Prognostic Value of PD-L1 mRNA Sequencing Expression Profile in Non-Small Cell Lung Cancer

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Background. **Immune checkpoint inhibitors that target the programmed cell death protein ligand 1 (PD-L1) pathway have shown benefit for the treatment of metastatic non-small cell lung cancer (NSCLC). However, the prognostic value of PD-L1 independent of immunotherapy is still unclear, with conflicting results reported between PD-L1 expression and patient survival. Our aim was to correlate PD-L1 mRNA level with clinical and pathologic factors and to investigative the prognostic value of PD-L1 mRNA in all stages of NSCLC.**

Methods. **Gene expression and clinical data were obtained from public repositories in The Cancer Genome Atlas from the National Cancer Institute. Genotype-Tissue Expression was used to compare with normal tissue expression analysis.**

Results. **A total of 985 patients met inclusion criteria, among whom 79.6% were stage I to II, 16.5% were stage III, and 3.5% were stage IV, representing 495 adenocarcinoma and 490 squamous cell carcinoma (SCC). PD-L1 mRNA gene expression in lung cancers was higher than in most other tumor and normal tissue types and was significantly higher in lung SCC than adenocarcinoma ($p < 0.001$). PD-L1 mRNA expression was associated with pathologic stage in SCC and with smoking status in adenocarcinoma of the lung. However, none of the cutoff values of PD-L1 mRNA expression were prognostic of overall survival.**

Conclusions. **Our results suggest that the value of PD-L1 mRNA in prognosticating outcome in lung cancer is limited. Further studies are needed to identify novel prognostic biomarkers other than PD-L1 that are associated with improved patient survival. Identification of further prognostically important biomarkers may prove useful in identifying patients suitable for immunotherapy.**

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Immune checkpoint inhibitors have shown remarkable efficacy in treating advanced non-small cell squamous and non-squamous lung cancers. Programmed death ligand 1 (PD-L1, B7-H1, CD274) has emerged as the most studied biomarker to predict response to checkpoint immunotherapy. The PD-L1 ligand, which may be present on tumor cells or immune cells alike, binds to a programmed cell death protein 1 (PD-1) and allows escape from cytotoxic T-cell action. Checkpoint inhibitors that target the PD-L1 pathway bind selectively to the PD-1 site and prevent this inhibitory interaction, thus allowing for cytotoxic T cells to execute their antitumor action. Previous studies have shown that with increased PD-L1 tumor expression, there is an increased likelihood of response to PD-1 checkpoint inhibitor therapy. Although the exact cutoff value of immunohistologic PD-L1 expression has not been clearly established, the Food and Drug Administration has approved pembrolizumab, an anti PD-1 agent for tumors with greater than 50% expression as the first-line therapy for patients with metastatic disease [1]; nivolumab is approved as a second-line treatment regardless of PD-L1 expression [2]. The interest in PD-L1 as a biomarker has expanded from its ability to predict response to immunotherapy to its role in estimating prognosis among all patients with non-small cell lung cancer (NSCLC). A number of retrospective surgical studies have attempted to correlate overall survival (OS) with PD-L1 expression levels among immunotherapy-naive early stage lung cancer (stages I to III). This endeavor was generated from the hypothesis that PD-L1 tumor expression at the time of operation would be a marker of the ability of the immune system to

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Abbreviations and Acronyms

CCLE = Cancer Cell Line Encyclopedia
GTEx = Genotype-Tissue Expression
HR = hazard ratio
IHC = immunohistochemical
NSCLC = non-small cell lung cancer
PD-1 = programmed cell death protein one
PD-L1 = programmed cell death protein ligand 1
OS = overall survival
SCC = squamous cell carcinoma
TCGA = The Cancer Genome Atlas

target the tumor. Interestingly, conflicting results have been reported in the literature on the value of PD-L1 in prognosticating survival among surgically resected lung cancer, with some studies citing high PD-L1 expression to be prognostic of worse outcome [3–6], and some studies claiming the opposite; that high PD-L1 expression was prognostic of better survival [7–10]. Most studies, as well as four meta-analyses, support the notion that high PD-L1 tumor expression results in worse OS [11–14], with the fifth meta-analysis showing no association with survival [15]. The association of high PD-L1 tumor expression with worse survival is intuitive, because PD-1 leads to tumor escape from the cytotoxic effect of the immune system, and higher expression of PD-L1 likely results in more robust immune suppression.

