



Programmed death ligand-1 expression in non-small cell lung cancer in a Western Australian population and correlation with clinicopathologic features

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Abstract

Immune checkpoint inhibition is an important therapeutic option in patients with non-small cell lung cancer. Programmed cell death ligand-1 (PD-L1) expression may serve as a predictive marker for anti-PD-1/PD-L1 therapies. The relationship between non-small cell lung cancer PD-L1 expression and clinicopathological characteristics remains unclear and there is no population level Australian data. We report the results of PD-L1 testing in patients with non-small cell lung cancer diagnosed at major Western Australian public hospitals served by a single state Pathology provider. We analyzed PD-L1 expression by immunohistochemistry in 241 non-small cell lung cancer specimens using the 22C3 clone on a Dako autostainer platform. Tumor cell PD-L1 expression was scored as Tumor Proportion Score and categorized using pre-specified subsets of 1%, 1–49% and $\geq 50\%$ for correlation with clinicopathologic features. PD-L1 Tumor Proportion Score was 1% in 65 (27%) cases, 1–49% in 100 (41%) cases and $\geq 50\%$ in 76 (32%) cases. PD-L1-positive rate was 92% in squamous cell carcinomas and 67% in adenocarcinomas. PD-L1 Tumor Proportion Score was higher in squamous cell carcinomas ($p = 0.004$) and lower in adenocarcinomas ($p = 0.003$). Of the 196 non-squamous carcinomas, 35% had rat sarcoma viral oncogene homolog (*RAS*) mutations, 13% had epidermal growth factor receptor (*EGFR*) mutations, 2% had anaplastic lymphoma kinase (*ALK*) translocations and 2% had *ROS1* translocations. Tumor Proportion Score $\geq 50\%$ was seen in 34% (23/68), 28% (7/25) and 25% (1/4) of *RAS*, *EGFR* mutant, and *ALK* translocated carcinomas, respectively. There was no significant correlation between PD-L1 expression and molecular or genetic abnormalities, or other parameters including age, gender, stage, and smoking status. In our patient cohort, PD-L1 Tumor Proportion Score was significantly higher in squamous cell carcinomas and lower in adenocarcinomas. The overall prevalence of Tumor Proportion Score $\geq 50\%$ is consistent with that reported in clinical trials.

Introduction

Recent advances in cancer immunotherapy have dramatically shifted the paradigm of lung cancer treatment. In particular, programmed cell death ligand-1 (PD-L1) and its interaction of the programmed cell death 1 (PD-1) receptor has been shown to play a crucial role in the down regulation of the host

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immune response and is thought to be a key strategy for tumor immune evasion [1–3]. Immune checkpoint inhibitors targeting the PD-1/PD-L1 pathway work by augmenting T-cell-mediated immune responses against cancer cells and have demonstrated significant durable clinical benefit in non-small cell lung cancer patients [4–7]. Some clinical trials have associated PD-L1 expression with higher overall response rates and improved survival outcomes when treated with PD-1/PD-L1 axis blockade suggesting that PD-L1 expression may serve as a predictive marker [4–6, 8–10].

Little is known about the intertumoral heterogeneity of PD-L1 expression in non-small cell lung cancer. In particular, there have been conflicting results on PD-L1 expression and its association with clinical, pathological, and molecular characteristics. Recent clinical series reported various frequencies of PD-L1 overexpression in non-small cell lung cancer ranging from 7.4% to 82.1% [11–28]. Numerous studies including large meta-analyses have attempted to correlate PD-L1 expression with clinicopathologic parameters with conflicting results. Some of the clinical and pathological features that have been linked with higher PD-L1 expression include smoking history, more advanced tumor stage and squamous cell histology [4, 14, 17–19, 26, 29–34]. Some studies have also observed an association between higher PD-L1 expression and epidermal growth factor receptor (*EGFR*), Kirsten rat sarcoma viral oncogene homolog (*KRAS*) and anaplastic lymphoma kinase (*ALK*) oncogenic mutations while others have shown differing results [4, 11, 15, 21, 35–38]. These discrepancies may be explained by differences in histological types and stages included in the studies, ethnicity of the patient population and heterogeneity in PD-L1 immunohistochemistry methods and cutoff values.

