Lung Cancer 129 (2019) 41–47

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

PD-L1 protein expression in non-small-cell lung cancer and its relationship with the hypoxia-related signaling pathways: A study based on immunohistochemistry and RNA sequencing data

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ARTICLE INFO

Keywords:
Non-small-cell lung cancer
Programmed death-ligand 1
Hypoxic-inducible factor 1α
Glucose transporter 1
Vascular endothelial growth factor A
Carbonic anhydrase 9

ABSTRACT

Objectives: Therapies that target programmed cell death protein-1 (PD-1)/programmed death-ligand 1 (PD-L1) have shown promising efficacy in non-small-cell lung cancer (NSCLC). Hypoxia-related genes are also important regulators of PD-L1, and the role of PD-L1 in NSCLC is still not clear. The objective of this study was to investigate PD-L1 expression and its correlation with hypoxic-inducible factor 1α (HIF1A), vascular endothelial growth factor A (VEGFA), glucose transporter 1 (GLUT1), and carbonic anhydrase 9 (CAIX) expression in NSCLC patients. The association between PD-L1 expression and survival was also determined.

Materials and methods: PD-L1/protein expression was evaluated in 295 resected NSCLCs and its correlation with HIF1A, VEGFA, GLUT1, CAIX expression and survival was determined based on immunohistochemical and RNA sequencing data obtained from The Cancer Genome Atlas (TCGA) database.

Results: PD-L1 protein expression was significantly correlated with HIF1A, VEGFA, GLUT1, and CAIX expression only in adenocarcinoma when a 10% or a 50% cut-off was used. PD-L1 mRNA expression was also significantly correlated with HIF1A, VEGFA, GLUT1, and CAIX expression in adenocarcinoma. Univariate analysis revealed that HIF1A expression was associated with poor recurrence-free survival (RFS), and GLUT1 was associated with poor overall survival (OS) and RFS. GLUT1 was an independent prognostic factor for OS in multivariate analysis of immunohistochemical and TCGA data ($p = 0.024$ and 0.029, respectively). Patients with low expression of both PD-L1 and GLUT1 had longer OS than other patterns in immunohistochemical and TCGA data ($p = 0.003$ and 0.051, respectively).

Conclusions: PD-L1 protein and mRNA expression were correlated with HIF1A, VEGFA, GLUT1, and CAIX expression in adenocarcinoma alone. Low expression of GLUT1 and low expression of both PD-L1 and GLUT1 were associated with improved prognosis. Our findings support the rationale for co-targeting hypoxia-related genes and PD-L1 in cancer therapy. Expression of hypoxia-related genes may be helpful in selecting patients appropriate for PD-L1 therapy.

1. Introduction

Blockers of programmed cell death protein-1 (PD-1)/programmed death-ligand 1 (PD-L1) are being actively studied in non-small-cell lung cancer (NSCLC). By expressing PD-L1 ligand on the surface of tumor cells and binding to the PD-1 receptor on T, B, and NK cells, tumors can bypass immunosurveillance [1]. In the field of lung cancer, anti-PD-1/PD-L1 antibodies have produced clinical response and survival improvement in advanced NSCLC in clinical trials, and two anti-PD-1 antibodies [2–4], nivolumab and pembolizumab, have been approved by the FDA for the treatment of advanced NSCLC. However, with a response rate of 15–20% in unselected patients with NSCLC, and 15–45% in patients with PD-L1-expressing NSCLC, these drugs can offer improved outcomes in only some patients but not all [5]. Other strategies, such as finding a better predictive marker for the response to PD-1 inhibitors or simultaneous inhibition, are needed.

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https://doi.org/10.1016/j.lungcan.2019.01.004
Received 1 October 2018; Received in revised form 10 January 2019; Accepted 15 January 2019

