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PD-L1, Vimentin and Ki-67 as predictive markers in pulmonary carcinomas

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PD-L1, Vimentina e Ki-67 como marcadores preditivos nos carcinomas pulmonares

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ABBREVIATIONS

ADC, adenocarcinoma;

ADSQC, adenosquamous carcinoma;

ALK, anaplastic lymphoma kinase;

BA, bronchioloalveolar;

BRAF, B-Raf proto-oncogene;

CI*,* confidence interval;

DAB, 3,3' – diaminobenzidine;

EGFR, epidermal growth factor receptor;

EMA, European Medicines Agency;

EML4-ALK, echinoderm microtubule-associated protein-like 4 and anaplastic lymphoma kinase;

EMT, epithelial-mesenchymal transition;

ER, epitope retrieval;

FDA, Food and Drug Administration;

FFPE, formalin-fixed paraffin-embedded;

HER2, human epidermal growth factor receptor 2;

IHC, immunohistochemistry;

KTN, keratinizing;

LI, labeling index;

MEK1, mitogen-activated protein kinase kinase 1;

MET, c-MET proto-oncogene;

mTOR, mechanistic target of rapamycin;

NCCN, National Comprehensive Cancer Network;

NGS, next-generation sequencing;

NTRK, neurotrophic tyrosine receptor kinase;

OR, odds ratio;

PD-1, programmed cell death 1;

PD-L1, programmed cell death ligand 1;

PFS, progression free survival;

PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha;

PTEN, phosphatase and tensin homolog;

RAS/MAPK, RAS/mitogen-activated protein kinase;

Ref., reference;

RET, rearranged during transfection;

ROS1, c-ros oncogene 1;

SQC, squamous cell carcinoma;

TBS, tris-buffered saline;

TCs, tumor cells;

TGF-β, transforming growth factor-β;

TILs, tumor-infiltrating lymphocytes;

TKis, tyrosine kinase inhibitors;

TMB, tumor mutation burden;

TME, tumor microenvironment;

TPS, tumor proportion score;

TTF1, thyroid transcription factor 1;

WHO, World Health Organization.

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ABSTRACT

Introduction: PD-L1 expression is currently approved as a biomarker of response to PD-1/PD-L1 inhibitors, and diverging parameters are emerging amongst PD-L1 scoring in response to immunotherapy agents. The aim of this study was to evaluate the association between PD-L1 expression and the routine panel applied in Pathology practice, in order to determine whether these antibodies might serve as biomarkers to guide patient selection for PD-1/PD-L1 blockade therapy.

Methods: A total of 97 lung cancer biopsies randomly selected were analyzed, where PD-L1 expression had been scored through Dako 22C3 pharmDx kit (Dako, Carpinteria, CA). CK7, TTF1, CK5.6, CD56, PAS-D and vimentin expression and ki-67 labeling index (LI) were retrieved from Pathology reports in association with PD-L1 status.

Results: PD-L1 positive 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 tumors with predominant lymphocytic stroma (9/10), adenocarcinoma solid subtype (21/23) and CK7-negative squamous cell carcinomas (8/13). In tumors with more than 50% stained PD-L1 TCs, the risk of vimentin expression was 3.85 times higher (OR=3.85; p=0.013) and the risk of ki-67 LI>30% 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 determined by vimentin staining analysis seem to be predictive biomarkers for the identification of tumors with higher percentage of PD-L1-positive TCs, more likely to benefit from PD-1/PD-L1 blockade therapy, overcoming the limitations of patient selection based on PD-L1 immunohistochemistry status.

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

RESUMO

Introdução: A expressão de PD-L1 foi aprovada como um biomarcador preditivo da resposta à terapêutica com inibidores do eixo PD-1/PD-L1, apesar dos parâmetros divergentes que têm vindo a surgir relativamente aos sistemas de quantificação da expressão de PD-L1 em resposta à imunoterapia. O objetivo deste estudo consistiu na avaliação da associação entre a expressão de PD-L1 e o painel de anticorpos de rotina utilizado na prática clínica, de modo a averiguar se estes anticorpos poderão vir a ser utilizados, como biomarcadores, na seleção de pacientes para imunoterapia com fármacos anti-PD-1/PD-L1.

Métodos: Foram analisadas 97 amostras aleatoriamente selecionadas, onde a expressão proteica de PD-L1 foi determinada aplicando o kit Dako 22C3 pharmDx (Dako, Carpinteria, CA). A expressão de CK7, TTF1, CK5.6, CD56, PAS-D, vimentina e o valor percentual de ki-67 foram obtidos retrospetivamente de análises prévias, tal como o nível de expressão de PD-L1.

Resultados: A expressão de PD-L1 foi identificada nas células tumorais de 56 amostras, estando significativamente relacionada com o género masculino (p=0.028), expressão de vimentina (p=0.018) e com um valor percentual de ki-67>30% (p=0.029). Foi identificada uma tendência para a expressão de PD-L1 nas amostras com um estroma predominantemente linfocítico (9/10), nas amostras de adenocarcinoma com padrão sólido (21/23) e nas amostras de carcinoma espinocelular negativas para a expressão de CK7 (8/13). Efetuando uma análise de risco, verificou-se que nas amostras com mais de 50% de expressão de PD-L1 nas células tumorais, o risco de expressão vimentina era 3.85 vezes superior (OR=3.85; p=0.013) e que o risco de apresentarem uma percentagem de ki-67>30% era 9.90 vezes superior (OR=9.90; p=0.033), comparativamente às amostras negativas para a expressão de PD-L1.