Here, we examined tumor cell PD-L1 expression in non-small cell lung cancer patients diagnosed at major Western Australian public hospitals served by a single Pathology provider and the association of PD-L1 expression with clinical features, histology and mutational profile of *EGFR* and *RAS* as well as structural rearrangements of *ALK* and *ROS1* genes. In cases with next generation sequencing (NGS) data we also examined association between PD-L1 expression and other genetic alterations, including *TP53*, *BRAF*, *MET*, *STK11*, *PIK3CA*, *APC*, *CTNNB1*, *PTEN*, *MAP2K1*, and *SMAD4*. The information generated from this study will provide better understanding of the pattern of PD-L1 expression in the Australian population.

Materials and methods

Patients and samples

This study included 241 patients with histologically confirmed non-small cell lung cancer who had PD-L1 testing on

tumor specimens at Pathwest Laboratory Medicine between April 2016 and August 2017. All but one tumor specimen were pre-treatment diagnostic samples. One tumor specimen was obtained from a mediastinal lymph node following disease recurrence after initial treatment with surgery and adjuvant chemotherapy. There were 112 core biopsy or endobronchial specimens, 103 cytology specimens, and 26 tumor resection specimens. Clinical data was retrospectively retrieved from medical records, including age at diagnosis, gender, histologic type, TNM stage (7th AJCC TNM Staging System) and smoking history.

Immunohistochemistry and assessment of PD-L1 expression

Immunohistochemistry for PD-L1 expression was performed using the PD-L1 22C3 pharmDx immunohistochemical assay on a representative section of tumor after assessment of adequacy. All tissue was fixed in 10% buffered neutral formalin and routinely processed into paraffin embedded blocks. Cytology specimens were assessed on cell block material, with aspirate concentrated by centrifugation (2000 rpm for 10 min). Plasma was added to the cell deposit and this cell suspension mixed with 0.25 mL of thrombin solution to form a clot, which was then fixed in 10% buffered neutral formalin and routinely processed into paraffin embedded blocks. Sections were cut at 4 µm on Matsunami Platinum Pro charged slides (Matsunami Glass Ind. Ltd, Japan), then dried in an oven at 60 °C for 60 min. Heat induced epitope retrieval was performed in EnVision™ Flex Target Retrieval Solution (TRS), at pH 6.1 and 95–99 °C for 20 min. Slides were incubated for 30 min in pre-diluted primary Ab (clone 22C3) and EnVision Flex + detection system, on a DAKO Autostainer Link 48 instrument. Verification of successful reaction on each slide was assessed with tonsil and placenta as external tissue controls.

PD-L1 expression was assessed based on the Tumor Proportion Score [39]. In brief only viable tumor cells are assessed, positivity is defined as any perceptible linear cell membrane staining (partial or complete), the score reflects percentage of positive tumor cells, and any associated immune cells are excluded from scoring.

Molecular and genetic analysis

Molecular studies were only performed on all non-squamous cases ($n = 196$). Representative sections of tumor were assessed for adequacy prior to DNA extraction from formalin fixed paraffin blocks. If tumor content was $> 50\%$ five 20 mm scrolls were placed in an Eppendorf tube, or alternately if the tumor comprised $< 50\%$ of the section, sections were cut and placed onto uncharged glass slides for microdissection. Following dewaxing with three washes of

xylene and three washes of 100% ethanol and removal of left over ethanol, the tissue was digested overnight with proteinase K and DNA was isolated from the digest using the QIAamp DNA FFPE tissue kit (Qiagen, Germany) according to the manufacturer's instructions.

