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ORIGINAL ARTICLE

The Clinicopathological and Molecular Associations of PD-L1 Expression in Non-small Cell Lung Cancer: Analysis of a Series of 10,005 Cases Tested with the 22C3 Assay

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Abstract

PD-L1 expression testing is mandatory prior to pembrolizumab prescription in non-small cell lung cancer. Our service offers PD-L1 testing using the PD-L1 IHC 22C3 pharmDx assay, in parallel with EGFR, ALK, ROS1 and (in some cases) KRAS testing. We correlate PD-L1 expression in 10,005 tumours with patient age and sex, with tumour histological subtypes, with the sampling modality and type of tissue, and with the presence of other molecular alterations. PD-L1 expression testing was performed using the aforementioned assay; tumour proportion scores (TPS) of 1 and 50% were taken as cut-offs for low and high positivity, respectively. EGFR testing was performed using the cobas® EGFR Mutation Test v2. ALK testing was performed using the VENTANA ALK (D5F3) CDx Assay. KRAS testing was performed using pyrosequencing. TPS <1% was seen in 44.4% of tumours, 1–49% in 25.0% and ≥ 50% in 30.6%. We identified no significant relationship with age. Female patients were slightly more likely to express PD-L1. Poorly-differentiated tumour histology and ALK translocation were significantly associated with PD-L1 expression. Rare EGFR mutations tended to be associated with PD-L1 expression. Pleural and nodal metastases were more likely to express PD-L1 than primary tumours, but biopsy and cytological specimens did not show different PD-L1 expression rates. Our data show that the means of acquiring a tumour sample (biopsy versus cytology) does not have a significant impact on PD-L1 expression. However, we found that certain metastatic sites were associated with significantly higher expression rates, which has substantial implications for selection of tissue for testing.

Keywords Lung cancer · PD-L1 · Immunotherapy · Pembrolizumab

Introduction

Non-small cell lung cancer (NSCLC) has, historically, had a dismal prognosis. Targeted therapies against EGFR mutations, ALK translocations and ROS1 translocations have certainly transformed the outlook of patients whose tumours bear these alterations, but – in Caucasian populations at least – this is the minority.

The Keynote trials demonstrated that a substantially larger proportion of patients with NSCLC show favourable responses to the anti-PD-1 agent pembrolizumab, and that expression of PD-L1 by tumour cells meaningfully predicts response to this therapy [1–3]. With a relatively low incidence of serious adverse events and – perhaps most importantly – proven efficacy in non-adenocarcinoma tumour types, this treatment paradigm has garnered a great deal of excitement.

Despite these impressive results, however, the relationship between PD-L1 expression and clinical, pathological and molecular tumour characteristics remains unclear. Though a number of investigators have already presented data, series have tended to be rather small, have used selected populations, and have made use of a range of antibodies with variable interpretations of positivity. Unsurprisingly, therefore, reported ranges of PD-L1 expression have varied enormously, and there have been numerous contradictory claims about the relationship of

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PD-L1 expression with various clinicopathological and molecular tumour features.

PD-L1 expression assessment is mandatory prior to prescription of anti-PD-1/PD-L1 therapy, and prescription of pembrolizumab can specifically be guided by PD-L1 expression assessment using the PD-L1 IHC 22C3 pharmDx assay (Agilent Technologies, Santa Clara CA). Our centre has been performing PD-L1 expression testing in NSCLC using this assay since April 2016. Here, we present the prospectively-collected data from a series of 10,005 unselected NSCLC specimens tested for PD-L1 expression in our routine clinical practice, using the assay employed in the Keynote trials. We correlate PD-L1 expression with patient age and sex, with tumour histological subtypes, with the modality and type of tissue sampled, and with the presence of ALK, EGFR and KRAS alterations.

