Original Investigation

Quantitative Assessment of the Heterogeneity of PD-L1 Expression in Non–Small-Cell Lung Cancer

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IMPORTANCE Early-phase trials with monoclonal antibodies targeting PD-1 (programmed cell death protein 1) and PD-L1 (programmed cell death 1 ligand 1) have demonstrated durable clinical responses in patients with non–small-cell lung cancer (NSCLC). However, current assays for the prognostic and/or predictive role of tumor PD-L1 expression are not standardized with respect to either quantity or distribution of expression.

OBJECTIVE To demonstrate PD-L1 protein distribution in NSCLC tumors using both conventional immunohistochemistry (IHC) and quantitative immunofluorescence (QIF) and compare results obtained using 2 different PD-L1 antibodies.

DESIGN, SETTING, AND PARTICIPANTS PD-L1 was measured using E1L3N and SP142, 2 rabbit monoclonal antibodies, in 49 NSCLC whole-tissue sections and a corresponding tissue microarray with the same 49 cases. Non–small-cell lung cancer biopsy specimens from 2011 to 2012 were collected retrospectively from the Yale Thoracic Oncology Program Tissue Bank. Human melanoma Mel 624 cells stably transfected with PD-L1 as well as Mel 624 parental cells, and human term placenta whole tissue sections were used as controls and for antibody validation. PD-L1 protein expression in tumor and stroma was assessed using chromogenic IHC and the AQUA (Automated Quantitative Analysis) method of QIF. Tumor-infiltrating lymphocytes (TILs) were scored in hematoxylin-eosin slides using current consensus guidelines. The association between PD-L1 protein expression, TILs, and clinicopathological features were determined.

MAIN OUTCOMES AND MEASURES PD-L1 expression discordance or heterogeneity using the diaminobenzidine chromogen and QIF was the main outcome measure selected prior to performing the study.

RESULTS Using chromogenic IHC, both antibodies showed fair to poor concordance. The PD-L1 antibodies showed poor concordance (Cohen κ range, 0.124-0.340) using conventional chromogenic IHC and showed intra-assay heterogeneity (E1L3N coefficient of variation [CV], 6.75%-75.24%; SP142 CV, 12.17%-109.61%) and significant interassay discordance using QIF (26.6%). Quantitative immunofluorescence showed that PD-L1 expression using both PD-L1 antibodies was heterogeneous. Using QIF, the scores obtained with E1L3N and SP142 for each tumor were significantly different according to nonparametric paired test ($P < .001$). Assessment of 588 serial section fields of view from whole tissue showed discordant expression at a frequency of 25%. Expression of PD-L1 was correlated with high TILs using both E1L3N ($P = .007$) and SP142 ($P = .02$).

CONCLUSIONS AND RELEVANCE Objective determination of PD-L1 protein levels in NSCLC reveals heterogeneity within tumors and prominent interassay variability or discordance. This could be due to different antibody affinities, limited specificity, or distinct target epitopes. Efforts to determine the clinical value of these observations are under way.

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PD-L1 (programmed cell death 1 ligand 1) expression is a major immune suppressive mechanism via engagement of the PD-1 (programmed cell death protein 1) and PD-L1 axis in non–small-cell lung cancer (NSCLC). After antigen recognition and activation of T cells through a T-cell receptor and major histocompatibility complex peptide-based interaction, PD-L1 can act as a coregulatory signal through binding of the inhibitory PD-1 receptor that ultimately leads to inactivation of lymphocytes and other immune cells.[1-3] Under certain circumstances, such as viral infections, this mechanism can act as a checkpoint to limit the immune response and avoid tissue damage.[4,5] This mechanism can also mediate immune tolerance as seen by placental trophoblastic expression, thereby preventing autoimmune-based destruction of this new immunologically foreign organ.[6-9] Similarly, tumor cells can evade the immune response through up-regulation of PD-L1, with diverse human malignant tumors showing elevated levels of PD-L1 protein, including NSCLC.[10-14]

Blockade of the PD-1 and PD-L1 interaction using monoclonal antibodies produces durable clinical responses in patients with diverse advanced tumor types.[15-18] While some studies have shown minimal predictive value for PD-L1 expression,[19,20] others have shown significantly increased response rates in expressers over nonexpressers.[15,16,21] Summarizing these initial studies, tumor PD-L1 protein expression by any assay with any distribution predicts a 3-fold increase in response to therapy compared with nonexpressers.[15-21] Most studies, however, also demonstrate a substantial response rate in tumors lacking PD-L1. Yet, initial drug labels for anti–PD-1 and anti–PD-L1 therapies, including pembrolizumab and nivolumab, currently do not require measurement of PD-L1 prior to administration of the drug.

The limited prognostic and predictive role of tumor PD-L1 protein expression is most likely due to the challenging nature of the assay. Contradictory findings have been published suggesting that different assay methods yield discordant results.[22-25] Currently, nearly every aspect of defining PD-L1 positivity using immunohistochemistry (IHC) is subject to lack of standardization and subjective interpretation. Additionally, some clinical trials have used PD-L1 assays that are not yet available to the research community. For instance, some trials measured PD-L1 in the epithelial cells[15,16,19] or even just the epithelial cell membrane,[21] whereas others included measurement of PD-L1 in immune cells of the peritumoral stroma.[26,27] The variability in the assays has been further complicated by the multiplicity of the reagents used to measure PD-L1. Diverse commercially available anti–PD-L1 antibodies have been used without thorough validation, resulting in a contradictory literature.[14,28] This is most often not due to antibodies that do not recognize PD-L1 but rather due to antibodies that recognize PD-L1 and, through cross-reactivity, other ill-defined proteins. Further complicating this situation, companies producing companion diagnostic tests have generated their own proprietary antibodies and testing platforms in preparation for US Food and Drug Administration submission without external validation or peer review. As a result, the interpretation of the literature and the data surrounding the predictive value of PD-L1 is challenging.