Conclusão: Uma alta taxa proliferativa, definida por um valor percentual de ki-67>30%, e um fenótipo de transição epitélio-mesênquima, definido pela expressão de vimentina, poderão ser biomarcadores preditivos relevantes na identificação de tumores com uma maior percentagem de células tumorais com expressão de PD-L1 e, consequentemente, mais propícios a desenvolverem uma resposta favorável aos fármacos inibidores do eixo PD-1/PD-L1, ultrapassando assim as limitações da seleção de doentes baseada apenas na determinação imuno-histoquímica da expressão tumoral de PD-L1.

Palavras-Chave: Carcinoma Pulmonar, PD-L1, Imunoterapia, Transição Epitélio-Mesênquima

INTRODUCTION

Lung cancer remains clinically asymptomatic in early stages and 75% of cases are diagnosed at an advanced stage, where a surgical resection is no longer an option, leading to a poor 5-year survival rate of approximately 15% [1–3]. Within the last two decades, targeted therapies with tyrosine kinase inhibitors (TKis) have become the standard of care to approximately 20% of patients with pulmonary carcinomas [3,4].

Programmed cell death 1(PD-1)/ programmed cell death ligand 1 (PD-L1) inhibitors, the base of immunotherapy, may be actually applied in combination with pemetrexed and carboplatin as first-line therapy in lung adenocarcinomas (ADCs), regardless of PD-L1 expression [5]. For pembrolizumab, PD-L1 expression determined by immunohistochemistry (IHC) stains is necessary for its approval as first-line therapy.

PD-L1 assessment remains challenging, since it is a continuous biomarker within tumoral heterogenous expression and there is no clear standardization among the different PD-L1 assays, concerning the antibodies referred in the published studies for the available drugs, after different detection methods and scoring systems [3,6]. The Blueprint Comparison Project demonstrated equivalency among 3 of the 4 currently used assays, with the limitation of including 39 tumor samples [7], and Blueprint Phase 2 corroborated this results using 81 samples [8].

Tumor mutation burden (TMB), as evaluated by next-generation sequencing (NGS) [6], is emerging as a predictive biomarker of response to immunotherapy, aiding to overcome the limitations of PD-L1 IHC expression [9–11]. Rizvi et al. demonstrated that progression free survival (PFS) and clinical response to PD-L1 inhibitors was higher in patients with tumors presenting high TMB, irrespective of PD-L1 status [12]. While for targeted therapy, higher TMB was associated with clinical resistance to epidermal growth factor receptor (EGFR)-TKis in previous investigations [13], and Singal et al. found that mutations in EGFR, anaplastic lymphoma kinase (ALK), c-ros oncogene 1 (ROS1) and rearranged during transfection (RET) proto-oncogene were correlated with significantly lower TMB [14]. Evaluation of TMB is not yet routinely used in clinical practice, due to elevated costs and interpretation complexity, and a threshold for classification of TMB levels as low *versus* high still remains to be found [9,10].

The most important method for diagnosis, classification and screening for therapeutical targets determination in pulmonary carcinomas remains to be morphology and IHC [15]. The benefits of defining tumoral histopathology with final diagnosis based on routine IHC panels include: a correct classification of the histopathological type (mainly among poorly represented tumors in small biopsy samples) in order to minimize diagnostic mistakes, excluding also metastatic

origin, and to select samples for molecular testing and therapy guidance [16], following recognition of histopathological subtyping patterns, namely solid, papillary, micropapillary, acinar and mucinous for ADCs; and keratinizing *versus* non-keratinizing for squamous cell carcinomas (SQCs) [16].

A consistent panel of IHC antibodies, such as thyroid transcription factor 1 (TTF-1) and NapsinA (both expressed in more than 85% of lung ADCs), CK5/6 and p63 (used to establish squamous cell differentiation), vimentin (as 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 a fast and cost-effective method applied in routine Pathology practice, aiding the identification of predictive biomarkers of response to lung cancer therapies [15].

Several studies have shown that high PD-L1 expression levels correlate with an increased response to PD axis blockade therapy [3]. However, some tumors harboring PD-L1-positive cells do not respond to therapy, while 10-20% of responses to anti-PD therapy occur after PD-L1-negative biopsies [9,17,18]. Hence, since PD-L1 expression alone is not 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 [19].

The aim of this study was to evaluate the association between the routine IHC panel, according to World Health Organization (WHO) 2015/2021 definitions, and PD-L1 status, considering also the proliferation marker ki-67 LI, the dedifferentiation marker vimentin and the tumoral stroma characteristics in association with PD-L1 expression, to guide patient selection for PD-1/PD-L1 blockade therapy in pulmonary carcinomas, based in biopsy tissue.

MATERIALS AND METHODS

Tumor samples

Based on biopsy diagnosis of non-surgical bronchopulmonary carcinomas, staged as pT3b or pT4 by the 2017 TNM system, a series of 97 cases concerning 16 SQCs, 64 ADCs, 7 adenosquamous carcinomas (ADSQCs), 3 possible large cell carcinomas and 7 pleomorphic carcinomas were included in this study. WHO 2015/2021 classification for lung tumors was applied to biopsy specimens belonging to the archives of the University Hospital of Coimbra.

ADC subtyping classification was determined according to the 2015/2021 WHO criteria as solid (23 cases), mucinous (22 cases), acinar (12 cases) and micropapillary (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. The study fulfilled the rules for an archival retrospective study defined by the Faculty of Medicine of the University of Coimbra Ethical Committee.

