similar area either in transthoracic and pleural biopsies, also represented in fragmented small surgical biopsies and pleural biopsies, consecutively collected in 2018/2020; neuroendocrine pattern/IHC (cluster of differentiation 56 (CD56)) expression were exclusive. WHO 2015/2020 classification for lung tumors, currently applied to biopsy specimens at the University Hospital of Coimbra, was well defined in this series of samples, according with representative tumor tissue, as referred.

ADC subtyping was registered according to the represented predominant pattern, as solid (23 cases), mucinous (22 cases) and acinar (12 cases); micropapillary designation was prevalent when this pattern was present (7 cases).

Median age of diagnosis was 68 years, ranging from 43 to 96 years; 75 patients were male and 22 were female. Descriptive data is summarized in Table 1. Informed consent was not applicable for this study and was waived by Faculty of Medicine of the University of Coimbra Ethical Committee. All methods were performed in accordance with relevant guidelines and regulations, and the study fulfilled the rules for archival retrospective study, also approved by the previously referred Ethical Committee.

Table 1  
Patients and sampling characteristics distribution according to lung carcinomas histopathological classification

	<b>SQC</b>	<b>ADC</b>	<b>ADCSQC</b>	<b>Carcinoma NOS</b>	<b>Pleomorphic</b>	<b>All patients</b>
	(n = 16)	(n = 64)	(n = 7)	(n = 3)	(n = 7)	(n = 97)
Age						
≤ 68	7	30	4	3	5	49
> 68	9	34	3	0	2	48
Gender						
Male	14	47	6	3	5	75
Female	2	17	1	0	2	22
Biopsy type						
Bronchial	12	25	4	3	1	45
Transthoracic	3	32	1	0	3	39
Surgical	1	5	2	0	3	11
Pleural	0	2	0	0	0	2
SQC squamous cell carcinoma, ADC adenocarcinoma, ADSQC adenosquamous carcinoma, NOS not otherwise specified						

## Immunohistochemistry

In order to ascertain tumor subgroups, CK7, TTF1, CK5.6, CD56, ki-67 LI and vimentin antibodies had been applied according to routine protocols (Supplementary Table 1). Periodic acid-Schiff (PAS-D) staining was performed in all carcinomas following the McManus Technique with diastase for glycogen digestion.

Concerning PD-L1, formalin-fixed paraffin-embedded (FFPE) serial sections of 3 µm were mounted on positively charged slides, deparaffinized and stained for PD-L1 using Food and Drug Administration (FDA)-approved Dako PD-L1 22C3 antibody (Dako, Carpinteria, CA). In Ventana platforms, sections were incubated in 3% diluted hydrogen peroxide for 5 minutes to neutralize endogenous peroxidase activity. Non-specific binding of primary antibodies and polymer were reduced with Protein Block. 22C3 Dako antibody, at 1:40 dilution, had been applied to tumor sections and then incubated for 52 minutes. After tris-buffered saline (TBS) washing, Post Primary Block was used to enhance penetration of anti-mouse/rabbit IgG HRP-polymer; 3,3' - diaminobenzidine (DAB) was used as chromogen. Finally, 0.02% diluted hematoxylin was used to counterstain the sections. Positive and negative controls were used, with human tonsil tissue as positive control for the PD-L1 staining.

The applied IHC panel, described in Supplementary Table 1, followed manufacturer indications. The slides were evaluated and scored in light microscopy by two experienced pathologists.

## **IHC scoring**

In general, 50% cut-off was defined for the applied routine antibodies, considering 3+ as high positivity. Positivity was near 100% for CK5.6 in SQCs and for CK7/TTF1 duet in ADCs. Vimentin expression cut-off was established also at 50% when expressed in TCs, as well as for PAS-D-positive cells interpretation, allowing two groups definition. Any expression of CD56 was considered for tumor exclusion from the present study, as referred.

