Medical Oncology (2019) 36:76
https://doi.org/10.1007/s12032-019-1299-4

ORIGINAL PAPER

Programmed death-1 receptor (PD-1) and PD-ligand-1 (PD-L1) expression in non-small cell lung cancer and the immune-suppressive effect of anaerobic glycolysis

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Received: 4 May 2019 / Accepted: 17 July 2019 / Published online: 24 July 2019

Abstract

The microenvironment of a tumor may regulate the anti-tumor immune response. Intratumoral acidosis and hypoxia may suppress lymphocyte proliferation and migration, and this may have important implications in modern immunotherapy. The expression of PD-L1 by cancer cells and of PD-1 by tumor infiltrating lymphocytes (TILs) was assessed in tissue specimens from 98 operable NSCLC patients. Their prognostic role and their association with makers of glycolysis and anaerobic metabolism were assessed. Strong cytoplasmic/membrane PD-L1 expression was noted in 45/98 cases. Intense presence of TILs was noted in 42/98 cases (high TIL-score), and intense presence of PD-1 expressing TILs (high PIL-score) in 17/98 cases. PD-L1 expression was directly correlated with high PIL-score ($p=0.005$). A significant inverse relationship was found between lactate dehydrogenase LDH5 expression and PIL-score ($p=0.008$). Similarly, low PIL-score was significantly linked with high-hexokinase HXKII and monocarboxylate transporter MCT2 expression ($p<0.04$). Cases with both intense TIL-score and PIL-score had significantly better survival ($p<0.05$). For patients with high TIL-score or high PIL-score, PD-L1 overexpression defined significantly poorer survival ($p=0.01$ and $p=0.03$, respectively). In multivariate analysis, stage ($p=0.002$, HR 3.33, 95%CI 1.4–4.5) and TIL-score ($p=0.02$, HR 2.12, 95%CI 1.1–4.0) were independent predictive variables of death events. Given the low specificity of PD-L1 as a biomarker for anti-PD-1/PD-L1 immunotherapy, a combined assessment of TIL, PD-L1, PD-1, and LDH5 provides a tool for an immunological/metabolic classification of NSCLC tumors, with a different prognosis and different expected response to anti-PD-1/PD-L1 immunotherapy, which should be considered in relevant clinical trials.

Keywords Non-small cell lung cancer · PD-1 · PD-L1 · Metabolism · TILs · LDH5

Introduction

The ability of the body to fight tumors was recognized at the end of the nineteenth century. In 1893, William Bradley Cooley, considered to be the father of immunotherapy, published a paper summarizing his attempts to immunize the human body by injecting erysipelas [1]. In 1907, Bashford, Murray, and Crammer published a key paper on the natural and induced resistance of mice against tumor cell transplantation [2]. Since then, many researchers have studied different types of immunotherapies in an attempt to treat cancer with vaccines prepared with autologous cancer cells, BCG, and other microbial extracts, or with interferons and interleukins in the 1990s. Although complete responses of metastatic tumors were recorded in a small percentage of patients treated with these immunotherapies that sought to

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activate the immune system, it was only recently that randomized trials established immunotherapy as a valuable therapy in the field of oncology [3]. This modern era of immunotherapy is based on the recognition and targeting of molecular pathways exploited by cancer cells that block the activity of cytotoxic T-cells or macrophages.

The programmed death-ligand 1 (PD-L1)/programmed cell death protein 1 (PD-1) pathway is one of the best understood and validated pathways in clinical practice [4], and several monoclonal antibodies have been approved for the treatment of various human carcinomas, including non-small cell lung cancer (NSCLC) [5]. The expression of PD-L1 in cancer cells, as assessed with immunohistochemistry [6], is a biomarker that guides immunotherapy in NSCLC patients; however, in the United States, the Federal Drug Administration (FDA) has approved anti-PD-L1/PD-1 agents for the treatment of head-neck or bladder cancer without demanding a pre-therapy evaluation of the PD-L1 expression status. PD-L1 (B7-H1) and PD-L2 (B7-DC) are ligands expressed on cancer cell membranes; when bound to the PD-1 receptor on CD8 regulatory T-cells, these ligands block their cytotoxic activity. This pathway plays an important physiological role in preventing a T-cell-mediated inflammatory response to infection; this pathway is also crucial for avoiding autoimmunity [5]. Because cancer cells can upregulate PD-ligands on their surface, T-cells are suppressed and the immune antitumor effect is abrogated. Because more aggressive tumors are more glycolytic [7], and there is a well-recognized suppressive effect of acidosis of most of the effector arm of the immune response [8–10], the current study analyzed the potential interactions of these pathways since they may be amenable to combination therapies.

We assessed the expression of PD-L1 by cancer cells and of PD-1 by tumor-infiltrating lymphocytes (TILs) in a series of operable NSCLC patients. Their prognostic role and their association with the markers of glycolysis and anaerobic metabolism were also assessed. This led to the formulation of an immunological/metabolic classification system with possible prognostic relevance and guidelines for therapy.

Materials and methods

Tissue samples of the primary tumor obtained from 98 NSCLC patients treated with surgery between 2002 and 2007 were retrieved from the archives of the Pathology Department at the Democritus University of Thrace. These cases were consecutive in terms of the surgical date. Patients who received pre- or postoperative radiotherapy or chemotherapy were excluded from the analysis. For all patients, the course of the disease was followed until the time of death or for a minimum of 5 years, although a follow-up of up to 10 years was available in a fraction of cases. The patients ranged in age from 32 to 81 (median age: 68). Twelve of the 98 patients were female. The Union for International Cancer Control (UICC) system was used for postoperative staging. Forty-six of the patients had stage I NSCLC, 22 had stage II, and 30 had stage III. Histologically, 58 cases were squamous cell carcinomas, 22 were adenocarcinomas, and 18 were undifferentiated large cell carcinomas. The time of follow-up ranged from 26 months to 112 months (median: 46 months). The end-point of the analysis was overall survival based on cancer-specific death events.

