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ORIGINAL ARTICLE

Assessment of TILs, IDO-1, and PD-L1 in resected non-small cell lung cancer: an immunohistochemical study with clinicopathological and prognostic implications

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Abstract

Several cancers, especially non-small cell lung cancer (NSCLC), are able to escape the immunosurveillance of tumor-infiltrating lymphocytes (TILs); among the molecules involved, the indoleamine 2,3-dioxygenase 1 (IDO-1) and the programmed cell death ligand-1 (PD-L1) play a crucial role. These aspects are of great interest in the current immunotherapeutic era, therefore the current study analyses the TILs, IDO-1, and PD-L1 interactions and their correlations with clinicopathological parameters and prognosis in NSCLC. One hundred ninety-three NSCLC surgical specimens, formalin-fixed, and paraffin-embedded were assessed for TILs density, TILs localization, IDO-1 (clone 4.16H1), and PD-L1 (clone E1L3N) immunohistochemical expressions. This data was correlated with clinicopathological parameters, disease free, and overall survivals. IDO-1 and PD-L1 high expressions were related to the solid pattern of adenocarcinomas (respectively $p = 0.036$ and $p = 0.026$); high PD-L1 expression was correlated with squamous histotype ($p = 0.048$). IDO-1 overexpression correlated with former smokers ($p = 0.041$), higher adenocarcinoma stages ($p = 0.039$), and with both higher TILs density and PD-L1 expression (respectively $p = 0.025$ and $p = 0.0003$). A better prognosis was associated with TILs intratumoral or mixed localizations ($p = 0.029$). TILs localization affects NSCLC prognosis; the higher expression of IDO-1 and PD-L1 in poorly differentiated and more aggressive lung adenocarcinomas, as well as the correlation between high PD-L1 expression and squamous cell histotype, confirm the more efficient immunoescaping of these NSCLC subgroups.

Keywords Non-small cell lung cancer · Tumor-infiltrating lymphocytes · IDO-1 · PD-L1 · Immunotherapy

Introduction

Lung cancer is the primary cause of cancer death among males and the secondary among females around the world. Non-small cell lung cancer (NSCLC) is the most common type, accounting for 85% of cases [1, 2]. Less than 20% of lung cancers carry a gene mutation which allows a targeted therapy, with a subsequent slight prognostic improvement; however, tumors invariably acquire mutation-driven drug resistance within 9–12 months [1–3]. Nevertheless, the immune system can recognize and eliminate proliferating cancer cells [4, 5]. The understanding, although still incomplete, of the regulatory mechanisms of the immune response has allowed the development of new antitumor treatments, without detrimental effects on healthy cells and without any loss of efficacy over time [6]. In particular, the presence of tumor-infiltrating lymphocytes (TILs) has been associated with a favorable clinical

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course and has been considered a positive prognostic factor in several cancers types [7–9].

On the other hand, tumors are able to escape the immune attack [2, 4, 6], using to their advantage the physiological interactions between ligands and their respective receptors expressed on cell membranes, both on tumor and on immune system cells, which act as "brakes" on the immune response at TILs level [6]. Among these mechanisms, a pivotal role could be played by the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase 1 (IDO-1), that is physiologically expressed in dendritic cells (DCs), but also in several human cancer cells. It is usually associated with the worst prognosis [10–16], especially in lung cancers with nodal and distant metastasis [11, 12]. Its effect on T lymphocytes is dual, being able to behave both as an enzyme and as signal-transducing molecule in the presence of a specific cytokine microenvironment, consequently being able to promote both a regulatory phenotype of immune response and tolerance interruption with amplification of inflammation [10, 11, 13, 17, 18]. Even if there are few studies about the prognostic role of this enzyme [11], many IDO-1 inhibitors are currently being tested in animal models and in ongoing clinical trials on humans, with encouraging, but still incomplete, results [10–12, 14].