One controversial aspect of PD-L1 biomarker use is whether to measure PD-L1 protein expression through immunohistochemical (IHC) staining with the use of either whole tumor sections or tissue microarrays. Other controversies include the interpretation and objective quantification of IHC staining intensity on the tumor cells and inflammatory cells. In addition, the influence of tumor microenvironment components or treatment-related factors (eg, after chemotherapy or radiation) on the dynamic expression of PD-L1 remains unknown. An alternative measure to IHC techniques that merits investigation is tumor mRNA PD-L1 expression. The aim of our study was to explore the association between PD-L1 mRNA tumor expression with histologic findings, stage, and other clinical variables and to also evaluate the role of PD-L1 mRNA to prognosticate OS among patients with all stages of NSCLC. We used data from publicly available lung adenocarcinoma and squamous cell carcinoma (SCC) data from The Cancer Genome Atlas (TCGA) [16, 17] project, and small cell and NSCLC line data from the Cancer Cell Line Encyclopedia (CCLE) [18].

Patients and Methods

RNA Sequencing and Clinical Data

Gene expression and clinical data from the lung adenocarcinoma [16] and lung SCC [17] data were obtained from TCGA, which is a public repository (https://tcga-data.nci.nih.gov). We used the following clinical variables: diagnosis, pathologic_stage, tobacco_smoking_history, age_at_initial_pathologic_diagnosis, gender, vital_status, days_to_death, and days_to_last_followup. Normal tissue expressions were downloaded from the Genotype-Tissue Expression (GTEx) [19] project's website (https://www.gtexportal.org/). CCLE cancer cell line mRNA expression data were obtained from the CCLE [18] (https://portals.broadinstitute.org/ccle/home).

Statistical Analyses

One-way analysis of variance tests were used to compare three or more groups. For comparisons of two groups, we used two-tailed Student's t tests. Cox proportional hazards regression was used to evaluate the hazard for death with PD-L1 expression and other clinical variables. To define the low and high PD-L1 expression groups, we used the median PD-L1 mRNA expression as the cutoff value. A sensitivity analysis was performed to check if the results would be different, depending on how PD-L1 mRNA level was modeled in the regression equation. PD-L1 was alternatively modeled with more than 20 varying cutoff levels and also as a continuous variable with diagnostic tests to evaluate for a linear relationship with survival. All differences were considered statistically significant when p was less than 0.05.

Visualization of Data

We used the Tableau business intelligence software [20] to prepare all figures.

Results

Demographic Detail

Demographic information of 495 adenocarcinoma and 490 SCC patients with RNA sequencing and clinical data is presented in Table 1. As expected, only a low percentage (14.4% of adenocarcinoma and 3.7% of SCC) of patients were lifelong nonsmokers. Most patients in our cohort were early stage (stages I to III), with only 5.5% of adenocarcinoma and 1.4% of SCC representing pathologic stage IV cancer.

PD-L1 Shows High Expression in Lung Adenocarcinoma and SCC

With the use of publicly available TCGA lung adenocarcinoma [16] and SCC [17], as well as GTEx normal tissue data [19], we analyzed PD-L1 expression in various normal and tumor tissues (Fig 1). For lung cancers, PD-L1 mRNA expression from mRNA sequencing was available for 517 adenocarcinoma and 501 SCC samples in the TCGA. Lung cancers demonstrated higher PD-L1 expression than most other cancers and normal tissues, in addition to normal lung tissue. In addition, we found that PD-L1 expression was significantly higher in SCC than in adenocarcinoma ($p < 0.001$).

PD-L1 Expression Correlates With Pathologic Stage in SCC and With Smoking Status in Adenocarcinoma

Among patients with stage II SCC, PD-L1 levels were significantly higher than with stages I ($p < 0.05$), III ($p < 0.05$), and IV ($p < 0.001$) SCC. Interestingly, stage IV