NGS was performed ($n = 86$) on cases with sufficient amount of DNA (> 150 ng) using the Illumina miSeq platform with TruSight Tumor 26 Sequencing Panel. Acceptance criteria for mutation calling was greater than x500 read depth and greater an 3% variant allele frequency, with data analysis pipeline using MiSeq Reporter and Illumina Variant Studio 2.2. The genes tested and exons covered by the TruSight tumor 26 panel are: *AKT* (exon 2), *ALK* (exon 23), *APC* (exon 1–3), *BRAF* (exon 11, 15), *CDH1* (exon 8,9,12), *CTNNB1* (exon 1,2), *EGFR* (exon 18,19,20,21), *ERBB2* (exon 20), *FBXW7* (exon 7,8,9,10,11), *FGFR2* (exon 6), *AKT* (exon 2), *FOXL2* (exon 1), *GNAQ* (exon 4,5,6), *GNAS* (exon 6,8), *KIT* (exon 9,11,13,17, 18), *KRAS* (exon 1,2,3,4), *MAP2K1* (exon 2), *MET* (exon 1,4,13,15,16,17,18,19,20), *MSH6* (exon 5), *NRAS* (exon 1,2,3,4), *PDGFRA* (exon 11,13,17), *PIK3CA* (exon 1,2,7,9,20), *PTEN* (exon 1,2,3,4,5,6,7,8,9), *SMAD4* (exon 8, 11), *STK11* (exon 1,4,6,8), *TP53* (exon 1,2,3,4,5,6,7,8,9,10,11), *SRC* [10].

If DNA content was <150 ng ($n = 110$), then step-wise mutation testing was initiated. First the Cobas 4800 real-time PCR system for *EGFR* mutations (covering exons 18, 19, 20, 21) was performed, then if this was negative pyrosequencing [Qiagen PyroMark Q24 instrument with Therascreen *KRAS* Pyro kit (Cat No 971460)] for *KRAS* mutations [covering exon 2 (codons 12 and 13) and exon 3 (codon 61)] was performed.

Immunohistochemical stains for *ALK1* protein (clone D5F3) and *ROS1* protein (clone D4D6) were performed in all non-squamous cases. If only wild-type *EGFR* and *KRAS* molecular profile was detected, or if immunohistochemical staining for *ALK1* or *ROS1* protein was not entirely negative, fluorescence in situ hybridization for *ALK* breakapart (Vysis probe) and *ROS1* breakapart (Vysis probe) was performed ($n = 114$).

Statistical analyses

PD-L1 expression Tumor Proportion Score was categorized into three groups based on expression values of $< 1\%$, $1\text{--}49\%$, and $\geq 50\%$. Descriptive statistics for patient demographics and clinical characteristics data were summarized using frequency distributions for categorical data and comparisons between the three PD-L1 expression category groups were made using Pearson's χ^2 or Fisher exact tests. Continuous outcome measures were summarized using means, standard deviations and ranges, and oneway ANOVA was used to compare groups. Factors

associated with PD-L1 expression categories and tumor types were evaluated using logistic regression analysis with effects summarized using odds ratios and their 95% confidence intervals. Data was analyzed using IBM SPSS version 24.0 (Armonk, NY). p -values < 0.05 were considered statistically significant.

Results

Tumor PD-L1 Tumor Proportion Score levels ranged from 0 to 95%. Of the 241 cases, 65 (27%) did not show any PD-L1 staining (Tumor Proportion Score $< 1\%$), 100 (41%) had partial PD-L1 expression of 1–49% and 76 (32%) had strong PD-L1 expression of $\geq 50\%$.

Clinical parameters

A total of 241 patients with non-small cell lung cancer were included in the study. The median age at diagnosis was 69 years (range 36–95), and 135 patients (56%) were male. Thirty-two patients (13%) were never smokers and 200 patients (83%) had a smoking history. Of these, 98 were former smokers (having quit smoking > 1 year prior to diagnosis) and 96 were current smokers (smoking at the time of diagnosis or within 1 year prior to diagnosis). Nine patients (4%) had unknown smoking status. Thirty-eight patients (16%) had stage I disease at the time of PD-L1 testing, 18 (8%) were stage II, 46 (19%) were stage III, and 136 (56%) were stage IV according to the 7th edition of the AJCC TNM staging system. Three patients were unstaged.

