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ARTICLE

Differential expression of PD-L1 and IDO1 in association with the immune microenvironment in resected lung adenocarcinomas

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Abstract

Like programmed cell death ligand 1 (PD-L1), indoleamine 2,3-dioxygenase 1 (IDO1) is known to exert immunosuppressive effects and be variably expressed in human lung cancer. However, IDO1 expression has not been well studied in lung adenocarcinoma. PD-L1 and IDO1 expression was evaluated in 261 resected lung adenocarcinomas using tissue microarrays and H-scores (cutoff: 5). We compared IDO1 and PD-L1 expression with clinical features, tumor-infiltrating lymphocytes, HLA class I molecule expression, molecular alterations, and patient outcomes. There was expression of PD-L1 in 89 (34%) and IDO1 in 74 (29%) cases, with co-expression in 49 (19%). Both PD-L1 and IDO1 were significantly associated with smoking, aggressive pathologic features, and abundant CD8+ and T-bet+ (Th1 marker) tumor-infiltrating lymphocytes. PD-L1 expression was also associated with preserved HLA class I molecule expression ($p = 0.002$). Compared to PD-L1 +/IDO1+ and PD-L1+ only cases, significantly fewer IDO1+ only cases had abundant CD8+ and T-bet+ tumor-infiltrating lymphocytes ($p < 0.001$, respectively). PD-L1 expression was significantly associated with *EGFR* wild-type ($p < 0.001$) and *KRAS* mutants ($p = 0.021$), whereas isolated IDO1 expression was significantly associated with *EGFR* mutations ($p = 0.007$). As for survival, PD-L1 was a significant predictor of decreased progression-free and overall survival by univariate but not multivariate analysis, while IDO1 was not associated with progression-free or overall survival. Interestingly, there was a significant difference in the 5-year progression-free and overall survival ($p = 0.004$ and 0.038, respectively), where cases without PD-L1 or IDO1 expression had the longest survival, and those with PD-L1 alone had the shortest survival. While PD-L1+/−IDO1 expression is observed in association with HLA class I expression, cytotoxic T lymphocyte/Th1 microenvironments, *EGFR* wild-type, and *KRAS* mutations, isolated IDO1 expression does not demonstrate these associations, suggesting that IDO1 may serve a distinct immunosuppressive role in lung adenocarcinomas. Thus, further investigation of IDO1 may demonstrate its role as a potential biomarker for patients who undergo anti-PD-1/PD-L1 therapy.

Introduction

Recent therapeutic strategies for non-small cell lung cancer have focused on targeting immune checkpoints to reinvigorate the host immune system and thus restore anti-tumor responses. Most notably and extensively studied across many malignancies is the programmed cell death 1 (PD-1) receptor/programmed cell death ligand 1 (PD-L1) interaction, which suppresses the CD8+ cytotoxic T-cell immune response, thus allowing tumor cells to bypass immune surveillance [1]. Multiple major clinical trials [2–6] have demonstrated significant improvements in patient survival in response to anti-PD-1 (i.e., nivolumab, pembrolizumab) and anti-PD-L1 (i.e., atezolizumab) agents. As a result, these agents are now FDA (Food and Drug Administration)

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approved for the treatment of patients with metastatic non-small cell lung cancer with disease progression during or after platinum-based chemotherapy. Of those, pembrolizumab has also been approved as the first-line therapy for advanced non-small cell lung cancer patients whose tumors express PD-L1 by immunohistochemistry on 50% or more of tumor cells and for advanced non-squamous non-small cell lung cancer patients in combination with platinum/pemetrexed regardless of PD-L1 expression [7, 8].

We have previously shown that PD-L1 expression in resected lung adenocarcinomas is associated with significant smoking history, aggressive pathologic features, the CD8+ cytotoxic T lymphocyte and T helper type 1 (Th1) immune microenvironment, and *KRAS* mutations and *EGFR* wild-type, using an individually-validated PD-L1 immunohistochemistry assay and a variety of scoring systems including H-score [9]. In that study, PD-L1 expression was predictive of decreased progression-free survival and overall survival by univariate but not multivariate analysis, though other previous studies have reported conflicting results regarding prognostication using PD-L1 expression [10–12].

Similar to PD-L1, indoleamine 2,3-dioxygenase 1 (IDO1) is known to exert a potent immunosuppressive effect and be variably expressed in a variety of human solid tumors, including non-small cell lung cancer [13, 14]. Furthermore, IDO1 has been shown to contribute to tumor progression in mouse models of lung cancer [15]. IDO1 is an enzyme with limited expression in normal adult tissues (lymphoid tissues and placenta) that catalyzes the rate-limiting step in the catabolic conversion of tryptophan to kynurenine. The depletion of tryptophan induces apoptosis/dysfunction of CD4+ T cells, and the concurrent accumulation of kynurenine promotes regulatory T-cell differentiation [16]. In vitro studies have shown that IDO1 expression is not associated with CD3+ and CD8+ T cells in a colorectal cancer cell line [17], but IDO1 inhibition in murine melanoma models lead to a marked increase in CD8+ tumor-infiltrating lymphocytes [18]. As such, tumor expression of IDO1 favors immune escape, and clinical investigation [16, 19, 20] of IDO1 inhibitors alone or in combination with other immune-checkpoint blockade are underway in hopes of reversing tumor-induced immunosuppression via tryptophan depletion. In terms of the tumor immune microenvironment, like PD-L1, IDO1 has also been shown to increase in response to interferon-γ (IFNγ) and to be associated with increased CD3+ and CD8+ tumor-infiltrating lymphocytes via quantitative immunofluorescence [21]. However, the correlation of IDO1 with additional clinicopathologic and immunologic parameters, molecular alterations, and patient outcomes in non-small cell lung cancer, as well as its relationship with PD-L1, have not been well characterized.

In this study, we compared the expression of PD-L1 and IDO1 in correlation to clinicopathologic variables, subtypes of tumor-infiltrating lymphocytes, human leukocyte antigen (HLA) class I expression, molecular alterations, and patient outcomes in a large cohort of molecularly annotated lung adenocarcinomas.

Materials and methods

Study population

The study was approved by the Massachusetts General Hospital Institutional Review Board. A total of 461 cases of primary lung adenocarcinomas resected between July 2010 and December 2012 were identified from the Massachusetts General Hospital surgical pathology database. After excluding post-neoadjuvant therapy resections, cases with concurrent multiple primaries, and those with no molecular testing or no sufficient tumor blocks available, 261 cases formed the study cohort. All cases in this cohort had undergone a multiplex polymerase chain reaction-based assay (SNaPshot platform; Applied Biosystems, Waltham, MA) to detect a panel of commonly mutated genes including *EGFR*, *KRAS*, *BRAF*, *Her2*, and *TP53*, as previously described [22]. Subsets of the cohort had also been tested for *ALK* ($n = 248$) or *ROS1* ($n = 65$) gene rearrangements, or *MET* ($n = 17$), *EGFR* ($n = 4$), or *Her2* ($n = 2$) amplifications. Demographic information, smoking history, and post-operative follow-up data were collected from patient records. Progression-free survival was measured from the time of resection to the development of clinical/radiographic progression or death; patients alive without documented disease progression were censored on the date of last follow-up. Overall survival was measured from the date of resection to death due to any cause.