## **Ki-67 LI scoring**

A binomial cut-off for ki-67 LI was defined at 30%, in accordance with previous studies, reporting this value as cut-off for prognosis assessment in pulmonary carcinomas instead of median ki-67 LI value, which is not clinically relevant according to literature [20].

## **PD-L1 scoring**

Immunohistochemical PD-L1 expression was scored after PD-L1 staining stratification through negative (0% expression in TCs), + (<5%), ++ (5-50%) and +++ (>50%). To make pathologists work reproducible, this estimation used the aforementioned four-point cut-off in order to approach the thresholds routinely employed in diagnostic settings, without interfering with criteria for pembrolizumab prescription [21, 22].

The binary PD-L1 expression score currently indicated for immunotherapy with pembrolizumab in advanced/metastatic lung cancer, establishing tumors with PD-L1 score of  $\geq 1\%$  but less than 50% to follow second-line therapy after one prior chemotherapy regimen and first-line treatment when 50% or more positive TCs are recognized in biopsies, had been clearly sustained in the reports [5, 17].

In this study, cases with PD-L1 positive TCs were then separated by +, ++ and +++ scores, and tumors with negative score (no stained TCs) formed another group.

The interpretation of PD-L1 immunostaining, as well as IHC correlations and final tumor diagnosis based in both histopathology predominant pattern and IHC panel expression, are represented in Table 2 and illustrated in Fig. 1. Carcinoma NOS diagnosis was consistent with representative bronchial biopsy cases where TTF1 and CK5.6 had no expression in TCs expressing CK7, with or without vimentin expression and without defined pattern, where giant and fusiform cells were also absent (Table 1).

Table 2  
Immunohistochemical and stromal characterization of SQC and ADC biopsies

	SQC		ADC			
	KTN (n = 7)	non-KTN (n = 9)	Solid (n = 23)	Micropapillary (n = 7)	Acinar (n = 12)	Mucinous (n = 22)
CK7						
Positive	0	3	23	7	12	22
Negative	7	6	0	0	0	0
TTF1						
Positive	0	0	22	7	10	17
Negative	7	9	1	0	2	5
PAS-D						
Positive	0	0	0	0	1	22
Negative	7	9	23	7	11	0
CK5.6						
Positive	7	9	1	0	0	0
Negative	0	0	22	7	12	22
Vimentin						
Positive	2	2	6	6	2	5
Negative	5	7	17	1	10	17
CD56						
Positive	0	0	0	0	0	0
Negative	7	9	23	7	12	22
Ki-67 LI						
≤30%	2	0	2	2	2	6
>30%	5	9	21	5	10	16
PD-L1 expression						
> 50%	2	3	10	5	1	3
5 - 50%	0	2	6	0	0	1

	SQC		ADC			
< 5%	1	1	5	0	0	5
Negative	4	3	2	2	11	13
Stroma subtype						
Immune-inflamed	1	1	5	0	1	1
Mixed	2	2	10	5	4	8
Fusiform	4	6	8	2	7	12
Lepidic	0	0	0	0	0	1
<i>SQC</i> squamous cell carcinoma, <i>ADC</i> adenocarcinoma, <i>KTN</i> keratinizing, <i>non-KTN</i> non keratinizing, <i>LI</i> labeling index						

## Tumoral stroma classification

Tumoral stroma subdivision was performed into four groups by light microscopy, following observation of bronchopulmonary carcinomas, in accordance with criteria adopted in previous studies [23–25].

Tumoral stroma was then classified as lymphocytic-predominant/immune-inflamed (when the infiltration of tumor-associated lymphocytes was predominant in the tumor stroma, positioned in the proximity to TCs), fusiform cells predominance and mixed type (when a balance between lymphocytes and fusiform cells was present). Lepidic pattern was represented in a transthoracic biopsy of one mucinous ADC, where TCs proliferated along the surface of intact or enlarged alveolar walls, consistent with lepidic tumoral pattern defined in WHO 2015/2020 criteria for ADCs. Stromal classification of ADC and SQC samples is described in Table 2.