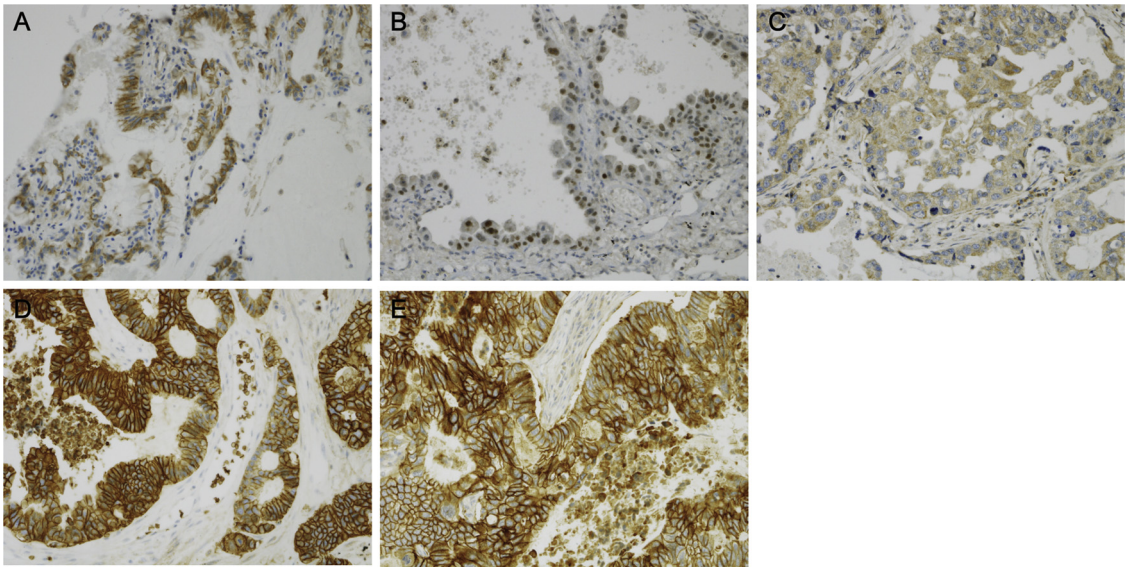

Fig. 1. Programmed death-ligand 1 (PD-L1), hypoxic-inducible factor 1α (HIF1A), vascular endothelial growth factor A (VEGFA), glucose transporter 1 (GLUT1), and carbonic anhydrase 9 (CAIX) expression in lung adenocarcinoma. (A) Positive PD-L1 expression on tumor cells. (B) Positive HIF1A expression on tumor cells. (C) Positive VEGFA expression on tumor cells. (D) Positive GLUT1 expression on tumor cells. (E) Positive CAIX expression on tumor cells.

Tumor cells can adapt to a hypoxic microenvironment by expressing hypoxic-inducible factor 1α (HIF1A) protein [6]. HIF1A upregulates several genes to promote survival under conditions of low oxygen. These include glucose transporter 1 (GLUT1) [7] (which allows adenosine triphosphate (ATP) synthesis in an oxygen-independent manner), vascular endothelial growth factor A (VEGFA) [8] (which promotes angiogenesis), and carbonic anhydrase 9 (CAIX) [9] (which contributes to acidification of the surrounding microenvironment). HIF1A is also associated with drug resistance and immune escape, thus limiting the therapeutic effect of chemotherapy and immunotherapy [10]. HIF1A is another important mediator of the tumor immune response. It may have a synergistic effect with PD-1/PD-L1. HIF1A promotes the expression of PD-L1 via binding to a specific hypoxia response element in the promoter of PD-L1 in mouse models of cancer [11]. A recent study revealed that a biomimetic core–shell nanoplatform with oxygen generation was able to downregulate the expression of HIF1A and further enhance the therapeutic effects of chemotherapy while reducing the expression of PD-L1 in murine melanoma models [12]. A significant correlation between PD-L1 and HIF1A has been identified in hepatocellular carcinoma [13] and oral squamous-cell carcinoma [14]. Several inhibitors of the HIF1A protein have been discovered and tested in clinical trials [6].

Previous studies have shown that PD-L1 is over-expressed in NSCLC and that it is associated with prognosis [15,16]. HIF1A is also over-expressed in NSCLC, and patients with high HIF1A expression have poor clinical outcomes [17]. Previous studies have also reported that GLUT1 [18], VEGFA [19], and CAIX [20] are frequently expressed in NSCLC and are associated with poor patient prognosis.

If PD-L1 is associated with hypoxia-related genes, co-targeting therapy may be available and may be helpful in selecting patients appropriate for PD-L1 therapy. However, HIF1A protein expression in NSCLC, and the relationship between HIF1A and PD-1/PD-L1, have not yet been reported. Therefore, the objective of this study was to investigate expression of hypoxia-related genes—including, HIF1A, VEGFA, GLUT1, and CAIX—in NSCLC patients by immunohistochemical and RNA sequencing data from The Cancer Genome Atlas (TCGA) database. We also analyzed the correlation between PD-L1 expression and expression of hypoxia-related genes, and conducted survival analysis of patients with NSCLC.

2. Materials and methods

2.1. Patients

This retrospective study was approved by the Institutional Review Board of Ajou University School of Medicine, Republic of Korea (AJIRB-BMR-KSP-18-263). Informed consent was waived due to the retrospective nature of this study. All analyses were performed in accord with ethical guidelines for clinical research at the respective institutions. A total of 295 patients with confirmed non-small-cell carcinoma after surgical resection between 2000 and December 2015 were enrolled. Bronchoscopy, pulmonary function testing, chest computed tomography (CT), and positron emission tomography/computed tomography (PET/CT) were performed preoperatively to determine the clinical stage. Patients who underwent induction chemotherapy when N2 was proven before surgery were excluded from the study.

2.2. Histopathological analysis and immunohistochemistry

Histological subclassification was performed by two pathologists (YWK and JHH), according to the 2015 World Health Organization Classification of Lung Tumors [21]. TNM stage was based on the 8th edition of the AJCC Cancer Staging Manual. We used tissue microarrays for immunohistochemical analysis. Representative paraffin blocks of tumor sections were prepared for each case, and two tumor cores 2 mm in diameter were obtained using a trephine apparatus. Formalin-fixed and paraffin-embedded tissue samples were arranged in a Benchmark XT automatic immunohistochemical staining device (Ventana Medical Systems, Tucson, AZ, USA). Samples were incubated with antibody to PD-L1 (rabbit monoclonal, clone 28-8, Abcam), HIF1A (mouse monoclonal, clone H1alpha 67, Santa Cruz Biotechnology), VEGFA (mouse monoclonal, clone G153-694, BD Pharmingen), GLUT1 (rabbit polyclonal, Cell Marque), and CAIX (rabbit monoclonal, clone EPR4151(2), Abcam).