Table 1. Demographic Characteristics of the Study Population

Characteristic	Adenocarcinoma (n = 495)	Squamous Cell Carcinoma (n = 490)
Age, median (range), years	66 (38–88)	68 (39–90)
Sex, n (%)		
Female	266 (53.7)	127 (25.9)
Male	229 (46.3)	363 (74.1)
Pathologic stage, n (%)		
I	266 (53.7)	239 (48.8)
II	120 (24.2)	159 (32.4)
III	80 (16.2)	83 (16.9)
IV	27 (5.5)	7 (1.4)
Not available	2 (0.4)	2 (0.4)
Tobacco smoking history, n (%)		
Current smoker	114 (23.0)	133 (27.1)
Current reformed smoker for ≤15 years	166 (33.5)	241 (49.2)
Current reformed smoker for >15 years	27 (25.7)	84 (17.1)
Lifelong nonsmoker	70 (14.1)	18 (3.7)
Not available/unspecified	18 (3.6)	14 (2.9)
PD-L1 level, median (25th–75th percentile)	3.63 (1.74–7.05)	4.39 (2.09–10.88)

PD-L1 = programmed cell death protein ligand 1.

SCC was associated with a lower PD-L1 expression compared with stages I, II, and III SCC ($p < 0.001$; Fig 2A). However, PD-L1 expression level was independent of pathologic stage among patients with lung adenocarcinoma. In addition, we found that among patients with lung adenocarcinoma, current or recently reformed smokers (15 years or less) had higher PD-L1 levels than either nonsmokers or reformed smokers for more than 15 years (both $p < 0.05$; Fig 2B). PD-L1 expression level, however, was independent of smoking history for patients who have SCC.

PD-L1 Expression Is Higher in NSCLC Cell Lines Than in Lung Small Cell Carcinoma Lines

With the use of the CCLE [18] cancer cell line data we identified that NSCLC cell lines show various expression of PD-L1 that were significantly higher than lung small cell carcinoma ($p < 0.001$; Fig 3).

Correlation of Clinical Variables With OS

Univariate analysis (Table 2) showed that increasing pathologic stage was associated with worse OS for both lung cancer types. Mean follow-up was 20.3 months (range: 0 to 223 months). Tobacco smoking history, age, and PD-L1 mRNA expression level were not statistically significantly associated with OS. In a multivariate analysis (Table 3), age was associated with worse OS (hazard ratio [HR] 1.023 and HR 1.027 per additional year of life for adenocarcinoma and SCC, respectively, $p < 0.05$). Paradoxically, current or recently reformed smokers for 15 years or less demonstrated significant association with improved survival in SCC (HR 0.344, $p < 0.05$). Of importance, PD-L1 mRNA expression was not associated with OS on multivariate analysis. A sensitivity analysis in which varying levels (analysis by quartiles, by less than and more than 1%, less than and more than %) of PD-L1