The correlation of PD-L1 expression with clinical characteristics is presented in Table 1. There was no significant correlation between PD-L1 expression and gender, age, tumor stage, or smoking history.

Histologic types

Histologic types were categorized as adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, and non-small cell lung cancer, not otherwise specified (defined by morphology and immunohistochemical profile [40]; the latter defined as non-small cell carcinoma not showing glandular or squamous morphology and lacked expression of TTF1 or p40/p63; designation of lung origin was based on clinical and radiological findings). One-hundred seventy-six cases (73%) were adenocarcinomas, 45 (19%) were squamous cell carcinomas, 9 (4%) were adenosquamous carcinomas, and 11 (5%) were non-small cell lung cancer, not otherwise specified.

In the squamous cell carcinoma cohort, 4 (9%) were PD-L1 negative, 27 (60%) were weakly positive, and 14 (31%) were strongly positive. Among adenocarcinomas, 58 (33%)

Table 1 Correlations between PD-L1 Tumor Proportion Score with clinical features in 241 cases

	No. of cases <i>n</i> = 241	PD-L1 < 1% <i>n</i> = 65 (27%)	PD-L1 1–49% <i>n</i> = 100 (41%)	PD-L1 ≥ 50% <i>n</i> = 76 (32%)	<i>p</i> -value
<i>N</i> (%)					
Age (years)	< 60	53 (22)	15 (28)	24 (45)	14 (26) 0.89
	60–69	75 (31)	19 (25)	31 (41)	25 (33)
	70–79	63 (26)	20 (32)	24 (38)	19 (30)
	≥ 80	50 (21)	11 (22)	21 (42)	18 (36)
Sex	Male	135 (56)	39 (29)	56 (41)	40 (30) 0.68
	Female	106 (44)	26 (25)	44 (41)	36 (34)
Smoking status ^a	Non-smoker	32 (14)	10 (31)	14 (44)	8 (25) 0.68
	Smoker	200 (86)	53 (27)	82 (41)	65 (32)
Stage ^b	I	38 (16)	9 (24)	20 (53)	9 (24) 0.43
	II	19 (8)	5 (26)	8 (42)	6 (32)
	III	45 (19)	9 (20)	22 (49)	14 (31)
	IV	136 (57)	43 (32)	47 (35)	46 (34)

^aNine patients had unknown smoking status

^bThree patients were unstaged

were PD-L1 negative, 67 (38%) were partially positive, and 51 (29%) were strongly positive.

PD-L1 expression significantly differed among histologic types (Table 2). There was higher PD-L1 expression in squamous cell carcinomas compared to other types ($p = 0.004$), whereas adenocarcinomas had lower PD-L1 expression compared to other types ($p = 0.003$).

Compared to non-squamous cell carcinomas, a significantly higher proportion of squamous cell carcinomas had PD-L1 expression of 1–49% ($p < 0.001$) and $\geq 50\%$ ($p = 0.025$). Using univariate logistic regression analysis, the odds of squamous cell carcinomas having partial or strong PD-L1 positivity were significantly higher than having negative PD-L1 expression, with an odds ratio of 5.64 (95% CI 1.87–17.01, $p = 0.002$) and 3.44 (95% CI 1.07–11.05, $p = 0.038$), respectively.

Conversely, adenocarcinomas were less likely to have PD-L1 Tumor Proportion Score of 1–49% ($p = 0.001$) and $\geq 50\%$ ($p = 0.001$) than other histological types. Adenocarcinomas were four times less likely to have PD-L1 Tumor Proportion Score 1–49% (odds ratio 0.25, 95% CI 0.10–0.60, $p = 0.002$) and $\geq 50\%$ (odds ratio 0.25, 95% CI 0.10–0.62, $p = 0.003$), compared to having negative PD-L1 expression.