The overexpression of the surface glycoprotein programmed cell death ligand-1 (PD-L1) on tumor cells is another mechanism of "immunoescape" [19]; this molecule is able to bind its receptor, programmed death protein 1 (PD-1), expressed at TILs level [2, 6, 20]. Several studies have attempted to correlate the expression of PD-L1 in NSCLC with a worse prognosis, but nowadays there are conflicting results [6, 19–22]. Studies about the efficacy of targeted therapies against PD-L1 [23] have emphasized the importance of tumor microenvironment; in particular, there are many efforts to stratify NSCLC based on the contemporary presence of PD-L1 and TILs, in order to select the best therapeutic combination for the patient [24].

It is not well known if the interactions between the aforementioned molecules and neoplastic microenvironment could have a correlation with patient's prognosis or if they could be potential therapeutic targets [2, 3, 7, 10–12, 15, 16, 19, 20, 25–35].

In this context, we carried out an immunohistochemical study on a series of resected NSCLC, in order to determine both the presence and the prognostic value of the interactions between TILs, IDO-1 and PD-L1.

Materials and methods

Patients' selection

One hundred ninety-three surgical specimens of resected NSCLC were examined in the period from 2009 to 2015. The mean follow-up was of 5 years. Clinical parameters recorded are showed in Table 1. We also recorded if the patients underwent adjuvant chemotherapy and/or radiotherapy, timing and localization of recurrences, cause of exitus, and months in between first diagnosis and death.

Histology and immunohistochemistry

The surgical specimens were formalin-fixed and paraffin-embedded (FFPE). Sections of 4 μm were performed and then stained with hematoxylin and eosin (H&E) or with immunostains, obtained from full-automated immunohistochemistry stainer BOND-III Leica Biosystems. The antibodies used were IDO-1 (clone 4.16H1 [16]; dilution 1:1000; courtesy of professor Benoit J Van den Eynde, Ludwing Institute for Cancer Research) and PD-L1 (clone E1L3N; dilution 1:200; Cell Signaling; research use only (RUO)—laboratory developed test (LDT)). The histotype and the predominant pattern of adenocarcinomas were assigned according to 2015 World Health Organization (WHO) classification for lung tumors. When a NSCLC was poorly differentiated, we classified it as adenocarcinoma or as squamous cell carcinoma according to TTF-1 (clone 8G7G3/1; dilution 1:100; Dako) and p40 (polyclonal; dilution 1:50; ScyTek Laboratories) immunohistochemical expression. TILs were evaluated on H&E stained sections, as previously described [36], considering their localization at tumoral site as absent, intratumoral or peritumoral if TILs were respectively absent or among tumoral cells or at the interface between the neoplasia and healthy lung parenchyma. We used the term "TILs mixed" to indicate a mixture of the last two localizations. We then evaluated the density of TILs as the percentage of lymphocytes observed in a given localization (absent or minimal 0–4%; mild 5–19%; moderate: 20–49%; intense ≥ 50%); a binary scoring system was used to consider the two first categories of density into a low TILs group and the last two into a high TILs group, as previously described [7]. Areas occupied by necrosis, regions with crush artifacts and/or stromal hyalinization were excluded by the latter evaluations [37].

Endothelium was the positive control of IDO-1 immunostains; both nuclear and/or cytoplasmic staining of tumoral cells were considered positive.

Macrophages membranes and/or lymphocyte labelling were the positive control of PD-L1 immunostains. Tumor cell membrane was considered as positive stain. IDO-1 and PD-L1 were evaluated on neoplastic cells using a scoring system obtained by the sum of the intensity of the stain (0, absent; 1, mild; 2, moderate; 3, intense) and the percentage of the tumoral cells labeled (0, 0%; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, 76–100%). We then grouped the stainings in a low expression class, with scores from 0 to 2, and a high expression class, with scores from 3 to 7.

