Received: 15 June 2022 | Accepted: 3 October 2022

DOI: 10.1111/1759-7714.14695

ORIGINAL ARTICLE

WILEY

Modifying factors of PD-L1 expression on tumor cells in advanced non-small-cell lung cancer

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Abstract

Background: Programmed death ligand-1 (PD-L1) expression predicts immunotherapy utility in nononcogenic addictive lung adenocarcinoma (ADC). However, its reproducibility and reliability may be compromised outside clinical trials. This study aimed to evaluate factors associated with PD-L1 expression in lung ADC.

Methods: This observational study assessed 547 tumor samples with advanced lung ADC from January 2016 to December 2020 in a single cancer institution. Tumor samples were stained by at least one approved PD-L1 clone, SP263 (Ventana) or 22C3 (Dako), and stratified in tumor proportion score (TPS) <1%, 1–49%, or ≥50%.

Results: Of all the tumor samples, positive PD-L1 staining was higher in poorly differentiated tumors (67.3% vs. 32.7%, $p < 0.001$). Analytical factors associated with a PD-L1 high expression (TPS ≥ 50%) were the SP263 clone (19.6% vs. 8.2%, $p < 0.001$), time of archival tumor tissue <12 months (15.3% vs. 3.8%, $p = 0.024$), whenever the analysis was performed in the most recent years (2019–2020) (19.0% vs. 8.3%, $p < 0.001$), and whenever the analysis was performed by pathologists in the academic setting (Instituto Nacional de Cancerologia, INCan) (19.9% vs. 11.9%, $p = 0.001$). In the molecular analysis, EGFR wild-type tumors had an increased proportion of PD-L1 positive and PD-L1 high cases (60.2% vs. 47.9%, $p = 0.006$ and 17.4% vs.8.5%, $p = 0.004$). A moderate correlation ($r = 0.69$) in the PD-L1 TPS% was observed between the two different settings (INCan vs. external laboratories).

Conclusion: Clinicopathological factors were associated with an increased PD-L1 positivity rate. These differences were significant in the PD-L1 high group and associated with the academic setting, the SPS263 clone, time of archival tumor tissue <12 months, and a more recent period in the PD-L1 analysis.

KEYWORDS
immunotherapy, lung adenocarcinoma, non-small-cell lung cancer, PD-L1 immunohistochemistry, programmed death-ligand 1

BACKGROUND

Programmed cell death-1 (PD-1) and programmed death ligand-1 (PD-L1) have become the most studied biomarkers in lung cancer.[1] Regardless of the expression site, both

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proteins are triggering immune checkpoints that negatively regulate the adaptive immune response of T cells, which maintains the peripheral tolerance.[2] PD-L1 is an immune regulatory protein that may be expressed on tumor cells as well as inflammatory cells, predominantly in tumor-associated macrophages. Binding to its two receptors, PD-1 and B7-1, inhibits T-cell proliferation, cytokine production, and cytolytic activity, leading to either inactivation or T-cell exhaustion.[3] Humanized antibodies directed to the PD-1/PD-L1 axis have changed the paradigm of treatment landscape, first, in the metastatic setting and, more recently, in the localized disease by restoring antitumor immunity.[4] However, as it is known, in most scenarios, only a minor proportion (20–40%) of patients will benefit from the anti-PD-1/PD-L1 blockade, and an even smaller proportion of patients (5.4–6.4%) will achieve a substantial durability (≥24 months) of treatment effect.[5]

Clinically, PD-L1 expression is detected by immunohistochemistry (IHC) techniques and is a helpful predictive biomarker of response to immune checkpoint inhibitors (ICIs) in patients with non-small-cell lung cancer (NSCLC).[6,7] For diagnosis, four antibody assays approved by the Food and Drug Administration are broadly used to assess PD-L1 expression, with a specific immunohistochemical assay (28–8, 22C3, SP263, and SP142) for each commercially available ICI.[8] Moreover, every PD-L1 clone demands a specific staining platform and scoring method, which leads to discrepancies between assays.[9] One previous meta-analysis evaluated the interassay concordance in NSCLC tumor cells and reported a high agreement between assays 28–8, 22C3-, and SP263.[4] In contrast, those comparisons which involved the SP142-based assays demonstrated a lower level of concordance, as it was, when the PD-L1 expression was evaluated on immune cells.[10]

In addition, many preanalytical and post-analytical factors contribute to irregularities in PD-L1 results, which might have direct implications for guiding therapy based on current treatment guidelines.[11] Even though some analytical characteristics and variability in staining procedures in noncontrolled environments have been assessed, there is no consensus about the impact on the reliability of PD-L1 reports in real-world settings.[12] Moreover, in low- and middle-income countries, due to a lack of resources, there is a need for surrogate PD-L1 analyses outside the main health facility, which risks introducing more variability in the staining procedure.

This study focused on assessing the clinicopathological and molecular characteristics associated with different levels of PD-L1 expression in lung adenocarcinoma (ADC) tumor samples. In addition, relevant analytical variables were examined to determine their contribution to PD-L1 expression in a real-world context.

METHODS

This study retrospectively analyzed data from patients with a confirmed diagnosis with recurrent or metastatic lung ADC with an available PD-L1 determination or with enough tissue to process this analysis. All patients were diagnosed and treated at a single cancer center, the Instituto Nacional de Cancerología (INCan) in Mexico, between January 10, 2016, and December 28, 2020. Medical records were evaluated by a multidisciplinary team that included medical oncologists, an expert pathologist, and a molecular biologist to obtain relevant data. Individual patient information remained confidential during the entire protocol and clinical decisions were not influenced based on these results. The Ethics Committee of INCan waived individual informed consent. The whole study was conducted following the Declaration of Helsinki and the Principles of Good Clinical Practice.

All clinical and histopathological variables of interest were included in the INCan database, comprising age, gender, smoking history, woodsmoke exposure history, targetable molecular alterations (*EGFR* and *ALK*), tumor differentiation grade, and predominant ADC pattern based on the International Association of the Study of Lung Cancer/American Thoracic Society/European Respiratory Society classification. Predominant histological subtype was defined based on the tumor architecture according to the lung ADC classification, which subdivides into lepidic (LEP) acinar (ACI), papillary (PAP), micropapillary (MIP), and solid (SOL).[13]

All variables of interest concerning PD-L1 expression were extracted from the electronic medical records and comprised the following: PD-L1 tumor proportion score (TPS), PD-L1 assay employed for the IHC analysis, location where the staining process took place (internal or external laboratories), and period that elapsed between the biopsy and the PD-L1 staining procedure (archived period). Patients without a complete PD-L1 report or unavailable archived tissue were automatically excluded and when missing data exceeded 20% of predetermined clinical or histopathological variables.