Pathology examination and tissue microarray construction

All tumor slides were reviewed, and tumor size, stage (pT and pN), histologic pattern, nuclear grade, pleural invasion, lymphatic invasion, and vascular invasion were evaluated in each case by three pathologists who were blinded to the clinical information [9]. The histology of the tumor was classified in accordance with the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society multidisciplinary classifications [23]. The tumor stage was diagnosed in accordance with the American Joint Committee on Cancer (AJCC) Cancer Staging Manual, 7th edition [24]. Nuclear grade was semi-quantitatively assessed on a scale of 1 to 3 [25].

Two-millimeter core tissue microarrays were constructed, with two cores from each of the study cases. If

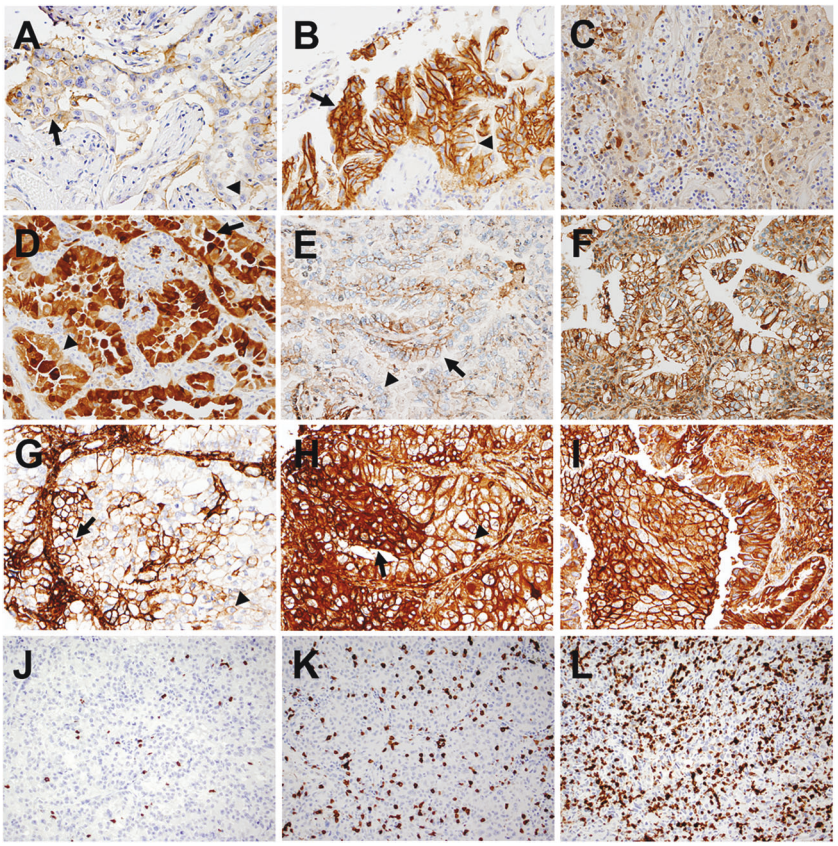

Fig. 1 Evaluation of immunohistochemical expression in lung adenocarcinomas using H-score (cutoff = 5) (magnification ×400 for **a–i**, ×200 for **j–l**). Membranous PD-L1: **a** 0 (arrowhead), 1 (arrow); **b** 2 (arrowhead), 3 (arrow). Cytoplasmic IDO1: **c** 1; **d** 2 (arrowhead), 3 (arrow). Membranous B2M: **e** 0 (arrowhead), 1 (arrow); **f** 2. Membranous HC10 (HLA-B/C): **g** 1 (arrowhead), 2 (arrow); **h** 2 (arrowhead), 3 (arrow). **i** Membranous HCA2 (HLA-A). Tumor-infiltrating lymphocytes were scored as the fraction of tumor cells with overlying positive T cells: 0, none/rare; 1, <5% (**j**); 2, >5 to <25% (**k**); 3, >25% (**l**)

available, one core was taken from the center of the tumor and the other from the invasive front. Two cores each of tonsil and placenta (positive controls) and normal lung parenchyma were also included.

Immunohistochemistry

Immunohistochemistry was performed on 5 µm sections cut from tissue microarray blocks using an automated stainer (Bond Rx; Leica Microsystems, Bannockburn, IL) and the following primary antibodies in accordance with the manufacturer's recommendations: PD-L1 (E1L3N (1:200), Cell Signaling Technology, Danvers, MA), IDO1 (1F8.2 (1:400), Millipore, Burlington, MA), B2M (D8P1H (1:8000), Cell Signaling Technology, Danvers, MA), HCA2 (IgG1 (1:100), Nordic-MUbio, The Netherlands), HC10 (IgG2a (1:100), Nordic-MUbio, The Netherlands), CD8 (4B11 (RTU), Leica Biosystems, Buffalo Grove, IL), T-bet (D6N8B (1:100), Cell Signaling Technology, Danvers, MA), and GATA3 (L50-823 (1:250), Biocare Medical, Pacheco, CA).

Interpretation of expression by immunohistochemistry

Two pathologists (MLZ and MM-K) who were blinded to the clinical and pathologic data evaluated cytoplasmic expression of IDO1 and membranous expression of B2M (the common HLA-A/B/C light chain required for surface expression), HCA2 (a specific antibody for HLA-A heavy chains), and HC10 (a specific antibody for HLA-B/C heavy chains) (Fig. 1) [26–28]. An H-score was recorded for each marker in each case. H-score was calculated based on the intensity (0–3) of membranous staining and extent (%) of positive tumor cells (3× percentage of strongly staining cells + 2× percentage of moderately staining cells + percentage of weakly staining cells), ranging from 0 to 300. The expression of each immunostain was dichotomized as negative and positive based on an H-score cutoff of 5 (≥5 vs. <5) [29]. If discrepant scores were given to the two different cores from the same case, the final score was determined following discussion under a multi-headed microscope.

Evaluation of PD-L1 membranous staining by immunohistochemistry was independently performed by two pathologists who were blinded to the clinical and pathologic data, as previously described [9]. Similarly, two of the authors previously evaluated tumor-infiltrating lymphocytes using cytoplasmic expression of CD8 (a marker for cytotoxic T lymphocytes) and nuclear expression of T-bet (a marker for Th1 pathway activation) and GATA3 (a marker for Th2 pathway activation) immunohistochemistry. tumor-infiltrating lymphocytes were scored as the fraction of

tumor cells with overlying positive T cells: 0, none or rare; 1, <5%; 2, ≥5 and <25%; and 3, ≥25%. Scoring of lymphocytes in the tumor stroma was performed by comparing the percentage of positive lymphocytes with the total number of nucleated cells in the stromal compartments [9].