At a Glance

- PD-L1 (programmed cell death 1 ligand 1) assays for predicting response to monoclonal antibodies targeting PD-1 (programmed cell death protein 1) and PD-L1 are not standardized, with different assay methods yielding discordant results.
- The purpose of this study is to evaluate the reproducibility, concordance, and heterogeneity of PD-L1 protein expression using both conventional and quantitative methods.
- Programmed-death ligand 1 protein was measured in 49 non–small-cell lung cancer cases with 2 validated PD-L1 antibodies (E1L3N [Cell Signaling Technology] and SP142 [Spring Bioscience]) using chromogenic immunohistochemistry (IHC) and quantitative immunofluorescence (QIF) to assess heterogeneity and determine assay concordance.
- The PD-L1 antibodies showed poor concordance (Cohen κ range, 0.124-0.340) using conventional chromogenic IHC and showed intra-assay heterogeneity (E1L3N coefficient of variation [CV], 6.75%-75.24%; SP142 CV, 12.17%-109.61%) and significant interassay discordance using QIF (26.6%).
- In the absence of standardization, PD-L1 assessment in companion diagnostic tests using different antibodies may be discordant and, thus, the assay for 1 drug may not predict response to others.

In addition to challenging assays and associated intellectual property limitations, PD-L1 has also been recognized to have a complex processing and heterogeneous expression. PD-L1 protein is expressed in a wide range of cell types and is stimulated by variable and incompletely understood mechanisms.[2,3,9,29] PD-L1 may also be expressed by lymphocytes, macrophages, or dendritic cells, and this may account for the observed stromal localization of expression.[26,27,30] It is also possible that PD-L1 could be detected in macrophages after its ingestion of the cell on which it was expressed.

In light of the confusing and often contradictory literature on the expression of PD-L1, we have begun a systematic effort to better understand its expression in lung cancer. Herein, we use 2 carefully validated PD-L1 rabbit monoclonal antibodies, E1L3N (Cell Signaling Technology) and SP142 (Spring Bioscience), to assess both reproducibility and concordance, as well as heterogeneity of PD-L1 protein expression using both quantitative and conventional methods.

Methods

Patient Cohorts and Control Preparations

Retrospectively collected formalin-fixed paraffin-embedded whole-tissue sections (WTS) from 49 NSCLC cases from the Yale Thoracic Oncology Program Tissue Bank were obtained from Yale Pathology archives. These specimens represented only resections and were processed in the routine manner in the Yale Surgical Pathology suite. These patients were not treated with PD-L1 axis therapies but rather selected as representative lung cancer cases for measurement of expression using multiple antibodies and detection systems for definition of heterogeneity. Cases were also represented in a tissue microarray (TMA) termed YTMA246. Clinicopathologic information from patients was collected from the clinical records and pathology

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reports. Whole-tissue sections and TMA preparations were serially cut from tissue blocks as described. A control TMA termed YTMA245 containing positive and negative control specimens was constructed for reagents titration, PD-L1 assay validation, and reproducibility assessment as described.[14] All cases obtained had signed consent for tissue use under approved human investigation committee protocols, and IRB approval for this study was obtained through Yale University.

PD-L1 Antibodies

PD-L1 expression in formalin-fixed paraffin-embedded WTS and TMA was performed using both chromogenic IHC and automated quantitative immunofluorescence (QIF) with 2 commercially available, validated PD-L1 antibodies: E1L3N, a monoclonal rabbit antibody (Cell Signaling Technology; catalog No. 13684S) and SP142, a monoclonal rabbit antibody (Spring Bioscience; catalog No. MM4420). Each antibody was validated for IHC analysis by proving expression in the syncytial trophoblast layer of the placenta but not in the stroma and by showing expression in PD-L1–transfected Mel 624 cells but not the parental Mel 624 cell lines (eFigure 1 in the Supplement).

IHC and Immunofluorescence

Whole-tissue sections and TMA slides were deparaffinized and then subjected to antigen retrieval. For E1L3N, the slides were treated with an antigen retrieval solution of sodium citrate buffer (Sigma-Aldrich) with 1M citric acid (pH, 6.0) and boiled for 20 minutes at 97°C in a PT Module pressure-boiling container (Laboratory Vision). For SP142, antigen retrieval was performed as recommended in the Tris-EDTA buffer data sheet (Sigma-Aldrich) with 1M sodium hydroxide (pH, 8.0) and boiled for 10 minutes at 97°C in a PT Module pressure-boiling container. Slides were then incubated with methanol and 30% hydrogen peroxide for 30 minutes at room temperature, and then treated with a bovine serum albumin blockade for 30 minutes at room temperature.