PD-L1 intensity was evaluated on a four-point intensity scale (0, none; 1, faint; 2, moderate; 3, strong) (Fig. 1A). The proportion of membrane expression of PD-L1 was evaluated. We referred to the previous studies of PD-L1 22-8, 22C3 or sp263 to define cut-offs. Previous clinical trials of 22-8, 22C3 or sp263 set the cut-off to 1%, 5%, 10% or 50% [22]. In our results, samples with PD-L1 expression < 5% were very rare. Therefore, a sample was considered PD-L1-positive if

≥10% or ≥50% of definitive tumor cells were found. Intensities of HIF1A (Fig. 1B), VEGFA (Fig. 1C), GLUT1 (Fig. 1D), and CAIX (Fig. 1E) were also evaluated on four-point intensity scales (0–3). Percentages (0–100%) of nuclear expression of HIF1A and cytoplasmic expression of VEGFA, GLUT1, and CAIX were also evaluated. Overall scores (0–300) were obtained by multiplying the intensity and percentage of positive cells.

2.3. Exploring cancer genomics data from cBioportal and kmplot.cOm

We obtained RNA-seq reads per kilobase million of transcript (RPKM) data and corresponding clinical records of 230 lung adenocarcinoma patients and 178 lung squamous-cell carcinoma patients of TCGA from cBioPortal for Cancer Genomics (http://cbioportal.org) [23,24]. The online tool, kmplot.com, has been used to assess the effect of 54,675 genes on survival using 10,461 cancer samples, including lung cancer [25]. Therefore, we used kmplot.com to analyze the survival rate according specific genes.

2.4. Statistical analyses

Overall survival (OS) and recurrence-free survival (RFS) were analyzed by Kaplan–Meier curves and compared by the log-rank test. Multivariate analysis of OS or RFS was performed using the Cox proportional hazards regression model. Important predictors with a p-value < 0.05 in univariate analysis were included in the final multivariate analysis. The enter method was employed to determine the final Cox model for multivariate analysis. Categorical variables were compared using the chi-squared test, while continuous variables were compared using the Mann–Whitney *U* test. Spearman correlation analysis was used to describe the correlation between quantitative variables. All statistical analyses were performed using SPSS statistical software version 18.0 (SPSS; Chicago, IL, USA), and a p-value < 0.05 was considered statistically significant.

3. Results

3.1. Patient demographics

Demographic data of patients included in this study are summarized in Table 1. Patient ages ranged from 31 to 86 years (median 64 years). There were 124 stage I patients (43.5%), 78 stage II patients (27.4%), and 83 stage III patients (29.1%). There were 10 pNx (3.4%), 168 pN0 (56.9%), 56 pN1 (19%), 59 pN2 (20%) and 2 pN3 patients (0.7%). Of the 59 pN2 stage patients, 37 patients were single-station pN2 and 22 were multiple-station pN2. The median OS was 73.1 months and the estimated 5-year OS was 57.2%. The median follow-up time was 37.7 months (range 0.5–123 months). R0 is 291 patients and R1 is four patients. Tumor recurrence or progression developed in 120 patients.

3.2. PD-L1 and hypoxia-related protein expression

When the cut-off was set at 10%, the PD-L1-positive rate was 43.1% (127/295). The PD-L1-positive rate was 40% (78/195) in adenocarcinoma and 49% (49/100) in squamous-cell carcinoma (Table 2). In adenocarcinoma, PD-L1 positivity correlated with high HIF1A (mean ± standard deviation: 30.02 ± 50.83 versus 13.76 ± 26.93, p = 0.046), VEGFA (72.02 ± 73.57 versus 43.21 ± 52.9, p = 0.017), GLUT1 (144.19 ± 124.52 versus 73.64 ± 107.28, p < 0.001), and CAIX expression (74.19 ± 94.7 versus 43.88 ± 80.09, p = 0.019). However, in squamous-cell carcinoma there was no correlation between PD-L1 positivity and hypoxia-related signaling pathway protein expression.

When the cut-off was set at 50%, the PD-L1-positive rate was 25.1% (74/295) (Table 2). The PD-L1-positive rate was 23.1% (45/195) in adenocarcinoma and 29% (29/100) in squamous-cell carcinoma. In adenocarcinoma, PD-L1 positivity was correlated with high HIF1A (38.13 ± 59.03 versus 14.93 ± 28.81, p = 0.014), GLUT1 (162.22 ± 124.77 versus 85.08 ± 112.46, p = 0.001), and CAIX expression (94.28 ± 102.16 versus 44.91 ± 80.04, p = 0.002). However, in squamous-cell carcinoma there was no correlation between PD-L1 positivity and hypoxia-related signaling pathway protein expression (Table 2).