PD-L1 immunohistochemistry

For histopathological diagnosis confirmation, each specimen was cut into 3-μm sections and stained with hematoxylin–eosin. The immunohistochemical analysis employed formalin-fixed, paraffin-embedded tissue sections to determine PD-L1 protein expression. Two PD-L1 assays were used for the immunohistochemical analysis: the 22C3 clone (PharmDx IHC assay, Dako North America, Inc.) and the SP263 (OptiView DAB IHC, Ventana). Both were processed according to the corresponding manufacturer manual by one or two expert pathologists. Whenever PD-L1 was analyzed in different settings or if two reports in the same sample were discordant, the highest PD-L1 TPS% was considered for the analysis.

Scoring PD-L1 expression

PD-L1 TPS was defined as the percentage of viable tumor cells showing partial or complete membranous staining of any intensity relative to the total number of viable tumor cells present in the sample.[8] PD-L1 TPS scores were

reported according to the most frequently clinically employed cut-off points: samples with <1% TPS were counted as PD-L1 negative, samples with a TPS of 1–49% were classified as PD-L1 positive, and samples with PD-L1 ≥50% were counted as high PD-L1 expression.[14,15]

Then we analyzed PD-L1 expression according to each setting in which it was performed, the year in which the PD-L1 expression was determined (2016–2018 vs. 2019–2020), archived period (< or ≥12 months) of FFPE tissue samples, and the PD-L1 clone (SP263 vs. 22C3) employed. Whenever the complete process (tissue biopsy, fixation, and staining) was performed in INCan, it was grouped in the academic or internal category. Otherwise, when the PD-L1 staining or the entire process was completed in external laboratories, the sample was categorized as external.

EGFR and ALK analysis

ALK gene rearrangement was determined using an LSI ALK Dual Color, Break Apart Rearrangements Probe from Vysis, and the assay was performed according to manufacturer instructions.[16] DNA was extracted from areas of paraffin slides using QIAamp DNA FFPE Tissue Kit (Qiagen). For EGFR mutational profile determination, all *EGFR* mutations (exons 18, 19, 20, and 21) were detected by Therascreen RGQ PCR Kit (Qiagen, Scorpions ARMS method), using real-time PCR performed in a Rotor-Gene Q 5-plex HRM (Qiagen) according to the manufacturer's instructions.

Statistical analysis

Categorical variables were reported as frequencies and percentages. All comparisons among categorical variables were analyzed by the chi-square test or Fisher's exact test. For continuous variables they were summarized as means and standard deviations or medians and percentiles 25–75, based on data distribution. Comparisons between two continuous variables [i.e., PD-L1 (−) vs. PPD-L1(+)] were evaluated using a Student's *t*-test for independent variables with normal distribution, otherwise the Mann Whitney *U*-test was used for those with an abnormal distribution. The parametric one-way ANOVA test was used for multiple group comparisons and the two-way ANOVA test was employed for comparisons of continuous variables with more than one independent variable or factor. The intra-class correlation coefficient (ICC) was performed to set correlations between two PD-L1 results as continuous variables. A predetermined $p < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS software platform, version 26 (SPSS Inc.).

RESULTS

Patients' baseline characteristics according to the PD-L1 TPS (%) expression range are shown in Table 1. The study included samples from 316 (57.8%) females and 231 (42.2%) males, of which 334 (61.1%) were 60 years of age or older. A total of 547 tumor samples with a confirmed advanced lung ADC diagnosis were eligible for the analyses. PD-L1 expression was determined by pathologists in INCan in 151 (27.6%) cases and 396 (72.4%) tumor samples by pathologists in external laboratories.

Clinical and pathological factors associated with each PD-L1 expression range

In total, 243 (44.4%) tumor samples were categorized as PD-L1 negative (PD-L1 < 1%), 227 (41.5%) had an intermediate expression (PD-L1 1–49%), and 77 (14.0%) were PD-L1 high (≥50%). None of the clinical variables (gender, age, or smoking status) were significantly associated with a PD-L1 expression range. In contrast, some histopathological variables were associated with a higher proportion of tumor samples with PD-L1 positive and PD-L1 high expression. Tumor samples classified as PD-L1 positive (≥1%) were significantly associated with poorly differentiated tumors (33.6% vs. 64.9%, $p < 0.001$). Those tumors with a PD-L1 high (TPS ≥ 50%) expression also occurred more frequently in poorly differentiated tumors than in moderately and well-differentiated tumors (19.5% vs. 15.7%, $p = 0.013$). These results agreed with the PD-L1 distribution according to the distinct lung ADC subtypes, in which the SOL and MIP pattern had the highest proportion of PD-L1 positive tumor samples (MIP 25%, SOL 32.5% vs. ACI 50% vs. LEP 62.5% vs. PAP 58.8%, $p < 0.001$). Also, the greatest number of cases classified as PD-L1 high occurred in the samples classified as SOL, compared with other subtypes (SOL 20.9% vs. ACI 10.6 vs. LEP 5.0 vs. PAP 3.9%, $p < 0.003$). None of the cases with a MIP pattern were classified as PD-L1 high, but this analysis was limited due to a low proportion of cases with a MIP pattern in this cohort. An invasive mucinous ADC was observed in 11 (2%) tumor samples; of these nine (81.8%) cases were classified as PD-L1 negative and two (18.2%) as PD-L1 positive. Table 1 summarizes the clinical and histopathological characteristics of the overall population according to its respective PD-L1 TPS (%) range.

After evaluating tumor samples corresponding to their mutational profile, those harboring an EGFR wild type had a higher proportion of cases classified as PD-L1 positive (60.9% vs. 34.5%, $p = 0.006$). Similarly, samples categorized as PD-L1 high expression remained more frequent in the EGFR wild-type subgroup (17.4% vs. 8.5%, $p = 0.005$), respectively.

Next, PD-L1 expression was evaluated as a continuous variable according to each differentiation grade. Hence, the mean PD-L1 expression was significantly higher in poorly differentiated tumors than in moderately ($p < 0.001$) and well-differentiated ($p < 0.001$) tumors, and no significant differences were observed between moderately and differentiated tumors (Figure 1).