Methods and Materials

Study Design

As part of routine clinical testing, a total of 11,167 NSCLC specimens were assessed for PD-L1 expression at the Queen Elizabeth Hospital Birmingham between 1st April 2016 and 1st October 2017. Clinicopathological features of the patients and tumours, together with the results of PD-L1 expression, EGFR mutation, ALK translocation and KRAS mutation analysis, were recorded in a prospectively-maintained database. The relationship between PD-L1 expression, and clinicopathological and molecular features was examined.

263 (2.4%) specimens were from our own centre; the remaining 10,904 (97.6%) specimens had been referred for testing from a total of 114 external centres (109 British NHS Trusts, Cyprus, Greece, Jersey, Kuwait and Malta).

1162 (10.4%) specimens could not be assessed for PD-L1 expression, for the reasons detailed in Table, Supplemental Data 1. 10,005 (89.6%) specimens were successfully tested and were included in the analysis.

Clinicopathological Details

Clinicopathological details (age, sex, sampling modality, tissue type, histological tumour type) were taken directly from the reports sent with the specimens by the referring pathologists; in any cases where such data were ambiguous or not entirely clear, the details were omitted from the database. Tumour histology was classified according to the 2015 WHO Classification of Lung Tumours; where reports gave histological subtypes not listed in the 2015 WHO Classification, these data were omitted from the analysis. Predominant adenocarcinoma growth patterns were included in the analysis only if the reporting pathologist clearly indicated a single predominant growth pattern. Where cytological specimens were used for PD-L1 assessment, testing was performed using formalin-fixed, paraffin-embedded clots/cell blocks produced from the fluid.

PD-L1 Immunohistochemistry

PD-L1 expression was assessed using formalin-fixed, paraffin-embedded tumour samples, using the PD-L1 IHC 22C3 pharmDx assay (Agilent Technologies, Santa Clara CA). All assays were performed at the Queen Elizabeth Hospital Birmingham, using the same procedures as those employed in the Keynote trials for pembrolizumab. Three assessors (BO, ME, PT), who had undergone formal training by Agilent, assessed the proportion of tumour cells showing membranous staining and gave a tumour proportion score (TPS); difficult cases were examined by at least two assessors and a consensus score agreed. As per the Keynote trials, cases were reported as being negative, low-positive or high-positive based on TPS of <1%, 1–49% or ≥ 50%, respectively.

EGFR, ALK and KRAS Testing

The results of EGFR, ALK and KRAS testing were recorded only in those cases where such testing was performed in our laboratory contemporaneously with, and using the same tissue block as PD-L1 expression assessment.

EGFR mutation testing was performed using the cobas® EGFR Mutation Test v2 (Roche, Basel, Switzerland). Results of plasma mutation testing were excluded. This technique detects 19 deletions in exon 19, T790M, L858R, G719X, S768I, L861Q and 5 insertions in exon 20.

ALK translocation testing was performed with immunohistochemistry using the VENTANA ALK (D5F3) CDx Assay (Roche, Basel, Switzerland). Immunohistochemistry was reported as negative or positive by three trained assessors (BO, ME, PT).

KRAS mutation testing was performed using pyrosequencing to detect mutations in exons 2, 3 and 4.

Statistical Analysis

The prevalence of PD-L1 positivity (TPS ≥ 1%) was compared between subgroups based on sex, age, sampling modality, tissue type, histological tumour subtype, EGFR status, ALK status and KRAS status, using chi-squared analysis or Fisher's exact test, as appropriate; the prevalence of PD-L1 low- and high-positivity (TPS 1–49% and ≥ 50%, respectively) were also compared between the same subgroups. Two-tailed T-testing was used to compare the mean age between PD-L1 negative and positive patients, and between low- and high-positive patients. All analyses were performed using IBM (Armonk NY) SPSS version 24, with p values less than 0.05 considered statistically significant.

Results

Clinicopathological and Molecular Characteristics

The clinicopathological and molecular characteristics of the tested cases are detailed in Table 1.