For IHC, slides were incubated overnight at 4°C with a solution containing the PD-L1 antibodies E1L3N (1:1600 dilution) and SP142 (1:500 dilution). Sections were placed for 1 hour at room temperature with rabbit amplification reagent (EnVision K4003; Dako), followed by incubation for 5 minutes at room temperature with diaminobenzidine (K3468; Dako) prepared at 1:50 in diaminobenzidine substrate buffer. Slides were then counterstained for 7 minutes at room temperature with hematoxylin (Automation Hematoxylin Histological Staining Reagent; Dako) and dehydrated for 1 minute in 70%, 85%, 95%, 100%, and 100% graded ethanol washes, followed by 5 minutes in xylene. Cytoseal (8310-4; Richard-Allan Scientific) was used as mounting media. Control slides were run for reproducibility alongside each experimental slide-staining run.

For QIF, slides were incubated overnight at 4°C with a solution containing the same primary PD-L1 antibody concentrations and mouse monoclonal antihuman pancytokeratin antibody (clone AE1/AE3, M3515; Dako) at 1:100 dilution. Sections were incubated for 1 hour at room temperature with conjugated goat antimouse secondary antibody (Alexa, A21089; Invitrogen Molecular Probes) diluted at 1:100 in rabbit amplification reagent (EnVision K4003). Cyanine 5 directly conjugated to tyramide (FP1117; Perkin-Elmer) at 1:50 dilution was used for target antibody detection. Mounting medium (ProLong Gold, P36931; Life Technologies) with 4′, 6-diamidino-2-phenylindole (DAPI) was used to stain nuclei. Control slides were run alongside each slide-staining experiment.

Determination of PD-L1 Positivity by IHC

The scoring of WTS slides for PD-L1 positivity with each PD-L1 E1L3N and SP142 antibody using chromogenic IHC was performed by a pathologist (K.A.S.) using the various cutoffs used in clinical trials.[15-18,21,26,27] PD-L1 positivity was defined by the presence of membranous and cytoplasmic staining in the tumor cells using different cutoffs (1%, 5%, and 50%) and/or in the stroma using 5% as threshold. Cases that could not be appropriately evaluated for technical reasons (eg, folded tissue, low tumor area) were designated as not evaluable.

Automated Quantitative Fluorescence

Quantitative immunofluorescence enables objective and sensitive measurement of targets within user-defined tissue compartments. The QIF measurements were performed using the AQUA (Automated Quantitative Analysis) (Genoptix Medical Laboratory) method as described.[31] The QIF score of PD-L1 signal for each antibody in the tumor and in the stroma was calculated by dividing the target PD-L1 pixel intensities in the area of the tumor and stroma compartment defined by the cytokeratin and DAPI positivity. Tumor-infiltrating lymphocytes (TILs) stroma was defined as cells that have DAPI stain but lack cytokeratin positivity. Scores were normalized to the exposure time and bit depth at which the images were captured, allowing scores collected at different exposure times to be comparable. For each WTS, between 5 to 24 fields of view (FOV) representative of the tumor were selected based on the amount of viable tissue available such that the 2 PD-L1 antibodies were examined in serial sections for each FOV. Each FOV measured 0.7 mm ×0.7 mm. All FOVs were visually evaluated and FOVs were systematically excluded when there were staining artifacts or presence of less than 2% tumor area.

Evaluation of Tumor-Infiltrating Lymphocytes

The scoring of TILs was performed in hematoxylin-eosin-stained WTS independently by 2 pathologists (D.C.H. and V.P.) using a recently reported system[32] for standardized evaluation of TILs in breast cancer. Cases that could not be appropriately evaluated for technical reasons (eg, folded tissue, low tumor area) were designated as not evaluable. Spots with discordance in TIL scoring between pathologists were reviewed jointly and a single consensus score was established.

Statistical Analysis

For each antibody, the PD-L1 QIF scores were compared between groups divided by clinical and pathologic characteristics using a 2-sided t test with $P = .05$ considered statistically significant. The concordance of PD-L1 positivity using chromogenic staining with different PD-L1 antibodies and 1%, 5%, and 50% cutoffs in tumor and a 5% cutoff in stroma were evaluated using weighted Cohen κ coefficient. Coefficients of variance were calculated for each individual case for both E1L3N