TABLE 1 Patients' baseline characteristics according to the PD-L1 TPS (%) expression range

		PD-L1 (−)	TPS 1%–49%	TPS ≥ 50%	*p*
Total population	547 (100)	243 (44.4)	227 (41.5)	77 (14.0)	
Gender					
Female	316 (57.8)	148 (46.8)	128 (40.5)	40 (12.7)	0.328
Male	231 (42.2)	95 (41.1)	99 (42.9)	37 (16.6)	
Age					
<60	213 (38.9)	94 (44.1)	83 (39.0)	36 (16.9)	0.283
≥60	334 (61.1)	149 (44.6)	144 (43.1)	41 (12.3)	
Smoking					
No	265 (48.5)	121 (45.8)	110 (41.7)	33 (12.5)	0.692
Yes	219 (40.0)	92 (43.4)	88 (41.5)	32 (15.1)	
Unknown	63 (11.5)				
Differentiation					
Poorly differentiated	220 (40.2)	74 (33.6)	103 (46.8)	43 (19.5)	**<0.001**
Moderately differentiated	254 (46.4)	130 (51.2)	98 (38.6)	26 (10.2)	
Well differentiated	37 (6.8)	24 (64.9)	11 (29.7)	2 (5.4)	
Unknown	36 (6.6)				
Histological subtype					
Solid	163 (29.8)	53 (32.5)	76 (46.6)	34 (20.9)	**< 0.001**
Acinar	160 (29.3)	80 (50.0)	63 (39.4)	17 (10.6)	
Lepidic	40 (7.3)	25 (62.5)	13 (32.5)	2 (5.0)	
Papillary	51 (9.3)	30 (58.8)	19 (37.3)	2 (3.9)	
Micropapillary	4 (0.7)	1 (25.0)	3 (75)		
Unknown	118 (21.5)				
Mucinous histology					
Yes	11 (2.0)	9 (81.8)	2 (18.2)		**0.037**
EGFR					
WT	333 (60.9)	133 (39.9)	142 (42.6)	58 (17.4)	**0.004**
Mutation	189 (34.5)	99 (51.9)	76 (39.7)	17 (8.5)	
Unknown	25 (4.6)				
ALK					
WT	336 (61.4)	153 (45.5)	130 (38.7)	53 (15.8)	0.057
Rearrangement	59 (10.8)	17 (28.8)	30 (50.8)	12 (20.3)	
Unknown	152 (27.8)				

Note: Data are shown as the percentage of the number *n* (%). Statistical analysis was performed by the parametric unpaired Student's *t*-test for independent samples, considering a 95% of confidence, with $p \leq 0.05$. Significance at $p \leq 0.05$. Statistically significant *p* values are in bold type.
Abbreviations: ALK, anaplastic lymphoma kinase; EGFR, epidermal growth factor receptor; PD-L1, programmed death ligand-1; TPS, tumor proportion score; WT, wild type.

PD-L1 expression according to analytical variables

We analyzed PD-L1 expression according to the PD-L1 assay employed (SP263 vs. 22C3), year (prior 2019 vs. 2019–2020), place in which the staining process was completed (internal INCan vs. external laboratories), and time of archived tissue (< or ≥12 months) (Table 2).

In the assessment according to the two PD-L1 clones (SP263 or 22C3), both showed similar performance in identifying tumor samples with a negative expression (41.8% vs. 47.2%), respectively. However, discrepancies emerged for cases with a high expression in which the SP263 clone identified a higher percentage of tumor samples classified as PD-L1 high (19.6% vs. 8.2%, $p = 0.001$). The PD-L1 expression also showed variations based on the clinical setting that completed the PD-L1 analysis. In the INCan sample we observed a higher proportion of cases with PD-L1 high (19.9% vs. 11.9%, $p < 0.001$) expression. No significant differences were observed between patients with a negative or an intermediate expression between both settings.

We then analyzed how the PD-L1 reports changed throughout the years, comparing two different periods, those analyzed before 2019 and those from 2019 to 2020.

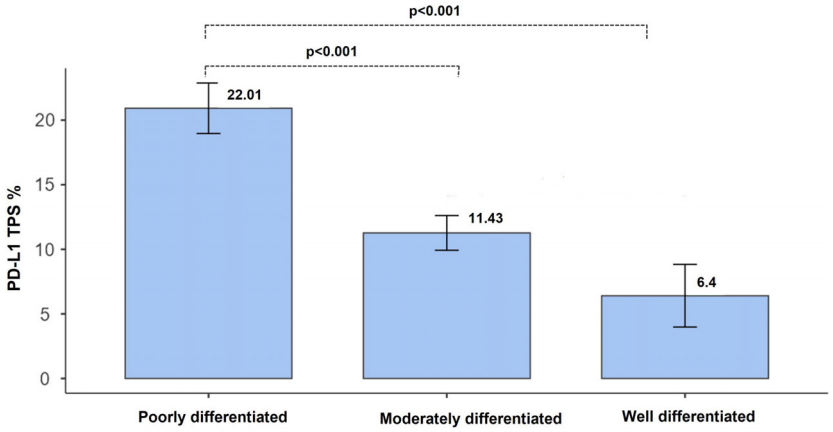

FIGURE 1 Mean PD- L1 TPS (%) expression according to each differentiation grade: poorly differentiated, moderately differentiated, and well differentiated. Statistical analysis was performed by parametric unpaired Student's *t*-test for independent samples, considering significance at $p \leq 0.05$.

TABLE 2 PD-L1 TPS (%) reports based on the PD-L1 clone, pathologists who accomplished the staining process, and the period in which the immunohistochemistry analyses were performed

		PD-L1 (−)	TPS 1–49%	TPS ≥ 50%	*p*
Total	547 (100)	243 (47.9)	227 (41.5)	77 (14.0)	
PD-L1 assay					
SP263	280 (51.2)	117 (41.8)	108 (38.6)	55 (19.6)	**<0.001**
22C3	267 (48.8)	126 (47.2)	119 (44.6)	22 (8.2)	
Pathologists (origin)					
Internal INCan (academic)	151 (27.6)	76 (50.3)	45 (29.8)	30 (19.9)	**0.001**
External laboratories	396 (72.4)	167 (42.1)	182 (45.9)	47 (11.9)	
Year					
<2019	253 (46.3)	123 (48.6)	109 (43.1)	21 (8.3)	**0.001**
2019–2020	294 (53.7)	120 (40.8)	118 (40.1)	56 (19.0)	
Time from diagnosis to IHC	495 (100)				0.073
<12 months		192 (43.4)	183 (41.3)	68 (15.3)	
≥12 months		27 (51.9)	23 (44.2)	2 (3.8)	

Note: Data are shown as the percentage of the number *n* (%). Statistical analysis was performed by the parametric unpaired Student's *t*-test for independent samples, considering a 95% of confidence, with $p \leq 0.05$. Significance at $p \leq 0.05$. Statistically significant *p* values are in bold type.
Abbreviations: IHC, immunohistochemistry; INCan, National Cancer Institute; PD-L1, programmed death ligand-1; TPS, tumor proportion score.