Statistical analysis

Associations between PD-L1 and/or IDO1 expression and demographics, clinicopathologic features, tumor-infiltrating lymphocytes (CD8+, T-bet+, and GATA3+), HLA class I molecule expression, and molecular alterations were examined using Fisher's exact tests for categorical variables and the two-sided *t*-test or Wilcoxon rank-sum test for continuous variables, as appropriate. Multivariate analysis was performed with a binary logistic regression model to evaluate for independent predictors of PD-L1 and IDO1 expression using an H-score cutoff of ≥5 to adjust for potential confounders. Survival analyses for progression-free and overall survival were performed using the Kaplan–Meier estimator and the log-rank test. Multivariate survival analysis was performed using the Cox proportional hazards regression model to adjust for potential confounders. The p values of <0.05 were considered statistically significant. Statistical analyses were performed using R version 3.2.2.

Results

Correlation of PD-L1 and IDO1 expression with clinicopathologic features

The study cohort ($n = 261$) had a mean age of 68 years (standard deviation: 10), and 171 (66%) were female. One case had no evaluable tumor for the assessment of IDO1 expression. Using an H-score cutoff of 5, 89/261 (34%) and 74/260 (29%) cases showed positive PD-L1 and IDO1 expression, respectively. Of the 260 cases, 146 (56%) were PD-L1−/IDO1−, 40 (15%) were PD-L1+/IDO1−, 25 (10%) were PD-L1−/IDO1+ and 49 (19%) were PD-L1+/IDO1+ (Fig. 2, Supplementary Table 1). The linear (Pearson) correlation coefficient between PD-L1 and IDO1 expression was $r = 0.4$ (weak correlation). Clinicopathologic and molecular characteristics of the study cohort were correlated with PD-L1, IDO1, and IDO1+/−PD-L1 expression (Table 1). PD-L1 expression was significantly associated with smoking history (>10 and >30 pack-years, $p < 0.001$ for both), higher pT stage ($p = 0.037$), higher pN stage ($p = 0.039$), solid-predominant pattern ($p < 0.001$), higher nuclear grade ($p < 0.001$), and the presence of lymphatic invasion and venous invasion ($p < 0.001$ for both). IDO1 expression was significantly associated with a history of heavy smoking (>30 pack-years, $p = 0.018$), acinar-predominant ($p = 0.003$) and solid-predominant ($p = 0.042$) patterns, higher nuclear grade ($p = 0.007$), and the presence of lymphatic invasion ($p = 0.011$) and vascular invasion ($p = 0.040$). There was no association between either PD-L1 or IDO1 and the overall AJCC pathologic stage or the presence of pleural invasion.

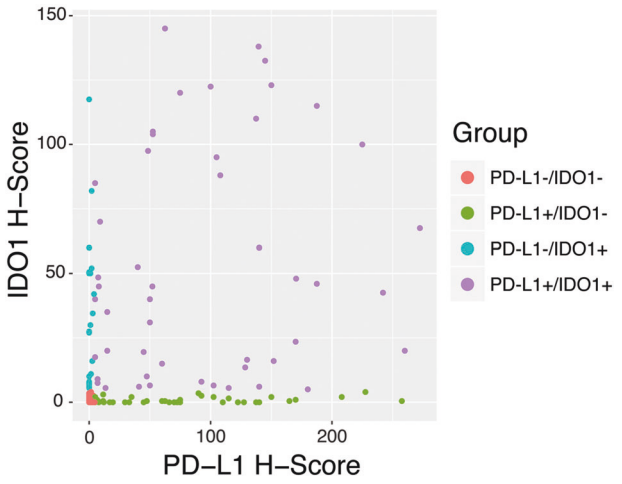

Fig. 2 Correlation between PD-L1 and IDO1 expression (cutoff of H-score = 5)

After stratifying the IDO1+ cases by PD-L1 expression, there was no longer a significant association between isolated IDO1 expression (IDO1+/PD-L1−) and heavy smoking history, solid-predominant histologic pattern, and the presence of lymphatic or vascular invasion (Table 1). However, isolated IDO1 expression was significantly associated with acinar-predominant histologic pattern ($p < 0.001$) and lower nuclear grade ($p < 0.001$). There were no differences between isolated PD-L1 expression and PD-L1/IDO1 co-expression.

Correlation of PD-L1 and IDO1 expression with tumor-infiltrating lymphocytes

As for tumor-infiltrating lymphocytes, both PD-L1 and IDO1 expression were significantly associated with abundant CD8+ tumor-infiltrating lymphocytes (66% vs. 4% and 47% vs. 16% of cases, $p < 0.001$, respectively), CD8+ lymphocytes in the tumor stroma (64% vs. 23% and 55% vs. 29% of cases, $p < 0.001$, respectively), and T-bet+ tumor-infiltrating lymphocytes (27% vs. 4% and 20% vs. 8% of cases, $p < 0.001$ and $p = 0.010$, respectively). There was no association of either PD-L1 or IDO1 with GATA3+ tumor-infiltrating lymphocytes, or stromal T-bet+ or GATA3+ lymphocytes. Isolated PD-L1 expression (PD-L1+/IDO−) demonstrated the same associations. Isolated IDO1 expression (IDO1+/PD-L1−) was no longer significantly associated with abundant T-bet+ tumor-infiltrating lymphocytes; all 25 (100%) isolated IDO1+ cases had rare-to-few CD8+ tumor-infiltrating lymphocytes

Table 1 Clinicopathologic characteristics and mutation status of patients with lung adenocarcinoma stratified by PD-L1 and IDO1 expression on tumor cells