Table 1 Clinical and pathological characteristics distribution according to lung carcinomas histopathological subtyping

	SQC (n = 16)	ADC (n = 64)	ADCSQC (n = 7)	Large cell (n = 3)	Pleomorphic (n = 7)	All patients (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

Immunohistochemistry

In order to ascertain tumor subgroups, IHC had been performed by applying CK7, TTF1, CK5.6, CD56, ki-67 LI and vimentin immunostaining, according to available protocols (Table 2). PAS-D staining was performed following the McManus Technique with diastase for glycogen digestion.

Formalin-fixed paraffin-embedded (FFPE) serial sections of 3 μm were mounted on positively charged slides, deparaffinized and stained for PD-L1 using the Food and Drug Administration (FDA)-approved Dako PD-L1 22C3 pharmDx kit (Dako, Carpinteria, CA). Sections were also 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 1:35 dilution, was applied to the sections and then incubated for 30 minutes. After washing with tris-buffered saline (TBS), Post Primary Block was used to enhance penetration of the 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, and human tonsil tissue was used as a positive control for the PD-L1 staining, as well as for all the other applied antibodies (Table 2). The slides were evaluated in light microscopy and scored by two experienced pathologists.

For assessment of PD-L1 protein expression, 22C3 Dako antibody was applied in Ventana autostainer, following Roche guidelines, with inclusion of positive and negative controls. The applied IHC panel, described in Table 2, followed manufacturer indications.

Table 2 Antibodies applied and immunohistochemistry method

Primary antibody	Clone	Manufacturer	Positive control	Method	Antigen retrieval	Dilution and incubation time
CK7	OV-TL12/30	Dako	Endometrium	BondMax	Enzym 1 (10')	1:800, 30'
TTF1	SPT24	Leica	Small cell carcinoma	BondMax	ER2 (20')	1:250, 30'
CK5.6	D5/16B4	Dako	Skin	BondMax	ER2 (32')	1:100, 28'
Vimentin	Vim 3B4	Dako	Colon	BondMax	ER1 (20')	1:250, 30'
CD56	CD564	Novocastra	Colon	BondMax	ER1 (20')	1:240, 20'
Ki-67	MIB-1	Dako	Small cell carcinoma	BondMax	ER2 (20')	1:150, 30'
PD-L1	22C3	Dako	Tonsil	BondMax	ER2 (45')	1:35, 60'

ER1 epitope retrieval solution 1, *ER2* epitope retrieval solution 2

IHC scoring

In general, 50% cut-off was defined for the applied routine antibodies, to be considered 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 tumor cells (TCs), and this criterion was also applied for CD56 and PAS-D positive cells, allowing two groups definition.

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 a cut-off for prognosis assessment in pulmonary carcinomas instead of the median ki-67 LI value, which is not clinically relevant according to literature [20].

PD-L1 scoring

Immunohistochemical expression of PD-L1 with 22C3 Dako assay 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 [21,22].

The binary PD-L1 expression score considered was based on the current indications for immunotherapy with pembrolizumab in advanced/metastatic lung cancer, establishing tumors with a PD-L1 tumor proportion score (TPS) of 1% to follow second-line therapy after one prior chemotherapy regimen, while for first-line treatment with pembrolizumab 50% or more positive TCs have to be recognized in biopsies [5,17]. In this study, tumors with PD-L1 positive cells, encompassing +, ++ and +++ scores, were separated from tumors with negative score (no stained TCs), where positive tumors fulfilled the 1% PD-L1 expression for second-line or first-line associated immunotherapy. Fig. 1 demonstrates the interpretation of PD-L1 immunostaining.

PD-L1 positivity had been detected in 56 cases, where 17 cases were classified between 1-5%, 10 cases between 5-50% and 29 cases over 50% of stained TCs (Table 3).