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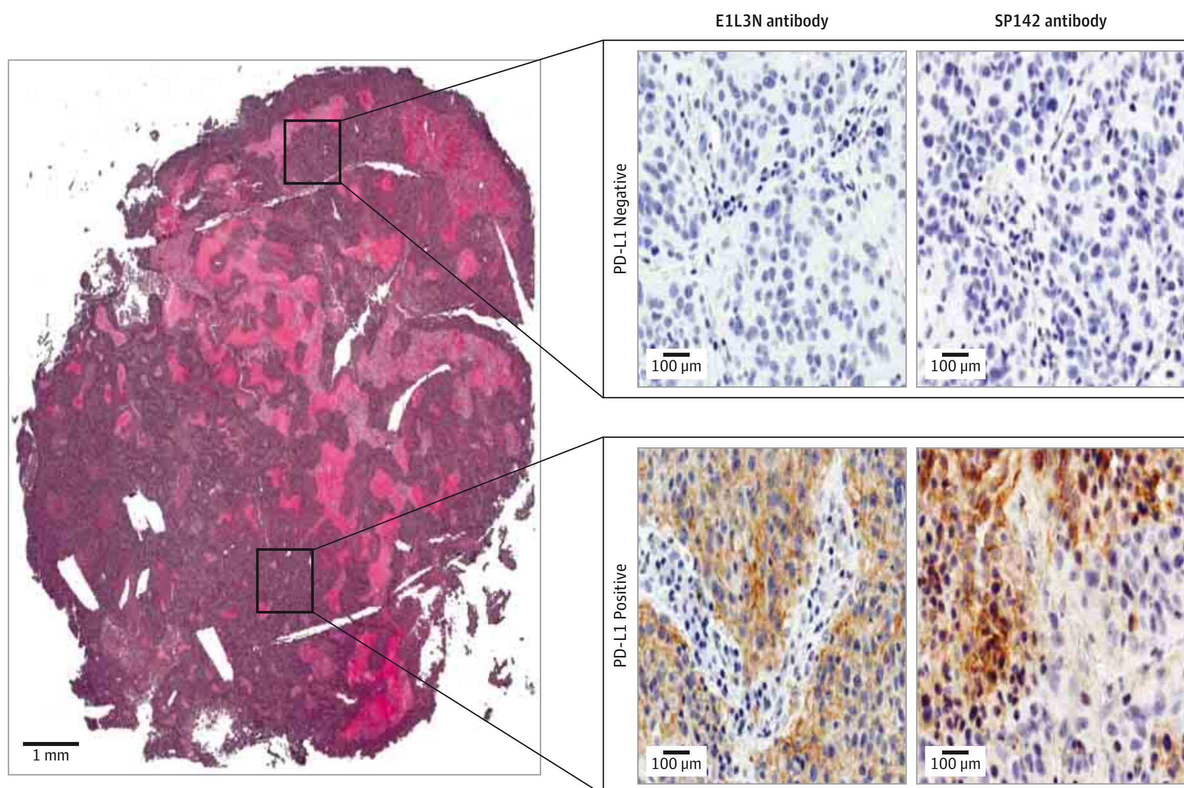
Figure 1. PD-L1 Protein Heterogeneity Using Diaminobenzidine



PD-L1 indicates programmed cell death 1 ligand 1.

and SP142. Total, mean, and maximum values of PD-L1 scores for each tissue sample were compared between the 2 antibodies based on: (1) Pearson and Spearman correlation coefficients, and (2) nonparametric paired tests including sign test and Wilcoxon signed-rank test. PD-L1 QIF scores for each antibody and TILs were compared using a 2-sided t test with $P = .05$ considered statistically significant. Statistical analyses were performed using GraphPad Prism (version 6.03; GraphPad Software) and SAS (version 9.4; SAS Institute Inc).

Results

Assessment of PD-L1 Expression

PD-L1 protein expression with 2 antibodies using chromogenic IHC (DAB) and quantitative immunofluorescence was heterogeneous. Representative cases are shown from different parts of the same tumor (WTS shown in hematoxylin-eosin), with both antibodies using DAB demonstrating positive staining in some regions of the tumor but negative in other regions (**Figure 1**). Distribution of expression was noted but could not be definitively or reproducibly defined as either leading edge or non-leading edge expression. Distribution of expression was frequently present near stromal tumor interfaces (eFigure 2 in Supplement). Coefficients of variation between FOVs for individual cases ranged from 6.75% to 75.24% for E1L3N, and from 12.17% to 109.61% for SP142 using QIF (eTable 1 in the Supplement).

Associations of PD-L1 Protein Using QIF With Clinicopathological Characteristics and TILs

The majority of patients were female smokers with early-stage (stage I) lymph node-negative lung adenocarcinomas (**Table**). PD-L1 protein QIF scores separated by clinicopathological features did not correlate with sex, age, smoking status, histological subtype, stage, or primary tumor size with either of the antibodies (Table). SP142 had significantly higher QIF scores in lymph node-positive patients than lymph node-negative patients ($P = .03$) (Table). E1L3N did not have significantly higher QIF scores in lymph node-positive vs lymph node-negative patients ($P = .06$) (Table). The majority of tumors (n = 42) had low TILs (<60%) (**Figure 2**). E1L3N and SP142 had significantly higher QIF scores in tumors with high TILs ($P = .007$) than low ($P = .02$).

PD-L1 Comparison Using Different PD-L1 Antibodies and IHC

Cohen κ coefficients were calculated between the antibodies at cutoffs of 1%, 5%, and 50% in tumors and 5% in stroma, which have all been previously defined as positive cutoffs used in clinical trials. Kappa concordance between antibodies was low, irrespective of the cutoff used (eTable 2 in the Supplement).