## Statistical analysis

Statistical analysis was performed using SPSS statistics 26.0 software for Windows (SPSS, Chicago, USA). Descriptive statistics included median with range for continuous variables, and count and frequency for categorical variables. Associations between PD-L1 expression and stratified PD-L1 score with clinicopathological variables, IHC markers and stromal subtype followed a multistep statistical approach. Firstly, the existence of association between the binary PD-L1 expression and these variables was analyzed using Pearson's  $\chi^2$  test and Fisher's exact test. Secondly, these tests were applied in order to investigate the association between the stratified PD-L1 staining (negative, +, ++ or +++) and the parameters that were significantly associated with binary PD-L1 expression. Finally, a logistics regression was performed to ascertain the effects of PD-L1 staining stratification on the likelihood of positivity of IHC markers selected in the previous tests. P-values <0.05 were considered statistically significant.

## Results

## PD-L1 expression and male gender tumors

PD-L1 positivity reported in Table 2 concerned 56 cases, where 17 cases were classified as < 5% expression in TCs, 10 cases between 5-50% and 29 cases over 50%, with complete/incomplete cytoplasmic membrane staining.

PD-L1 positive expression was significantly associated with male gender ( $p=0.028$ ): 48 of the 56 samples positive for PD-L1 expression were found among male individuals, while among the 22 female patient samples, 14 tumors were scored as PD-L1-negative (Table 3). However, gender was not significantly associated with the stratified PD-L1 score (Table 3).

Table 3  
Patients, pathological factors and PD-L1 positivity in tumor cells

	Negative	PD-L1 pos. cases		Stratification of PD-L1 pos. cases			P value
		Total	P value	+	++	+++	
	(n = 41)	(n = 56)		(n = 17)	(n = 10)	(n = 29)	
Gender			<i>0.028</i>				0.131
Male	27 (65.85)	48 (85.71)		15 (88.24)	9 (90.00)	24 (82.76)	
Female	14 (34.15)	8 (14.29)		2 (11.76)	1 (10.00)	5 (17.24)	
IHC markers							
Vimentin			<i>0.018</i>				0.049
Positive	8 (19.51)	24 (42.86)		5 (29.41)	5 (50.00)	14 (48.28)	
Negative	33 (80.49)	32 (57.14)		12 (70.59)	5 (50.00)	15 (51.72)	
Ki-67 LI			<i>0.029</i>				<i>0.026</i>
≤30%	11 (26.83)	5 (9.26)		4 (25.00)	0 (0.00)	1 (3.57)	
>30%	30 (73.17)	49 (90.74)		12 (75.00)	10 (100.00)	27 (96.43)	
Stroma subtype			0.151				0.506
Immune-inflamed	1 (2.50)	9 (16.07)		3 (17.65)	1 (10.00)	5 (17.24)	
Mixed	14 (35.00)	21 (37.50)		5 (29.41)	4 (40.00)	12 (41.38)	
Fusiform	24 (60.00)	25 (44.64)		8 (47.06)	5 (50.00)	12 (41.38)	
Lepidic	1 (2.50)	1 (1.79)		1 (5.88)	0 (0.00)	0 (0.00)	
Data presented as n(%). Pearson's $\chi^2$ and Fisher's exact test results.							
<i>IHC</i> immunohistochemistry, <i>LI</i> labeling index, <i>pos.</i> positive							

ADC solid pattern and higher PD-L1 expression: among ADC cases evaluated for PD-L1 positivity, 21 of the 23 cases with solid pattern expressed PD-L1; 11 of the 12 acinar ADC cases and 13 of the 22 mucinous ADC cases were negative for PD-L1 expression (Supplementary Table 2).