Variable	PD-L1+	PD-L1−	*P*1	IDO1+	IDO1−	*P*2	IDO1+/PD-L1−	IDO1+/PD-L1+	*P*3
N[a]	89	172		74	186		25	49	
Mean age (SD)	68 (10)	68 (10)	0.630	67 (10)	69 (10)	0.170	64 (10)	68 (10)	0.380
Sex, *n* (%)			0.784			0.148			0.383
F	57 (64)	114 (66)		43 (58)	127 (68)		13 (52)	30 (61)	
M	32 (36)	58 (34)		31 (42)	59 (33)		12 (48)	19 (39)	
Smoking status, *n* (%)									
≥10 PPY	81 (92)	123 (72)	**<0.001**	63 (86)	141 (76)	0.089	19 (76)	44 (90)	0.082
≥30 PPY	65 (75)	84 (49)	**<0.001**	51 (70)	98 (54)	**0.018**	14 (56)	37 (76)	0.105
Tumor size, cm (IQR)	2.1 (2)	1.9 (2)	0.092	2.0 (1)	2.0 (2)	0.169	1.5 (1)	2.0 (2)	0.070
T status (pT), *n* (%)			**0.037**			0.252			**0.011**
T1	51 (57)	116 (67)		53 (72)	113 (61)		20 (80)	33 (67)	
T2	33 (37)	38 (22)		18 (34)	53 (29)		3 (12)	15 (31)	
T3	5 (6)	13 (8)		3 (4)	15 (8)		2 (8)	1 (2)	
T4	0	5 (3)		0	5 (3)		0	0	
N status (pN)[b], *n* (%)			**0.039**			0.310			1.000
N0	65 (73)	147 (86)		59 (80)	152 (82)		20 (80)	39 (80)	
N1	11 (12)	8 (5)		8 (11)	11 (6)		2 (8)	6 (12)	
N2	8 (9)	7 (4)		5 (7)	10 (5)		2 (8)	3 (6)	
Pathologic stage (AJCC), *n* (%)			0.438			0.917			1.000
I	66 (74)	135 (79)		59 (80)	141 (76)		20 (80)	40 (82)	
II	11 (12)	23 (13)		8 (11)	26 (14)		2 (8)	5 (10)	
III	11 (12)	11 (6)		6 (8)	16 (9)		2 (8)	4 (8)	
IV	1 (1)	3 (2)		1 (1)	3 (2)		1 (4)	0	
Predominant pattern (IASLC), *n* (%)			**<0.001**			**<0.001**			**<0.001**
MIA	2 (2)	18 (11)		3 (4)	17 (9)		1 (4)	2 (4)	
Lepidic	8 (9)	23 (13)		7 (10)	24 (13)		2 (8)	5 (10)	
Acinar	39 (44)	65 (38)		40 (54)	63 (34)		17 (68)	23 (47)	
Papillary	5 (6)	34 (20)		3 (4)	36 (19)		2 (8)	1 (2)	
Micropapillary	1 (1)	2 (1)		1 (1)	2 (1)		1 (4)	0	
Solid	34 (38)	9 (5)		18 (24)	25 (13)		0	18 (37)	
IMA	0	18 (11)		1 (1)	17 (9)		1 (4)	0	
Fetal	0	3 (2)		1 (1)	2 (1)		1 (4)	0	
Nuclear grade, *n* (%)			**<0.001**			**0.007**			**<0.001**
1	0	7 (4)		0	7 (4)		0	0	
2	34 (38)	131 (76)		39 (53)	126 (68)		21 (84)	18 (37)	
3	55 (62)	34 (20)		35 (47)	53 (29)		4 (16)	31 (63)	
Pleural invasion, *n* (%)			0.207			0.490			0.533
0	71 (80)	149 (87)		60 (81)	159 (86)		19 (76)	41 (84)	
1	18 (20)	23 (13)		14 (19)	27 (15)		6 (24)	8 (16)	
Lymphatic invasion, *n* (%)			**<0.001**			**0.011**			0.051
0	35 (39)	122 (71)		35 (47)	121 (65)		16 (64)	19 (39)	
1	54 (61)	50 (29)		39 (53)	65 (35)		9 (36)	30 (61)	
Vascular invasion, *n* (%)			**<0.001**			**0.040**			0.290
0	57 (64)	152 (88)		53 (72)	155 (83)		20 (80)	33 (67)	
1	32 (36)	20 (12)		21 (28)	31 (17)		5 (20)	16 (33)	
CD8+ tumor-infiltrating lymphocytes, *n* (%)			**<0.001**			**<0.001**			**<0.001**
0–1	30 (34)	166 (97)		39 (53)	156 (84)		25 (100)	14 (29)	
2–3	59 (66)	6 (4)		35 (47)	30 (16)		0	35 (71)	
CD8+ stroma, *n* (%)			**<0.001**			**<0.001**			**0.001**
≤10%	32 (36)	133 (77)		33 (45)	132 (71)		18 (72)	15 (31)	
>10%	57 (64)	39 (23)		41 (55)	54 (29)		7 (28)	34 (69)	
T-bet+ tumor-infiltrating lymphocytes, *n* (%)			**<0.001**			**0.010**			0.073
0–1	65 (73)	166 (97)		59 (80)	171 (92)		23 (92)	36 (74)	
2–3	24 (27)	6 (4)		15 (20)	15 (8)		2 (8)	13 (27)	
T-bet+ stroma, *n* (%)			0.055			0.228			1.000

Table 1 (continued)

Variable	PD-L1+	PD-L1−	*P*1	IDO1+	IDO1−	*P*2	IDO1+/PD-L1−	IDO1+/PD-L1+	*P*3
≤5%	68 (76)	149 (87)		58 (78)	159 (86)		20 (80)	38 (78)	
>5%	21 (24)	23 (13)		16 (22)	27 (15)		5 (20)	11 (22)	
GATA3+ tumor-infiltrating lymphocytes, *n* (%)			1.000			1.000			1.000
0–1	88 (99)	170 (99)		73 (99)	184 (99)		25 (100)	48 (98)	
2[c]	1 (1)	2 (1)		1 (1)	2 (1)		0	1 (2)	
GATA3+ stroma, *n* (%)			0.105			0.149			1.000
≤5%	70 (79)	150 (87)		58 (78)	161 (87)		20 (80)	38 (78)	
>5%	19 (21)	22 (13)		16 (22)	25 (13)		5 (20)	11 (22)	
Molecular alterations, *n* (%)			**<0.001**			0.832			**0.018**
KRAS mutations	46 (52)	62 (36)		29 (39)	78 (42)		7 (28)	22 (45)	
EGFR mutations	5 (6)	49 (29)		13 (18)	41 (22)		9 (36)	4 (8)	
ALK rearrangements	1 (1)	3 (2)		1 (1)	3 (2)		1 (4)	0	
Other alterations	4 (5)	9 (5)		4 (5)	9 (5)		1 (4)	3 (6)	
SNaPshot wild type	33 (37)	49 (29)		27 (37)	55 (30)		7 (28)	20 (41)	

PD-L1 programmed cell death ligand 1, *IDO1* indoleamine 2,3-dioxygenase 1, *IQR* interquartile range, *PPY* pack per year, *AJCC* American Joint Committee on Cancer, *IASLC* International Association for the Study of Lung Cancer, *MIA* minimally invasive adenocarcinoma, *IMA* invasive mucinous adenocarcinoma, *stroma* lymphocytes in the tumor stroma

[a]One case did not have assessable IDO1 staining

[b]pN data were not available for $N = 15$ cases

[c]No case with a score of 3 for GATA3+ TILs was identified in this cohort

Bold values are significant *P* values

($p < 0.001$) and most cases (18/25, 72%) had rare-to-few CD8+ lymphocytes in the tumor stroma ($p = 0.001$).

Correlation of PD-L1 and IDO1 expression with HLA class I molecules

In the entire cohort, 62 (24%), 234 (90%), and 250 (96%) had positive membranous expression of B2M, HCA2, and HC10, respectively, and all cases with positive B2M membranous expression exhibited HCA2 and HC10 staining. Given that B2M is required for surface expression of HLA-A/B/C, the B2M status was considered to represent the overall HLA class I molecule expression. The HLA class I molecule expression was seen in 32 (37%) of 87 cases with PD-L1 expression and 30 (18%) of 166 cases without; thus, PD-L1 expression was significantly associated with the overall HLA class I molecule expression ($p = 0.002$). Similarly, it was seen in 24 (33%) of 73 IDO1+ and 38 (21%) of 180 IDO1− cases, and IDO1 expression was marginally associated with overall HLA class I expression ($p = 0.054$). After stratifying the IDO1+ group by PD-L1 expression, there was no significant association between isolated IDO1 expression and overall HLA class I expression ($p = 0.300$).