Table 1
Demographic and clinical characteristics of patients.

Variable	Number (%)
Age, median (range) (years)	64 (31–86)
Male sex	206 (69.8%)
Smoking history[a]	183 (67%)
Operation	
Pneumonectomy	21 (7.1%)
Lobectomy	242 (82%)
Sublobar resection	32 (10.8%)
Histological subtype	
Adenocarcinoma	195 (66.1%)
Squamous-cell carcinoma	100 (33.9%)
pT stage	
T1/T2	52 (17.6%)/187 (63.4%)
T3/T4	35 (11.9%)/21 (7.1%)
pN stage	
Nx/N0/N1	10 (3.4%)/168 (56.9%)/56 (19%)
N2/N3	59 (20%)/2 (0.7%)
pTNM 8th edition[b]	
Stage I	124 (43.5%)
Stage II	78 (27.4%)
Stage III	83 (29.1%)
Recurrence or progression after surgery	120 (40.6%)
Adjuvant chemotherapy	90 (30.5%)
Adjuvant radiotherapy	46 (15.5%)

[a] Smoking history was collected for 273 patients.
[b] pTNM was collected for 285 patients.

In PD-L1 expression, overall scores (0–300) were obtained by multiplying the intensity and percentage of positive cells. Next, we performed correlation analyses between PD-L1, HIF1A, VEGFA, GLUT1, and CAIX expression using continuous variables. In adenocarcinoma, PD-L1 showed a positive correlation with HIF1A scores (rho = 0.172, p = 0.016), VEGFA scores (rho = 0.166, p = 0.047), GLUT1 scores (rho = 0.310, p < 0.001), and CAIX scores (rho = 0.239, p = 0.004). In squamous-cell carcinoma PD-L1 showed no correlation with HIF1A scores (Spearman's rho = –0.04, p = 0.695), VEGFA scores (rho = 0.11, p = 0.324), GLUT1 scores (rho = 0.125, p = 0.236), or CAIX scores (rho = –0.155, p = 0.141).

3.3. mRNA expression profile of PD-L1 and hypoxia-related genes

We obtained RPKM data for PD-L1, HIF1A, VEGFA, GLUT1, and CAIX from the TCGA data sets. We performed a correlation analysis to determine relationships among PD-L1, HIF1A, VEGFA, GLUT1, and CAIX mRNA expression in 230 lung adenocarcinoma samples. PD-L1 showed a positive correlation with HIF1A scores (rho = 0.451, p < 0.001, Fig. 2A), VEGFA scores (rho = 0.134, p = 0.042, Fig. 2B), GLUT1 scores (rho = 0.249, p < 0.001, Fig. 2C), and CAIX scores (rho = 0.150, p = 0.023, Fig. 2D).

We also performed a correlation study to determine relationships between PD-L1, HIF1A, VEGFA, GLUT1, and CAIX mRNA expression in 178 lung squamous-cell carcinoma samples. PD-L1 showed a significant positive correlation with HIF1A scores (rho = 0.239, p = 0.001). PD-L1 showed a significant negative correlation with VEGFA scores (rho = –0.155, p = 0.039) and CAIX scores (rho = –0.236, p = 0.002). However, there was a negative correlation between PD-L1 and GLUT1 scores (rho = –0.016, p = 0.832).

Table 2
Correlations among programmed death-ligand 1 (PD-L1), hypoxic-inducible factor 1α (HIF1A), vascular endothelial growth factor (VEGF), glucose transporter 1 (GLUT1), and carbonic anhydrase 9 (CAIX) expression.

Characteristic (10% cutoff)	Adenocarcinoma		p	Squamous-cell carcinoma		p
	PD-L1-negative (*n* = 117)	PD-L1-positive (*n* = 78)		PD-L1-negative (*n* = 51)	PD-L1-positive (*n* = 49)	
HIF1A (mean ± SD)	13.76 ± 26.93	30.02 ± 50.83	0.046[a]	42.35 ± 60.87	46.93 ± 64.07	0.851[a]
VEGF (mean ± SD)	43.21 ± 52.9	72.02 ± 73.57	0.017[a]	45.95 ± 62.89	57.31 ± 68.52	0.469[a]
GLUT1 (mean ± SD)	73.64 ± 107.28	144.19 ± 124.52	< 0.001[a]	281.08 ± 50.25	283.04 ± 61.25	0.502[a]
CAIX (mean ± SD)	43.88 ± 80.09	74.19 ± 94.7	0.019[a]	65.06 ± 86.66	41.08 ± 80.08	0.054[a]
Characteristic (50% cutoff)	Adenocarcinoma		*p*	Squamous-cell carcinoma		*P*
	PD-L1-negative (*n* = 150)	PD-L1-positive (*n* = 45)		PD-L1-negative (*n* = 71)	PD-L1-positive (*n* = 29)	
HIF1A (mean ± SD)	14.93 ± 28.81	38.13 ± 59.03	0.014[a]	47.32 ± 62.42	37.93 ± 62.18	0.364[a]
VEGF (mean ± SD)	53.73 ± 62.6	63.88 ± 70.31	0.472[a]	46.21 ± 60.63	64.01 ± 75.71	0.383[a]
GLUT1 (mean ± SD)	85.08 ± 112.46	162.22 ± 124.77	0.001[a]	275.15 ± 65.34	297.85 ± 11.33	0.415[a]
CAIX (mean ± SD)	44.91 ± 80.04	94.28 ± 102.16	0.002[a]	50.02 ± 78.8	60.12 ± 95.56	0.234[a]

SD, standard deviation.
[a] Mann–Whitney *U* test.