SQC variable PD-L1 expression: in the 16 SQC samples, 3 cases expressed CK7 and of the 13 SQC CK7-negative samples, 8 expressed PD-L1, and 4 of these cases had PD-L1 expression over 50% in TCs (Supplementary Table 3).

## **Vimentin expression as an independent marker for immunotherapy selection**

Relationship between vimentin expression and PD-L1 positive expression was also significant ( $p=0.018$ ) (Table 3). Vimentin expression was positive in 32 cases, 24 of which also showed positive PD-L1 expression; and among the 41 PD-L1-negative samples, 33 cases were also negative for vimentin expression.

The stratified PD-L1 score was found significantly associated with vimentin expression ( $p=0.049$ ): in 24 vimentin-positive/PD-L1-positive samples, 14 had PD-L1 expression over 50% in TCs (Table 3). Vimentin was also significantly associated with histopathological subtyping ( $p=0.037$ ): 5 of the 7 pleomorphic carcinomas had vimentin expression over 50% in TCs (Supplementary Table 4).

A logistic regression was performed to ascertain the effects of PD-L1 expression on the likelihood that tumors were positive for vimentin expression. Samples with more than 50% of PD-L1 stained TCs were 3.85 times more likely to be vimentin-positive than PD-L1-negative specimens ( $OR=3.85$ ;  $p=0.013$ ) (Table 4).

Table 4

Risk of vimentin positivity and ki-67 LI &gt; 30%

		PD-L1 stratified score			
		Negative	+	++	+++
Vimentin expression					
		Ref.	1.72	4.13	3.85
OR	-	0.47 - 6.29	0.957 - 17.77	1.33 - 11.13	
p-value	-	0.413	0.057	0.013	
ki-67 LI > 30%					
		Ref.	1.10	592340775.71	9.90
OR	-	0.29 - 4.14	-	1.20 - 81.83	
p-value	-	0.888	-	0.033	
Logistics regression results					
Modeling index, Ref. reference, OR odds ratio, CI confidence interval					

## Ki-67 30% cut-off applicable in ADCs

A significant association was found between ki-67 LI and PD-L1 expression (p=0.029), where 49 of 54 positive PD-L1 cases had ki-67 LI>30% (Table 3).

The stratified PD-L1 score was also significantly associated with ki-67 LI (p=0.026), as 37 from 38 samples with PD-L1 score > 5% presented ki-67 LI>30% (Table 3).

A logistic regression was used to determine the relationship between PD-L1 expression and ki-67 LI>30%. Cases with PD-L1 expression over 50% in TCs showed a 9.90 times higher probability of having ki-67 LI>30%, *versus* PD-L1 negative tumors (OR=9.90; p=0.033) (Table 4).

## Immune-inflamed stroma and PD-L1 expression correlation

Patients' age, immunohistochemistry panel, PAS-D and carcinoma histopathological subtyping did not show a significant association with PD-L1 expression (Supplementary Table 5).

A tendency to PD-L1 positive expression came up in lymphocytic-predominant/immune-inflamed stroma samples ( $p=0.151$ ), where 9 of 10 samples with this stroma subtype showed positive PD-L1 expression in TCs (Table 3).

## Discussion

As heterogenous diseases, either at cellular and histopathological perspective, with distinct diagnostic, prognostic and therapeutic features [26], ADC and SQC keep being the most prevalent bronchopulmonary carcinomas, responsible for approximately 50% and 30% of cases, respectively [3].

With 5-year survival rate still under 20% [3], near 30% of patients with tumors in non-surgical stages have mutations amenable to targeted therapy [27], and PD-1/PD-L1 inhibitors have been prolonging patients survival with acceptable toxicity, proving undoubted superiority over chemotherapy and targeted therapy in terms of efficacy [9, 28].