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Table. Clinicopathological Characteristics of NSCLC Cohort

Characteristic	Patients, No. (%)	E1L3N QIF, Mean (SD), AU	*P* Value	SP142 QIF, Mean (SD), AU	*P* Value
All patients	49 (100)				
Age, y			.36		.14
<70	24 (49)	719.2 (131.6)		194.0 (45.7)	
≥70	25 (51)	1140.0 (425.3)		1083.0 (583.3)	
Sex			.29		.28
Male	19 (38.8)	627.2 (128.5)		911.8 (489.9)	
Female	30 (61.2)	1128.0 (358.3)		230.1 (46.9)	
Smoking status			.93		.42
Smoker	43 (87.8)	686.6 (84.5)		275.4 (49.1)	
Never	4 (8.2)	662.4 (212.6)		141.7 (73.4)	
Unknown	2 (4.0)				
Histology			.85		.57
Adenocarcinoma	36 (73.5)	894.0 (299.9)		665.5 (390.0)	
Squamous	10 (20.4)	1002.0 (202.2)		235.7 (108.0)	
Other	3 (6.1)				
Stage			.30		.21
I	32 (65.3)	760.4 (119.4)		380.3 (154.5)	
II-IV	17 (34.7)	1261.0 (616.4)		1199.0 (872.1)	
Tumor size, cm			.41		.48
<3	29 (59.2)	764.1 (139.7)		450.7 (189.8)	
≥3	20 (40.8)	1142.0 (476.4)		889.0 (636.8)	
Lymph node status			.06		.03
Negative	40 (81.6)	731.6 (98.4)		339.2 (128.1)	
Positive	9 (18.4)	1833.0 (1156.0)		2018.0 (1531.0)	

Abbreviations: AU, arbitrary units; NSCLC, non–small-cell lung cancer; QIF, quantitative immunofluorescence.

PD-L1 Comparison Using Different PD-L1 Antibodies and QIF

PD-L1 QIF scores were obtained from 5 to 24 FOV for each WTS, with nearly every case having at least 10 FOV. Distribution of QIF for each of the 49 different cases is shown. The majority of cases showed a wide variation in QIF scores and differences between PD-L1 protein expression within the same case. When QIF scores for 588 FOVs were compared using E1L3N vs their serial sections using SP142, we found 26.6% discordance, including 51 (8.6%) positive by SP142 and negative by E1L3N, and 106 (18.0%) positive by E1L3N and negative by SP142. These FOVs included both epithelial and stroma FOVs.

The total, mean, and maximum QIF scores measured in the tumor for each case and QIF scores from the corresponding TMA were compared between the PD-L1 antibodies (eFigure 3 in the Supplement). Between the antibodies E1L3N and SP142, the Pearson correlation coefficient was calculated to be 0.81 ; mean, 0.91 ; and maximum, 0.83 ($P < .001$ for all). The scores are driven by few high values. The Spearman rank correlation coefficient, however, was estimated to be 0.21 ($P < .001$); mean, 0.14 ($P = .34$); and maximum, 0.13 ($P = .37$). This indicates that the significance in the Pearson correlation coefficients was mainly driven by outliers (eFigure 3 in the Supplement). The majority of the measurements from the 2 antibodies were not significantly correlated according to the Spearman correlation as a rank-based metric. For paired means, medians, and maximums from the 2 antibodies, both the sign test and the Wilcoxon signed-rank test led to *P* values less than 0.001 for the total, mean, and maximum scores, indicating significant discrepancy between measurements from the 2 antibodies. Quantitative immunofluorescence scores were also measured in the stroma and compared with scores measured in the tumor for each antibody. Quantitative immunofluorescence scores between the stroma and tumor showed a high association for each PD-L1 antibody (data not shown).

The QIF score (**Figure 3**) of each TMA case was compared with the corresponding mean WTS QIF score for the same case (eFigure 4 in the Supplement). The Pearson correlation coefficients were calculated to be 0.75 ($P < .001$) for E1L3N and 0.98 ($P < .001$) for SP142 but were driven by few high values. The Spearman rank correlation coefficients, however, were estimated to be 0.20 ($P = .17$) for E1L3N and 0.52 ($P < .001$) for SP142. This indicates that the significance in the Pearson correlation coefficients was mainly driven by outliers.

Discussion

Recent studies suggest that determination of PD-L1 status in tumors may help predict response to novel anti–PD-1 and anti–PD-LI monoclonal antibody therapy.[15-21,33] However, each therapeutic study used its own companion diagnostic methods and antibodies without reference to a common standard. Our results suggest that PD-L1 protein expression is heterogeneous and that different antibody assays may yield discordant results. As shown in **Figure 4**, 2 different antibodies showed that over 25% of patients who were positive by 1 an-