Immunohistochemistry

Immunohistochemistry analysis was performed on formalin-fixed paraffin-embedded (FFPE) tissue sections of 3 μm that were placed on positively charged slides. The slides were then deparaffinized by xylene and rehydrated using graded ethanol solutions. The heat-induced epitope retrieval process was performed in a microwave oven using Dako EnVision FLEX Target Retrieval Solution (pH 6.0).

A polymer detection method was employed to test the antibodies using the UltraVision Quanto Detection System (Thermo Fischer Scientific). Nonspecific background staining was blocked by preincubation using UltraVision Protein Block for 5 min, after buffer washing the specimen twice for 6 min each time. Then, the slides were incubated with primary antibody. The mouse monoclonal PD-1 antibody (clone: NAT105, Biocare Medical, CA, USA) was used at a dilution of 1:50 and 60 min incubation at room temperature. Similarly, the rabbit monoclonal PD-L1 antibody (clone CAL10, Biocare Medical) was used at a dilution of 1:100 and 60 min incubation at room temperature (validation of the antibody is reported in [11]). After primary antibody incubation, the tissue sections were buffered washed (2 × 6 min), and then the UltraVision Hydrogen Peroxide Block was applied for 10 min in order to neutralize the endogenous peroxide activity. The slides were washed with buffer (2 × 6 min), and then incubated with Primary Antibody Amplifier Quanto for 10 min. Then, HRP Polymer Quanto was applied and the slides were incubated for another 10 min. Subsequently, thorough buffer washing was performed (3 × 6 min), and the tissue sections were incubated using diaminobenzidine (DAB) Quanto Chromogen for 6 min. Ultimately, the slides were buffer washed, counterstained with hematoxylin QS (Vector H-3404), dehydrated using graded ethanol solutions and xylene, and then mounted in synthetic resin.

For double PD-L1/PD1 expression, the following procedure was used. Following DAB staining for PD-L1, as previously described, a thorough buffer washing (3 × 7 min) was carried out, and the tissue sections were incubated for 60 min at room temperature with primary antibody PD-1 (Biocare, clone: NAT105), at a dilution of 1/25. The double staining protocol was performed using DAKO, Agilent

EnVision G/2 System/AP Rabbit/Mouse Kit (Permanent Red) (Code K5355), which is a high-sensitivity alkaline/phosphatase-based 2nd generation visualization kit. After primary antibody incubation, the sections were buffer washed (1 × 7 min), and Vial 1 Rabbit/Mouse (LINK) was applied for 30 min. Then, the slides were washed with phosphate-buffered saline (PBS) solution 1 × 7 min, and Vial 2 AP Enzyme (ENHANCER) was applied for another 30 min. Subsequently, the tissue sections were buffer washed 1 × 7 min and incubated with Permanent Red Chromogen for 20 min. Then, the slides were buffer washed 1 × 7 min, counterstained with Hematoxylin QS (Vector H-3404) for 2 min, and then rinsed, first with buffer (1 × 7 min) and then with distilled water (1 × 7 min). The tissue sections were left to air-dry completely, and then they were mounted with DPX mounting medium.

After establishing the methodology, assessment of immunohistochemistry was performed independently by two experienced observers who were blinded to the clinical data and outcome of the patients. A conference microscope was used to resolve any discrepancies. The entire tissue section was examined in all optical fields (o.f). The lymphocyte parameters were scored in the tumor stroma; intraepithelial lymphocytes were only occasionally observed and only in a small number of cases. Assessment of double staining was performed using the same methodology applied for single staining separately.

Assessment of PD-L1 expression

The percentage of cancer cells with strong cytoplasmic and/or membrane expression was recorded in all × 200 o.f., and the mean value was used to score each case. Cases were grouped into four categories: negative (lack of reactivity), low (expression in 1–9% of the cancer cells), medium (expression in 10–49% of the cancer cells), and high (expression in ≥ 50% of the cancer cells). PD-L1 expression was also assessed in the stroma-infiltrating lymphocytes.

TIL assessment

TILs were assessed in the PD-1 immunostaining slides as lymphocytes stained with hematoxylin (with or without staining with PD-1). This does not result in any bias because the lymphoid population is mainly stained with hematoxylin even after hematoxylin and eosin staining. Moreover, in immunohistochemical investigation of different types of lymphomas, the lymphoid population is stained only with hematoxylin in addition to specific immunohistochemical markers that stain neoplastic lymphoid cells. The number of TILs was assessed in all × 40 o.f., and the mean value was used to obtain a final score. The four different groups of TILs present in the tumor stroma were defined and scored (TIL score) as: 1 (minimal, 1–10 lymphocytes/o.f.), 2 (low, 10–70 lymphocytes/o.f.), 3 (medium, 70–150 lymphocytes/o.f.), and 4 (high, > 150 lymphocytes/o.f.).