Correlation of PD-L1 and IDO1 expression with molecular alterations

Molecular alterations were identified in 179 (69%) of the cohort and included 108 (41%) *KRAS* mutations, 54 (21%) *EGFR* mutations, 4 (2%) *ALK* rearrangements, and 13 (5%) alterations other than *KRAS*, *EGFR*, and *ALK*. PD-L1 expression was present in 46 (43%) of *KRAS* mutants and was significantly associated with *KRAS* mutations ($p = 0.021$). Conversely, PD-L1 was positive in only 5 (9%) of *EGFR* mutants and was inversely associated with *EGFR* mutations ($p < 0.001$). In contrast, IDO1 expression was not significantly associated with any molecular alterations. Of the four cases with *ALK* rearrangements, one expressed PD-L1 (weak-to-moderate expression in 100% of tumor cells) and another expressed IDO1 (weak-to-moderate expression in 40% of tumor cells). Among the SNaPshot wild-type group, 33/82 (40%) showed PD-L1 expression and 27/82 (33%) showed IDO1 expression. There were no differences in the molecular mutational profile between isolated PD-L1 expression and PD-L1/IDO1 co-expression. While there was no association of overall IDO1 expression with molecular alterations, isolated IDO1 expression was seen in 9/13 (69%) of *EGFR* mutants and was significantly associated with *EGFR* mutations ($p = 0.007$).

Logistic regression analysis for PD-L1 and IDO1 expression

Logistic regression analysis revealed that high-grade nuclei (grade 3 vs. grades 1–2, $p = 0.005$), abundant CD8+ tumor-infiltrating lymphocytes ($p < 0.001$), and abundant CD8+ lymphocytes in the tumor stroma ($p = 0.032$) were independent predictors of PD-L1 expression (Table 2). However, only abundant CD8+ tumor-infiltrating lymphocytes independently predicted isolated PD-L1 (PD-L1+/IDO1−)

Table 2 Logistic regression analysis for correlation of clinicopathologic characteristics and molecular alterations with PD-L1 expression

Predictors of PD-L1 expression	OR	95% CI	*P*
Male	0.54	0.20–1.34	0.196
Age	1.00	0.95–1.05	0.987
Smoking (≥10 PPY)	1.33	0.39–5.12	0.664
T stage (pT2–4 vs. pT1)	0.93	0.34–2.46	0.888
Nodal stage (pN1–2 vs. pN0)	2.19	0.67–7.19	0.192
Histologic pattern (solid vs. non-solid)	2.02	0.60–6.96	0.254
Nuclear grade (3 vs. 1–2)	3.80	1.50–9.81	**0.005**
Lymphatic invasion (present vs. absent)	1.93	0.65–5.69	0.230
Vascular invasion (present vs. absent)	1.56	0.50–4.87	0.437
CD8+ tumor-infiltrating lymphocytes (2–3 vs. 0–1)	29.53	9.74–108.02	**<0.001**
CD8+ stroma (>10% vs. ≤10%)	2.65	1.08–6.51	**0.032**
T-bet+ tumor-infiltrating lymphocytes (2–3 vs. 0–1)	0.70	0.13–3.99	0.676
KRAS mutations	1.99	0.75–5.57	0.177
EGFR mutations	0.29	0.06–1.21	0.107

PD-L1 programmed cell death ligand 1, *PPY* pack per year, *OR* odds ratio, *CI* confidence interval, *stroma* lymphocytes in the tumor stroma

Bold values are significant *P* values

Table 3 Logistic regression analysis for correlation of clinicopathologic characteristics and molecular alterations with IDO1 expression

	OR	95% CI	*P*
Male	1.65	0.87–3.13	0.126
Age	0.97	0.94–1.00	**0.033**
Smoking (≥30 PPY)	1.58	0.82–3.07	0.172
Histologic pattern (acinar vs. non-acinar)	2.42	1.31–4.53	**0.005**
Nuclear grade (3 vs. 1–2)	1.12	0.56–2.20	0.746
Lymphatic invasion (present vs. absent)	1.01	0.49–2.03	0.976
Vascular invasion (present vs. absent)	1.36	0.62–3.00	0.441
CD8+ tumor-infiltrating lymphocytes (2–3 vs. 0–1)	2.94	1.31–6.64	**0.009**
CD8+ stroma (>10% vs. ≤10%)	1.71	0.87–3.34	0.118
T-bet+ tumor-infiltrating lymphocytes (2–3 vs. 0–1)	1.26	0.48–3.27	0.638

IDO1 indoleamine 2,3-dioxygenase 1, *PPY* pack per year, *OR* odds ratio, *CI* confidence interval, *stroma* lymphocytes in the tumor stroma

Bold values are significant P values

expression (odds ratio (OR) = 4.20, 95% confidence interval (CI) 1.40–13.02, $p = 0.011$). For IDO1, logistic regression analysis revealed that younger age ($p = 0.033$), acinar-predominant histological pattern ($p = 0.005$), and abundant CD8+ tumor-infiltrating lymphocytes ($p = 0.009$) were independent predictors of expression (Table 3). However, the association with abundant CD8+ tumor-infiltrating lymphocytes disappeared with isolated IDO1 (IDO1+/PD-L1−) expression, while the association with acinar-predominant histologic pattern remained significant (OR = 5.31, 95% CI 1.84–16.91, $p = 0.003$). The analyses were adjusted for sex, age, and factors that were significantly associated with PD-L1 or IDO1 expression, respectively, using univariate analysis.

Survival analysis

Patients with lung adenocarcinomas expressing PD-L1 had significantly lower 5-year progression-free survival (65% vs. 75%, log rank $p = 0.005$) and overall survival (74% vs. 83%, log rank $p = 0.011$) compared to patients whose tumors did not express PD-L1 (Fig. 3). However, by multivariate analysis, PD-L1 was no longer significantly associated with progression-free and overall survival. In the multivariate model, higher T stage (pT2–4 vs. pT1) was associated with shorter progression-free and overall survival; histologic pattern (solid vs. non-solid) and lymphatic and vascular invasion were associated with shorter progression-free survival. There was no significant relationship between CD8+ tumor-infiltrating lymphocytes or CD8+ lymphocytes in the stroma with progression-free or overall survival (Supplementary Table 2).