Table 1 Clinicopathological characteristics

Clinical parameters	Patients cohort	
	n. 193	
	N	%
Gender		
M	139	72.02
F	54	27.98
Age		
< 68 years	89	46.11
≥ 68 years	104	53.89
Smoking		
Current smokers	78	40.40
Former smokers	99	51.30
Never smokers	16	8.30
Stage		
I	101	52.33
II	56	29.01
III	34	17.62
IV	2	1.04
Histopathological parameters		
Histotype		
Squamous cell carcinomas	69	35.75
Adenocarcinomas	122	63.21
Adenosquamous carcinomas	2	1.04
Adc[a] pattern		
Acinar	77	63.11
Solid	28	22.95
Papillary	11	9.02
Mucinous	4	3.28
Lepidic	2	1.64
Adenocarcinomas stage		
I	74	60.66
II	24	19.67
III	22	18.03
IV	2	1.64
Squamous cell carcinomas stage		
I	27	39.13
II	31	44.93
III	11	15.94
TILs		
Localization		
Intratumoral	90	46.63
Mixed	84	43.52
Peritumoral	14	7.26
Absent	5	2.59
Density		
Low	97	50.26
High	96	49.74
Adenocarcinomas		
Localization		
Intratumoral	66	54.10
Mixed	45	36.88
Peritumoral	7	5.74
Absent	4	3.28
Density		
Low	62	50.82
High	60	49.18
Squamous cell carcinomas		
Localization		
Intratumoral	24	34.78
Mixed	37	53.62
Peritumoral	7	10.15
Absent	1	1.45
Density		
Low	35	50.72
High	34	49.28

[a] *Adc*, adenocarcinoma

Two trained pathologists separately performed a semiquantitative evaluation of both TILs and immunostains; afterwards, they convened to compare discrepant cases.

Statistical analysis

The presence of TILs, IDO-1, and PD-L1 expression were correlated with clinicopathological parameters and with follow-up data. Statistical analysis was performed using Pearson's χ^2 test and log-rank test, considering as significant the p values less than 0.05.

Results

Patients' cohort

Patients' average age was 67.59 years (range 38–84). One hundred thirty-nine (72.02%) patients were male. Seventy-eight (40.40%) patients were current smokers and 99

(51.30%) former smokers. According to the 8th edition for cancer staging by American Joint Committee on Cancer (AJCC) and regrouping patients in four group (Table 1), we had 101 (52.33%) cases belonging to stage I, but only 2 (1.04%) cases to stage IV; because of the small impact of these last two cases, we did not consider them in subsequent elaborations involving staging. One hundred forty-one (73.06%) patients have not received adjuvant therapy, 52 (26.94%) have received either radiotherapy or chemotherapy or both, according to international guidelines applied at the time of the diagnosis. Seventy-one (36.79%) NSCLC relapsed after surgery, 121 (62.69%) had no relapse, and 1 (0.52%) patient was lost at follow-up. Sixty-one (31.60%) patients died between 1 and 83 months from initial diagnosis; among them, 43 (70.49%) died from lung carcinoma.

Pathological findings

One hundred twenty-two (63.21%) neoplasms were adenocarcinomas. Among these, 28 (22.95%) presented a predominant solid pattern.

More than half of adenocarcinomas (74; 60.66%) belonged to stage I, whereas the majority of squamous cell carcinomas (31; 44.93%) were in stage II. Data about tumoral histotype and stage are summarized in Tables 1 and 2.

TILs evaluation

TILs were present in 188 (97.41%) carcinomas. The localizations observed were reported in Table 1 and some examples are shown in Fig. 1 a, b.

As regards density, regrouping TILs in low and high categories, 97 (50.26%) of the tumors fell into the first group (Table 1).

Both TILs localization and density were correlated with clinicopathological parameters, without statistically significant results.

Immunohistochemistry

IDO-1 staining evaluation showed heterogeneity in different neoplastic areas (Fig. 1c). One hundred and six (54.92%) cases belonged to the high expression class of IDO-1, as well as 66 (54.10%) adenocarcinomas and 38 (55.07%) squamous cell carcinomas (Table 3). Moreover, adenocarcinomas most frequently belonged to the high expression class if the predominant pattern was solid ($p = 0.036$) and if the tumor stage was more advanced ($p = 0.039$).

We found that there was a greater frequency of former smoker patients among tumors within high expression class of IDO-1 ($p = 0.041$) (Table 2).