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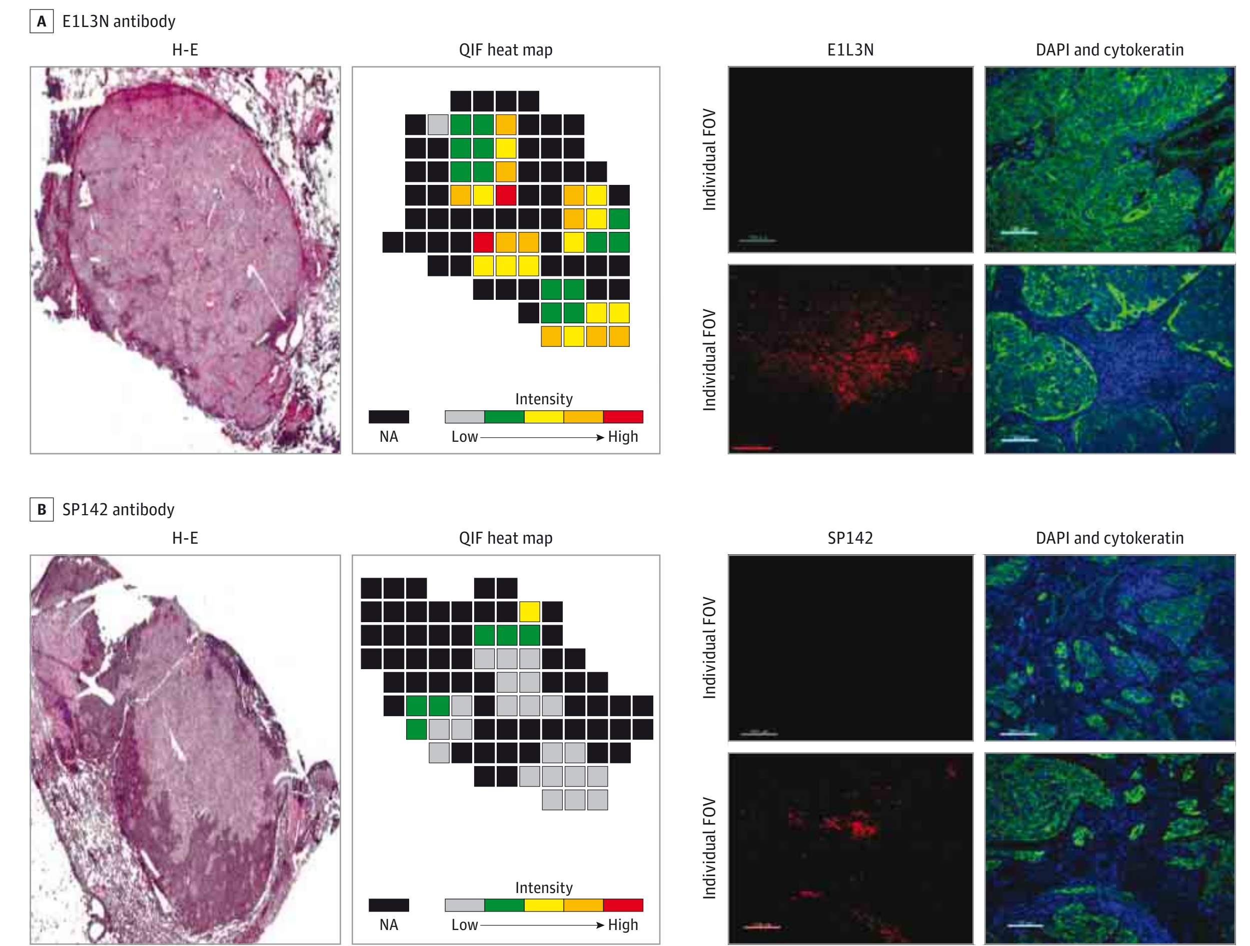
Figure 2. PD-L1 Protein Heterogeneity Using QIF

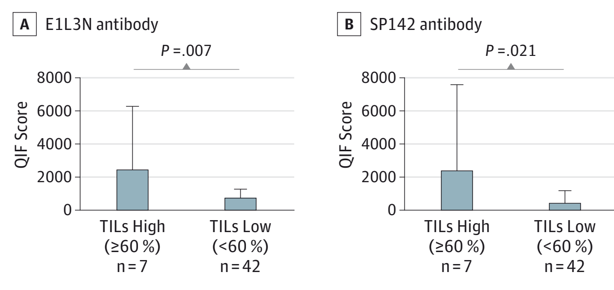


Intratumor PD-L1 protein heterogeneity with QIF in different cases using antibodies E1L3N (A) and SP142 (B). Heat map QIF scores range from gray (low) to high (red), and black squares represent FOV in which QIF scores were not calculated (lack of tumor, poor quality, etc). Representative positive and negative FOV from each heat map case are shown using immunofluorescence. PD-L1 protein represented in the red channel; DAPI (nuclear), blue channel; and cytokeratin (tumor), green channel. DAPI indicates 4', 6-diamidino-2-phenylindole; FOV, fields of view; H-E, hematoxylin-eosin; PD-L1, programmed cell death 1 ligand 1; QIF, quantitative immunofluorescence.

tibody were negative (below the threshold) by the other. This is concerning because PD-L1 antibodies and platforms used by pharmaceutical companies for their clinical trials are proprietary, and thus, there is no opportunity for comparison between methods or reagents. Recently, Ventana made their SP142 companion diagnostic antibody available. It is tested herein but shown not to be concordant with another validated PD-L1 antibody not currently used in any companion diagnostic test. Two new rabbit monoclonal antibodies, PD-L1 28-8 (Abcam) and SP263 (Ventana), were recently released and comparisons with these antibodies is under way.

The PD-L1 protein expression has been noted to be heterogeneous, though it has not been demonstrated in a quantitative, validated assay.[34] While this heterogeneity is difficult to objectively demonstrate using traditional IHC methods, QIF allows for more objective measurement. The inherent heterogeneity of PD-L1 may partly explain the contradictory role of PD-L1 as a predictive biomarker to anti-PD-1 and anti-PD-L1 antibodies seen in clinical trials.[1,15-21,26,27] For instance, some tumors deemed PD-L1 negative may be negative at the biopsied site but may be positive at another location. This phenomenon may also partly explain so-called mixed responses

Figure 3. PD-L1 Protein Correlation With TILs Using QIF



Error bars represent standard deviation. QIF scores measured in arbitrary units. PD-L1 indicates programmed cell death 1 ligand 1; TILs, tumor-infiltrating lymphocytes; QIF, quantitative immunofluorescence.