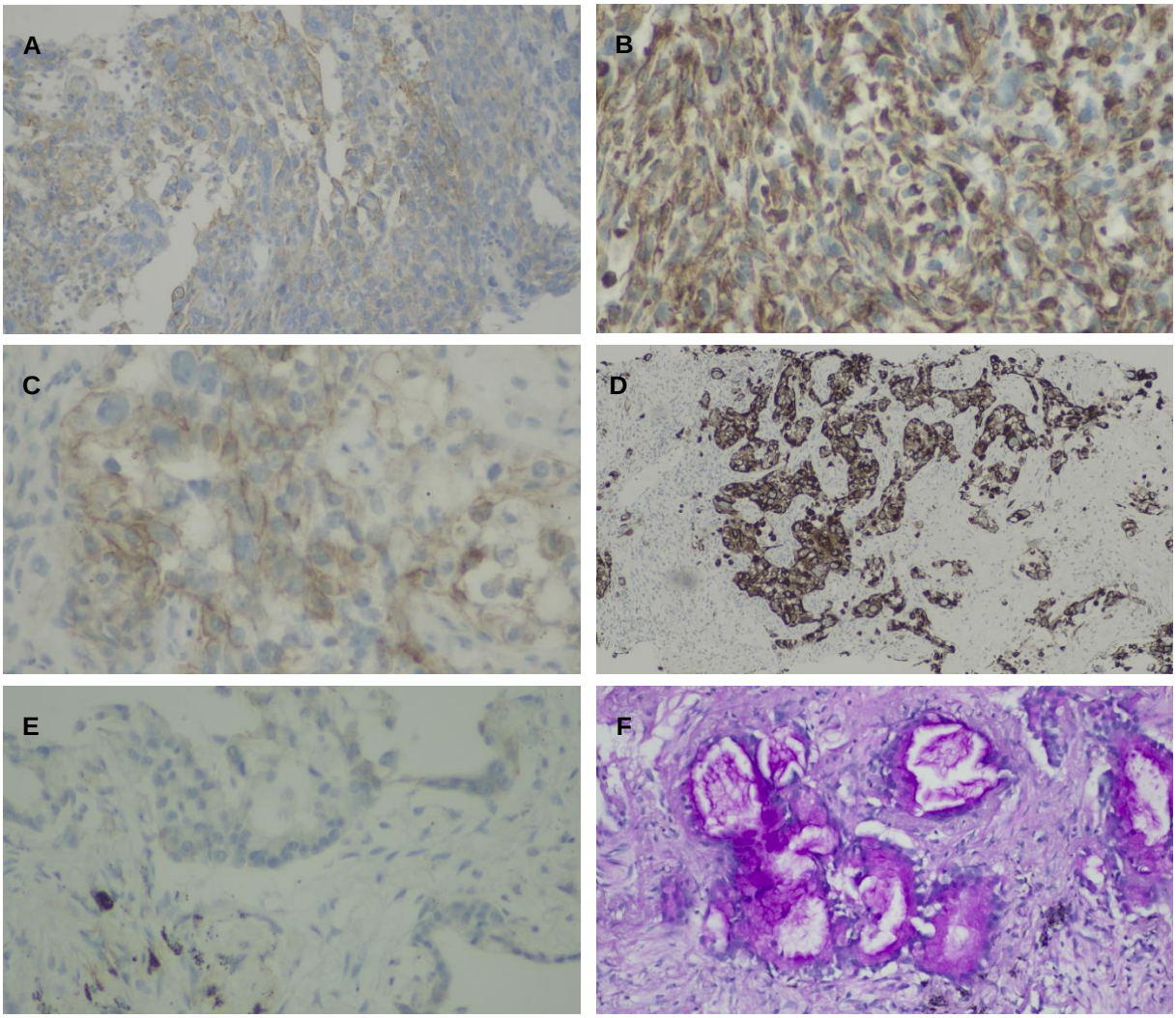

Fig. 1 PD-L1 22C3 Dako immunoexpression is scored in routine Pathology following tumor cells complete and/or incomplete cytoplasmatic membrane immunostaining independent from intensity – squamous cell carcinoma with malignant spindle cells suggesting pleomorphic carcinoma in bronchial biopsy was scored 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≥80%, x400 (C) and cytoplasmatic CK7 expression, x100 (D); transthoracic biopsy of mucinous adenocarcinoma with PD-L1 5% weak intensity, x400 (E) and PAS-D mucin demonstration, x200 (F).

The final tumor diagnosis based in both histopathology predominant pattern and IHC panel expression is described in Table 3. Large cell carcinoma 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 absent.

Table 3 IHC 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
1 - 5%	1	1	5	0	0	5
Negative	4	3	2	2	11	13
Stroma subtype						
Limphocytic	1	1	5	0	1	1
Mixed	2	2	10	5	4	8
Fusiform	4	6	8	2	7	12
BA	0	0	0	0	0	1

SQC squamous cell carcinoma, *ADC* adenocarcinoma, *KTN* keratinizing, *non-KTN* non keratinizing, *LI* labeling index, *BA* bronchioloalveolar

Tumoral stroma classification

Tumoral stroma subdivision was performed into four groups by light microscopy, following experienced observation of bronchopulmonary carcinomas, in accordance with criteria adopted in previous studies [23,24]. Tumoral stroma was classified as lymphocytic (where predominance of background lymphocytes was possible to consider in biopsies), fusiform cells predominance and mixed type (where a balance between lymphocytes and fusiform cells was present). The bronchioloalveolar/lepidic type (BA) was represented in the transthoracic biopsy of one mucinous ADC, where TCs proliferated along the surface of intact or enlarged alveolar walls, consistent with bronchioloalveolar/lepidic tumoral pattern defined in WHO 2015/2021 criteria for ADCs. Stromal classification of ADC and SQC samples is described in Table 3.

Statistical analysis

Statistical analysis was performed using the 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 intensity 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 the 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 intensity (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 intensity on the likelihood of positivity of IHC markers selected in the previous tests. P-values <0.05 were considered statistically significant.

RESULTS

PD-L1 in male gender tumors

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 were scored PD-L1-negative (Table 4). However, gender was not found to be significantly associated with the stratified PD-L1 score (Table 4).

ADC solid pattern and higher PD-L1 expression

Among the ADC specimens 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 (Table 5).

SQC with variable PD-L1 expression

Among the 16 SQC samples, 3 cases expressed CK7 (Supplementary table 1) and of the 13 SQC CK7-negative samples, 8 expressed PD-L1, and 4 of these cases had PD-L1 expression in over 50% of TCs.

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 4). Vimentin expression was positive in 32 cases, 24 of which showed PD-L1 expression ≥ 1%; and among the 41 PD-L1-negative samples, 33 were also negative for vimentin expression.

The stratified PD-L1 score was found significantly associated with vimentin expression (p=0.049), and in the 24 vimentin-positive/PD-L1-positive samples, 14 had PD-L1 expression in over 50% of TCs (Table 4). Vimentin was also significantly associated with the histological subgroup (p=0.037), as 5 of the 7 less differentiated pleomorphic carcinoma samples were also positive for vimentin expression (Supplementary Table 2).