Regarding PD-L1 expression, 57 (29.50%) cases were positive for this marker (Fig. 1d) and there was heterogeneous immunostaining in different neoplastic areas; in this group, 43 tumors (75.43%) showed a high expression of PD-L1. Both among adenocarcinomas and squamous cell carcinomas, the majority of tumors showed a low expression of PD-L1 (Table 3). The high expression class of PD-L1 correlated with squamous cell histotype ($p = 0.048$) and with solid pattern of adenocarcinomas ($p = 0.026$) (Table 2).

Tumoral microenvironment interactions

There was an increased frequency of tumors with IDO-1 high expression among the high-density TILs class ($p = 0.025$), in particular, in the squamous cell carcinomas subgroup ($p = 0.002$). On the other hand, comparing PD-L1 expression and TILs density, we did not reach a statistically significant result, as well as for TILs localization and its correlations with IDO-1 and PD-L1 (Table 3).

Furthermore, we found a strong relationship between 34 (17.62%) cases presenting a co-expression of both high IDO-1 and PD-L1 ($p = 0.0003$). This finding was present both in squamous cell carcinomas ($p = 0.004$) and in adenocarcinomas ($p = 0.015$) (Table 4). In addition, 21 (61.76%) cases with co-expression of high IDO-1 and PD-L1 had a high TILs density and showed an intratumoral or mixed localization of TILs.

Disease free and overall survivals

Among all NSCLC, cases with intratumoral or mixed TILs had significantly longer overall survivals (OS) compared with those presenting a peritumoral TILs ($p = 0.029$) (Fig. 2). As regards TILs localization, we did not obtain statistically significant results for disease-free survival (DFS), similarly concerning TILs density groups, both for DFS and OS.

Analyzing separately the squamous cell carcinomas group, cases with low IDO-1 expression presented a better OS ($p = 0.012$).

As regards correlations between survivals and PD-L1 expression classes, there were no any statistical significant results. We noticed only a worse OS for that cases which presented ≥ 80% of PD-L1 positive cells ($p = 0.048$).

Discussion

The present immunohistochemical study examined 193 resected NSCLC, in order to evaluate the interactions between TILs, IDO-1, and PD-L1, correlations with clinicopathological parameters and potential prognostic impact.

Concerning IDO-1, we have found a statistically significant correlation between high IDO-1 expression and solid pattern of adenocarcinomas. Most of the studies about IDO-1 were performed on murine models and none exists about its

Table 2 Correlations between IDO-1, PD-L1 expression, and clinicopathological parameters

Parameter	IDO-1 low		IDO-1 high			PD-L1 low		PD-L1 high		
	N	%	*N*	%	*p*	*N*	%	*N*	%	*p*
	87	45.08	106	54.92		150	77.72	43	22.28	
Gender										
M	62	44.60	77	55.40	n.s.	104	74.82	35	25.18	n.s
F	25	46.30	29	53.70		46	85.19	8	14.81	
Age										
< 68 years	51	49.51	52	50.49	n.s.	67	75.28	22	24.72	n.s.
≥ 68 years	36	40.00	54	60.00		83	79.81	21	20.19	
Smoking										
Current smokers	43	55.13	35	44.87	*0.041*	60	76.92	18	23.08	n.s.
Former smokers	36	36.37	63	63.63		77	77.78	22	22.22	
Never smokers	8	50.00	8	50.00		13	81.25	3	18.75	
Stage										
Adenocarcinomas stage										
I	40	54.05	34	45.95	*0.039*	65	87.84	9	12.16	n.s.
II–III	16	34.78	30	65.22		35	76.09	11	23.91	
Squamous cell carcinomas stage										
I	10	37.04	17	62.96	n.s.	19	70.37	8	29.63	n.s.
II–III	21	50.00	21	50.00		29	69.05	13	30.95	
Histotype										
Squamous cell carcinomas	31	44.93	38	55.07	n.s.	48	69.57	21	30.43	*0.048*
Adenocarcinomas	56	45.90	66	54.10		100	81.97	22	18.03	
Adc[a] *pattern*										
Other than solid	48	51.06	46	48.94	*0.036*	81	86.17	13	13.83	*0.026*
Solid	8	28.57	20	71.43		19	67.86	9	32.14	

[a] *Adc*, adenocarcinoma

correlation with human NSCLC histotypes [11, 15, 16, 38–40]. The increased IDO-1 expression might facilitate immunotolerance in solid adenocarcinomas and could partly explain because this pattern is usually related to a worse prognosis [1, 39].