We then compared strong (Tumor Proportion Score $\geq 50\%$) PD-L1-positive tumors with those with partial or negative PD-L1 expression, and did not observe any statistically significant differences in the adenocarcinoma and

Table 2 Correlations between PD-L1 Tumor Proportion Score with histological type

Histology	No. of cases <i>n</i> = 241	PD-L1 < 1% <i>n</i> = 65 (27%)	PD-L1 1–49% <i>n</i> = 100 (41%)	PD-L1 ≥ 50% <i>n</i> = 76 (32%)	<i>p</i> -value
<i>N</i> (%)					
Adenocarcinoma	176 (73)	58 (33)	67 (38)	51 (29)	0.003
Other	65 (27)	7 (11)	33 (51)	25 (38)	
Squamous cell	45 (19)	4 (9)	27 (60)	14 (31)	0.004
Other	196 (81)	61 (31)	73 (37)	62 (32)	
Adenosquamous	9 (4)	2 (22)	3 (33)	4 (44)	0.70
Other	232 (96)	63 (27)	97 (42)	72 (31)	
NSCLC, NOS ^a	11 (5)	1 (9)	3 (27)	7 (64)	0.06
Other	230 (95)	64 (28)	97 (42)	69 (30)	

^aNSCLC, NOS: non-small cell lung cancer, not otherwise specified

Statistically significant *p* values

squamous cell carcinoma subgroups. However, among the 11 patients with non-small cell lung cancer, not otherwise specified, 7 (64%) had PD-L1 Tumor Proportion Score $\geq 50\%$, which was significantly higher compared to other histologic subtypes ($p = 0.026$). The odds of having strong PD-L1 positivity was significantly higher in this group (odds ratio 4.08, 95% confidence interval 1.16–14.40, $p = 0.02$).

Molecular and genomic mutations

All adenocarcinomas, adenosquamous carcinomas, and non-small cell lung cancer, not otherwise specified, were tested for the oncogenic driver mutations of *EGFR* and *RAS*, and gene rearrangements of *ALK1* and *ROS1*. Of a total of 196 cases, 96 (49%) had no detectable abnormalities, 68 (35%) had *RAS* mutations, 25 (13%) had *EGFR* mutations, 4 (2%) had an *ALK* translocation, and 3 (2%) had a *ROS1* translocation. These four driver abnormalities were mutually exclusive.

Among *EGFR* mutant tumors, exon 19 deletion (delExon19) was the most common variant and was identified in 14 patients (56%). Exon 21 variant (Exon21) was identified in 9 patients (36%) and 2 patients had other *EGFR* variants [an Exon 18 codon 719 variant and an exon 20 insertion p.(Val769_Asp770insASV)].

Of the 68 *RAS* mutant tumors, there were 67 *KRAS* and 1 *NRAS* variants. p.Gly12Cys *KRAS* was the most common variant arising in 24 patients (35%) followed by p.Gly12Val *KRAS* variant, seen in 14 patients (21%).

The correlation of PD-L1 Tumor Proportion Score with oncogenic driver mutation status in 196 non-squamous carcinoma cases is presented in Tables 3–5. There was no significant correlation between PD-L1 expression and any of the molecular alterations detected.

Table 3 Correlations between PD-L1 Tumor Proportion Score with oncogenic driver mutation status in 196 non-squamous carcinoma cases

	No. of cases n = 196	PD-L1 < 1% n = 61 (31%)	PD-L1 1–49% n = 73 (37%)	PD-L1 ≥ 50% n = 62 (32%)	p-value
<i>N (%)</i>					
<i>EGFR</i> mutation	25 (13)	11 (44)	7 (28)	7 (28)	0.69
<i>RAS</i> mutation	68 (35)	18 (26)	27 (40)	23 (34)	
<i>ALK</i> translocation	4 (2)	2 (50)	1 (25)	1 (25)	
<i>ROS1</i> translocation	3 (2)	0 (0)	1 (33)	2 (67)	
Pan-wildtype	96 (49)	30 (31)	37 (39)	29 (30)	

Table 4 Correlations between EGFR and RAS mutation genotypes and PD-L1 Tumor Proportion Score