PD-L1 Expression and Clinical Characteristics

There was no significant difference in mean age between TPS < 1%, 1–49% and ≥ 50%; subgroup analysis, however, revealed that PD-L1 expression at TPS ≥ 1% was significantly commoner in patients aged 90 years and older compared to all younger age groups. No difference in PD-L1 expression at any cut-off was identified between males or females (Table 2).

PD-L1 Expression and Pathological Characteristics

Cytological specimens were significantly more likely to express PD-L1 at both cut-offs than were biopsy specimens; biopsy specimens were, in turn, more likely to express PD-L1 at both cut-offs than were resection specimens.

Significant differences in rates of PD-L1 expression were identified between different tissues tested. Of note, samples from the lung parenchyma showed low rates of PD-L1 positivity at both the 1% and 50% cut-offs; in contrast, lymph node and pleural metastases showed significantly higher rates of positivity at both cut-offs.

Subgroup analysis (Table, Supplemental Data 2) revealed that the high rates of PD-L1 expression in both pleura and lymph nodes were seen regardless of the sampling modality.

There was no significant difference in PD-L1 expression rates between adenocarcinomas, squamous cell carcinomas or adenosquamous carcinomas. However, neuroendocrine carcinomas were significantly less likely to express PD-L1 at either cut-off; sarcomatoid carcinomas were significantly more likely to express PD-L1, with this difference being driven by a very high number of tumours with TPS ≥ 50%. There were significant differences in PD-L1 expression between predominant adenocarcinoma growth patterns. High rates were seen in solid and micropapillary adenocarcinomas; low rates were seen in lepidic, mucinous lepidic, invasive mucinous and papillary adenocarcinomas. A particularly high proportion of solid carcinomas had TPS ≥ 50%. Particularly low proportions of lepidic, lepidic mucinous, invasive mucinous and papillary carcinomas had such high TPS (Table 3).

Table 1 The clinicopathological and molecular characteristics of the tested cases

	All cases tested	Successfully-tested cases
Overall	11,167	10,005
Age		
Mean (SD)	68.4 (9.9)	68.4 (9.9)
Age group		
< 50 years	431 (3.9)	393 (3.5)
50–59 years	1503 (13.5)	1349 (12.1)
60–69 years	3699 (33.1)	3281 (29.4)
70–79 years	4081 (36.5)	3681 (33.0)
80–89 years	1196 (10.7)	1066 (9.5)
≥ 90 years	70 (0.6)	65 (0.6)
Unknown	187 (1.7)	170 (1.5)
Sex		
Male	5848 (52.4)	5262 (52.6)
Female	5028 (45.0)	4470 (44.7)
Unknown	291 (2.6)	273 (2.7)
Sampling modality		
Biopsy	5450 (48.8)	4876 (48.7)
Cytology	2276 (20.4)	1931 (19.3)
Resection	799 (7.2)	796 (8.0)
Unknown	2642 (23.7)	2402 (24.0)
Tissue type		
Lung	4362 (39.1)	3963 (39.6)
Bronchus	1256 (11.2)	1140 (11.4)
Pleura	757 (6.8)	653 (6.5)
Lymph node	2132 (19.1)	1858 (18.6)
Liver	200 (1.8)	189 (1.9)
Bone	313 (2.8)	281 (2.8)
Brain	126 (1.1)	124 (1.2)
Others	517 (4.6)	473 (4.7)
Unknown	1504 (13.5)	1324 (13.2)
Histological subtype		
NSCLC NOS	1152 (10.3)	997 (10.0)
Adenocarcinoma	5197 (46.5)	4660 (46.6)
Squamous cell carcinoma	2447 (21.9)	2272 (22.7)
Adenosquamous carcinoma	99 (0.9)	93 (0.9)
Neuroendocrine carcinoma	37 (0.3)	36 (0.4)
Sarcomatoid carcinoma	20 (0.2)	20 (0.2)
Others	11 (0.1)	10 (0.1)
Unknown	1759 (15.8)	1519 (15.2)
Predominant adenocarcinoma growth pattern		
Adenocarcinoma NOS	4050 (86.5)	4044 (86.9)
Acinar adenocarcinoma	207 (4.4)	205 (4.4)
Solid adenocarcinoma	139 (3.0)	137 (2.9)
Lepidic adenocarcinoma	117 (2.5)	112 (2.4)
Invasive mucinous adenocarcinoma	74 (1.6)	66 (1.4)
Papillary adenocarcinoma	46 (1.0)	46 (1.0)