The TIL score describes, semi-quantitatively, the absolute mean lymphocytic density in the stroma of tumors. PD-1 and PD-L1 stain a fraction of TILs so that two distinct parameters can be retrieved. One parameter is the relative density of the positive lymphocytes in the total TIL population (regardless of the TIL density) and the other parameter is the absolute density of these PD-1 or PD-L1 positive lymphocytes in the stroma of tumors.

To assess the relative density, the relative PD-1 score (percentage of PD-1 expressing TILs) and the relative PD-L1 score were calculated after counting the PD-1 or PD-L1 expressing lymphocytes per o.f., and, subsequently, by determining the percentage of their presence among the overall TIL counts. Three PD-1 and PD-L1 scores were identified: 0 (lack of lymphocytes with PD-1 expression), 1 (% of PD-1+ lymphocytes ranging between 1 and 9%), and 2 (% of PD-1+ lymphocytes > 10%).

To semi-quantitatively provide an index of the absolute density, the relative PD-1 and the relative PD-L1 scores were multiplied by the TIL score to produce the total PIL score and the total PDL-IL score. Thus, three scores were identified: 0 (product 0), 1 (product 1–4), and 2 (product 5–8).

Assessment of metabolism-related proteins

The expression of the multiple proteins involved in glucose absorption (GLUT1,2), glycolysis (Hexokinase II, aldolase) and monocarboxylate trafficking (MCT1,2) were assessed, as previously reported [7]. We also examined the expression of two major isoenzymes of lactate dehydrogenase, namely the LDH5 and the LDH1 [12]. The LDH molecule is a tetramer composed of four polypeptide chains, of the M and/or the H type. The M (muscle) chain is encoded by the LDHA gene and the H (Heart) by the LDHB gene. The LDH-1 (H4) is composed of four H-subunits, and the LDH-5 (M4) of four M-subunits. The higher the amount of the M-subunits in the LDH molecule, the stronger its ability to catalyze pyruvate transformation to lactate. On the contrary, H-prevailing isoenzymes catalyze the inverse transformation of lactate to pyruvate. Thus LDH5 is the LDH isoenzyme with the strongest role in the anaerobic pyruvate metabolism. The methodology of immunohistochemistry has been previously reported [7].

Strong cytoplasmic expression in more than 50% of cancer cells was used to classify cases as having high reactivity. The following primary antibodies were used: GLUT1 ab15309, GLUT2 ab111117, MCT1 ab85021, MCT2 ab129290, HXKII ab 103836, aldolase ab54770, LDH5 ab9002 and LDH1 ab81485 (all from Abcam, UK), as previously described [7].

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5.0 package and the SPSS (v14.0, SPSS, Inc.) program. Either the χ^2 test or Fisher's exact t test was used to compare the categorical variables, as appropriate. Kaplan–Meier survival curves were used to assess the impact of the assessed variables on the disease-specific overall survival of patients. A Cox's proportional hazard regression model was applied to compare the categorical variables, using dichotomized variables, including the TIL (1 vs. 2, 3, 4), PD-L1 expression by cancer cells (negative vs. positive), lymphocyte PD-1 score (0, 1 vs. 2), lymphocyte PIL-score (0, 1 vs. 2), together with stage (1, 2 vs. 3) and histology type (squamous vs. non-squamous), using backward elimination to assess the effect of the parameters on the death events. A p value < 0.05 was considered to be statistically significant.

Results

Expression in normal lung tissue

PD-L1 and PD-1 were not expressed by the normal bronchial epithelium or lung alveolar tissue. Bronchial cartilage and seromucinous bronchial glands were also negative (Fig. 1a).

PD-LI and PD-1 expression in cancer cells

The expression of PD-L1 was membranous, always accompanied by a strong cytoplasmic expression in cells with membrane reactivity. Lack of reactivity was noted in 49/98 cases. Weak cytoplasmic expression with a lack of membranous staining was noted in 4/98 cases. The remaining 45/98 cases showed strong cytoplasmic and membranous staining in a varying percentage of cancer cells. None of the cases showed PD-1 reactivity by cancer cells, while PD-1 was expressed by a varying percentage of TILs.

The distribution of tumors according to the percentage of cells with membranous/cytoplasmic immunostaining is shown in Table 1a. Figure 1b–e shows the typical immunohistochemical patterns of PD-L1 expression (negative, low, medium, and high).

Expression in TILs

Table 1b shows the distribution of cases according to the TIL score (number of infiltrating lymphocytes per o.f.), the PD-1 score (% of TILs with PD-1 positive expression), the PIL score (product of TIL score × PD-1 score), the PD-L1-score (% of TILs with PD-L1 positive expression), and the PDL-LI score (product of TIL score × PD-L1 score), following the assessment explained in the methods section. Figure 1f–h shows tumors with an intense TIL presence (hematoxylin stained) and those with a lack of, limited, or extensive PD-1 expression, respectively.