Durable host immune anti-neoplasm responses and long-term remissions of several tumor types proved favorable benefit-to-risk of anti-PD therapy [17, 29]. For advanced carcinomas without EGFR/ALK mutations, European Medicines Agency (EMA) and FDA approved pembrolizumab monotherapy after PD-L1 score  $\geq 50\%$  or in combination with pemetrexed and platinum chemotherapy in carcinomas other than SQCs as first-line treatment, and as monotherapy for ADCs with PD-L1 expression between 1% and 50% after at least one prior chemotherapy regimen. [5, 17, 27].

Relationship between PD-L1 expression and gender remains contradictory [30, 31]. Concerning a limited series, our findings demonstrated that PD-L1 expression was significantly associated with gender, as 48 of 56 positive PD-L1 cases belonged to male patients.

It is becoming evident that histopathological subtyping relates to PD-L1 expression in TCs, particularly among solid ADC subgroup, still with worse prognosis. Driver et *al.* and Mandarano et *al.* demonstrated that lung ADCs defined by PD-L1 expression in TCs and tumor-infiltrating immune cells correlated with solid pattern, while acinar, mucinous and papillary patterns presented lower PD-L1 expression in TCs [4, 32]. As in literature [26, 30], our study confirmed that positive PD-L1 expression was relevant in solid pattern of ADCs (21/23), defining a clear relationship, while among acinar and mucinous subtypes, less than 50% of cases showed PD-L1 positive TCs.

EGFR mutations and ALK rearrangements keep being rare in advanced lung SQC, while immunotherapeutic strategies have been particularly effective [33, 34]. In CheckMate 017 trial, nivolumab improved survival, PFS and response rate *versus* docetaxel [35]. Following progression after first-line chemotherapy, PD-L1 inhibitors are the preferred treatment, according to U.S. National Comprehensive Cancer Network (NCCN) guidelines [33].

Cytokeratin 7, complementary gut differentiation marker present in normal glandular and transitional epithelium but not in squamous epithelium and expressed in 60-100% of ADCs [36, 37], is used to

subclassify lung SQC into two groups: pure SQC (CK7-negative), and non-pure SQC with CK7 expression, according to WHO 2015/2020 criteria for lung tumors. In considered pure SQC subgroup, EGFR and ALK mutations are almost absent and targeted therapy is much more limited, while for non-pure SQCs, molecular pathology may define therapy [38].

In our preliminary results, 8 cases out of 13 CK7-negative SQCs expressed PD-L1, with 4 cases over 50% of positive TCs. This tendency to high PD-L1 expression in pure lung SQC cases (CK7-negative) needs to be further characterized in future for a more personalized application of PD-1/PD-L1 immune checkpoint inhibitors in so-called pure SQCs.

The applicability of tumor microenvironment (TME) as a diagnostic, prognostic or predictive biomarker in bronchopulmonary carcinomas seems to correlate with tumorigenesis, heterogeneity, resistance to immunotherapy and tumor progression [2, 39], and also stromal cells may express the ligand PD-L1, with still unclear meaning [39].

The tendency of high PD-L1 expression in TCs among cases with lymphocytic-predominant/immune-inflamed stroma was 9/10 samples. As in previous studies [40], induction of TCs PD-L1 expression by interferon- $\gamma$  produced by T lymphocytes present in the TME corroborates our results. Tumor-infiltrating lymphocytes (TILs) have also been proposed as biomarker of response for PD-1/PD-L1 inhibition therapy [41, 42]. Anti-PD therapy seems less effective in non-inflamed tumors (low lymphocyte infiltration/PD-L1 expression) with increased levels of transforming growth factor- $\beta$  (TGF- $\beta$ ), inducer of resistance to anti-PD-L1 therapy, and fusiform cells rich stroma [43].