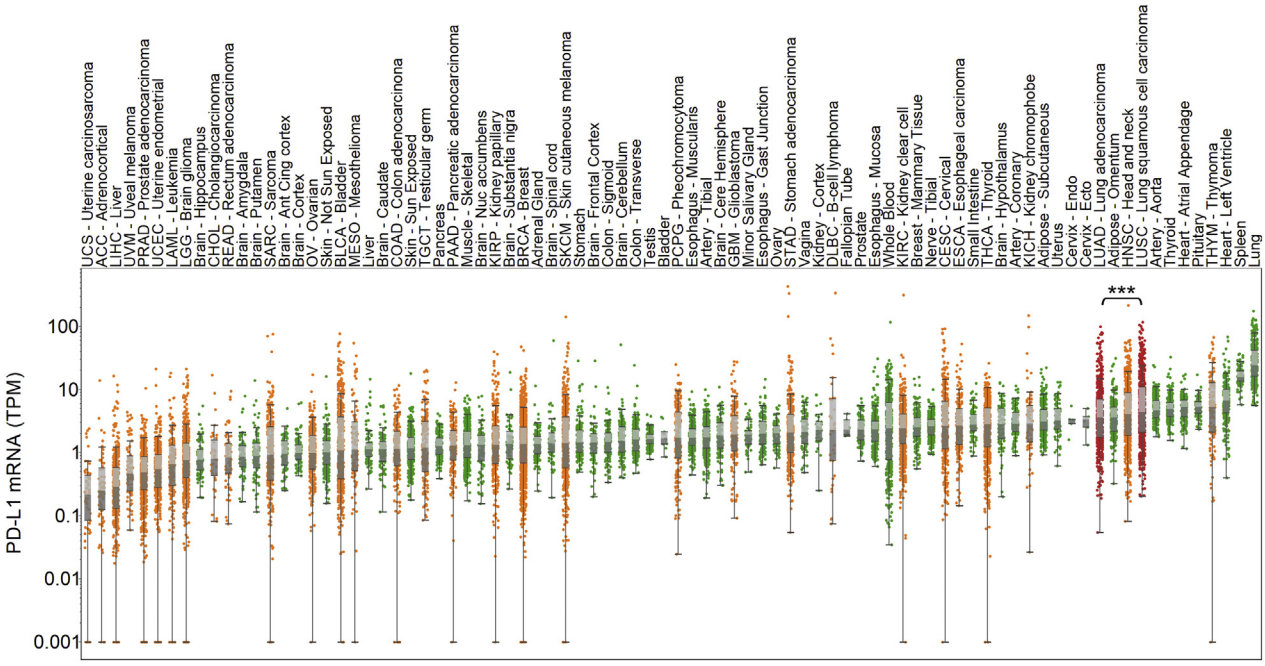

*Fig 1. Programmed cell death protein ligand 1 (PD-L1) expression in tumor and normal tissues. Expression of PD-L1 is shown for The Cancer Genome Atlas tumor samples (orange and red dots) and Genotype-Tissue Expression normal tissues (green). ***Denotes the statistically significant difference ($p < 0.001$) between lung adenocarcinoma and squamous cell carcinoma. Tissue types were sorted by median expression of samples. (TPM = transcripts per million.)*

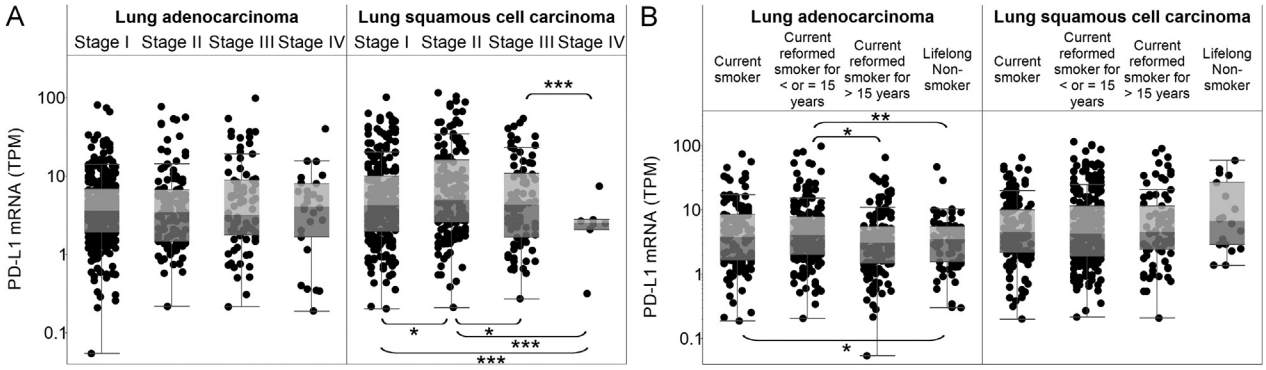

*Fig 2. Programmed cell death protein ligand 1 (PD-L1) expression associations with pathologic stage and smoking status. (A) The mRNA expression of PD-L1 in various stages (stages I, II, III, and IV) is displayed for lung adenocarcinoma and squamous cell carcinoma. (B) Similarly, PD-L1 expression is shown for three types of smoker and lifelong nonsmoker patients. Statistically significant differences are denoted by *p < 0.05, **p < 0.01, and ***p < 0.001. (TPM = transcripts per million.)*

mRNA expression was used as cutoff for high or low values was performed, and we observed no difference in results (see Methods).

Comment

Immune checkpoint blockade by the PD-1/PD-L1 pathway has changed the treatment paradigm for a number of solid tumors, including lung cancers [21]. The magnitude of PD-L1 expression has been shown to have prognostic value in multiple cancers; however, conflicting results remain about its prognostic value with surgically resected lung cancers [22]. In this report, we analyzed publicly available data within the National Cancer Institute TCGA repositories and GTEx. Although PD-L1 mRNA gene expression was higher in lung cancers than in most other tumors and normal tissue types, PD-L1 mRNA level was not associated with OS in this cohort of surgically treated lung cancer patients.