On the contrary, patients with lung adenocarcinomas expressing IDO1 did not show a significant difference in 5-year progression-free survival (67% vs. 73%, log rank $p = 0.147$) or overall survival (77% vs. 81%, log rank $p = 0.199$) compared to those with negative IDO1 expression (Fig. 4). Similarly, multivariate analysis did not reveal a significant association between IDO1 and progression-free or overall survival. In this model, lymphatic and vascular invasion were associated with shorter progression-free survival, and significant smoking history was associated with shorter overall survival. As with PD-L1, there was no significant relationship between CD8+ tumor-infiltrating lymphocytes or CD8+ lymphocytes in the stroma with progression-free or overall survival (Supplementary Table 3).

Interestingly, stratification into the four expression groups (PD-L1−/IDO1−, PD-L1+/IDO1−, PD-L1−/IDO1+, and PD-L1+/IDO1+) revealed a significant difference in 5-year progression-free survival ($p = 0.004$) and overall survival ($p = 0.038$) among the four groups (Fig. 5). The 5-year progression-free survival rates were 77%, 55%, 62%, and 71% for PD-L1−/IDO1−, PD-L1+/IDO1−, PD-L1−/IDO1+, and PD-L1+/IDO1+ tumors, respectively. The 5-year overall survival rates were 84%, 68%, 77%, and 78% for PD-L1−/IDO1−, PD-L1+/IDO1−, PD-L1−/IDO1+,

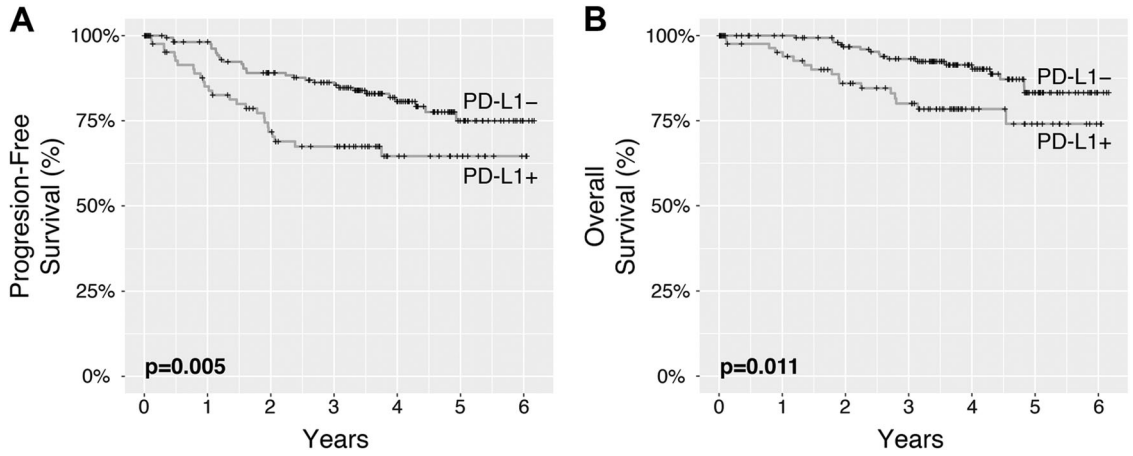

Fig. 3 Kaplan–Meier curves showing (**a**) progression-free survival and (**b**) overall survival of lung adenocarcinoma patients with positive and negative PD-L1 expression (cutoff of H-score = 5)

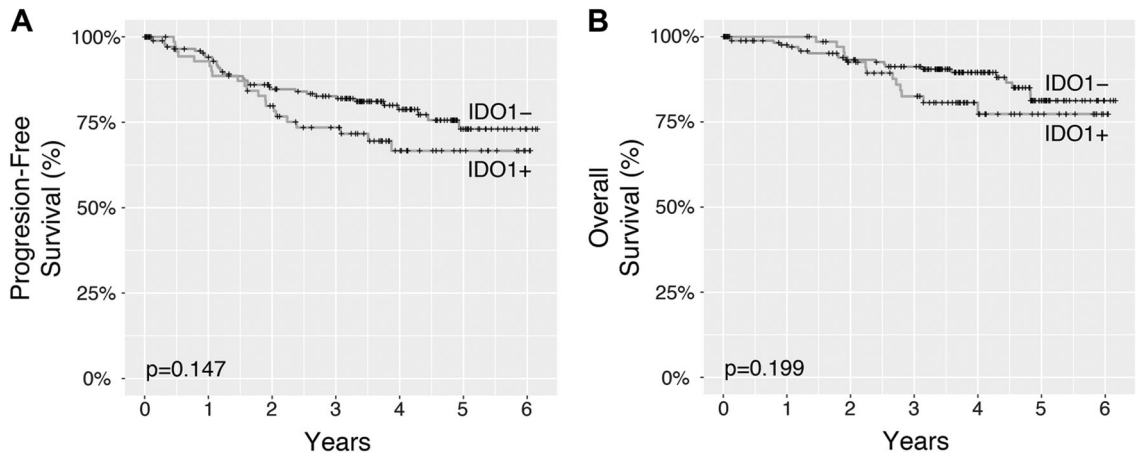

Fig. 4 Kaplan–Meier curves showing (**a**) progression-free survival and (**b**) overall survival of lung adenocarcinoma patients with positive and negative IDO1 expression (cutoff of H-score = 5)

and PD-L1+/IDO1+ tumors, respectively. Tumors that were negative for both PD-L1 and IDO1 had the best survival, while those with isolated PD-L1 expression had the worst survival; expression of IDO1 conferred an intermediate survival.

Discussion

In this study, we confirm that PD-L1 expression (with or without IDO1 co-expression) is observed in association with CD8+ and T-bet+ tumor-infiltrating lymphocytes, HLA class I expression, and *KRAS* mutations [9, 30]. We also corroborate the inverse association of PD-L1 with *EGFR* mutations, which is consistent with data suggesting that the *EGFR*-mutant status confers diminished response to PD-1 blockade [2, 31]. This, together with the association between PD-L1 and the cytotoxic T lymphocyte/Th1 microenvironment, supports the role of PD-L1 upregulation in adaptive immune resistance as opposed to innate immune resistance, the latter of which may be seen in *EGFR*-mutant and *ALK*-rearranged non-small cell lung cancer [32, 33]. In contrast to PD-L1, isolated IDO1 expression was significantly associated with the absence of CD8+ tumor-infiltrating lymphocytes along with a lower pT stage and lower nuclear grade. Furthermore, significantly more cases with *EGFR* mutations had IDO1 expression without co-expression of PD-L1.