Forty-three cases of the present series (22.28%) showed a high PD-L1 expression, similarly to the results obtained by Schalper et al. [12], who employed the same antibody clone. Moreover, the study by Brunnström et al. [41], in spite of using different PD-L1 clones, confirmed that about 16–44% of the NSCLC are positive to this marker. It should be clarified that other studies showed a higher percentage of PD-L1 positive cases, but the evaluation was not performed on surgical specimens, which may display a not-optimal fixation and a subsequent worse antigenic preservation than small biopsies. Furthermore, most of studies consider the percentage of PD-L1 positive cells only for predictive aims [23].

Another interesting finding was that among the high PD-L1 expression group, there were more squamous cell carcinomas than adenocarcinomas. Some previous studies confirmed this latter result, although not showing a statistically significant meaning [19, 29, 30, 39]. However, it is well known that squamous cell carcinoma is driven by suppressor genes alterations, which lead to chromosomal instability and to accumulation of somatic mutations, resulting in the increased production of neoantigens which act as one of the major stimuli to activate innate immune response. The subsequent inflammation allows immune checkpoint activation, including overexpression of PD-L1 in this NSCLC histotype [42].

Furthermore, we found that in the subgroup of adenocarcinomas with solid pattern, there was a higher PD-L1 expression. In fact, a recent meta-analysis [34] has evidenced the association between high PD-L1 expression and poorly neoplastic differentiation. As known, poor differentiation is an independent negative prognostic factor in NSCLC; perhaps, the higher PD-L1 expression in these circumstances enables the tumoral immunoescape, as abovementioned about increased IDO-1 expression, and could explain a greater aggressiveness, confirming the results of recent studies [39, 43].