Table 1 (continued)

	All cases tested	Successfully-tested cases
Lepidic mucinous adenocarcinoma	23 (0.5)	22 (0.5)
Micropapillary adenocarcinoma	18 (0.4)	18 (0.4)
Adenocarcinoma in situ	6 (0.1)	6 (0.1)
EGFR mutation status		
Wild-type	3148 (28.2)	2966 (29.6)
Mutated	323 (2.9)	303 (3.0)
Not tested	7696 (68.9)	6736 (67.3)
ALK translocation status		
Negative	3634 (32.5)	3320 (33.2)
Positive	59 (0.5)	56 (0.6)
Not tested	7474 (66.9)	6629 (66.3)
KRAS mutation status		
Wild-type	70 (0.6)	63 (0.6)
Mutated	31 (0.3)	29 (0.3)
Not tested	11,066 (99.1)	9913 (99.1)

PD-L1 Expression and Molecular Characteristics

In our series, EGFR wild-type tumours were significantly more likely to express PD-L1 than mutated tumours; furthermore, wild-type tumours were significantly more likely to express PD-L1 at TPS ≥ 50%. Owing to small numbers of rarer mutations, there was no significant difference in the rate of PD-L1 expression between individual mutations; however, there was a trend for rare EGFR mutations (G719X, L861Q, exon 20 insertions, S768I) to show higher expression rates than the classical mutations. Further analysis demonstrated that tumours with compound mutations showed the highest rates of PD-L1 expression at TPS ≥ 1%, followed by those with rare singlet mutations, followed by those with classical singlet mutations.

We found that the presence of ALK translocation was strongly associated with PD-L1 expression, both at TPS ≥ 1% and TPS ≥ 50%.

There was no significant association between KRAS mutation and PD-L1 expression in our series, which is not surprising given the very small number of cases available for analysis (Table 4).

Discussion

Reported rates of PD-L1 expression, as detected by the 22C3 assay, vary enormously (Table 5). Our positivity rates are broadly similar to those reported in the Keynote trials [1–3], but our rate of low positivity was conspicuously lower than in all three trials. No clear explanation is evident from our data: even allowing for the inclusion of cytology in our series, and for different age, sex and histological distributions, this difference persists. There is clearly a need for further large, prospectively-collected series to establish whether this is a general phenomenon.

Previous reports of the relationship between PD-L1 expression and patient age have been conflicting, with some studies reporting a positive relationship with age [8], others reporting a negative relationship [10, 13], but most reporting no significant association [6, 7, 9, 14–28]. In our series, there was no overall significant relationship between PD-L1 expression and age; however, subgroup analysis revealed a statistically-significant spike in PD-L1 expression in those patients aged ninety years and older. There was no difference in patient sex or histological subtypes between this

Table 2 The relationship between PD-L1 expression and patient clinical characteristics