Tumor samples categorized as PD-L1 high occurred more frequently in those analyzed in more recent years (2019–2020) than in previous years (19.0% vs. 8.3%, $p < 0.001$). In contrast, those samples classified as PD-L1 negative and with an intermediate expression were similar. Finally, the time elapsed from diagnosis to the IHC analysis was also associated with an increased fading of PD-L1 expression. Those patients with archived tumor tissue >12 months showed fewer cases classified as PD-L1 high (15.3 vs. 3.8%, $p = 0.001$) (Table 2).

Mean PD-L1 TPS (%) was significantly higher whenever the PD-L1 analysis was by the internal INCan laboratory compared with external laboratories ($p = 0.002$). Similarly, it was higher whenever the SP263 clone was the chosen assay for the analysis ($p = 0.006$) when the analysis was performed in the more recent period, 2019–2020 ($p = 0.009$), and when the archival tumor tissue was processed within the first 12 months after biopsy ($p = 0.007$) (Figure 2).

Next, we performed a separate analysis for the INCan and external laboratory samples. Both PD-L1 assays (SP263 and 22C3) were comparable, identifying each subgroup based on the predefined PD-L1 expression range within INCan, but the proportion of cases classified as PD-L1 high was higher for the 22C3 assay (21.5% vs. 6.3%), although this was not significant. For those samples evaluated in external laboratories, the SP263 clone showed a higher proportion of PD-L1 high tumor samples (17.9% vs. 8.4%, $p < 0.08$). In the case of those samples analyzed at INCan, the period in which the PD-L1 analysis was performed did not show significant differences, but tumor samples classified with high expression were more common between 2019-2020 (21.1% vs. 11.1%). In external laboratories, PD-L1 reports showed an increased variability over time, reporting a higher proportion of PD-L1 high cases in 2019–2020 compared with previous years (17.4% vs. 8.4%, $p < 0.001$) (Table 3).

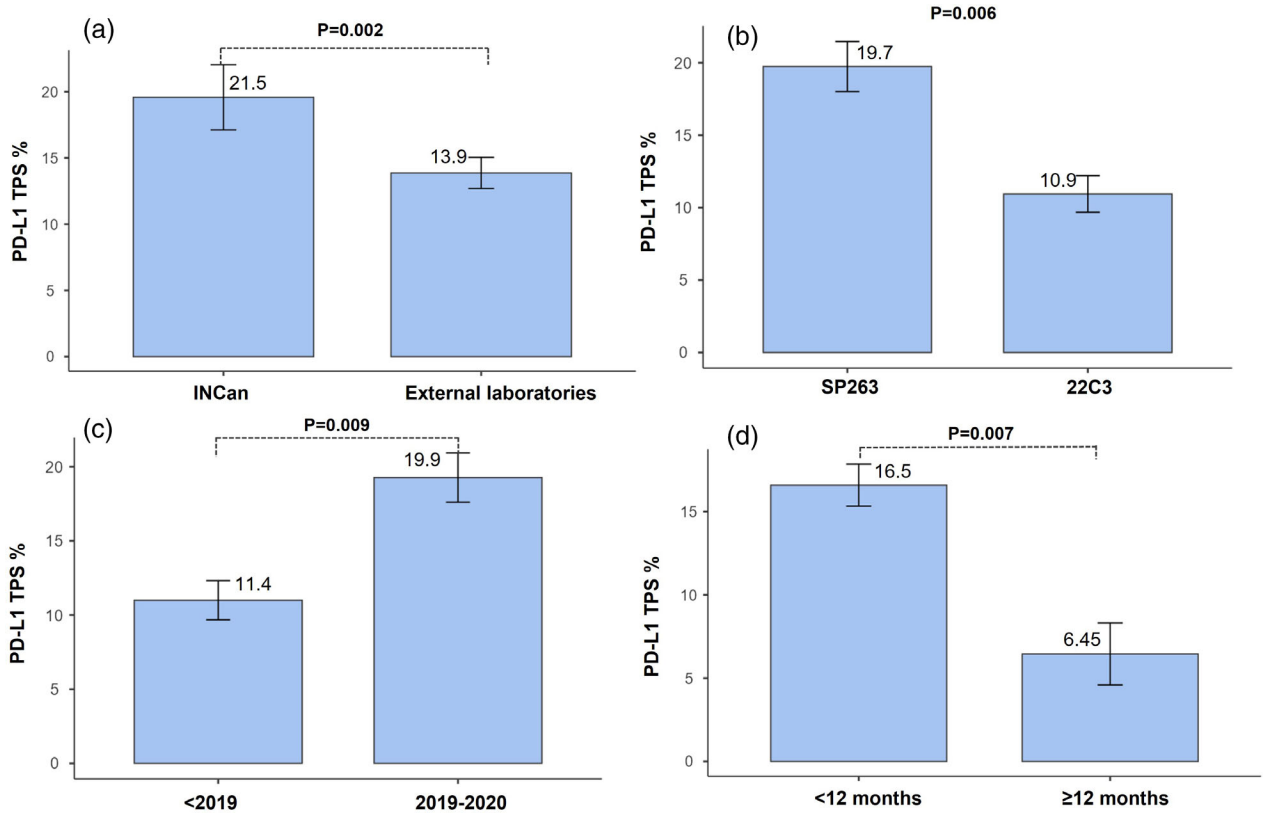

FIGURE 2 Mean PD-L1 TPS stratified by (a) the place (internal INCan vs. external laboratories) in which the staining process was completed, (b) the PD-L1 clone employed (SP263 vs. 22C3), (c) the year (prior to 2019 vs. 2019–2020), and (d) the time of archived tissue (< or ≥12 months). Statistical analysis was performed by parametric unpaired Student's *t*-test for independent samples, considering significance at $p \leq 0.05$.