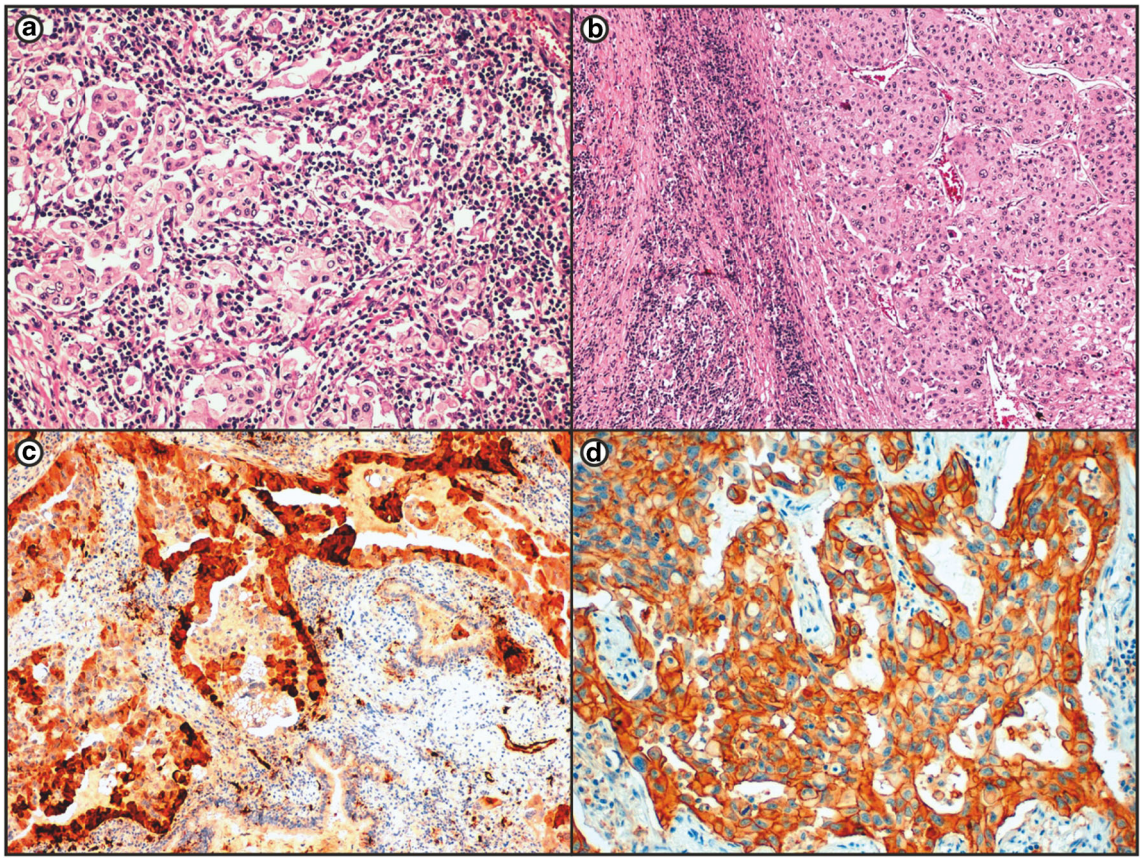

Fig. 1 **a** Intratumoral TILs (H&E 200X). **b** Peritumoral TILs (H&E 200X). **c** Heterogeneous stain for IDO-1 (100X). **d** Intense immunostaining (3 +) for PD-L1 (200X)

Table 3 Correlations between IDO-1, PD-L1 expression, and TILs

Parameter	IDO-1 low		IDO-1 high			PD-L1 low		PD-L1 high		
	N	%	*N*	%	*p*	*N*	%	*N*	%	*p*
Adenocarcinomas	56	45.90	66	54.10		100	81.97	22	18.03	
TILs density										
Low	33	53.23	29	46.77	n.s.	52	83.87	10	16.13	n.s.
High	23	38.33	37	61.67		48	80.00	12	20.00	
TILs Localization										
Intratumoral	30	45.45	36	54.55	n.s.	56	84.85	10	15.15	n.s.
Mixed	19	42.22	26	57.78		36	80.00	9	20.00	
Peritumoral	4	57.14	3	42.86		5	71.43	2	28.57	
Absent	3	75.00	1	25.00		3	75.00	1	25.00	
Squamous cell carcinomas	31	44.93	38	55.07		48	69.57	21	30.43	
TILs Density										
Low	22	62.86	13	37.14	*0.002*	26	74.29	9	25.71	n.s.
High	9	26.47	25	73.53		22	64.71	12	35.29	
TILs Localization										
Intratumoral	12	50.00	12	50.00	n.s.	16	66.67	8	33.33	n.s.
Mixed	14	37.84	23	62.16		26	70.27	11	29.73	
Peritumoral	4	57.14	3	42.86		5	71.43	2	28.57	
Absent	1	100.00	0	0.00		1	100.00	0	0.00	

Table 4 Correlations between IDO-1 and PD-L1 expression classes

Parameter		Adenocarcinomas (n. 122)					Squamous cell carcinomas (n. 69)					
		IDO-1 low		IDO-1 high				IDO-1 low		IDO-1 high		
		n. 56		n. 66				n. 31		n. 38		
		N	%	*N*	%	*p*		*N*	%	*N*	%	*p*
PD-L1 low	n. 100	51	51.00	49	49.00	*0.015*	n. 48	27	56.25	21	43.75	*0.004*
PD-L1 high	n. 22	5	22.73	17	77.27		n. 21	4	19.05	17	80.95	

Another relevant finding was that the majority of cases with high TILs density presented concomitant higher IDO-1 expression, in particular, in squamous cell carcinomas group. This outcome demonstrated how the antitumor activity of TILs could be opposed by IDO-1 [8, 16, 17] and is in agreement with the results obtained in previous studies on human NSCLC [12, 44]. On the other hand, evidence exists, in other carcinomas types, such as colorectal cancer, that the higher the IDO-1 expression, the lower the presence of TILs [25]. This reflects the versatile nature of IDO-1, which is able to induce immunotolerance or magnify the immune response depending on the presence of other molecules in the microenvironment [11–13, 16–18]. It could also explain another finding that we have observed about the correlation between former smokers' tumors and higher IDO-1 expression: the chronic lung inflammation, along with the elevation of mutational load induced by smoking, resulted in an increasing tumoral immunoescape [39] probably induced by IDO-1 overexpression [13, 15–17]. On the other hand, in murine series, the induction of IL-6, implied in tumor growth in an inflamed microenvironment and in IDO-1 degradation, is related with KRAS pathway [15], a characteristic molecular alteration in smokers' lung cancers [1]. In fact, we observed a lower IDO-1 expression in current smoker patients. However, these aspects need further studies to better understand the mechanisms regulating IDO-1 expression.