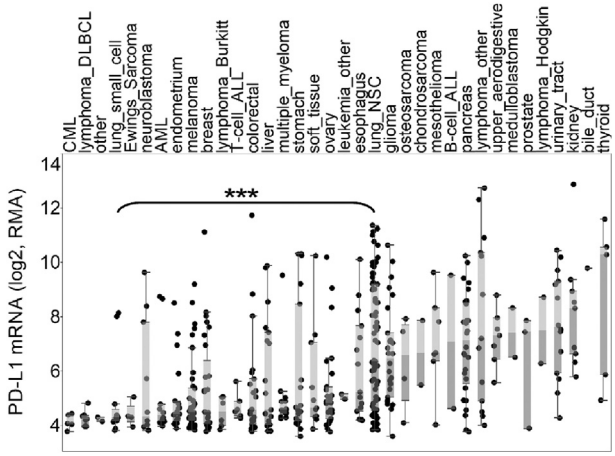

*Fig 3. Cell line expression of programmed cell death protein ligand 1 (PD-L1). Cancer Cell Line Encyclopedia (CCLE) cell line PD-L1 expressions are shown for all CCLE tumor types. Cell lines are sorted by median PD-L1 expression. ***Denotes the statistically significant difference (p < 0.001) between small cell and non-small cell lung cancer cell lines. (ALL = acute lymphoblastic leukemia; AML = acute myelocytic leukemia; CML = chronic myelogenous leukemia; DLBCL = diffuse large B-cell lymphoma; NSC = non-small cell; RMA = robust multi-array average.)*

The value of PD-L1 expression in prognosticating survival in anti–PD-1 naive patients appears to be limited, given the widely heterogeneous results reported in the literature. A prognostic biomarker needs to be reliable; unfortunately, PD-L1 levels have not been reliably shown to be associated with survival. Given the variation in methodology between studies, a potential explanation for observed heterogeneity may lie with a complex biologic interaction between PD-L1 levels and other clinical or tumor microenvironmental factors. Alternatively, especially in the light of PD-L1 being associated with other prognostic factors, PD-L1 may act as a confounder, serving as a surrogate for another clinical factor that affects survival. Regardless of the cause, the lack of reliable results limits the utility of PD-L1 level in establishing prognosis in patients who undergo upfront surgical resection for lung cancer. However, negative studies that established the lack of a relationship between PD-L1 level and survival also need to be published to prevent a publication bias.

We chose to analyze PD-L1 expression with the use of mRNA analysis, given that PD-L1 mRNA expression is less well studied, as well as the potential limitations of IHC staining. The reported publications have shown a high concordance between mRNA levels and IHC staining [8, 23]. As with IHC staining, mixed results have been shown with investigating the role of PD-L1 mRNA as a prognostic factor, with one publication showing improved survival with increasing PD-L1 [8], and two others showing no relationship with survival [23, 24], indicating that PD-L1 level is not reliably associated with survival regardless of the method of detection.

We also showed that PD-L1 expression is significantly higher in SCC than in adenocarcinoma; this finding has been generally true in previous studies [25]. PD-L1 mRNA level correlated with pathologic stage in SCC but not in adenocarcinoma. Of interest, stage 2 SCC showed the highest PD-L1 expression, and stage 4 showed the lowest. In the agreement with earlier results [26], our analyses identified that smoking status in

Table 2. Univariate Predictors of OS

	Adenocarcinoma (n = 495)		Squamous Cell Carcinoma (n = 490)	
Predictor	OS HR (95% CI)	*p* Value	OS HR (95% CI)	*p* Value
Age, continuous variable	1.016 (0.997 to 1.034)	0.10	1.017 (0.997 to 1.036)	0.10
Sex				
Female	Reference	…	…	…
Male	0.922 (0.592 to 1.252)	0.66	1.040 (0.659 to 1.421)	0.83
Pathologic stage				
I	Reference	…	…	…
II	2.261 (1.224 to 3.297)	<0.001	1.226 (0.761 to 1.692)	0.29
III	4.322 (2.340 to 6.303)	<0.001	1.501 (0.893 to 2.109)	<0.05
IV	3.131 (1.033 to 5.229)	<0.001	2.116 (−0.347 to 4.580)	0.21
Tobacco smoking history				
Lifelong nonsmoker	Reference	…	…	…
Current smoker	0.742 (0.244 to 1.240)	0.38	0.594 (−0.018 to 1.205)	0.32
Current reformed smoker for ≤15 years	0.967 (0.367 to 1.567)	0.92	0.439 (−0.005 to 0.884)	0.11
Current reformed smoker for >15 years	0.963 (0.331 to 1.595)	0.91	0.475 (−0.0283 to 0.977)	0.17
PD-L1 expression				
Low (less than median)	Reference	…	…	…
High (more than median)	1.096 (0.705 to 1.486)	0.62	1.033 (0.701 to 1.366)	0.84