TABLE 3 PD-L1 TPS (%) categorization according to the PD-L1 clone employed and the evaluation period when pathologists belonged to an academic center or external laboratories

		PD-L1 (−)	TPS 1–49%	TPS ≥ 50%	*p*
Internal INCan (academic)	**231 (100)**	**116 (50.2)**	**75 (32.5)**	**40 (17.3)**	
Clone					
SP263	135 (89.4)	67 (49.6)	39 (28.9)	29 (21.5)	0.341
22C3	16 (10.6)	9 (56.3)	6 (37.5)	1 (6.3)	
Year					
<2019	18 (11.9)	7 (38.9)	9 (50.0)	2 (11.1)	0.128
2019–2020	133 (88.1)	69 (51.9)	36 (27.1)	28 (21.1)	
External laboratories	**396 (100)**	**211 (46.6)**	**194 (42.8)**	**48 (10.6)**	
Clone					
SP263	145 (36.6)	50 (34.5)	69 (47.6)	26 (17.9)	**0.005**
22C3	251 (63.4)	117 (46.6)	113 (45.0)	21 (8.4)	
Year					
<2019	235 (59.3)	116 (49.4)	100 (42.6)	19 (8.1)	**<0.001**
2019–2020	161 (40.7)	51 (31.7)	82 (50.9)	28 (17.4)	

Note: Data are shown as the percentage of the number *n* (%). Statistical analysis was performed by the parametric unpaired Student's t-test for independent samples, considering a 95% of confidence, with $p \leq 0.05$. Significance at $p \leq 0.05$. Statistically significant *p* values are in bold type.
Abbreviations: INCan, National Cancer Institute; PD-L1, programmed death ligand-1; TPS, tumor proportion score.

Qualitative results were consistent with the assessment of the mean PD-L1 TPS. The mean TPS% was higher for those samples analyzed with the SP263 clone compared with the 22C3 (20.9% vs.8.44%), but it was not significant. Also, the analysis which considered the period in which the IHC analysis was accomplished showed a trend favoring the group analyzed

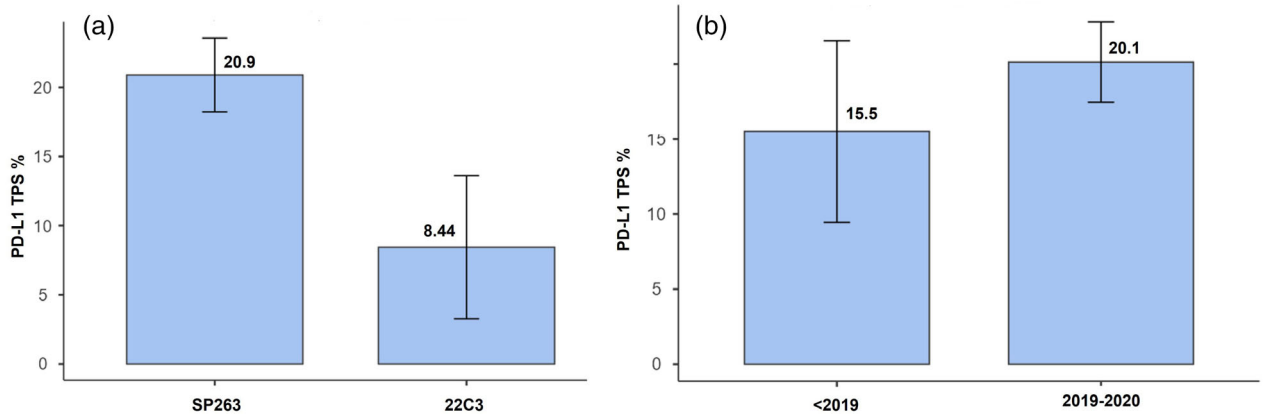

FIGURE 3 Mean PD-L1 TPS (%) evaluated in internal laboratories. (a) When the SP263 clone was the chosen assay for the analysis and (b) when the analysis was performed in a recent period. $p \leq 0.05$ was statistically significant. Statistical analysis was performed by parametric unpaired Student's *t*-test for independent samples, considering significance at $p \leq 0.05$.