Epithelial-mesenchymal transition (EMT) with epithelial cells becoming mesenchymal cells by losing cell-cell adhesion and polarity and acquiring invasive/migratory properties, might contribute to drug resistance and hence poor prognosis [2, 39, 44, 45]. E-cadherin downregulation and overexpression of vimentin, N-cadherin,  $\alpha$ -actin and fascin depending on EMT genes, such as snail family transcriptional repressor 2 (Slug), twist family bHLH transcription factor 1 (TWIST), zinc finger E-box binding homeobox 1 (ZEB1), nuclear-translocated  $\beta$ -Catenin and TGF- $\beta$ , have been associated with TKi resistance, namely to EGFR-TKis [31, 42, 46].

To Kim et al., PD-L1 expression may be responsible for EMT oncogenesis and immune evasion during tumor development [47], contradictory with EMT-induced PD-L1 expression in pulmonary carcinomas [48]. PD-L1 and EMT bidirectional cross-talk has since then been proposed to promote tumor aggressiveness [47, 49]. Neurotrophic tyrosine receptor kinase (NTRK) gene rearrangements present in 0.1–1% of lung carcinomas, assessed by NGS and with effective targeted therapy [50, 51], are associated with microscopically high grade features and less differentiated phenotype in mesenchymal tumors [51], needing further studies to be correlated with EMT phenotype in carcinomas, where vimentin and other EMT markers expression might become relevant.

A significant association was found between PD-L1 expression and high vimentin expression in TCs, with 24 of 32 vimentin-positive cases expressing PD-L1, *versus* 33 of 41 PD-L1-negative samples without

vimentin expression. This result was consistent with previous observations that PD-L1 expression was positively correlated with vimentin expression and EMT phenotype in lung ADC, extrahepatic cholangiocarcinoma, breast carcinoma and head/neck/esophageal squamous carcinoma, suggesting that tumors with EMT status stand as potential targets for immunotherapy agents [47, 49]. In fact, Ancel et al. proposed vimentin as canonical marker and actor of EMT [31], verified in this series in 24 vimentin-positive/PD-L1-positive cases where 14 had PD-L1 expression in over 50% TCs, becoming a frequent event upon increasingly higher PD-L1 expression, significantly leading the risk of 3.85 times higher expression among cases with more than 50% PD-L1 stained TCs, *versus* PD-L1 negative samples.

Proliferation marker ki-67 keeps being associated with tumor aggressiveness and metastization in solid tumors [52]. A significant association was present between ki-67 LI and both positive PD-L1 expression and stratified PD-L1 score, as 49 of 54 PD-L1-positive cases had ki-67 LI>30% and 37 from 38 samples with more than 5% of PD-L1 stained TCs showed ki-67 LI>30%. Association between PD-L1 status and tumor cell proliferation was also confirmed by the tendency of PD-L1 positivity in the solid pattern of ADC samples, in accordance with literature [21, 53], still contradictory regarding association between PD-L1 expression and ki-67 LI in SQCs [53]. Similarly to vimentin, the risk of ki-67 LI>30% is 9.90 times higher in samples with more than 50% of PD-L1 stained TCs, *versus* PD-L1 negative specimens.

To the best of our knowledge, our study was the first to investigate the relationship between PD-L1 expression and EMT status, evaluated by vimentin included in IHC panel applied in Pathology practice, with perspective of risk analysis. Similarly to previous investigations [45, 47], EMT status by vimentin staining can be relevant in selecting patients more likely to have higher TCs PD-L1 expression. Favorable response to PD-1/PD-L1 immune checkpoint blockade in bronchopulmonary carcinomas would be then more accurate based on two biomarkers expressed in TCs, namely in follow-up of targeted therapy acquired resistance [44]. Also, as 10-20% of unselected patients with advanced carcinomas benefit from anti-PD therapy [18, 29, 41, 43], combined therapy of PD-1/PD-L1 inhibitors with EMT targeted therapies might become an ultimate therapeutical option and vimentin might work as a fundamental biomarker.