		No. of cases	PD-L1 < 50%	PD-L1 ≥ 50%	p-value
<i>EGFR</i> mutation	DelExon19	14 (56)	11 (79)	3 (21)	0.43
	Exon21	9 (36)	6 (67)	3 (33)	
	Other	2 (8)			
<i>RAS</i> mutation	p.Gly12Cys <i>KRAS</i>	24 (35)	15 (62)	9 (38)	0.64
	p.Gly12Val <i>KRAS</i>	14 (21)	11 (79)	3 (21)	
	p.Gly12Asp <i>KRAS</i>	10 (15)	5 (50)	5 (50)	
	p.Gly12Ser <i>KRAS</i>	7 (10)	5 (71)	2 (29)	
	p.Glu61His <i>KRAS</i>	5 (7)	3 (60)	2 (40)	
	p.Gly12Arg <i>KRAS</i>	4 (6)	3 (75)	1 (25)	
	p.Gly12Ala <i>KRAS</i>	2 (3)	2 (100)	0 (0)	
	p.Gly13Cys <i>KRAS</i>	1 (2)	0 (0)	1 (100)	
	p.Gln61Leu <i>NRAS</i>	1 (1)	1 (100)	0 (0)	

In our population of *EGFR* mutant tumors, strong PD-L1 expression was seen 28%, which is consistent with the prevalence reported in clinical trials. There was strong PD-L1 expression in 3/14 (21%) of 19delExon and 3/9 (33%) of Exon21 mutant cases, with no significant difference seen between the two genotypes. In tumors harboring *RAS* variants, there was no significant difference in PD-L1 expression across the different *RAS* variants cases.

Eighty-six tumors were analyzed for additional gene variants via NGS (Table 5). The most commonly found variant was in the tumor suppressor gene *TP53*, seen in 40 of the 86 tumor specimens (47%). Variants were detected in *BRAF*, *MET*, *STK11*, *PIK3CA*, *APC*, *CTNNB1*, *PTEN*, *MAP21K*, and *SMAD4* genes in a small number of patients.

By far the other most common genetic alteration detected via NGS was in the *TP53* gene. 30% (12/40) of *TP53*-positive tumors had PD-L1 Tumor Proportion Score ≥ 50% compared to 28% (13/46) of *TP53*-negative tumors ($p = 0.52$).

Discussion

The major significant finding from our study is the differential PD-L1 expression in non-small cell lung cancer

Table 5 Correlations between PD-L1 Tumor Proportion Score and genetic alterations identified by next generation sequencing in 86 cases

	No. of cases n = 86	PD-L1 < 50% n = 61 (71%)	PD-L1 ≥ 50% n = 25 (29%)	p-value
<i>N (%)</i>				
<i>TP53</i> Positive	40 (47)	28 (70)	12 (30)	0.52
<i>TP53</i> Negative	46 (53)	33 (72)	13 (28)	
Other mutations				
<i>BRAF</i>	4	3 (75)	1 (25)	
<i>MET</i>	4	4 (100)	0 (0)	
<i>PIK3CA</i>	3	1 (33)	2 (67)	
<i>STK11</i>	3	3 (100)	0 (0)	
<i>APC</i>	2	1 (50)	1 (50)	
<i>CTNNB1</i>	2	2 (100)	0 (0)	
<i>PTEN</i>	2	2 (100)	0 (0)	
<i>MAP21K</i>	1	1 (100)	0 (0)	
<i>SMAD4</i>	1	1 (100)	0 (0)	

subtypes including adenocarcinoma and squamous cell carcinoma, due to increased proportion of partial (1–49%) PD-L1 rather than strong (≥ 50%) expression. PD-L1 expression was more frequently observed in squamous cell carcinomas and less frequently observed in

adenocarcinomas. However, when analyzed separately, there was no significant difference between adenocarcinoma and squamous cell carcinoma subtypes in those with Tumor Proportion Score $\geq 50\%$.