	TPS < 1%	95% CI	TPS ≥ 1%	95% CI	p value	TPS 1–49%	95% CI	TPS ≥ 50%	95% CI	p value
Overall	4447 (44.4)	43.6–45.3	5558 (55.6)	54.7–56.4		2501 (25.0)	24.3–25.7	3057 (30.6)	29.8–30.6	
Age										
Mean (SD)	68.4 (9.6)		68.3 (10.1)		0.716	68.4 (10.3)		68.3 (9.9)		0.684
Age group					0.004					0.004
< 50 years	164 (41.7)	37.6–46.0	229 (58.3)	54.0–62.4		118 (30.0)	26.2–34.1	111 (28.2)	24.5–32.2	
50–59 years	593 (44.0)	41.7–46.2	756 (56.0)	53.8–58.3		315 (23.4)	21.5–25.3	441 (32.7)	30.6–34.9	
60–69 years	1442 (44.0)	42.5–45.4	1839 (56.0)	54.6–57.5		818 (24.9)	23.7–26.2	1021 (31.1)	29.8–32.5	
70–79 years	1707 (46.4)	45.0–47.7	1974 (53.6)	52.3–55.0		900 (24.4)	23.3–25.6	1074 (29.2)	27.9–30.4	
80–89 years	455 (42.7)	40.2–45.2	611 (57.3)	54.8–59.8		277 (26.0)	23.8–28.3	334 (31.3)	29.0–33.8	
≥ 90 years	15 (23.1)	14.8–33.3	50 (76.9)	66.7–85.2		24 (36.9)	26.9–47.8	26 (40.0)	29.8–51.0	
Sex					0.088					0.272
Male	2380 (45.2)	44.1–46.4	2882 (54.8)	53.6–55.9		1289 (24.5)	23.5–25.5	1593 (30.3)	29.2–31.3	
Female	1946 (43.5)	42.3–44.8	2524 (56.5)	55.2–57.7		1132 (25.3)	24.3–26.4	1392 (31.1)	30.0–32.3	

Table 3 The relationship between PD-L1 expression and pathological characteristics

	TPS < 1%	95% CI	TPS ≥ 1%	95% CI	p value	TPS 1–49%	95% CI	TPS ≥ 50%	95% CI	p value
Sampling modality					0.000					0.000
Biopsy	2227 (45.7)	44.5–46.9	2649 (54.3)	53.1–55.5		1237 (25.4)	24.3–26.4	1412 (29.0)	27.9–30.0	
Cytology	762 (39.5)	37.6–41.3	1169 (60.5)	58.7–62.4		412 (21.3)	19.8–22.9	757 (39.2)	37.4–41.1	
Resection	428 (53.8)	50.8–56.7	368 (46.2)	43.3–49.2		189 (23.7)	21.3–26.4	179 (22.5)	20.1–25.1	
Tissue type					0.000					0.000
Lung	1926 (48.6)	47.3–49.9	2037 (51.4)	50.1–52.7		979 (24.7)	23.6–25.9	1058 (26.7)	25.5–27.9	
Bronchus	470 (41.2)	38.8–43.7	670 (58.8)	56.3–61.2		326 (28.6)	26.4–30.9	344 (30.2)	27.9–32.5	
Pleura	215 (32.9)	29.9–36.1	438 (67.1)	63.9–70.1		160 (24.5)	21.7–27.4	278 (42.6)	39.3–45.9	
Lymph node	760 (40.9)	39.0–42.8	1098 (59.1)	57.2–61.0		429 (23.1)	21.5–24.8	669 (36.0)	34.2–37.9	
Liver	104 (55.0)	48.8–61.2	85 (45.0)	38.8–51.2		39 (20.6)	15.9–26.1	46 (24.3)	19.3–30.0	
Bone	141 (50.2)	45.1–55.2	140 (49.8)	44.8–54.9		66 (23.5)	19.4–28.0	74 (26.3)	22.0–31.0	
Brain	60 (48.4)	40.7–56.1	64 (51.6)	43.9–59.3		25 (20.2)	14.4–27.0	39 (31.5)	24.6–39.0	
Histological subtype					0.000					0.000
NSCLC NOS	378 (37.9)	35.4–40.5	619 (62.1)	59.5–64.6		242 (24.3)	22.0–26.6	377 (37.8)	35.3–40.4	
Adenocarcinoma	2149 (46.1)	44.9–47.3	2511 (53.9)	52.7–55.1		1070 (23.0)	21.9–24.0	1441 (30.9)	29.8–32.1	
Squamous cell carcinoma	985 (43.4)	41.6–45.1	1287 (56.6)	54.9–58.4		691 (30.4)	28.8–32.0	596 (26.2)	24.7–27.8	
Adenosquamous carcinoma	34 (36.6)	28.2–45.6	59 (63.4)	54.4–71.8		25 (26.9)	19.4–35.5	34 (36.6)	28.2–45.6	
Neuroendocrine carcinoma	29 (80.6)	66.6–90.5	7 (19.4)	9.5–33.4		3 (8.3)	2.3–20.2	4 (11.1)	3.9–23.6	
Sarcomatoid carcinoma	5 (25.0)	10.4–45.6	15 (75.0)	54.4–89.6		2 (10.0)	1.8–28.3	13 (65.0)	44.2–82.3	
Adenocarcinoma growth patterns					0.000					0.000
Adenocarcinoma NOS	1780 (44.0)	42.7–45.3	2264 (56.0)	54.7–57.3		946 (23.4)	22.3–24.5	1318 (32.6)	31.4–33.8	
Acinar adenocarcinoma	118 (57.6)	51.6–63.4	87 (42.4)	36.6–48.4		60 (29.3)	24.0–34.9	27 (13.2)	9.5–17.7	
Solid adenocarcinoma	35 (25.5)	19.5–32.4	102 (74.5)	67.6–80.5		21 (15.3)	10.5–21.3	81 (59.1)	51.8–66.2	
Lepidic adenocarcinoma	92 (82.1)	75.1–87.8	20 (17.9)	12.2–24.9		17 (15.2)	9.9–21.9	3 (2.7)	0.7–6.8	
Invasive mucinous adenocarcinoma	51 (77.3)	67.2–85.4	15 (22.7)	14.6–32.8		12 (18.2)	10.8–27.8	3 (4.5)	1.3–11.3	
Papillary adenocarcinoma	37 (80.4)	68.3–89.4	9 (19.6)	10.6–31.7		7 (15.2)	7.4–26.7	2 (4.3)	0.8–13.1	
Lepidic mucinous adenocarcinoma	20 (90.9)	74.1–98.4	2 (9.1)	1.6–25.9		2 (9.1)	1.6–25.9	0	0–12.7	
Micropapillary adenocarcinoma	8 (44.4)	24.4–65.9	10 (55.6)	34.1–75.6		4 (22.2)	8.0–43.9	6 (33.3)	15.6–55.4	
Adenocarcinoma in situ	6 (100)	60.7–100	0	0–39.3		0	0–39.3	0	0–39.3	