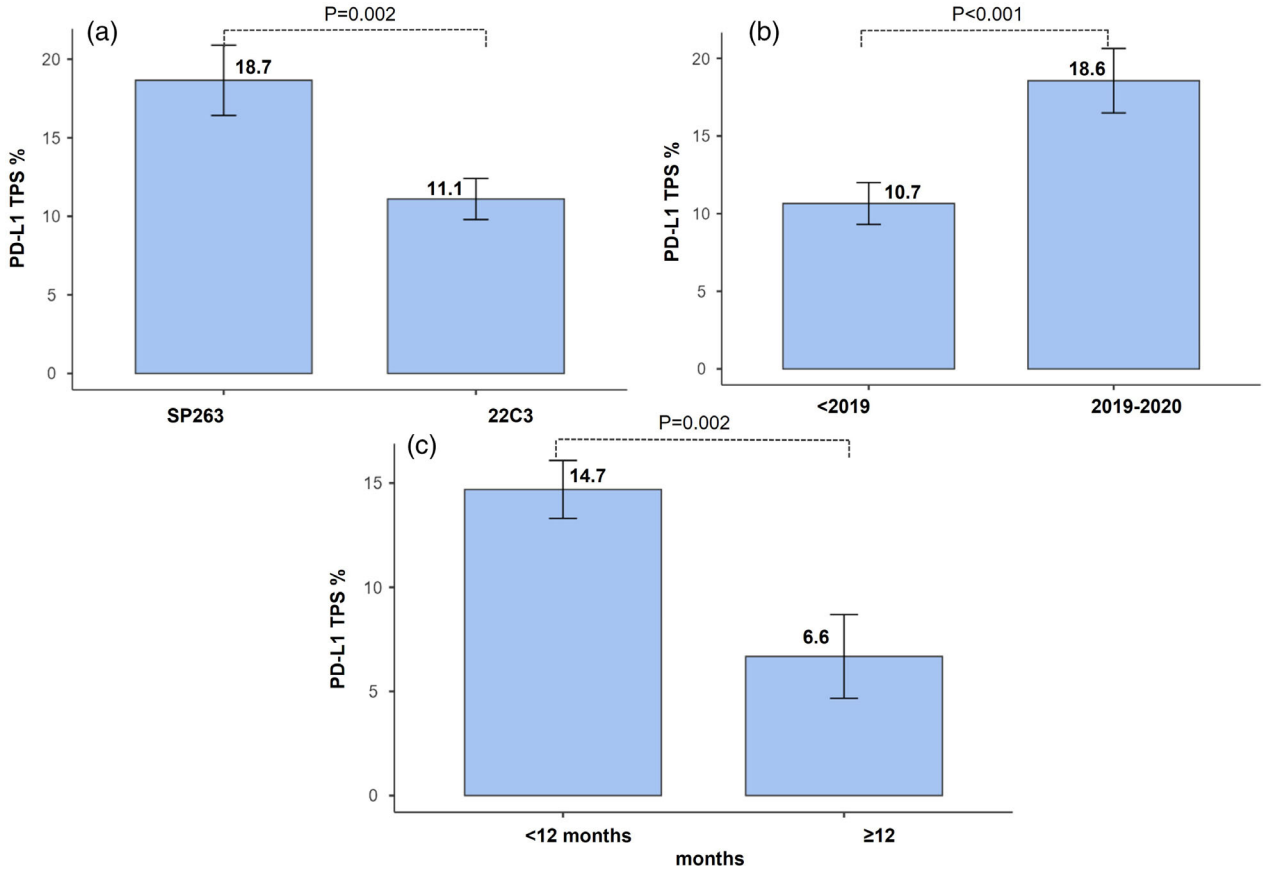

FIGURE 4 Mean PD-L1 TPS (%) evaluated in external laboratories. (a) When the SP263 clone was the chosen assay for the analysis, (b) when the analysis was performed in a recent period, and (c) when the time of archival tissue was <12 months. $p \leq 0.05$ was statistically significant. Statistical analysis was performed by parametric unpaired Student's *t*-test for independent samples, considering significance at $p \leq 0.05$.

in more recent years compared with previous years (20.1% vs. 15.5%), but it was still not significant (Figure 3).

In contrast, in external laboratories, the mean PD-L1 TPS % was significantly higher with the SP263 clone than with the 22C3 clone ($p = 0.002$). Similarly, the mean PD-L1 was higher in more recent years ($p < 0.001$) and when the archived period was less than 12 months ($p = 0.002$) (Figure 4).

Associations with PD-L1 high (≥50%) expression

Additionally, we carried out another analysis exclusively for those tumor samples with PD-L1 high expression compared with those with intermediate or negative expression. Factors associated significantly with high expression were the

academic setting, the SP263 clone, more recent evaluation period (2019 to 2020), and tumor tissue archived <12 months (Table 4).

Correlation between INCan and external laboratories

We evaluated the correlation between PD-L1 TPS% reports assessed by pathologists at INCan and pathologists in external laboratories. Overall, 116 tumor samples were evaluated in both clinical settings. Of these tumor samples, 46 (39.6%) were reported with a different PD-L1 TPS% threshold and 36 (31.0%) were completely discordant (i.e., reported as PD-L1 positive in one setting and PD-L1 negative in the other). Approximately 10 (8.6%) samples had the same PD-L1 positivity in both assays but discrepancies in the PD-L1 threshold (i.e., reported as PD-L1 high in one setting and with an intermediate expression in the other). Of note, 70 (60.3%) tumor samples showed complete concordance (i.e., same positivity and PD-L1 range) between pathologists. Figure 5a illustrates the correlation of PD-L1 reports between pathologists of different settings ($n = 116$). Hence, we found an intraclass correlation coefficient (ICC = 0.625) among PD-L1 TPS% between pathologists at INCan (y axis) and pathologists in external laboratories (x axis). Interestingly, the correlation was stronger across tumor samples with negative expression and more discrepancies arose in samples with PD-L1 high expression. In the INCan samples, the correlation in the PD-L1 evaluation between two expert pathologists in the field significantly increased the strength of the correlation (ICC = 0.90) (Figure 5b).

The correlation between the PD-L1 TPS (%) assessed between pathologists at INCan and from external laboratories was moderate and the correlation of PD-L1 TPS (%) between two expert pathologists within INCan was very strong.

TABLE 4 PD-L1 high (TPS > 50%) expression distribution according to the PD-L1 assay, evaluation setting, evaluation period, and time elapsed between biopsy and PD-L1 assay

	547 (100)	TPS ≥ 50%	*p*
Institution			
INCan (academic)	151 (27.6)	30 (19.9)	**0.016**
External laboratories	396 (72.4)	47 (11.9)	
Clone			
SP263	280 (51.2)	55 (19.6)	**<0.001**
22C3	267 (48.8)	22 (8.2)	
Year			
<2019	253 (46.3)	21 (8.3)	**<0.001**
2019–2020	294 (53.7)	56 (19.0)	
Time elapsed from biopsy to IHC analysis			
0–12 months	443 (89.5)	68 (15.3)	**0.024**
≥12 months	52 (10.5)	2 (3.8)	

Note: Data are shown as the percentage of the number *n* (%). Statistical analysis was performed by the parametric unpaired Student's *t*-test for independent samples, considering a 95% of confidence, with $p \leq 0.05$. Significance at $p \leq 0.05$. Statistically significant *p* values are in bold type.
Abbreviations: IHC, immunohistochemistry; INCan, National Cancer Institute.

DISCUSSION

Numerical factors may influence variations in PD-L1 expression reports, which may have significant consequences for clinicians in guiding treatment with ICIs.[8,16,17] This observational retrospective study evaluated, in a single cancer center, the PD-L1 expression stratified in more