The correlation between PD-L1 expression and squamous histology is concordant with the results of other studies including two recently published meta-analyses [26, 29, 32–34]. These findings suggest that the PD-1/PD-L1 pathway may be utilized more commonly by squamous cell carcinomas to achieve host immune evasion. However, studies have shown that PD-L1 expression in non-small cell lung cancer does not always select patients who are more likely to benefit from checkpoint blockade. For example, PD-L1 expression in squamous cell carcinoma did not impact response rate and patient outcome when treated with nivolumab [7], and first line chemoimmunotherapy combinations significantly improved survival compared to chemotherapy regardless of PD-L1 status [41, 42]. This indicates that PD-L1 alone is not a good predictive biomarker for treatment response and that there are other factors that can affect tumor immunogenicity and response to PD-1/PD-L1 antibodies, such as somatic mutational and neoantigen burden as shown recently by the Checkmate 227 trial [43], as well as other differences in the tumor immune landscape.

Non-EGFR mutated or non-ALK translocated carcinomas with PD-L1 Tumor Proportion Score $\geq 50\%$ (22C3 pharmDx assay) have been shown to benefit from first line PD-1 inhibitor therapy with pembrolizumab when compared with platinum doublet chemotherapy, with a higher overall response rate of 44.8% and greater progression free survival of 10.3 months [44]. In our patient cohort, we did not observe any significant predictors of strong PD-L1 expression except for the non-small cell lung cancer, not otherwise specified histologic type. It is not possible to ascertain the significance of this given the small sample in our cohort.

The association between PD-L1 expression and molecular profiles in non-squamous carcinomas has been studied extensively and remains controversial. Several reports suggest that *EGFR* mutations were associated with higher PD-L1 expression [11, 15, 21, 35, 36]. Conversely other studies show that PD-L1 positivity was more frequent in *EGFR* wild-type tumors [45–47]. Recent reports have also shown a significant association between PD-L1 expression and *KRAS* mutations [4, 37] and *ALK* translocations [38], while other studies have failed to find such relationships [11, 14, 20, 27, 29, 37, 48, 49]. Our study did not show any significant correlation between PD-L1 expression and underlying molecular aberrations.

Our cases mostly consisted of adenocarcinomas and *TP53* was the most commonly detected genetic abnormality identified via NGS. Although three groups have demonstrated that *TP53* mutated adenocarcinomas expressed

higher levels of PD-L1, especially in the presence of a coexisting *KRAS* mutation [50–52], we did not see a significant difference when using the PD-L1 Tumor Proportion Score cutoff of 50%. Our cohort only had eight patients with synchronous *TP53* and *KRAS* mutations, limiting the statistical power of the analysis.

Finally, some studies have shown that PD-L1 expression correlates with smoking history [4, 17, 29–31]; however, other studies have failed to demonstrate such associations [20–22, 45, 48, 53]. PD-L1 expression has also been linked with more advanced tumor stage [14, 18, 19, 26]. In our patient cohort, we did not find a significant link between PD-L1 expression and any of the clinical variables.

There are a few limitations to our study. This study included patients treated at a single center, which can limit the generalizability of the results. Some of the tumor subsets such as those with *ALK1* and *ROS1* alterations had a small number of patients which limits the statistical analysis power in these groups. A common drawback shared with other studies is the lack of standardized tumor PD-L1 expression cutoff values. To increase the clinical translational relevance of this study, we chose three PD-L1 expression cutoffs that are used in clinical trials and are relevant to the current method of selecting patients for anti-PD-1/PD-L1 therapy. Furthermore, due to intratumoral and intra-patient tumor heterogeneity, especially in patients with metastatic disease who underwent tumor sampling of only a single site, the PD-L1 expressions observed might not be representative of the patient's disease. Lastly, the dataset was collected retrospectively and subject to bias as a result.

Our study showed significant differences in PD-L1 expression between lung squamous cell carcinomas and adenocarcinomas, supporting the notion of biological and immunological diversity among non-small cell lung cancer subtypes. How this influences tumor response to immune checkpoint blockade is still unclear and further research is needed to advance our understanding of the complexities of the tumor microenvironment and tumor immunology.

Compliance with ethical standards

Conflict of interest Michael Millward—Advisory Board for Merck Sharpe & Dohme; Benhur Amanuel—Advisory Board for Merck Sharpe & Dohme. The other authors declare that they have no conflict of interest.

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