A logistic regression was performed to ascertain the effects of PD-L1 expression on the likelihood that samples 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 6).

Ki-67 30% cut-off applicable for 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 4).

The PD-L1 stratified 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 4).

A logistic regression was used to determine the relationship between PD-L1 expression and ki-67 LI>30%. The cases with PD-L1 expression over 50% on TCs showed a 9.90 times higher probability of having ki-67 LI>30%, *versus* PD-L1 negative specimens (OR=9.90; p=0.033) (Table 6).

Lymphocytic stroma and PD-L1 expression correlation

Patients' age, immunohistochemistry panel, PAS-D and carcinoma histological subtyping did not show a significant association with PD-L1 expression (Supplementary Table 3).

A tendency to PD-L1 positive expression came up in lymphocytic stroma samples (p=0.151), where 9 of the 10 of samples with a lymphocytic stroma showed positive PD-L1 expression (Table 4).

Table 4 Clinical and pathological factors by PD-L1 positivity in tumor cells

	Negative	PD-L1 pos. cases		Stratification of PD-L1 pos. cases			
		Total	P value	+	++	+++	P value
	(n = 41)	(n = 56)		(n = 17)	(n = 10)	(n = 29)	
Gender			*0.028*				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			*0.018*				*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			*0.029*				*0.026*
≤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
Limphocytic	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)	
BA	1 (2.50)	1 (1.79)		1 (5.88)	0 (0.00)	0 (0.00)	

Data presented as n(%). Pearson's χ2 and Fisher's exact test results.

IHC immunohistochemistry, *LI* labeling index, *BA* bronchioloalveolar, *pos.* positive

Table 5 PD-L1 expression according to histological and stromal subtype

	SQC		ADC							
	(n = 16)		Solid (n = 23)		Micropapillary (n = 7)		Acinar (n = 12)		Mucinous (n = 22)	
	PD-L1 (n)		PD-L1 (n)		PD-L1 (n)		PD-L1 (n)		PD-L1 (n)	
	neg.	pos.	neg.	pos.	neg.	pos.	neg.	pos.	neg.	pos.
Stroma subtype										
Lymphocytic	0	2	0	5	0	0	1	0	0	1
Mixed	1	3	1	9	1	4	4	0	6	2
Fusiform	6	4	1	7	1	1	6	1	6	6
BA	0	0	0	0	0	0	0	0	1	0
P value	0.202		0.725		1.000		0.677		0.336	

Fisher's exact test results

BA bronchioloalveolar, *SQC* squamous cell carcinoma, *ADC* adenocarcinoma, *neg.* negative, *pos.* positive

Table 6 Risk of vimentin positivity and ki-67 LI > 30%

	PD-L1 stratified intensity			
	Negative	+	++	+++
Vimentin expression				
OR	Ref.	1.72	4.13	3.85
95% CI	-	0.47 - 6.29	0.957 - 17.77	1.33 - 11.13
P value	-	0.413	0.057	*0.013*
Ki-67 LI > 30%				
OR	Ref.	1.10	592340775.71	9.90
95% CI	-	0.29 - 4.14	-	1.20 – 81.83
P value	-	0.888	-	*0.033*

Logistics regression results

LI labeling index, *Ref.* reference, *OR* odds ratio, *CI* confidence interval

DISCUSSION

Bronchopulmonary carcinomas classification in biopsies concerns the wide accepted criteria for routine interpretation and data registries according with WHO 2015/2021 criteria, in order to interpret molecular pathology. As an heterogenous disease, either at cellular and histopathological perspective, with distinct diagnostic, prognostic and therapeutic features [25], ADC and SQC are currently the two most prevalent histopathological subtypes, accounting for approximately 50% and 30% of cases, respectively [3].

EGFR and echinoderm microtubule-associated protein-like 4 (EML4)-ALK gene mutations in lung ADC paved the way for the development of targeted therapies using TKis [3], together with B-Raf proto-oncogene (BRAF) mutations, human epidermal growth factor receptor 2 (HER2) amplification, c-MET proto-oncogene (MET) amplification, ROS1 rearrangements, RET fusions, neurotrophic tyrosine receptor kinase (NTRK) fusion, mitogen-activated protein kinase kinase 1 (MEK1) mutations and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) mutations as less frequent targets [3,26]. International Association for the Study of Lung Cancer and Association for Molecular Pathology recommends testing for EGFR, ALK and ROS1 mutations in all patients who have metastatic tumors, irrespective of clinical features [16].

However, most of the targetable alterations described in ADCs are rarely present in pure SQCs [27], and clinical trials in patients with SQCs involving drugs targeting these kinases have been disappointing when compared with ADCs [3,28]. Targeted therapy for SQCs is currently under active research, with the PIK3CA mutation and loss of function of phosphatase and tensin homolog (PTEN) tumor suppressor gene being some of the most promising targets [28].

With 5-year survival rate still under 20% [3], only approximately 30% of patients with tumors in non-surgical stages have mutations in considered driver genes that are amenable to targeted therapy [29]. In stage IV lung cancer, immune checkpoint blockers, including PD-1/PD-L1 inhibitors, prolonged patients survival with an acceptable toxicity, proving undoubted superiority over chemotherapy and targeted therapy in terms of efficacy [9,30].