3.4. Prognostic significance of PD-L1 and hypoxia-related genes in immunohistochemistry data

Because there was no correlation between PDL1 and hypoxia-related genes in squamous-cell carcinoma, we performed survival analyses in adenocarcinoma only. In the survival analysis, the cut-off of PDL1 was 10%. Mean values of HIF1A (20.8), VEGFA (59.7), GLUT1 (103.1), and CAIX (60.8) protein expressions were used as cut-offs.

PD-L1 expression was not associated with OS or RFS rate (p = 0.848; Fig. 3A and p = 0.312; Fig. 3B, respectively). HIF1A expression was not associated with OS rate (p = 0.129; Fig. 3C). HIF1A-positive patients with adenocarcinoma had lower 5-year RFS rates than HIF1A-negative patients (42% versus 53%, p = 0.044; Fig. 3D). VEGFA expression was not associated with OS or RFS rate (p = 0.784 and p = 0.39, respectively). GLUT1-positive patients with adenocarcinoma had lower 5-year OS and RFS rates than GLUT1-negative patients (51% versus 82%, p < 0.001, Fig. 3E and 42% versus 71%, p = 0.001, Fig. 3F, respectively). CAIX expression was not associated with OS or RFS rate (p = 0.116 and p = 0.597, respectively). Univariate analysis revealed that OS was associated with sex, stage, smoking history, lymphovascular invasion, and GLUT1, while RFS was associated with stage, smoking history, solid histology, lymphovascular invasion, GLUT1, and HIF1A. In multivariate analysis, GLUT1 expression was an independent prognostic marker for OS (hazard ratio = 2.214, p = 0.024; Table 3), but not for RFS (hazard ratio = 1.743, p = 0.113; Table 3) in adenocarcinoma. However, HIF1A expression was not an independent prognostic marker for RFS (Table 3).

We also found that patients with low expression of both PD-L1 and GLUT1 had significantly longer OS than patients with high expression of either PD-L1 or GLUT1, or both PD-L1 and GLUT1 (p = 0.003, Fig. 3G). However, such patients did not have significantly better RFS (p = 0.071, Fig. 3H).

Because GLUT1 and HIF1A were associated with prognosis, we combined GLUT1 and HIF1A expression for survival analysis in adenocarcinoma. Patients with low levels of expression of both GLUT1 and HIF1A had significantly longer OS than patients with high levels of

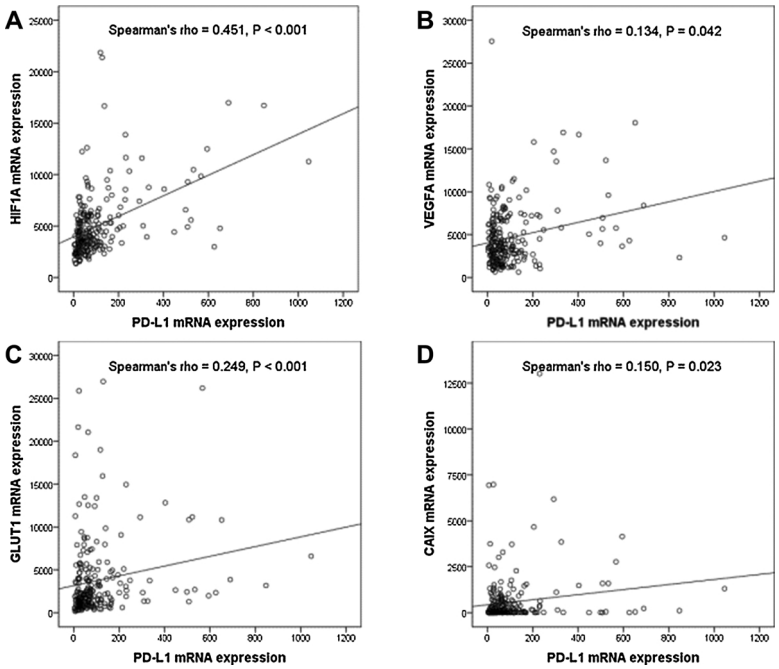

Fig. 2. Spearman correlation analysis in RNA-sequencing data (A) Programmed death-ligand 1 (PD-L1) and hypoxic-inducible factor 1α (HIF1A). (B) PD-L1 and vascular endothelial growth factor A (VEGFA). (C) PD-L1 and glucose transporter 1 (GLUT1). (D) PD-L1 and carbonic anhydrase 9 (CAIX).