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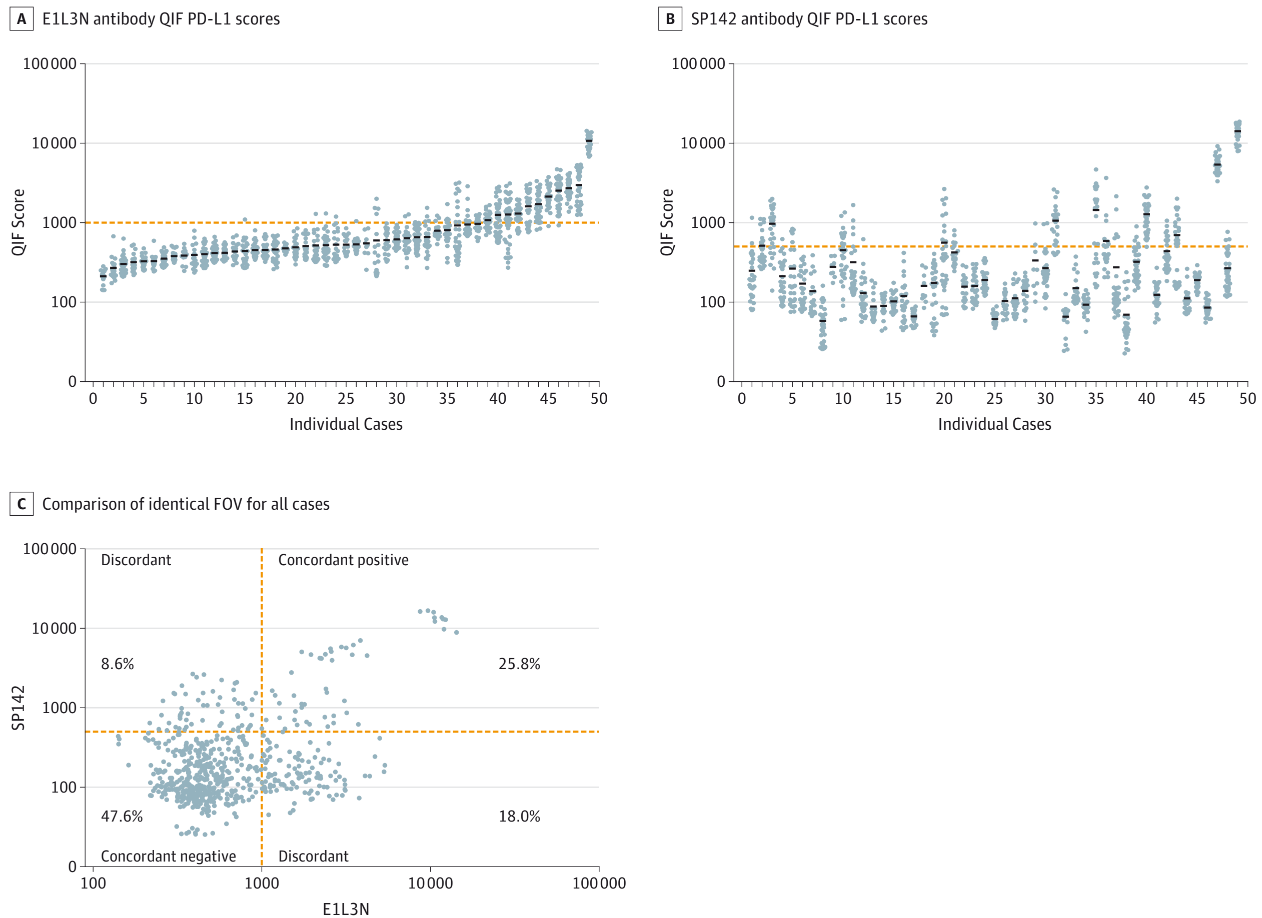
Figure 4. FOV Comparison of 2 PD-L1 Antibodies Using QIF



A and B, Mean score represented by black bar. Scores represented in arbitrary units. C, Dotted red line represents visual detection threshold for both antibodies. Scores represented in arbitrary units. FOV indicates fields of view; PD-L1, programmed cell death 1 ligand 1; QIF, quantitative immunofluorescence.

seen between different tumor sites in clinical trials. Despite the PD-L1 heterogeneity, it is notable that PD-L1 expression, as detected by either antibody, is significantly correlated with TILs using QIF.

Inherent differences between validated PD-L1 antibodies have also not been reported. Many of the commercially available PD-L1 antibodies have not been thoroughly validated, leading to conflicting results regarding PD-L1 expression and correlation to overall survival and the presence of TILs.[14,35-38] We compared 2 different validated PD-L1 antibodies: E1L3N and SP142. Using traditional IHC methods, we show that concordance between 2 rigorously validated antibodies is fair to poor. Comparison of both antibodies using QIF demonstrated that the 2 antibodies have low correlation and are statistically different than each other in identical FOVs in the same cases. While both E1L3N and SP142 reportedly bind to the intracellular domain of PD-L1, the difference between the 2 antibodies raises concerns and suggests antibody validation data should be shown in future clinical trial reports. The assay performance data has been largely absent in clinical trial reports to date, even those reporting benefit in IHC-selected groups.[39]

Our analysis has a number of limitations that could not be addressed in this first quantitative study. One major limitation is that it only includes recent retrospectively collected cases and that mature survival information is therefore not yet available. A second is that we selected only 49 cases for analysis. The low number of cases makes it difficult to draw conclusions in terms of associations with clinicopathological characteristics and outcomes, although the cases are representative of an average population of NSCLC and the study is sufficiently powered for the comparison of 2 antibodies. A third limitation is the lack of data available for response to anti-PD-1 and anti-PD-L1 monoclonal antibodies in this patient population. In the future, we hope to be able to do similar studies on material from patients treated with anti-PD-1 therapy.