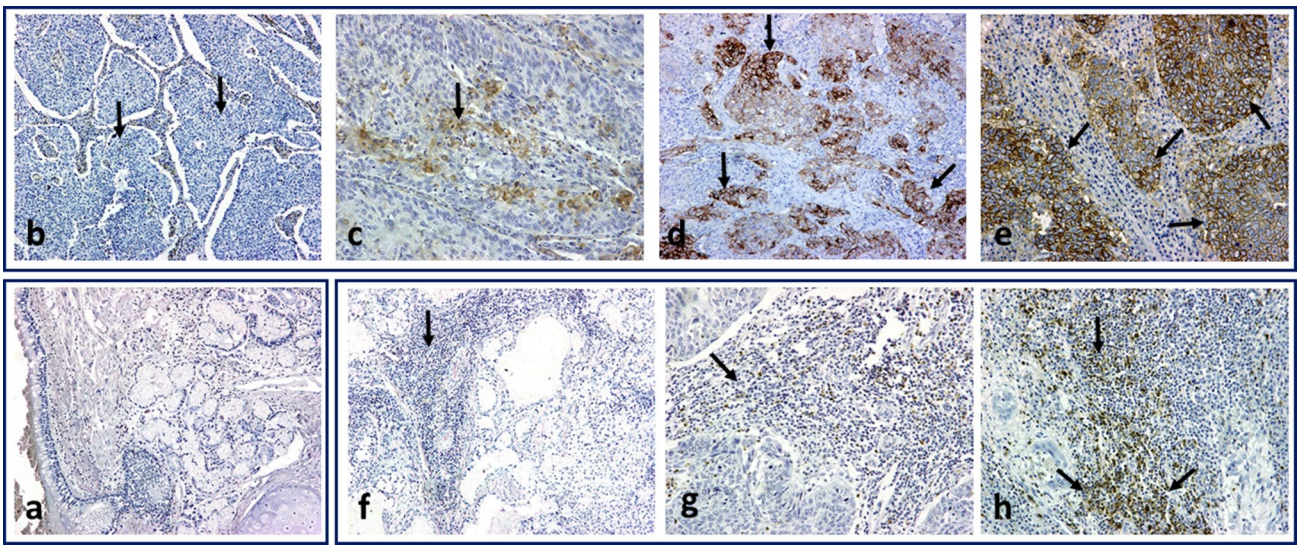

Fig. 1 Tissue images of PD-L1 and PD-1 immunostaining (magnification × 100 for **a**, **b**, **d** and **f** and × 200 for **c**, **e**, **g** and **h**): **a** Normal bronchus and submucosa tissue (seromucinal glands, vessels and cartilage) with lack of any PD-L1 reactivity; **b**, **c**, **d**, **e** squamous cell lung cancer tissue sections with negative, low, medium and high PD-L1 expression by cancer cells, respectively (arrows); **f–h** lung cancer cases with intense TIL infiltration and lack, limited and intense expression of PD-L1 by lymphocytes, respectively (arrows)

Table 1 Distribution of cases according to (a) expression of PD-L1 and PD-1 in cancer cells and (b) according to tumor-infiltrating lymphocytes (TIL) and PD-1 expression parameters

Group	% + cells	PD-L1 No pts (%)	PD-1 No pts (%)
(a)			
Negative	0	53[a] (54.0)	98 (100)
Low	1–9	22 (21.4)	0 (0.0)
Medium	10–49	11 (12.3)	0 (0.0)
High	50–90	12 (12.3)	0 (0.0)

TIL-score [no pts (%)]	PD-1-score [no pts (%)]	PIL-score [no pts (%)]	PD-L1-score [no pts (%)]	PDL-IL score [no pts (%)]
(b)				
1 [20 (20.4)]	0 [48 (48.9)]	0 [54 (55.1)]	0 [61 (62.2)]	0 [61 (62.2)]
2 [36 (36.7)]	1 [28 (28.6)]	1 [27 (27.6)]	1 [31 (31.6)]	1 [32 (32.3)]
3 [26 (26.6)]	2 [22 (22.5)]	2 [17 (17.3)]	2 [6 (9.2)]	2 [5 (5.1)]
4 [16 (16.3)]				

[a]Four of them with weak cytoplasmic staining

The recorded range and median % of PD1+ lymphocytes in the four TIL score categories are: TIL score 1: range 0–5%, median 0%; TIL score 2: range 0–20%, median 0%; TIL score 3: range 0–30%, median 2%; TIL score 4: range 0–40%, median 10%. The recorded range and median % of PD-L1+ lymphocytes in the four TIL score categories are: TIL score 1: range 0–5%, median 0%; TIL score 2: range 0–10%, median 0%; TIL score 3: range 0–20%, median 0%; TIL score 4: range 0–20%, median 1.5%.

Double staining

The expression of PD-L1 in cancer cells and of PD-1 in the stroma lymphocytes was repeated blindly in the slides of the tumor samples that underwent double immunostaining, confirming a high intra-observer concordance ($p < 0.0001$, $r > 0.96$). Thus, this method is feasible and allows a reliable evaluation of both variables in the same slide.

Association between cancer cell PD-L1 and lymphocyte parameters

Table 2 shows the association between PD-L1 expression and the other parameters. All 20 tumors with a lack of PD-L1 expression had a very low intratumoral presence of TILs ($p = 0.05$). Medium/high PD-L1 expression was significantly linked with a high PIL score ($p = 0.005$) and a high PD-1 score ($p = 0.004$). No statistically significant association with PD-L1+ lymphocytes was noted.

Table 2 Association of cancer cell PD-L1 expression with lymphocyte variables

	Negative/low	PD-L1 Medium/high	*p* value
TIL-score			
1	20	0	
2	26	10	0.05
3	18	8	
4	11	5	
PD-1-score (lymphocytes)			
0	43	5	
1	20	8	0.004
2	12	10	
PIL-score (lymphocytes)			
0	48	6	
1	16	11	0.005
2	11	6	
PD-L1-score (lymphocytes)			
0	49	12	
1	21	10	0.37
2	5	1	
PDL-LI-score (lymphocytes)			
0	49	12	
1	21	11	0.12
2	5	0	

Association with the histopathological variables

Analysis based on the patient's NSCLC stage, histology type, age, and sex did not reveal any statistically significant

association with PD-L1 expression, TIL score, PD-1 score, PIL score, or PD-L1 score (data not shown).