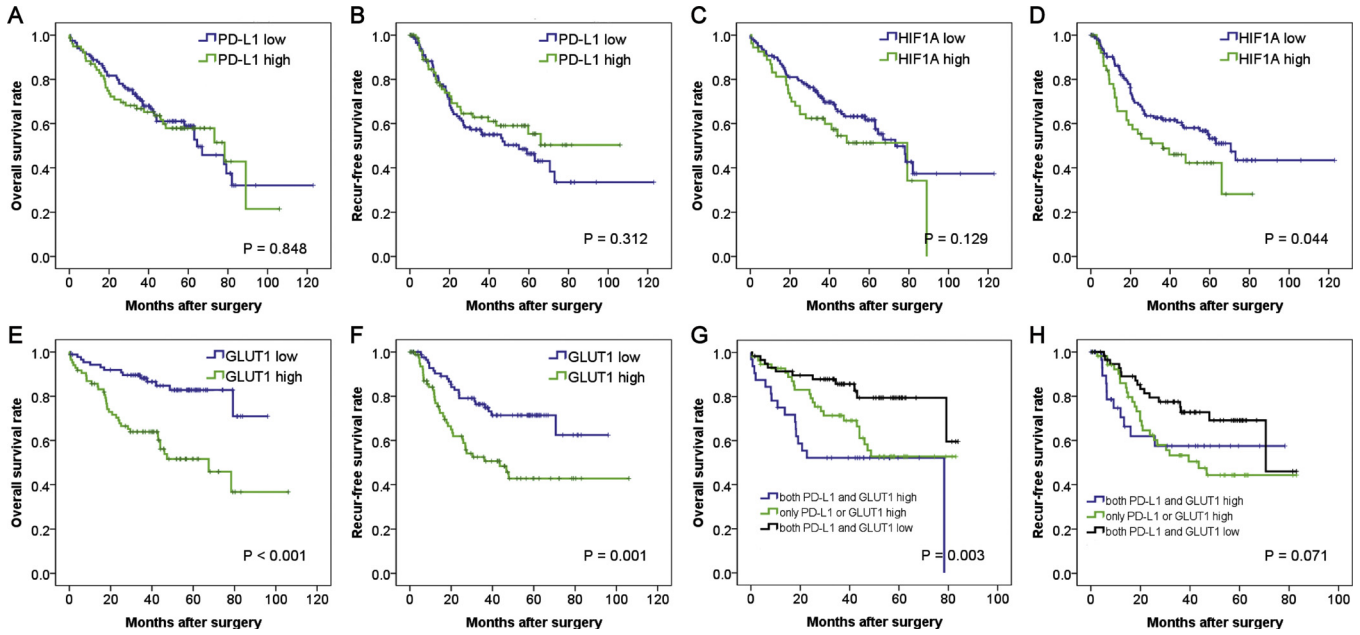

Fig. 3. Comparison of survival rates according to programmed death-ligand 1 (PD-L1), hypoxic-inducible factor 1α (HIF1A), and glucose transporter 1 (GLUT1) protein expression in patients with adenocarcinoma. (A) Overall survival (OS) and PD-L1. (B) Recurrence-free survival (RFS) and PD-L1. (C) OS and HIF1A. (D) RFS and HIF1A. (E) OS and GLUT1. (F) RFS and GLUT1. (E) OS, PD-L1 and GLUT1. (F) RFS, PD-L1 and GLUT1.

expression of either GLUT1 and HIF1A, or both GLUT1 and HIF1A (p = 0.002, Supplementary Fig. S1A). Patients with low expression of both GLUT1 and HIF1A had significantly better RFS than patients with other expression patterns (p = 0.019, Supplementary Fig. S1B). In multivariate analysis, low expression of both GLUT1 and HIF1A was not an independent prognostic marker for OS (hazard ratio = 0.649, p = 0.229; Supplementary Table S1). Both GLUT1 and HIF1A low expression had a borderline prognostic significance for RFS in adenocarcinoma (hazard ratio = 0.532, p = 0.075; Supplementary Table 1).

3.5. Prognostic significance of PD-L1 and hypoxia-related genes in RNA sequencing data

In survival analysis, mean mRNA expression values of PD-L1 (105.5), HIF1A (4999), VEGFA (4651.6), GLUT1 (3752.8), and CAIX (573.2) were used as cut-offs. We performed survival analyses for adenocarcinoma only.

PD-L1 mRNA expression was not associated with OS rate (p = 0.347; Supplementary Fig. S2A). Patients with high expression of

Table 3
Multivariate analyses of recurrence-free survival and overall survival.