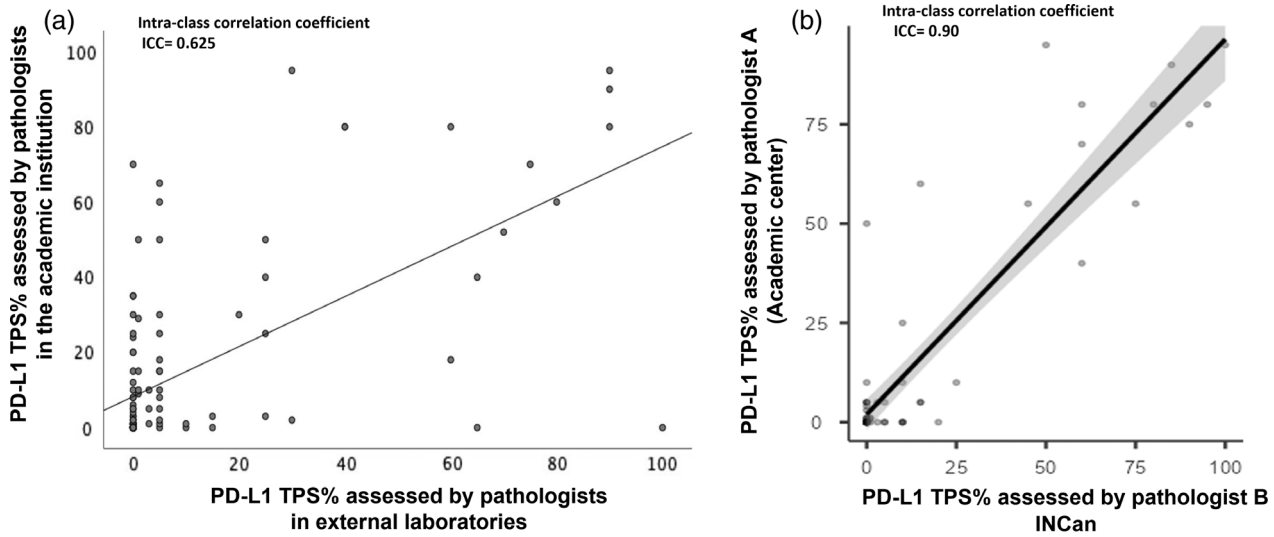

FIGURE 5 (a) The correlation between PD-L1 TPS (%) assessed as a continuous variable between pathologists in INCan and in external laboratories. (b) The correlation of PD-L1 TPS (%) between two expert pathologists within INCan.

relevant cut-off points and associated them with the main clinicopathological and molecular characteristics in lung ADC specimens.

In the present study, a significant association between the SOL predominant lung ADC subtype and a higher proportion of tumor samples classified as PD-L1 positive was observed. In addition, those samples classified as PD-L1 high were significantly more prevalent in the SOL pattern. Although the MIP predominant subtype was associated with a positive PD-L1 expression, we considered that the low proportion of cases with this pattern. The current results are consistent with Miyazawa and colleagues, who evaluated 307 resected lung ADC samples in which the PD-L1 positive rate was 73.7% in the SOL pattern and PD-L1 high samples were enriched with SOL predominant subtypes in 53.5% of cases. In other words, 62.5% of tumor samples categorized as PD-L1 high corresponded to a SOL predominant pattern.[18] In this study, the SOL pattern found a PD-L1 positive rate of 69.1% and a PD-L1 high of 21.6%. Notably, <10% samples were categorized as PD-L1 negative in the SOL pattern. These results align with previous findings in this population.[17]

The SOL predominant pattern has been associated with a higher tumor burden, higher copy number amplifications, higher fraction of genome altered, higher rate of whole-genome doubling, and higher number of oncogenic pathways altered than other ADC subtypes.[19] We hypothesized that the association between the SOL predominant ADC and the increased PD-L1 expression might be related to alterations in main oncogenic pathways (TP53, WNT, and MYC) which are usually impaired in this tumor subtype.[19,20] In this regard, TP53 has been the most studied oncogenic pathway, but there are still no specific mechanisms by which TP53 regulates PD-L1 expression. Preclinical data revealed that P53 regulates PD-L1 through microRNAs implicated in adaptative immunity.[21] In the same way, at a clinical level, Yanagawa and colleagues correlated TP53 overexpression, evaluated by IHC, and positively associated it with PD-L1 upregulation. In contrast, other studies suggest that the aberrant expression P53 or mutations in TP53 might positively alter PD-L1 expression.[20,22] Characterizing the molecular and biological basis in advanced LADC could lead to identify mechanisms of PD-L1 regulation in the SOL predominant subtype and might help to select ICIs candidates more efficiently.

Previous reports have stated that PD-L1 positivity was frequently associated with poorly differentiated tumors, closely related to high-grade tumors, and PD-L1 overexpression.[23] Also, PD-L1 expression may vary within the whole tumor due to intratumoral heterogeneity across tumor areas with different grades of differentiation.[23] In this study, poorly differentiated tumors showed higher PD-L1 positivity and a more significant proportion of PD-L1 high positive samples. Of note, we do not observe differences among smokers, who have been associated with PD-L1 overexpression,[24] PD-L1 high samples, and improved responses to ICIs.[25,26]

Controversy remains about the mechanisms by which EGFR mutant tumors regulate PD-L1 expression.[27] Most evidence supports the downregulation effect that predominates in this molecular subtype. In this regard, one recent metanalysis included 32 studies and calculated the association between the EGFR mutant status and PD-L1 expression. Those tumor samples harboring EGFR mutations were associated with lower PD-L1 expression rates, thus indicating that EGFR mutants might not be good candidates for ICIs as monotherapy after progression to targeted therapy.[28] This study also reported lower rates of PD-L1 positive and high PD-L1 positive samples in EGFR mutant compared with EGFR wild-type tumors.

PD-L1 reproducibility and performance in a real-world context have not been explored previously. In this regard, the Blueprint Phase 2 study demonstrated high comparability in interpreting tumor cell staining at any intensity with 28–8, 22C3, and SP263-based assays in lung cancer samples. In contrast, the SP142 clone exhibited the lowest sensitivity for determining TPS%, whereas the best performance was observed with the 73–10 assay. One metanalysis brought together more than 42 studies that investigated the analytical concordance of IHC assays utilizing two or more PD-L1 antibodies. Concordance was high between 28–8, 22C3, and SP263-based assays across different types of neoplasms, including lung cancer, when PD-L1 was assessed in tumor cells.[10] In this study, PD-L1 expression was evaluated by two different assays (SP263 and 22C3), and distribution among different cut-offs was in line with previous reports: negative and positive expression of PD-L1 was observed in 44.4% and 55.6%. PD-L1 high positive was identified in 14.0% of samples, which is in line with previous reports from INCan.[17] We found significantly more cases with PD-L1 high expression whenever the chosen assay was SP263 (19.6%) and pathologists determined the PD-L1 staining in the INCan samples (19.9%), which is an internal and academic setting.