To date, the biological role of additional potentially actionable immune inhibitory targets and their associations with PD-L1 are extremely limited in lung cancer. Clinical trials of IDO1 inhibitors as a single agent (NCT01219348 [19], NCT03164603, NCT03208959) and in combination with pembrolizumab/nivolumab (NCT02752074,

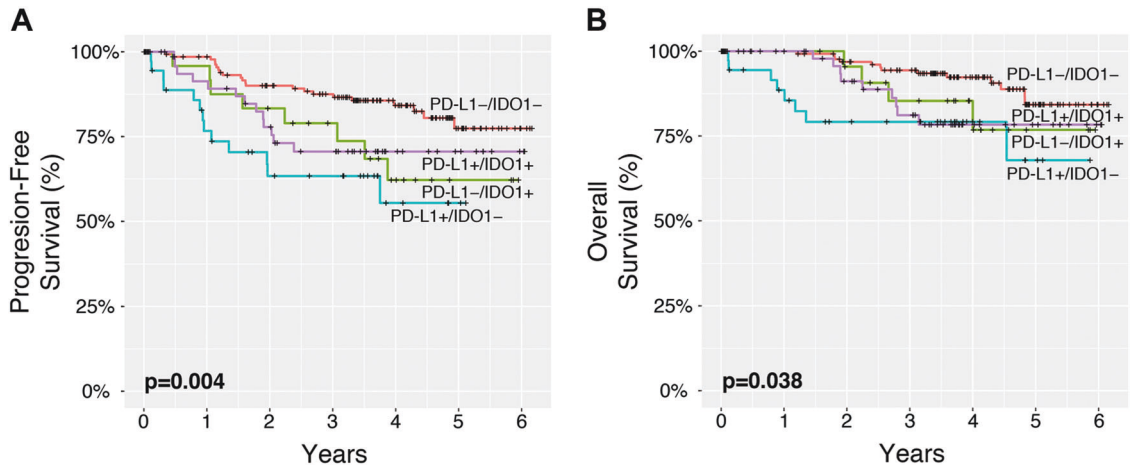

Fig. 5 Kaplan–Meier curves showing **a** progression-free survival and **b** overall survival of lung adenocarcinoma patients with PD-L1−/IDO1−, PD-L1+/IDO1−, PD-L1−/IDO1+, and PD-L1+/IDO1+ expression (cutoff of H-score = 5)

NCT03301636, NCT02658890, NCT02178722) for patients with non-small cell lung cancer are currently underway. However, the only evidence for the biopathological role of IDO1 in lung cancer are that IDO1 promotes tumorigenesis in mouse models of primary *KRAS*-induced lung adenocarcinoma [15] and that out of carcinomas, human non-small cell lung cancer is the third most frequent expresser of IDO1 after endometrial/cervical carcinomas and renal carcinomas [13]. In our cohort, 49/260 (19%) cases co-expressed PD-L1 and IDO1, while 40 (15%) and 25 (10%) cases expressed only PD-L1 and only IDO1, respectively. Interestingly, recent studies have reported that 7–29% of tumors co-express PD-L1 and IDO1 in 4 non-small cell lung cancer cohorts (Table 4) [21, 34, 35]. The discrepant results could be attributed to the differences in the study cohorts (adenocarcinoma only vs. all types of non-small cell lung cancer, ethnicity), the detection assays used (immunohistochemistry vs. immunofluorescence), and the cutoff values. It is reasonable to see some extent of PD-L1 and IDO1 co-expression in tumor cells, however, given that the IFNγ can induce expression of those immune inhibitory proteins [21, 36].

In the current study, overall IDO1 expression was significantly associated with a heavy smoking history (≥30 pack-years, but not ≥ 10), more aggressive pathological factors, and abundant CD8+/T-bet+ tumor-infiltrating lymphocytes. A recent study of a smaller non-small cell lung cancer cohort also showed that upregulation of IDO1 expression was significantly correlated with a higher pathologic stage as well as lymph node metastasis (Table 4) [37], which was not seen in the present study. There was no association between overall IDO1 expression and molecular alterations. Younger age and abundant CD8+ tumor-infiltrating lymphocytes were independent predictors of overall IDO1 expression by multivariate analysis. At first glance, these initial analyses suggest close similarities between the relationships of PD-L1 and IDO1 expression with clinicopathologic factors; however, further stratification into isolated IDO1 expression versus IDO1 with PD-L1 co-expression elucidated notable differences. Isolated IDO1 expression did not show an association with significant smoking history. Interestingly, some previous associations with overall IDO1 expression changed with isolated IDO1 expression: IDO1 expression alone was significantly associated with less aggressive pathologic factors (i.e., lower nuclear grade) and rare/few CD8+ tumor-infiltrating lymphocytes/stromal lymphocytes, and was no longer associated with T-bet+ tumor-infiltrating lymphocytes. Abundant CD8+ tumor-infiltrating lymphocytes were not seen in any cases with isolated IDO1 expression. These findings suggest that a mechanism(s) distinct from the cytotoxic T lymphocyte/Th1 microenvironment may facilitate the isolated IDO1 expression. For instance, activation of RAS and PAMP (pathogen-associated molecular pattern) along with IFNγ/JAK2/STAT (interferon-γ/Janus kinase 2/signal transducer and activator of transcription) pathways in tumor cells can induce IDO1 [36]. Of those, the latter is shared with PD-L1 expression. In the current cohort, the isolated IDO1 expression was associated with *EGFR* mutations that activate RAS signaling and often exhibit lower nuclear grade and limited inflammatory infiltrate [38–40]. The association raises the possibility of treatment with IDO1 inhibitors for *EGFR*-mutated non-small cell lung cancer, for which PD-1/PD-L1 blockade is generally not effective. These findings, however, need to be confirmed by additional studies with larger IDO1+ cohorts.

PD-L1 expression was significantly associated with expression of HLA class I molecules, while abundant CD8+ tumor-infiltrating lymphocytes were also associated with HLA class I expression (data not shown). This is consistent

Table 4 Literature review of IDO1 in human lung cancer

Reference	Tumor Type	Study cohort (*N*)	Antibody clone	IDO1+ cutoff	IDO1+ *N* (%)	Results/findings
Schalper et al. [21]	Non-small cell lung cancer	Resection specimens: Cohort #1 (202) Cohort #2 (350)	IDO1 (1:250, 1F8.2, Millipore)	Determined by a quantitative immunofluorescence assay[a]	78/183 (43%) of cohort #1167/335 (50%) of cohort #2	IDO1 and PD-L1 were co-expressed in 7 and 11% of cohort #1 and #2, respectively; PD-L1 and IDO1 expression were associated with increased CD3+ and CD8+ tumor-infiltrating lymphocytes and IFNγ stimulation
Tang et al. [37]	Non-small cell lung cancer	Resection specimens (64)	Rabbit anti-IDO1 mAb (1:50, Abnova) with BioGenex i6000 automated stainer	Absent (0), weak (1), moderate (2), strong (3)	Reported median expression levels for different comparison groups	IDO1 expression was increased in lung cancer compared to corresponding non-tumor tissues; upregulation of IDO1 was significantly associated with higher stage and lymph node metastasis
Volaric et al. [34]	Non-small cell lung cancer	Resection specimens (102): 51 adenocarcinomas; 42 squamous cell carcinomas; 9 adenosquamous carcinomas	IDO1 (1:2000, HPA 023072, Sigma Prestige)	Cytoplasmic staining in ≥1% of tumor cells	21/51 (42%) adenocarcinomas18/42 (43%) squamous cell carcinomas4/9 (44%) adenosquamous carcinomas	IDO1 was frequently co-expressed with PD-L1 (27% of adenocarcinomas); IDO1 expression was not significantly associated with clinicopathologic factors or survival
Kozuma et al. [35]	Lung adenocarcinoma	Resection specimens (427)	Mouse anti-IDO1 mAb (1:200, UMAB126, Origene Technologies)	Cytoplasmic and membranous staining in ≥1% of tumor cells	260/427 (61%) using 1% cutoff63/427 (15%) using 50% cutoff	IDO1 and PD-L1 were co-expressed in 29% of tumors; IDO1 expression was significantly associated with aggressive clinicopathologic features (e.g., higher stage, higher grade, lymphovascular invasion, perineural invasion); IDO1 expression was significantly associated with shorter disease-free and overall survival