Association with the metabolism parameters

High LDH5 was noted in 67/98 cases, high HXKII was noted in 45/98 cases, and high MCT2 was noted in 15/98 cases. A significant inverse relationship was confirmed between LDH5 expression and the PD-1 score ($p = 0.006$) or the PIL score ($p = 0.008$). Similarly, a low PIL score and a low PD-1 score were significantly linked with high HXKII and MCT2 expression ($p < 0.04$). A low TIL score was linked with high MCT2 expression ($p = 0.01$). These associations are presented in Table 3. There was no statistically significant association with GLUT1,2 expression, aldolase, or MCT1 expression. The PD-L1 and PDL-LI scores were also inversely related to LDH5 expression ($p = 0.06$ and $p = 0.04$, respectively). High LDH1 expression was noted in 30/98 cases. There was no association between LDH1 and the immunological or histopathological variables assessed in the current study.

Survival analysis

The Kaplan–Meier disease-specific overall survival analysis results (Fig. 2a–d) showed a trend for the cases with PD-L1 expression to have a poorer prognosis, but the difference was not statistically significant ($p = 0.10$). Cases with intense lymphocytic infiltration (TIL score 4) had significantly better survival than cases with very low-lymphocytic presence (TIL score 1) ($p = 0.007$). Similarly, patients with a high PIL score of 2 had a significantly better prognosis than patients with a PIL score of 1 ($p = 0.04$). LDH-5 was also linked with poor survival ($p = 0.02$). No association was noted between the PD-L1 score or the PDL-L score and survival. The prognostic role of metabolism-related variables has been previously reported [7].

Double stratification analysis revealed that, in cases with a high TIL score (3, 4) or a high PIL score (2), PD-L1 overexpression was correlated with significantly poorer survival ($p = 0.01$ and $p = 0.03$, respectively); Fig. 2e, f. PD-L1 expression was not related with survival

Table 3 Association of PD-L1 and lymphocyte variables with metabolism markers

	LDH5			HXKII			MCT2		
	Low	High	*p* value	Low	High	*p* value	Low	High	*p* value
PD-L1 (cancer cells)									
Neg	16	37		26	27		44	9	
Low	8	14		12	10		20	2	
Med	2	9	0.62	5	6	0.17	8	3	0.48
High	5	7		10	2		11	1	
TIL-score									
1	5	15		6	14		14	6	
2	9	27	0.12	20	16	0.07(**)	29	7	0.01(**)
3	9	17		16	10		25	1	
4	8	8		11	5		15	1	
PD-1-score (lymphocytes)									
0	11	37		22	26		38	10	
1	8	20	0.006(*)	15	13	0.04(*)	24	4	0.11(*)
2	12	10		16	6		21	1	
PIL-score (lymphocytes)									
0	12	42		26	28		43	11	
1	9	18	0.008(*)	14	13	0.04(*)	13	4	0.05(*)
2	10	7		13	4		17	0	
PD-L1-score (lymphocytes)									
0	15	46		31	30		48	13	
1	12	19	0.06	18	13	0.65	29	2	0.1
2	4	2		4	2		6	0	
PDL-LI-score (lymphocytes)									
0	15	46		31	30		48	13	
1	13	19	0.04	19	13	0.7	30	2	0.09
2	3	2		3	2		5	0	