PD-1/PD-L1 pathway blockade has become the base of immunotherapy and created durable host immune anti-neoplasm responses and long-term remissions in a subset of patients with several tumor types [17,31]. Due to the proved favorable benefit-to-risk profile of anti-PD therapy, the European Medicines Agency (EMA) and FDA approved pembrolizumab monotherapy for the first-line treatment of metastatic carcinomas in tumors with PD-L1 TPS ≥ 50%, without EGFR or ALK genomic aberrations [5,29]. It is also approved in combination with pemetrexed and platinum chemotherapy as first-line treatment of metastatic carcinomas

other than SQCs, without EGFR or ALK positive mutations, and as monotherapy for treatment of advanced/metastatic ADCs in tumors with PD-L1 TPS between 1% and 50% who had at least one prior chemotherapy regimen [5,17].

Association between PD-L1 expression and clinicopathological characteristics shows contradictory results in literature, namely the relationship between PD-L1 expression and gender [32,33]. Although concerning a limited series, our findings demonstrated that PD-L1 expression was significantly associated with gender, with 48 of the 56 positive PD-L1 expression samples belonging to male gender.

Investigating the potential interest of clinicopathological correlations with PD-L1 expression, Driver et al. demonstrated that lung ADC samples defined by PD-L1 expression in TCs or tumor-infiltrating immune cells correlated with solid pattern, while the patterns with the lowest PD-L1 levels included acinar, mucinous and papillary subtypes [34]. Mandarano et al. also reinforced that high PD-L1 expression levels were associated with the solid pattern of ADCs [4]. Our study confirmed that PD-L1 positive expression (over 1% stained TCs) was relevant in the solid pattern of ADCs (21/23), while among the acinar and mucinous subtypes, less than 50% of cases showed PD-L1 protein expression.

It is becoming evident that histopathological subtyping is related to the PD-L1 TPS on TCs, particularly among the solid ADC subgroup, which is also related with worse prognosis. Therefore, our results support current literature in which a clear relationship between solid pattern and PD-L1 protein expression was described [25,32].

Available therapeutic options for advanced lung SQC remain limited when compared to those for ADCs [27]. Given the rarity of EGFR mutations and ALK rearrangements in advanced lung SQCs, the majority of these patients do not receive targeted therapies [27]. However, immunotherapeutic strategies aimed at negative costimulatory receptors were found to be particularly effective in SQCs [28]. CheckMate 017 trial showed that nivolumab improved survival, PFS and response rate *versus* docetaxel in patients with SQC [26]. In fact, following progression after first-line chemotherapy, PD-L1 inhibitors are the preferred treatments for advanced lung SQC, according to the U.S. National Comprehensive Cancer Network (NCCN) guidelines [27].

CK7 as a glandular and anterior gut differentiation marker, present in most normal glandular and transitional epithelium but not in squamous epithelium [35,36], is expressed in 60-100% of ADCs and in up to 25% of SQC samples [36] and is used to subclassify lung SQC into two groups: pure SQC (CK7-negative, without any invasive glandular component) and non-pure SQC (CK7-positive). Among the pure SQC subgroup, EGFR and ALK mutations are almost

absent, whereas non-pure SQCs with a small cellular representation of CK7 may benefit from targeted therapy [37]. For pure SQC, targeted therapy is much more limited, and although it has been reported the presence of EGFR and ALK translocations on in situ hybridization analysis, its frequency does not justify testing for these mutations routinely [38].

Our results showed that approximately 20% of SQC samples expressed CK7, which is in accordance with current literature. Among the 13 CK7-negative SQC samples, 8 of them expressed PD-L1 and half of these (4/8) had PD-L1 expression in more than 50% of TCs. These findings evidenced a tendency to high PD-L1 expression intensity in pure lung SQC cases (CK7-negative), which may be further characterized in future for a more personalized application of PD-1/PD-L1 immune checkpoint inhibitors in pure SQCs and, according to Socinski et al., possibly in combination with targeted treatments [27].

The applicability of tumor microenvironment (TME) as a diagnostic, prognostic or predictive biomarker in bronchopulmonary carcinomas is under investigation, as it may help to identify patients with higher chances to benefit from immunotherapy [2]. Studies focusing on the tumoral stroma raised evidence correlating it with tumorigenesis, heterogeneity, resistance to immunotherapy and tumoral progression [39]. Stromal cells may express the ligand PD-L1, but the effect of stromal expression of PD-L1 on immunotherapy response is still unclear [39].

In this study, we demonstrate a tendency to PD-L1 positive expression among lymphocytic stroma samples, where 9/10 samples with a lymphocytic stroma had positive PD-L1 expression. This result might be partially explained by the mechanism of induction of tumor PD-L1 expression, in which the interferon-γ produced by T lymphocytes present in the TME induces the expression of PD-L1 on TCs [40]. Furthermore, our observation corroborates evidence from previous studies in which tumor-infiltrating lymphocytes (TILs) have been proposed as a biomarker of response for PD-1/PD-L1 inhibition therapy [41]. Literature also suggests that anti-PD therapy is less effective in non-inflamed tumors (with poor lymphocyte infiltration and low PD-L1 expression) and in the presence of increased levels of transforming growth factor-β (TGF-β), which induces resistance to anti-PD-L1 therapy [42].