PD-L1 programmed cell death ligand 1, *IDO1* indoleamine 2,3-dioxygenase 1, *mAb* monoclonal antibody

[a]The cutoff was established from scores in negative control preparations and visual detection threshold by a quantitative immunofluorescence assay

with the mechanism of adaptive immune resistance, whereby antigen presentation via HLA class I molecules on the surface of tumor cells recruits CD8+ cytotoxic T lymphocytes that subsequently produce IFNγ and drive upregulation of PD-L1 in tumor cells, leading to the deactivation of CD8+ tumor-infiltrating lymphocytes via PD-L1/PD-1 interactions. Thus, it follows that downregulation of HLA class I expression likely confers a worse prognosis in cancers, including non-small cell lung cancer [41, 42]. In contrast, neither overall IDO1 nor isolated IDO1 expression were associated with HLA class I molecule expression in this study, supporting a different mechanism of immune suppression within the tumor microenvironment, possibly involving other types of T lymphocytes such as regulatory T cells, B cells and/or myeloid-derived suppressor cells [16, 21, 36].

Regarding patient outcomes, PD-L1 expression was associated with decreased progression-free and overall survival by univariate but not multivariate analysis, while multiple studies have reported conflicting results regarding the prognostic utility of PD-L1 expression, including correlations with poorer [10, 43, 44], improved [11, 45, 46], and no difference [12, 21] in overall survival. In this study, there was no difference in progression-free or overall survival between tumors expressing IDO1 and those without IDO1 expression, confirming the results of the non-small cell lung cancer study by Volaric et al. [34]. In contrast, a few other studies involving lung adenocarcinoma and other cancer types have reported a correlation between high IDO1 expression in tumor cells and decreased survival [35, 47, 48]. Thus, additional large-scale studies are warranted to determine the prognostic role of IDO1 in non-small cell lung cancer. Interestingly, however, after separating expression of PD-L1 and IDO1 in these cases, PD-L1 +/IDO1− tumors showed the shortest progression-free and overall survival, whereas PD-L1/IDO1 double-negative tumors showed the longest progression-free and overall survival and tumors expressing IDO1 had an intermediate survival outcome. These findings raise the possibility that while IDO1 expression does contribute to immune resistance and tumor progression, it acts via mechanisms separate and not necessarily synergistic with that of PD-L1, thus conferring a slight survival advantage over tumors expressing PD-L1 but not IDO1.

Our study has several important limitations. First, there is currently no gold standard in PD-L1 or IDO1 testing, and there exist a number of different antibodies and scoring systems [49, 50]. To account for this, we used a commercially available anti-PD-L1 antibody that has been independently validated [10, 12, 51] and an IDO1 antibody that was validated in a previous study using non-small cell lung cancer tissue microarrays [21]. Second, we chose to use H-scoring and a cutoff of H-score ≥5 as the definition of positive expression for both PD-L1 and IDO1 immunohistochemistry to account for both the extent and intensity of expression. However, there is heterogeneity of the scoring systems and cutoffs for positive expression used across different studies, making it difficult for direct comparison. Previously on this cohort, Huynh et al. [9]. analyzed PD-L1 expression using H-score (cutoff ≥5), immune score (3 vs. 0–2), and extent of expression of any intensity (cutoffs of 1%, 5%, and 50%), and achieved similar results across the different scoring systems. Using an H-score cutoff of ≥5 performed most similarly to an expression cutoff of ≥5%. Another issue is the use of tissue microarrays, which only represent small portions of the tumor and thus makes it difficult to evaluate for heterogeneous expression within a tumor, as recently reported [52–55]. To overcome the issue of heterogeneity, we constructed tissue microarrays of two larger-caliber cores from each of the cohort cases, where one core was taken from the tumor center and the other from the invasive front. The final H-score was determined based on their collective assessment. Of note, the two cores had discrepant scores in 7% (PD-L1) and 5% (IDO1) of our study cases (data not shown), and the discrepancy rate of PD-L1 was smaller than those observed between biopsies or tissue microarray cores and whole tumor sections [52, 53]. Furthermore, corresponding whole sections from a subset of the study cohort (28 cases, 11%) with a range of IDO1 expression on tissue microarray (including the vast majority of those with discrepant cores) were evaluated for IDO1 expression and showed strong, significant correlation in H-score with the tissue microarray cores (Pearson's $r = 0.7$, $p < 0.001$). Using an H-score cutoff of ≥5, only one case (4%) showed discrepant IDO1 expression (H-score = 4 on tissue microarray and 8 on the corresponding whole section). Finally, our study cohort consisted of mostly white, female patients with early-stage tumors (reflective of the resected primary lung adenocarcinoma population at our institution) and limited molecular data regarding *ALK* rearrangement-positive cases (only $n = 4$) and *ROS1* rearrangement-positive cases ($n = 65$ evaluated by *ROS1* fluorescence in situ hybridization, but none positive).

Further large-scale studies are needed to elucidate the roles and interactions of PD-L1 with IDO1 and other immunosuppressive markers. Additional questions include the expression of IDO1 after anti-PD-1/PD-L1 blockade [56] and whether it may play a role in immunotherapy resistance, the relationship between IDO1 and HLA class II markers, and the role of PD-L1 and/or IDO1 expression in rearrangement-positive non-small cell lung cancer.

Overall, our results confirm that PD-L1 expression with or without IDO1 co-expression is associated with the cytotoxic T lymphocyte/Th1 immune microenvironment. IDO1 appears to serve an immunosuppressive role distinct from and possibly not synergistic to that of PD-L1,

supporting the notion that lung adenocarcinomas use a combination of mechanisms to evade tumor immunity. As there remains a need for biomarkers to identify tumors that may be resistant to PD-1/PD-L1 blockade, further investigation of IDO1 as a possible biomarker may be warranted.

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Compliance with ethical standards

Conflict of interest AJI has equity interest and royalties from exclusive license of AMP technology to ArcherDx and served as a scientific advisory board member for ArcherDx. JFG served as a compensated consultant or received honoraria from Bristol-Myers Squibb, Merck, Genentech/Roche, Ariad, Takeda, Pfizer, Amgen, Agios, Array Biopharma, Novartis, Incyte, and Loxo. MM-K served as a compensated consultant for Merrimack Pharmaceuticals and H3 Biomedicine. The other authors declare that they have no conflict of interest.

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