One key unaddressed issue is the potential of a quantitative assay (eg, protein, mRNA) to predict response to anti-PD-1 and anti-PD-L1 therapies along with the potential differences between PD-L1 binding antibodies. However, access to tissue from treated patients is still challenging because the PD-1 axis therapies have only recently been approved in lung cancer.

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Conclusions

Future studies measuring PD-L1 protein quantitatively in patients treated with anti–PD-1 and anti–PD-L1 therapies may better address the prognostic and/or predictive value of these biomarkers. Determination of the optimal assay, PD-L1 antibody, and the best cut-point for PD-L1 positivity, will require further rigorous studies including tissues with known response to anti–PD-1 and anti–PD-L1 therapies.

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Invited Commentary

PD-L1 Expression as a Predictive Biomarker

Is Absence of Proof the Same as Proof of Absence?

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As targeted therapies are superior to standard chemotherapy in select patients, effective predictive biomarkers are needed to identify patients who are more likely to respond to targeted therapies. Using a tumor sample, biomarker testing is often protein- or nucleic acid-based. In cases of non-small-cell lung cancer (NSCLC), tumor cells are tested for epidermal growth factor receptor mutations and anaplastic lymphoma kinase rearrangements, and the results help the oncologist to tailor therapy based on individual tumor genetics.

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For immune-based therapies, especially those that target the PD-1 (programmed cell death 1 protein) and PD-L1 (programmed cell death 1 ligand 1) axis, immunohistochemical staining is a companion diagnostic tool to facilitate therapeutic decision making. The PD-1/PD-L1 pathway and its role in human cancers is complex. The PD-1 receptor binds PD-L1 and PD-L2 (programmed cell death 2 ligand 2) ligands and hinders immune destruction of tumor cells. Moreover, when PD-L1 is upregulated, certain tumor types are more biologically aggressive, leading to poor patient outcomes. Thus, emerging anti–PD-1/PD-L1 immune therapies aim to block PD-1/PD-L1 interaction, allowing T lymphocytes to attack tumor cells, improving clinical outcomes in a subset of patients. Early-phase trials[1] demonstrated that patients with tumors that lacked PD-L1 expression were unlikely to respond to anti–PD-1 targeted therapy (ie, PD-L1 had a high negative predictive value). Subsequent work has established, however, that across many cancer types, patients with PD-L1–negative tumors show an aggregate 15% response rate, compared with a 48% response rate among patients with PD-L1–positive tumors.[2] Unfortunately, some anti–PD-1/PD-L1 clinical trials still exclude patients with PD-L1–negative tumors. Accurate immunohistochemical interpretation is essential to avoid false-negative PD-L1 interpretation and subsequent therapeutic exclusion.

While most studies focus on PD-L1 expression among tumor cells, it is increasingly recognized that inflammatory cells within, adjacent to, or outside of the tumor mass may also express PD-L1, and these PD-L1–positive cells may represent the host immune response to the tumor cells. Interestingly, some microsatellite instable colon cancers also demonstrate upregulation of PD-L1 among other immune checkpoints, which represents an additional potential therapeutic biomarker.[3]

The current study by McLaughlin et al[4] addresses several factors that contribute to false-negative PD-L1 findings. First, tumor samples may be inadequate or not representative of the entire tumor mass. While diagnostic sampling usually represents only a small fraction of a patient's tumor, complete surgical resections are similarly limited, as only representative sections are submitted for microscopic evaluation. Typically, only 0.001% of a 5 cm spherical tumor volume is examined microscopically. As McLaughlin et al[4] demonstrate, PD-L1 expression is heterogeneous and very focally expressed in NSCLC biopsy samples. As illustrated by Figure 1 in the article and eFigure 2 in the supplement,[4] there is heterogeneous expression in different regions of the same tumor specimen and in various sections of the same tumor. Interestingly, they report a statistically nonsignificant trend of PD-L1 expression at tumor and nontumor interfaces, suggesting that the interface may represent the host immune reaction to invading tumor cells.

Second, as there are at least 12 different anti–PD-L1–directed antibodies, it is not surprising to see considerable variation in the targeted epitope, isotype, and source (eg, recombinant vs polyclonal antibodies). In some cases, the details of the antibody are proprietary and therefore undisclosed. As the performance of different antibodies on identical tumor samples has not been examined to date, McLaughlin et al[4] address this issue by comparing the performance of 2 commonly used rabbit monoclonal antibodies (SP142 and E1L3N) and report low κ concordance rates regardless of the 1%, 5%, and 50% cut points used to determine if a tumor sample expressed PD-L1. As shown in Figure 4 in the article by McLaughlin et al, nearly 25% of tumor samples that were positive for PD-L1 using one antibody were negative using the second antibody. Despite current planning to make cross platform com-

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