Interestingly, a relevant percentage of tumors with high PD-L1 expression presented concurrently high IDO-1 expression. In literature, studies on murine models and on human melanomas and NSCLC have demonstrated that those two molecules are closely interconnected [11, 39, 40]. As known, both IDO-1 and PD-L1 overexpression are induced by T_H1/IFNγ signaling, implemented at TILs level [6, 17, 21, 44, 45], supporting our result. However, further studies are required to understand additional signaling events occurring in the tumor microenvironment, in order to establish the benefits of a combined immunotherapy against these two potential biomarkers.

The relationship between TILs peritumoral localization and worst overall survivals (OS) is in agreement with current literature, which confirms a prognostic role of TILs localization both in NSCLC [8] and in other cancers, such as colorectal ones [36, 46]. Furthermore, lymphocytes direct attack on tumoral cells is probably the most effective mechanism to contrast cancer growth [4, 7–9, 36, 37, 47], reasonably because the contact between lymphocytes and tumoral cells better activate molecular signaling to control tumoral aggressiveness.

Although the prognostic role of the TILs density has been demonstrated in many kind of tumors [37, 46], we did not reach any statistically significant results as regards correlations between TILs density and survivals [7–9, 37, 46]. However, it should be pointed out that our study, according to that by Brambilla et al. [7], examined TILs only on H&E sections. On the other hand, the use of TILs immunophenotyping, which is not considered essential by some authors [7], has produced

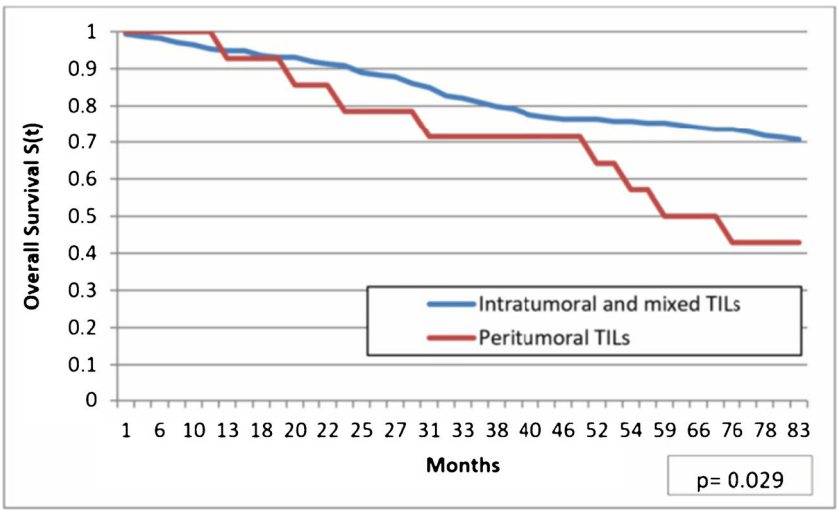

Fig. 2 Overall survival curves according to TILs localization

conflicting prognostic results, probably because of the unknown role of single lymphocytic subtype in immune adaptive answer to tumor growth [7–9, 37, 47, 48]. Nevertheless, tumor cells are able to escape immune attack with various mechanisms [8, 48] and TILs are only one of the possible ways to fight against cancer.

Furthermore, we noticed better OS of squamous cell carcinomas with low IDO-1 expression. Even though there are conflicting data about the IDO-1 prognostic role [10, 11, 15, 26–28], other studies [11, 16, 25] have demonstrated that high IDO-1 expression by neoplastic cells correlates with higher rates of metastatization and worse prognosis. On the other hand, the not univocal selection of antibody clones, the absence of standardized cutoffs and scores for immunohistochemical analysis, and the intrinsic stain heterogeneity in the tumoral bed could explain the conflicting results of the various studies.