Combination of mitogen-activated protein kinase kinase (MEK) inhibitors with PD-L1 inhibitors improved tumor regression, where MEK inhibitors may sensitize TCs to immunotherapy agents [49]. Mechanistic target of rapamycin (mTOR) promotes EMT phenotype and immune evasion through upregulation of PD-L1 expression and the effect of mTOR inhibition combined with PD-L1 blockade was also reported in preclinical lung cancer trials [49]. Finally, combination of PD-1 inhibitors with EGFR TKis in PD-L1-positive carcinomas with EGFR activating mutations raised promising results in preclinical trials, as EGFR activation up-regulated PD-L1 expression, probably making these tumors more susceptible to PD-1/PD-L1 blockade therapy [27, 44, 49].

Limitations of this study, concerning 97 biopsies, and the utility of the applied IHC panel, deserve replication with additional EMT markers, to support EMT-phenotype as a new potential predictive biomarker to define immunotherapy in pulmonary carcinomas. Research on combined therapies with

EMT targeted agents and PD-L1 inhibitors to improve patients outcome and survival deserve to be implemented.

## Conclusion

PD-L1 expression was significantly associated with vimentin expression and ki-67 LI>30% and this association was maintained when stratified according to increasing intervals of PD-L1 expression score.

PD-L1 positive samples with more than 50% stained TCs had a significantly increased risk of expressing vimentin and presenting with high proliferation status defined by ki-67 LI>30%.

Consequently, ki-67 LI>30% and vimentin expression may become rationale biomarkers that can be used to identify tumors more likely to benefit from PD-1/PD-L1 axis blockade, overcoming the limitations of single PD-L1 IHC scoring due to tumoral heterogeneity and high staged carcinomas associated with resistance to targeted therapy.

Vimentin expression and ki-67 LI may also complement the evaluation of TMB as cost-effective and available markers. Reinforcing EMT targeted therapy agents combined with PD-L1 inhibitors in bronchopulmonary carcinomas, an EMT phenotype is raising as a promising field for future research.

## Declarations

**Acknowledgments** The authors thank Dr. Ana Ladeirinha (Institute of Anatomical and Molecular Pathology, Faculty of Medicine, University of Coimbra, Portugal) for support with statistical analysis and execution of immunohistochemistry.

**Conflicts of interest** The authors report no conflicts of interest.

**Funding** The authors did not receive funding for conducting this study.

**Data availability** Data sets from this study are available upon request from the corresponding author.

**Code availability** Not applicable.

**Ethics approval** The study fulfilled the rules for archival retrospective study defined by the Faculty of Medicine of the University of Coimbra Ethical Committee.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Compliance with Ethical Standards** The study fulfilled the rules for archival retrospective study defined by the Faculty of Medicine of the University of Coimbra Ethical Committee.