(*) score 0, 1 vs. 2; (**) score 1, 2 vs. 3, 4

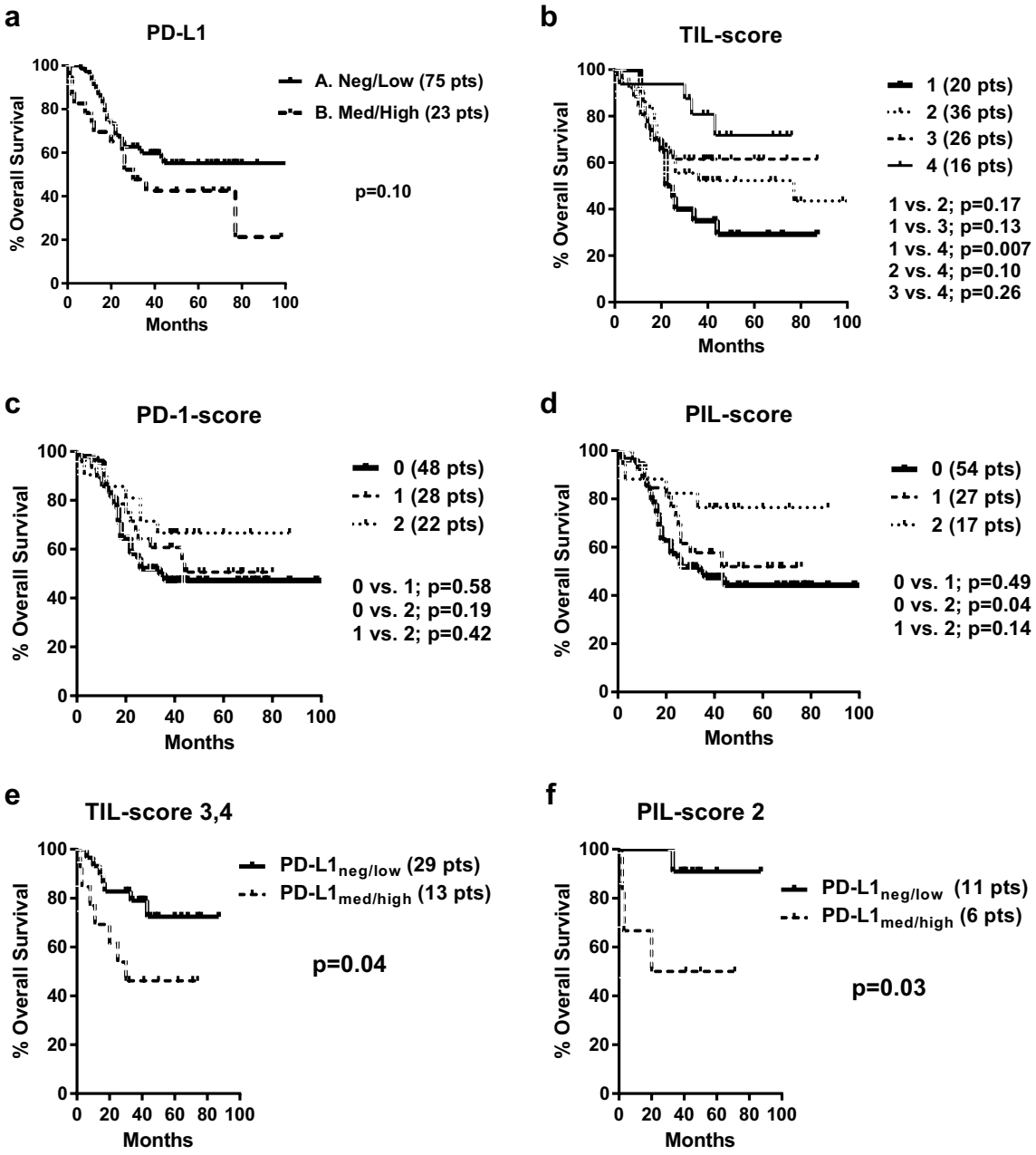

Fig. 2 Kaplan–Meier disease-specific overall survival curves, stratified for PD-L1 cancer cell expression (**a**), TIL-score (**b**), PD-1 score (**c**), PIL-score (**d**), PD-L1 cancer cell expression in cases with high TIL score (**e**) and PD-L1 cancer cell expression in cases with high PIL score (**f**)

in the groups of patients with low TIL score or low PIL score (data not shown).

In multivariate analysis, stage (3 vs. 1, 2; $p = 0.002$, HR 3.33, 95%CI 1.4–4.5) and TIL score (1 vs. 2, 3, 4; $p = 0.02$, HR 2.12, 95%CI 1.1–4.0) were independent predictive variables of death events.

Discussion

For decades, pathologists have recognized that infiltration of tumors by lymphocytes is an important feature that deserves reporting in the context of diagnosis, as this is

considered a direct marker of host anti-tumor response and, potentially, of a better prognosis. In a meta-analysis by Gooden et al., it was concluded that TILs' density has a moderately positive impact on survival of cancer patients, although the ratio of specific sub-population (e.g., CD4/CD8 or CD8/FOXP3 cells) seems to be important in defining prognosis [13]. As the authors stated, 'the exact magnitude of the effect of TILs on prognosis remains mysterious'.

In the current study, intense TIL density was linked to a better postoperative prognosis of patients with NSCLC. In a recent study, Lin et al. reported on the prognostic relevance of TILs in a series of patients with NSCLC [14]. In contrast, other studies found a positive association between TIL density and a good prognosis [15], even in stage I patients [16]. Therefore, it seems that TIL is a term that refers to the density of lymphocytic infiltration, while the composition of TILs (which may include various subtypes and ratios between regulatory and suppressive T-cells), defines the anti-tumor effectiveness of the host immune response. For example, it has been reported that CD8+ or PD-1+ TILs are strong independent positive prognostic markers in NSCLC [17–19], while FOXP3 + TILs is related to a poor prognosis [20, 21].

However, the biology of immune response is far more complex. In addition to the sub-population of TILs, cancer-derived proteins may neutralize their cytotoxic activity. e.g., PD-L1 expression by cancer cells [4]. In the current study, the multivariate analysis results showed that extensive PD-L1 expression by cancer cells was associated with poor survival in NSCLC. This effect was more prominent when the tumors had intense TIL infiltration. Chen et al. found that PD-L1 expression was significantly linked with poor prognosis in NSCLC and related to advanced stage and poor differentiation [22]. In our study, PD-L1 expression was independent of histology, differentiation, or stage of the disease.

In 2014, Velcheti et al. found a direct association between PD-L1 expression and TILs density and a better prognosis [23]. Our study only agrees with the finding of the direct association between PD-L1/TILs, as none of the 20 patients with very low TILs density expressed PD-L1. The same year, a study by Zhang et al. showed poor survival in patients overexpressing PD-L1 and PD-L2 [24]. A subsequent study by Cooper et al. reported no association between PD-L1 and stage or histology; however, in accordance with [23], PD-L1 was linked with better overall survival [25]. Interestingly, Schmidt et al. found that PD-L1 is associated with a good prognosis in patients with squamous cell histology who received adjuvant chemotherapy, showing that postoperative therapy may be a confusing factor in the attempt to identify the prognostic role of PD-L1 [26]. However, Tokito et al. reported a poorer survival of PD-L1+ patients with stage III NSCLC that were treated with concurrent chemoradiotherapy [27]. In 2016, a study by Sun et al. on 1070 surgically resected NSCLC specimens found an adverse prognostic role of PD-L1 expression [28]. In the same year, Ameratunga et al. found no prognostic relevance in a cohort of 522 resected NSCLC cases [29]. In 2017, Mori et al. reported a direct association between PD-L1 expression and poor prognosis only in patients with wild-type epidermal growth factor receptor (EGFR) function [30]. Several more recent studies support an ominous prognostic role of PD-L1 expression in NSCLC [31–33].