Univariate analysis						
Covariate	Overall survival			Recurrence-free survival		
	HR	95%CI	p-value[a]	HR	95%CI	p-value[a]
Age (≥70 y versus < 70 y)	1.323	0.781–2.240	0.297	1.126	0.653–1.942	0.67
Sex (male versus female)	2.136	1.308–3.489	0.002	1.330	0.858–2.063	0.202
Stage (III versus I–II)	2.601	1.660–4.076	< 0.001	2.624	1.701–4.406	< 0.001
Smoking history (+ versus –)	1.692	1.051–2.724	0.03	1.715	1.090–2.699	0.02
Micropapillary histology (+ versus –)	1.771	0.862–3.636	0.12	1.585	0.780–3.222	0.203
Solid histology (+ versus –)	1.767	0.969–3.221	0.063	2.090	1.204–3.626	0.009
LVI (+ versus –)	1.89	1.213–2.944	0.005	2.615	1.724–3.967	< 0.001
GLUT1 (+ versus –)	3.064	1.726–5.441	< 0.001	2.404	1.392–4.513	0.002
HIF1A (+ versus –)	1.621	0.881–2.982	0.121	1.589	1.007–2.509	0.047
Multivariate analysis						
covariate	Overall survival			Recurrence-free survival		
	HR	95%CI	p-value[a]	HR	95%CI	p-value[a]
Sex (male versus female)	1.675	0.534–5.253	0.376	–	–	–
Smoking history (+ versus –)	1.215	0.436–3.389	0.709	1.155	0.598–2.230	0.668
Stage (III versus I–II)	2.077	1.103–3.911	0.022	1.687	0.877–3.244	0.117
Solid histology (+ versus –)	–	–	–	1.179	0.551–2.521	0.671
LVI (+ versus –)	0.981	0.523–1.839	0.952	1.364	0.722–2.577	0.339
GLUT1 (+ versus –)	2.214	1.108–4.424	0.024	1.743	0.876–3.470	0.113
HIF1A (+ versus –)	–	–	–	1.078	0.549–2.119	0.826

CI, confidence interval; GLUT1, glucose transporter 1; HIF1A, hypoxia-inducible factor 1α; HR, hazard ratio; LVI, lymphovascular invasion.
[a] Cox proportional hazard model.

GLUT1 mRNA had lower 5-year OS rates than patients with low expression of GLUT1 (26% versus 36%, p = 0.004; Supplementary Fig. S2B). Additionally, we found that patients with low expression of both PD-L1 and GLUT1 had longer OS than patients with high expression of either PD-L1 or GLUT1, or both PD-L1 and GLUT1, although the difference in OS was not statistically significant (p = 0.051, Supplementary Fig. S2C). Univariate analysis revealed that OS was associated with T stage. In multivariate analysis, GLUT1 expression was an independent prognostic marker for OS (hazard ratio = 1.785, p = 0.029; Supplementary Table S2). In agreement with our findings, the online tool kmplot.com also revealed that lung adenocarcinoma patients with high levels of expression of GLUT1 had lower OS rates than patients with low expression of GLUT1 (n = 866) (p < 0.001; Supplementary Fig. S2D).

4. Discussion

To the best of our knowledge this is the first study to analyze PD-L1 protein expression in patients with NSCLC, and to describe correlations between PD-L1 and hypoxia-related genes (including HIF1A, VEGFA, GLUT1, and CAIX). Additionally, we performed correlation analyses between PD-L1 expression and expression of hypoxia-related genes using RNA sequencing data.

We found that PD-L1 protein expression was correlated with protein expression of HIF1A, VEGFA, GLUT1, and CAIX in adenocarcinoma only. PD-L1 mRNA expression was also correlated with HIF1A, VEGFA, GLUT1, and CAIX mRNA expression in adenocarcinoma. However, there was no correlation between PD-L1, HIF1A, VEGFA, GLUT1, and CAIX in squamous-cell carcinoma. Our findings correlating PD-L1 with hypoxia-related genes are supported by previous research linking PD-L1 and hypoxia-related genes. In a mouse model of Lewis lung carcinoma, hypoxia significantly increased the expression of PD-L1 on macrophages, dendritic cells, and tumor cells [11]. HIF1A can bind directly to the hypoxia-response element in the proximal promoter of PD-L1 and upregulate its expression under hypoxic conditions [11]. Biomimetic multifunctional nanoplatforms can catalyze H_2O_2 to produce O^2, relieve tumor hypoxia, and reduce the expression of HIF1A in murine melanoma models [12]. In addition, with decreased expression of HIF1A, the expression of PD-L1 in tumors cells was also downregulated [12]. Dual inhibition of the PD-L1/PD-1 axis by PD-1 antibody and nanoplatform can induce a significant immune response, prolong tumor recurrence time, and inhibit tumor metastasis [12]. These results suggest that simultaneous blockade of HIF1A and PD-L1 may represent a novel therapeutic approach for cancer immunotherapy. Significant correlations have also been reported between PD-L1 and HIF1A in hepatocellular carcinoma [13], pheochromocytoma [26], and oral squamous-cell carcinoma [14]. VEGF was shown to upregulate inhibitory receptor T-cell immunoglobulin and mucin domain 3 (TIM-3) on CD8 + T cells and induce resistance to PD-1 blockade in a mouse model of small-cell lung cancer [27]. Combined anti-VEGF/anti-PD-L1 target therapy can improve treatment outcomes with synergistic effects, compared to anti-PD-L1 or anti-VEGF monotherapy [27]. VEGF expression has been correlated with PD-L1 in patients with glioma [28]. GLUT1 expression has been correlated with PD-L1 expression in clear-cell renal-cell carcinoma [29]. CAIX was also associated with PD-L1 expression in clear-cell renal-cell carcinoma [29].