**Contributions** All authors contributed to the study development and design. Data collection, preparation and analysis were performed by Maria Inês Figueiredo, Vítor Sousa and Lina Carvalho. The first draft of the manuscript was written by Maria Inês Figueiredo and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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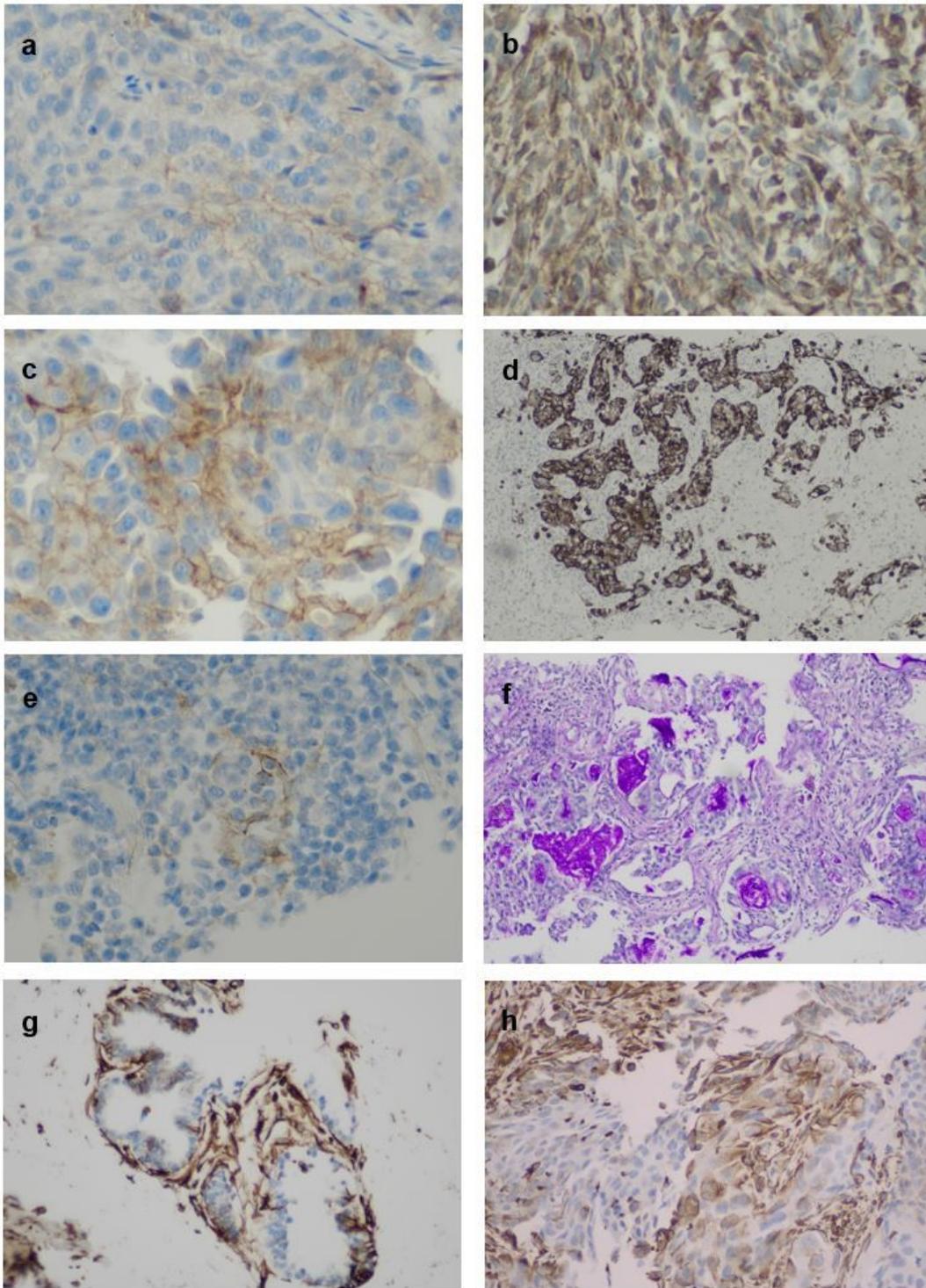
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## Figures

**Fig. 1**



**Figure 1**

PD-L1 22C3 Dako immune expression is scored in routine Pathology following tumor cells complete and/or incomplete cytoplasmic membrane immunostaining independent from intensity – squamous cell carcinoma with malignant spindle cells suggesting pleomorphic carcinoma in bronchial biopsy was scored with PD-L1 of 60%, x200 (a), sustained by CK5.6 expression, x400 (b); adenocarcinoma with relevant solid pattern in transthoracic biopsy with PD-L1  $\geq$ 50%, x400 (c) and cytoplasmic CK7

expression, x100 (d); transthoracic biopsy of mucinous adenocarcinoma with PD-L1 5% score, x400 (e) and PAS-D mucin demonstration, x200 (f); transthoracic biopsy of adenocarcinoma – acinar pattern (CK7/TTF1 3+) with > 50% vimentin expression in tumor cells, x200 (g) and vimentin >50% expression in bronchial biopsy of keratinizing squamous cell carcinoma, x400 (h).

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