There was no significant data about the correlations between survivals and PD-L1 expression using our score for staining evaluation. We only observed that 4 cases with a very high percentage of PD-L1 positive tumor cells (≥ 80%) showed a worst OS. All of these 4 NSCLC belonged to squamous cell histotype group and only 1 patient is still alive. In literature, there are many conflicting results about the correlation between PD-L1 expression and NSCLC prognosis [19, 20, 23, 29–35, 48]. This could be due to the fact that the majority of current papers consider only the predictive role of PD-L1 immunostaining, so a lower percentage of stained tumoral cells is enough to regard that tumor as expressing PD-L1 and candidates it to immune blockade treatment. Moreover, they do not take into account the role of immunostains intensity, which should be relevant for a prognostic evaluation of a potential biomarker. In addition, recent comparative studies confirmed the reproducibility of 3 out of 4 of different antibodies clones [49, 50], including the clone we had used, going beyond the concept that differences in PD-L1 antibody clones and platforms could contribute to conflicting results [29, 31, 48]. The discrepancy seen in previous studies is likely attributable to the high levels of tumor heterogeneity for expression of PD-L1 [12, 51], as we encountered in our analysis, rather than the ability of the various clones to bind PD-L1 itself, which is highly similar [49]. Furthermore, the different kind of specimens used for the evaluation of these markers, as well as the different techniques adopted (immunohistochemistry versus quantitative immunofluorescence) [12, 43] contribute to heterogeneous results, which should be overcome in the future establishing common protocols to assess PD-L1 staining for prognostic purposes.

In conclusion, our study highlights some potential prognostic role of TILs, IDO-1, and PD-L1 in NSCLC. In particular, IDO-1 and PD-L1 high expressions are closely linked with the worst prognostic pattern (solid) of lung adenocarcinomas. IDO-1 seems to act as an important modulator of immune system response, as the correlations between this molecule, smoking and higher cancer stages partly confirmed, suggesting the need for further studies to better determine the potential role of IDO-1 immunohistochemistry as prognostic biomarker.

Moreover, the close interactions evidenced between TILs and IDO-1, together with the frequent co-expression of IDO-1 and PD-L1 and the better prognosis of NSCLC with intratumoral TILs, could support the use of a combined immunotherapy, stimulating the TILs and inhibiting IDO-1 and/or PD-L1.

However, further studies are needed to better understand the intimate connections between these three main actors of tumor microenvironment and to improve our knowledge about these still unknown, but potentially beneficial, pathways.

Contribution statement Martina Mandarano, Guido Bellezza, Maria Laura Belladonna, Benoit J Van den Eynde, Rita Chiari, Vienna Ludovini, Francesco Puma, and Angelo Sidoni conceived and designed the study, edited, and review the manuscript. Jacopo Vannucci, Lucio Cagini, and Francesco Puma provided the resected surgical specimens, edited, and reviewed the manuscript. Martina Mandarano, Guido Bellezza, Maria Laura Belladonna, Rita Chiari, Jacopo Vannucci, Giada Mondanelli, Vienna Ludovini, Elisa Albini, Rachele del Sordo, and Giulio Metro researched and analyzed data, edited, and reviewed the manuscript. Benoit J Van den Eynde provided the IDO-1 antibody and Ivana Ferri performed the immunohistochemical stains. Martina Mandarano, Guido Bellezza, Rachele del Sordo, and Angelo Sidoni analyzed the histopathological aspects, wrote, edited, and reviewed the manuscript. Martina Mandarano and Fortunato Bianconi performed the statistical analysis. All authors gave final approval for publication.

Martina Mandarano and Guido Bellezza take full responsibility for the work as a whole, including the study design, access to data, and the decision to submit the manuscript.

Compliance with ethical standards

This manuscript is not under consideration elsewhere. There are no financial disclosures for all authors or funding sources for the manuscript. The work has been prepared according to ethical adherence regarding the informed consent of the involved human participants (Number of Local Ethic Committee Decision: N. 2216/13).

Conflict of interest The authors declare that they have no conflict of interest.

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