The epithelial-mesenchymal transition (EMT) is a reversible biological process in which epithelial cells become mesenchymal cells by losing their cell-cell adhesion and polarity and acquire invasive/migratory properties, thereby contributing to a reduction in response to therapy, drug resistance and hence poor prognosis [2,39,43,44]. During EMT, the expression of E-cadherin is downregulated, whereas the expression of vimentin as mesenchymal protein marker of the EMT is upregulated [33,45]. The EMT has been associated with TKi resistance,

namely with resistance to EGFR-TKis, compromising the first-line therapy of patients harboring EGFR activating mutations [45].

According to Kim and colleagues, PD-L1 expression may be the mechanism responsible for EMT oncogenesis and immune evasion during tumor development [46]. However, it has also been reported that EMT is also capable of inducing PD-L1 expression in pulmonary carcinomas [47]. Consequently, a PD-L1 and EMT bidirectional cross-talk has been proposed to promote tumor aggressiveness [46,48]. More recently, NTRK gene rearrangements, assessed by NGS, emerged as a new valuable target to highly effective targeted therapies, being present in 0.1% to 1% of lung carcinomas [49,50]. As new reports suggest an association between NTRK mutations and microscopically high grade features and undifferentiated phenotype in mesenchymal tumors [50], the need to further characterize the association between NTRK rearrangements and EMT phenotype in lung carcinomas, evaluated through vimentin expression, becomes a field for future research.

In this study, the association between PD-L1 expression in lung cancer cells and the EMT phenotype evaluated through immunohistochemical expression of vimentin allowed us to dichotomize the studied samples into PD-L1-positive and PD-L1-negative groups, where a significant association was found between PD-L1 expression and vimentin expression, with PD-L1 positivity on TCs being a more frequent event (24/32) among samples with high vimentin expression (mesenchymal phenotype), *versus* those without PD-L1 expression (33 of the 41 PD-L1-negative samples were also negative for 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, head and neck and esophageal squamous carcinoma, suggesting that tumors with an EMT status stand as potential targets for immunotherapy agents [46,48].

Our results also demonstrated that the significant association between vimentin and PD-L1 expression was maintained when the cases were regrouped by stratified PD-L1 intensity: among the 24 vimentin-positive/PD-L1-positive cases, 14 had PD-L1 expression in over 50% of TCs. These findings evidence that vimentin expression is not only associated with PD-L1 positivity, but it also becomes a more frequent event with increasingly higher PD-L1 expression intensity, being significantly associated with PD-L1 overexpression in TCs. Additionally, we further demonstrated that the risk of vimentin positivity is 3.85 times higher among cases with more than 50% PD-L1 stained TCs, *versus* PD-L1 negative samples.

Proliferation marker ki-67 is still the standard marker routinely used in clinical practice and has been associated with tumor aggressiveness and metastization in several solid tumors [51]. Still without recognized relevance in pulmonary carcinoma, other than small cell carcinoma, our

results highlight a significant association between ki-67 LI with both positive PD-L1 expression and stratified PD-L1 intensity, 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 a ki-67 LI>30%. This observation highlights the association between PD-L1 status and tumor cell proliferation, also confirmed by the tendency to PD-L1 positivity in the solid pattern ADC samples. These results support current literature reporting a significant association between PD-L1 expression and increased ki-67 LI in lung ADC [21,52]. Opposite, in lung SQC, there are contradictory results in literature regarding the association between PD-L1 expression and the ki-67 LI [52]. Furthermore, similarly to vimentin, we found that 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.

Interestingly, our findings demonstrated that EMT-based biomarkers such as vimentin and elevated ki-67 LI may be useful for identifying lung cancer patients with higher chances to benefit from PD-1/PD-L1 immune checkpoint inhibitors, once these biomarkers were associated with elevated percentage of PD-L1 stained TCs.

To the best of our knowledge, our study was the first to investigate the relationship between PD-L1 expression and EMT status in a perspective of risk analysis. Taking this into account and similarly to previous investigations [44,46], we propose that the identification of an EMT status by IHC staining analysis can be relevant in selecting patients who are more likely to have higher PD-L1 TPS and thus develop a more favorable response to PD-1/PD-L1 immune checkpoint blockade, contributing for the optimization of treatment with this class of immunotherapy drugs in bronchopulmonary carcinomas.

Drug resistance is becoming a major barrier for targeted therapy and immunotherapy in lung cancer due to acquired resistance and disease progression [43]. Unselected patients with advanced carcinomas benefit from anti-PD therapy in only 10% to 20% of cases [18,31,41,42], and new strategies to overcome this situation need to be developed, including possible combination therapies. Considering the results from past investigations together with our findings, we propose that the combination therapy of PD-1/PD-L1 inhibitors with EMT targeted therapies might become an ultimate therapeutical option.

The synergistic therapeutic effects of EMT targeted agents combined with PD-L1 inhibitors have been reported in previous preclinical and clinical trials [26,29]. Combination therapy of galunisertib (a TGF-β receptor kinase I inhibitor) with nivolumab is currently being investigated in clinical trials, as TGF-β is one of the primary EMT inducers, and recent results demonstrated a significantly greater tumor regression with this combination therapy *versus* with either agents in monotherapy [48]. Combination of MEK inhibitors (who interfere with RAS/mitogen-activated protein kinase (RAS/MAPK) signaling pathway involved in EMT regulation) with PD-L1

inhibitors also improved tumor regression, which might be due to the role of MEK inhibitors in sensitizing TCs to immunotherapy agents [48]. Mechanistic target of rapamycin (mTOR) promotes the EMT phenotype and immune evasion through the upregulation of PD-L1 expression, and the effect of mTOR inhibition combined with PD-L1 blockade was also reported in preclinical lung cancer trials [48]. Finally, the combination of PD-1 inhibitors with EGFR TKis in PD-L1-positive carcinomas with EGFR activating mutations raised promising results in preclinical trials, as the EGFR activation up-regulated PD-L1 expression, making these tumors more susceptible to the PD-1/PD-L1 blockade therapy [29,43,48].