Thus, it seems that, despite the indisputable role of PD-L1 in suppressing host immune response, the clinical validation of PD-L1 as a postoperative prognostic marker remains controversial. An obvious explanation is the methodology used for staining and scoring. This raises the question of how to apply a score clinically. A more important consideration is the existence of subgroups of tumors with highly aggressive or non-metastasizing disease, defined by molecular pathways beyond immune biology. Even if immune surveillance applies to all tumors, PD-L1 is certainly not the only pathway involved. For example, in our study, 20% of the cases were negative for PD-L1 and had poor infiltration by lymphocytes, implying that PD-1/PD-L1 immunity has little to do with these types of tumors.

Another explanation revealed in our study is based on the fact that PD-L1 is associated with both high TIL and PD-1+ TIL density [current study, 14, 26, 34] (a marker of good prognosis), while, at the same time, PD-L1 l is correlated with poor prognosis. This apparent discrepancy is resolved by the observation that PD-L1 expression segregates patients with high-TIL density into two categories with a significantly different prognosis, where a particularly ominous prognosis applies for patients with PD-L1 overexpression. When evaluating the PD-1+ infiltrating TIL density, the median survival of patients with high density dropped from 90 to 50% when PD-L1 was overexpressed.

Consequently, it seems that the prognostic role of PD-L1 should be investigated from the perspective of its biological context. The prognostic role of PD-L1 is relevant only in immunogenic tumors that bear high PD-1+ lymphocytic infiltration. PD-L1 performs the well-validated function of blocking the cytotoxic PD-1+ T-cell response, allowing migration and metastasis that has already occurred at an earlier stage before surgery.

Despite the direct association between the expression of PD-L1 by cancer cells and the stroma infiltration by TILs and PD-1 expressing TILs, a significant percentage of tumors with high TIL and PIL score did not express PD-L1. Although it has been suggested that cytokine released by TILs are involved in up-regulation of PD-L1 gene expression in cancer cells [35, 36], genetic and epigenetic regulation of PD-L1 expression is also involved in certain tumors [37]. An