Additionally, we performed two separate analyses, one for INCan and a second one for the external laboratories. This analysis was crucial due to the insufficient number of samples assessed in both settings. Of note, most patients received different immunotherapy-based therapies based on a single PD-L1 report. Although the distribution among the different PD-L1 ranges was comparable between both assays, SP263 and 22C3, discrepancies were more evident when detecting samples with high PD-L1 expression. Remarkably, these differences occurred in the INCan and external laboratories, but the main difference arose in external settings.

Another interesting observation occurred in those samples analyzed externally, in which the proportion of cases classified as PD-L1 high was higher when the IHC analysis was performed in more recent years (2019–2020) than in previous years. In other words, the proportion of PD-L1 high samples increased significantly from one year to another. This could be explained by the increasing gain experience in Mexico with immunotherapy after 2019, in

agreement with the approval of new ICIs indications in many solid tumors, especially in private medicine.

We should also be aware of crucial factors that must be considered when interpreting these results. Although the concordance is high in resected samples, this cohort focus on an exclusively metastatic population, therefore most patients underwent small biopsies by noninvasive procedures that may not represent the whole tumor. Also, the number of cores obtained on each biopsy procedure was unknown and might introduce more variability to the analysis. However, in the INCan is not standard practice to obtain more than two core biopsies, which have been demonstrated to increase concordance between the biopsy and the whole tumor.[29] We also observed a clear imbalance in the use of PD-L1 assays. In external laboratories, both assays were employed in almost equal proportions in 396 samples, but in the INCan academic setting the 22C3 assay was the chosen clone in just 16 patients, representing less than 11% of the IHC analyses processed in this setting. Although SP263 and 28–8 are considered interchangeable with 22C3 for TPS % assessment in NSCLC, some evidence suggests SP263 assays might stain slightly more tumor cells, mainly when assessing the mean difference.[30]

Finally, we evaluated the PD-L1 correlation between pathologists from INCan and the external laboratories. The ICC was 0.62, showing a moderate correlation, which is acceptable considering the many external factors that might introduce variability. Notably, the correlation between two expert pathologists in the field within INCan was very strong (ICC 0.90). The stronger correlation between pathologists in the INCan could be related to post-analytical factors such as the pathologists' expertise and interobserver variability. Before the approval of pembrolizumab in the PDL-1 high population (2017), pathologists had limited experience in discriminating between PD-L1 positive and PD-L1 high, especially in centers without clinical research activities. Thus, insufficient training and less experience outside academic centers introduced substantial interobserver variability.[31] Moreover, the interobserver variability in external laboratories might be more significant considering the increased number of pathologists involved in the staining process compared with INCan, in which two expert pathologists complete the staining process. Interestingly, in the present study, the intrinsic source of error introduced by interobserver variability occurred at higher PD-L1 scores rather than lower ones, as is described in other reports.[32]

Previous work has clarified the relevance of performing a proper IHC analysis as close as possible to the biopsy date to avoid fading PD-L1 expression. In the ATLANTIC trial, in which patients with pre-treated advanced-stage NSCLC received durvalumab, samples archived more than 3 years had markedly diminished rates of PD-L1 positivity. Moreover, older samples might no longer accurately represent the immunological status, thus compromising their likelihood of benefiting from ICIs. However, other studies have demonstrated that PD-L1 expression fades intumor tissue archivaed more than 12 months.[33] Concordantly, we detected lower PD-L1 expression 1 year after the biopsy date, which was more evident in those samples categorized with high PD-L1 expression. This situation was significant in external laboratories, which may contribute to the differences in performance between the two settings. Hence, more than 60.3% of tumor samples showed complete concordance across all PD-L1 thresholds.

The present study had some limitations that must be considered. Due to a lack of access to immunotherapy, we could not evaluate the response to immunotherapy and oncological outcomes to identify the predictive role of ICIs in this population. Additionally, multiple preanalytical variables that may influence results were unknown. Notably, INCan followed strict recommendations based on international guidelines for PD-L1 staining. In contrast, we cannot rule out that some preanalytical aspects in external settings impacted the reliability of IHC assays, such as cold ischemia time, fixative type, and fixation duration. Prolonged cold ischemia time, unbuffered formalin, and either inadequate or excessive fixation duration can all lead to diminished tissue antigenicity and increase the risk of false-negative results.

CONCLUSION

PD-L1 is an essential biomarker guiding treatment in non-oncogene addicted advanced lung ADC, and many factors may impact its reproducibility and performance. This study demonstrated an increased probability of identifying PD-L1 positive samples in poorly differentiated, solid predominant, and EGFR wild-type tumors. Discrepancies were higher in evaluating the highest PD-L1 threshold (≥50%), which represents the main target population due to its eligibility for ICI monotherapy. Moreover, the academic setting, SPS263 clone, time of archival tumor tissue (<12 months), and a more recent evaluable period are all factors associated with an increased proportion of cases with PD-L1 high expression. Although more confounding factors exist in external settings, the correlation between PD-L1 reports in the four years examined was moderate. This supports the reliability of laboratories guiding oncological therapy in a real-world context.

ACKNOWLEDGMENTS

Role of the funding source. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CONFLICT OF INTEREST

Dr. Oscar Arrieta has received honoraria as an advisor, speaker, and given expert opinions to Pfizer, AstraZeneca, Boehringer-Ingelheim, Roche, Lilly, and Bristol-Myers Squibb. Dr. Cardona reports grants from Merck Sharp & Dohme, Boehringer Ingelheim, Roche, Bristol-Myers Squibb, and the Foundation for Clinical and Applied Cancer

Research (FICMAC), and personal fees from Merck Sharp & Dohme, Boehringer Ingelheim, Roche, Bristol-Myers Squibb, Pfizer, Novartis, Celldex Therapeutics, Foundation Medicine, Eli Lilly, and FICMAC outside the submitted work. Dr. Luis Lara-Mejía has participated as speaker and receive personal fees from Roche, Pfizer, and Boehringer Ingelheim. Dr. David Heredia has participated as speaker and receive personal fees from Roche and Astra Zeneca. The other authors declare no potential conflicts of interest.

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How to cite this article: Avilés-Salas A, Flores-Estrada D, Lara-Mejía L, Catalán R, Cruz-Rico G, Orozco-Morales M, et al. Modifying factors of PD-L1 expression on tumor cells in advanced non-small-cell lung cancer. Thorac Cancer. 2022;13(23):3362–73. https://doi.org/10.1111/1759-7714.14695