However, the main concern regarding this combination therapy is the relatively high incidence of treatment-related adverse events. In recent trials using MEK inhibitors and osimertinib (EGFR-TKi) combined with PD-L1 inhibitors, grade 3-4 adverse effects happened in 44% and 67% of patients, respectively [29,48]. Additionally, 10% to 20% of responses to PD-1/PD-L1 inhibitors occur in tumors classified as PD-L1-negative by IHC, due to PD-L1 expression being heterogenous in large tumors [9,17,18,31]. Finally, the early trials that did not report a synergistic effect of the combination therapy with PD-L1 inhibitors and EGFR-TKi probably was due to a low TMB combined with low cytotoxic T cell infiltration [48].

Limitations of this study concerned the limited size of our data set (97 samples). Hence, a replication of our findings in other patient populations support the need to further investigate the EMT-phenotype as a new potential predictive biomarker to help guide the selection of patients with higher chances of benefiting from immunotherapy in bronchopulmonary carcinomas [53], and to foster research on the development of combination therapies with EMT targeted agents and PD-L1 inhibitors to improve the outcomes of bronchopulmonary carcinomas therapy.

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.

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

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

Vimentin expression and ki-67 LI may also overtake the evaluation of TMB as a more cost-effective and available method. Combination therapy of EMT targeted therapy agents with PD-L1 inhibitors in bronchopulmonary carcinomas with an EMT phenotype is also a promising field for future research.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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APPENDICES

Supplementary Table 1 PD-L1 intensity in SQC samples (n = 16) by CK7 expression

	PD-L1 intensity				
	0	+	++	+++	All PD-L1+ samples
CK7					
Positive	2	0	0	1	1
Negative	5	2	2	4	8

Supplementary Table 2 Vimentin expression by histological subtype

	n(%) Vimentin		
	Negative	Positive	P value
Histological subtype			*0.037*
Bronchopulmonary carcinomas	63 (96.92)	27 (84.38)	
Pleomorphic carcinoma	2 (3.08)	5 (15.63)	

Fisher's exact test results

Supplementary table 3 Clinical and pathological factors by PD-L1 expression in tumor cells

	n (%) PD-L1		
	Negative (n = 41)	Positive (n = 56)	P value
Age			1.000
≤ 68	21 (51.22)	28 (50.00)	
>68	20 (48.78)	28 (50.00)	
IHC markers			
TTF1			0.369
Positive	27 (65.85)	42 (75.00)	
Negative	14 (34.15)	14 (25.00)	
CK7			0.772
Positive	36 (87.80)	47 (83.93)	
Negative	5 (12.20)	9 (16.07)	
CK 5.6			1.000
Positive	11 (26.83)	14 (25.45)	
Negative	30 (73.17)	41 (74.55)	
PAS-D			0.074
Positive	16 (39.02)	12 (21.82)	
Negative	25 (60.98)	43 (78.18)	
Histological subtype			0.549
SQC	7 (17.07)	9 (16.07)	
ADC	28 (68.29)	36 (64.29)	
ADCSQC	3 (7.32)	4 (7.14)	
Large cell	2 (4.88)	1 (1.79)	
Pleomorphic	1 (2.44)	6 (10.71)	

Pearson's χ2 and Fisher's exact test results
TTF1 thyroid transcription factor 1, *SQC* squamous cell carcinoma, *ADC* adenocarcinoma, *ADSQC* adenosquamous carcinoma

Virchows Archiv Instructions for Authors (abbreviated)

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In print, original articles should not exceed 10 printed pages. This equals about 3500 words, scientific documentation (tables, images) and a reference list, which for original articles as a rule should not exceed 50 references. The abstract, legends and references are not included in the word-count. Manuscripts exceeding 3500 words might be returned to the author for abbreviation. As a rule, a number of 5 figures and/or tables is acceptable. Any documentation in excess of this quantity is subject to approval by the Editors. Authors should specify the word count of their manuscript (excluding abstract and references) on the title page of the manuscript. Documentation in excess of the maximum word count or images/tables can be submitted as supplementary material available on-line only.

All manuscripts are subject to copy editing. Extensively edited manuscripts will be returned to authors prior to production for formal approval.

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The title should be concise and informative.

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- ✓ The name(s) of the author(s)
- ✓ The affiliation(s) of the author(s), i.e. institution, (department), city, (state), country
- ✓ A clear indication and an active e-mail address of the corresponding author
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Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

Funding (information that explains whether and by whom the research was supported)

Conflicts of interest/Competing interests (include appropriate disclosures)

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Manuscripts should be submitted in Word.

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- ✓ Use the automatic page numbering function to number the pages.
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- ✓ Use tab stops or other commands for indents, not the space bar.
- ✓ Use the table function, not spreadsheets, to make tables.
- ✓ Use the equation editor or MathType for equations.
- ✓ Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

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Please use no more than three levels of displayed headings.

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Abbreviations should be defined at first mention and used consistently thereafter.

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Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

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The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

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- ✓ Book
    - South J, Blass B (2001) The future of modern genomics. Blackwell, London
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- ✓ Online document
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