CI = confidence interval; HR = hazard ratio; OS = overall survival; PD-L1 = programmed cell death protein ligand 1.

adenocarcinoma (but not in SCC) was associated with PD-L1 expression, a finding that may be related to the high mutational burden among smokers [27]. Indeed, tumors from lifelong nonsmokers and reformed smokers for more than 15 years expressed lower levels of PD-L1 than current smokers and reformed smokers for 15 years or less. These results may help establish the validity of the use of PD-L1 expression from mRNA sequencing in predicting which lung cancer disease stages and subgroups may potentially benefit from checkpoint blockade-based immunotherapies in the adjuvant setting. Currently, no evidence suggests that adjuvant immunotherapy improves survival in lung cancer patients after standard-of-care treatment of surgical resection and chemotherapy. This question is studied in a nationwide ANVIL (Adjuvant Nivolumab in Resected Lung Cancers) trial (NCT02595944) [28].

The levels of PD-L1 expression in various disease stages and subgroups of lung cancer patients also provide rationale for neoadjuvant or window-of-opportunity immunotherapy trials, which would enable us to sort out the mechanisms and to identify patients best suited

Table 3. Multivariate Predictors of OS

	Adenocarcinoma (n = 495)		Squamous Cell Carcinoma (n = 490)	
Predictor	OS HR (95% CI)	*p* Value	OS HR (95% CI)	*p* Value
Age, continuous variable	1.023 (1.002 to 1.044)	<0.05	1.027 (1.006 to 1.048)	<0.05
Sex				
Female	Reference	…	…	…
Male	0.867 (0.511 to 1.223)	0.50	1.008 (0.627 to 1.389)	0.97
Pathologic stage				
I	Reference	…	…	…
II	2.738 (1.391 to 4.084)	<0.001	1.183 (0.712 to 1.653)	0.41
III	4.782 (2.437 to 7.127)	<0.001	1.630 (0.949 to 2.310)	<0.05
IV	4.124 (1.206 to 7.043)	<0.001	1.959 (−0.368 to 4.286)	0.27
Tobacco smoking history				
Lifelong nonsmoker	Reference	…	…	
Current smoker	0.827 (0.228 to 1.425)	0.61	0.483 (−0.035 to 1.001)	0.18
Current reformed smoker for ≤15 years	1.400 (0.464 to 2.336)	0.32	0.344 (−0.017 to 0.704)	<0.05
Current reformed smoker for >15 years	1.198 (0.365 to 2.031)	0.61	0.348 (−0.043 to 0.739)	0.07
PD-L1 expression				
Low (less than median)	Reference	…	…	
High (more than median)	1.117 (0.679 to 1.555)	0.58	1.022 (0.683 to 1.361)	0.90

CI = confidence interval; HR = hazard ratio; OS = overall survival; PD-L1 = programmed cell death protein ligand 1.

for immunotherapy. After all, immunotherapy is more effective with tumor antigens in place. A phase 2 study of induction checkpoint blockade for untreated stage I to IIIA NSCLC amenable for surgical resection is currently under way at our institution (NCT03158129). Because mRNA sequencing is becoming less expensive and it is included in an increasing number of studies, PD-L1 gene expression associations may become increasingly useful in guiding immunotherapies.

Limitations of this analysis include its retrospective nature and the lack of detailed treatment or patient-related information, which could not be obtained from the TCGA database, and may have influenced survival analysis. Because the PD-L1 mRNA was not prognostic of survival in this cohort of lung cancer patients, it is unlikely that a positive result would have been observed if we had available additional known prognostic variables in the multivariable model. Furthermore, there is a potential for database errors within TCGA, as with any database study. However, TCGA is produced by a joint effort by the National Human Genome Research Institute and the National Cancer Institute and the likelihood of database errors are low.

In summary, we found that PD-L1 mRNA is a poor prognosticator of OS among all stages of NSCLC; however, PD-L1 mRNA was associated with clinical and tumor-related factors, indicating that it may be useful for identifying subgroups that are more likely to respond to immunotherapy. The identification of further biomarkers that reliably prognosticate survival beyond the current staging system or predict response to immunotherapy still need to be discovered [22, 29].

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