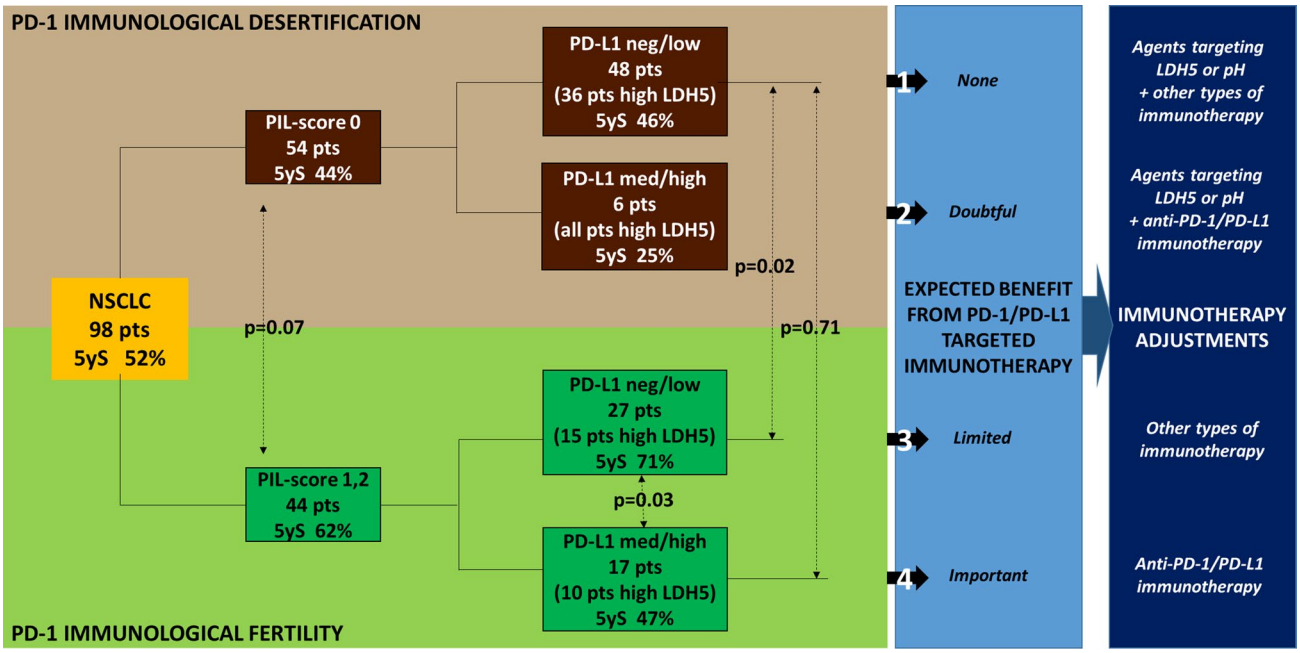

Fig. 3 Immunological/metabolical classification of non-small cell lung cancer: Type 1: includes the vast majority of tumors with immunological desertification, where no PD-1 cytotoxic lymphocytes can transmigrate into the tumoral stroma. PD-L1 expression is seldom in such tumors (89% are negative or have limited expression). These are mainly tumors with high anaerobic glycolytic metabolism and low intratumoral pH due to high amounts of lactate released by cancer cells with intense LDH5 activity. Anti-PD-1/PD-L1 are not expected to have any activity due to a combined lack of PD-L1 expression and PD-1 T-cell infiltration. Metabolical interference with agents targeting LDH5 or agents that may increase pH may prove of importance in the re-introduction of immunotherapy as a treatment option. Type 2: includes a minority of tumors with immunological desertification, still expression PD-L1. Again, metabolic therapy may restore TIL infiltration in the tumor environment, and patients may benefit from PD-1/PD-L1 immunotherapy. Type 3: includes one fourth about of NSCLC cases, and half of the tumors bearing an immune fertile microenvironment with intense PD-1 expressing T-cell infiltration and in the absence of PD-L1 expression by cancer cells. This subgroup has the best post-operative overall survival, and PD-1/PD-L1 immunotherapy may have only a limited role. However, intensification of the immunological anti-tumor activity with other immune stimulating agents or agents targeting different immune pathways may be of value. Type 4: includes the other half of immunologically fertile tumors with an extensive expression of PD-L1 by cancer cells. The postoperative survival of these patients is severely compromised, and anti-PD-1/PD-L1 immunotherapy can be the most effective way to restore immune surveillance and improve outcomes

additional subgroup of tumors with discordant TIL-density and PD-L1 expression has been also identified in the current study. This concerns non-immunogenic tumors with intense anaerobic glucose metabolism and lactate production, as predicted by the overexpression of LDH5 enzyme. LDHA gene, and its product LDH5 (tetramer iosenzyme composed by 4 M-subunits) is responsible for the transformation of pyruvate to lactate under anaerobic conditions or mitochondrial dysfunction [38]. Two-thirds of tumors with LDH5 overexpression had a total absence of PD-1+ TILs in their stroma, implying that acidic microenvironmental conditions block the access of cytotoxic lymphocytes in the tumor environment [8–10]. Hypoxia and its metabolic consequence, anaerobic metabolism, may therefore, drive a cancer phenotype that combines PD-L1 overexpression with cold immune environment as, indeed, a direct control of PD-L1 gene by the hypoxia inducible factor HIF1α has been reported [39].

It is important to note that, in a study of melanoma patients treated with a vaccine from autologous melanoma cells and dendritic cells, lactate dehydrogenase (LDH) serum levels predicted poor response to immunotherapy [40]. Despite the fact that half of the tumors with high LDH5 expression do express PD-L1 in cancer cells, the immunological desert noted in these cases suggests that targeted immunotherapies are unlikely to have any activity. However, high LDH5 is also noted in approximately 50% of immunologically hot tumors. It may be suggested that, in these tumors, the amount of lactate released does not result in a low enough pH to block T-cell migration, potentially due to the adequate buffering offered by stroma metabolic activity, as suggested by previous work from our group [41, 42].

The effects of acidity on T-cell anti-tumor activity in vivo is poorly studied. However, acidic conditions seem to block the stimulatory activity of IL-2 on the proliferation of lymphocytes [43]. Lymphokine-activated killer cells and natural killer cell activity is also suppressed under acidic conditions [44, 45], and the T-cell cytotoxic activity against cancer cell lines is also diminished [46]. Calcinotto et al. showed that pH levels ranging from 6 to 6.5 induce an anergic state in CD8+ T-lymphocytes, impairing their cytolytic activity and blocking secretion of cytokines [47]. The macrophage function is also affected because TNF-α secretion is diminished under acidic conditions [48]. Nevertheless, in animal models, reversing tumor acidity has been shown to be beneficial in tumor immunotherapy [10].

Conclusions

The current pilot study provides preliminary evidence that the combined assessment of TILs, PD-1, PD-L1, and LDH5 provides a tool for an immunological/metabolic classification of NSCLC tumors. Validation of this tool in a large series of NSCLC patients is required to confirm its value in identifying subgroups of patients with different prognoses and different expected responses to anti-PD-1/PD-L1 immunotherapy. The immunological/metabolic classification applied in our small series of patients is summarized in Fig. 3, together with suggested therapeutic manipulation for the optimization of the immunotherapy outcome. Given the low specificity of anti-PD-L1 immunostaining in predicting the response to anti-PD-1/PD-L1 therapy in NSCLC, and the lack of any predictive importance in other tumors, an overall assessment of the PD-1 lymphocytic infiltration variables could prove to have clinical value. The results suggest that combinations of therapies should be tried to reduce tumor acidosis, for example, CA9 or LDH inhibitors and immunotherapy. Whether automated digital scoring of PD-L1/PD1 double immunostaining can simplify the assessment procedures and easily provide immunological scoring is an issue that should be examined in future studies.

Funding The study has been funded by the Tumour and Angiogenesis Research Group, Heraklion, Greece

Compliance with ethical standards

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

Ethical approval Ethical approval was obtained from the Internal Scientific Committee and the Ethic Research Committee of the University Hospital of Alexandroupolis (study Approval Number ES11-26-11-18). The study was conducted according to the criteria set by the declaration of Helsinki.

Informed consent The study is retrospective on 'existing holdings' and no informed consent is demanded for examining anonymously archival material (material archived between 2002 and 2007). (Human Tissue Authority, E Research, Code of Practice and standards; https://www.hta.gov.uk/sites/default/files/Code%20E%20-%20Research%20Final.pdf; page 15, Consent exceptions paragraph 56 and 57).

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