We also conducted survival analyses for RFS and OS. HIF1A was related to inferior RFS, while GLUT1 was related to inferior RFS and OS by analysis of immunohistochemical results. In RNA sequencing data, patients with high GLUT1 mRNA expression had lower 5-year OS rates than patients with low GLUT1 mRNA expression. Furthermore, patients with low expression of both PD-L1 and GLUT1 had longer OS than patients with high expression of either PD-L1 or GLUT1, or both PD-L1 and GLUT1 in analyses of both immunohistochemical and RNA sequencing data. This was consistent with previously reported results. HIF1A levels in tumor cells are closely correlated with disease-free survival in NSCLC [30]. In meta-analysis of 1665 NSCLC patients, GLUT1 overexpression was associated with poor OS and disease-free survival [31]. No previous study has reported dual expression of PD-L1 and GLUT1 in NSCLC. Based on the fact that simultaneous blockade of HIF1A and PD-L1 has a significant treatment effect in murine melanoma models [12], simultaneous blockade of PD-L1 and GLUT1 could be a promising combinatorial strategy for human cancer.

Currently, many target agents for HIF1A, VEGFA, GLUT1, and CAIX have been developed. Q6, a novel hypoxia-targeted drug, downregulates HIF1A via an autophagy-dependent mechanism in hepatocellular carcinoma [32]. Icaritin inhibits the invasion and epithelial-to-mesenchymal transition of glioblastoma cells via HIF1A signaling [33]. M410, a combretastatin A4 analogue, disrupts microtubules and downregulates HIF1A expression in human breast cancer cells [34]. Bevacizumab, a well-known VEGFA blocker, can be used to treat a number of types of cancer, including colon cancer [35], lung cancer [36], and renal-cell carcinoma [37]. Furthermore, combined anti-VEGF/anti-PD-L1 target therapy can improve the survival rate with synergistic effects in small-cell lung cancer [27]. Apigenin, a GLUT1-specific inhibitor, can induce growth retardation and apoptosis through metabolic and oxidative stress by inhibiting glucose utilization in lung cancer and adenoid cystic carcinoma [38]. WZB117, a small-molecule inhibitor of GLUT1, also downregulates glycolysis, induces cell-cycle arrest, and inhibits cancer cell growth in breast and lung cancer cell lines [39]. Sulfonamide, a CAIX inhibitor, can significantly reduce proliferation and increase apoptosis in colorectal carcinoma cell lines [40]. Considering significant correlations of PD-L1 with HIF1A, VEGFA, GLUT1, and CAIX, based on immunohistochemical analysis and RNA sequencing data, simultaneous blocking of HIF1A and hypoxia-related genes might be absolutely necessary.

In our study, PD-L1 protein expression was not correlated with HIF1A, VEGFA, GLUT1, and CAIX protein expression in squamous-cell carcinoma. However, PD-L1 mRNA showed a significant positive correlation with HIF1A mRNA, and a negative correlation with VEGFA and CAIX mRNA. Protein levels are influenced by a variety of factors. Alternative splicing has been reported in HIF1A, VEGFA and CAIX, and this process may result in different expressions of protein [41–43]. Translation rates can be modulated by the binding of non-coding RNA such as microRNAs [44]. A complex ubiquitin–proteasome pathway or autophagy can affect the protein concentration, regardless of transcription level [45]. Therefore, several protein modulators can cause discrepancies between protein and mRNA levels.

This study has some limitations. First, because of its retrospective design, our study was missing information about some driver mutations. Second, the most commonly used antibodies in current clinical trials of PD-L1 are dako 28-8, dako 22C3, ventana sp263, and ventana sp142 [22]. However, we did not use those antibodies because we did not have the proper auto-stainer. Anti-PD-L1 antibody (rabbit monoclonal, clone 28-8, Abcam) is also a very widely-used antibody which has been used in many previous studies [46–48]. Third, we used a tissue microarray design for the immunohistochemical study. Unfortunately, the design of tissue microarrays cannot reflect the entire tumor section. Fourth, our study had a relatively small sample size.

5. Conclusion

In conclusion, hypoxia-related signaling pathways are crucial for cancer immunotherapy. However, synergistic interactions between PD-L1 and hypoxia-related genes in NSCLC are not yet fully understood. Simultaneous blockade of PD-L1, HIF1A, and VEGFA has shown remarkable treatment outcomes in other cancers. In this study, we found clinical correlation of PD-L1 expression with expression of HIF1A, VEGFA, GLUT1, and CAIX in immunohistochemical and RNA-sequencing data in adenocarcinoma. Low expression of both PD-L1 and GLUT1 was correlated with improved survival outcome. HIF1A and GLUT1 were related to poor prognosis.

Our findings support the rationale for co-targeting hypoxia-related genes and PD-L1 in cancer therapy. Expression of hypoxia-related genes may be helpful in selecting patients appropriate for PD-L1 therapy.

Conflicts of interest

The authors have disclosed that they have no significant relationships with, or financial interest in, any commercial companies pertaining to this article.

Source of funding

None.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.01.004.

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