age group and the population at large. We initially hypothesised that the increased rate of PD-L1 positivity in older patients may be a result of higher rates of sampling from serous effusions or lymph nodes in this age group, as discussed below, but high positivity rates were seen even in long biopsies from this age group. The cause for this phenomenon therefore remains unexplained.

Similarly, studies have variously reported higher rates in males [7, 8, 16, 20, 27–30], in females [14, 31], and no difference between sexes [6, 9–11, 13, 15, 17–19, 21–26, 32–37]. Our data demonstrated that rates of PD-L1 expression were very similar in males and females.

In our series, there was no significant difference in PD-L1 expression between adenocarcinoma and squamous cell carcinoma. Again, the literature is conflicting, with reports of no significant difference [6, 9, 10, 15, 18, 32, 33, 36], of higher rates in adenocarcinoma [14, 23, 31, 33, 34], and of higher rates in squamous cell carcinoma [7, 8, 13, 30, 35, 38].

In contrast, we found that it was the rarer NSCLC subtypes which demonstrated distinct patterns of PD-L1 expression. As previously described [26], neuroendocrine carcinomas were significantly less likely to express PD-L1 at both cut-offs than other subtypes. In contrast, poorly-differentiated sarcomatoid carcinomas were significantly more likely to express PD-L1 and, furthermore, were much more likely to express PD-L1 at the 50% cut-off – a finding which has previously been reported [17, 26].

In examining growth patterns of adenocarcinoma, the number of analysed cases was unfortunately small owing to our decision only to include cases where the reporting pathologist

Table 4 The relationship between PD-L1 expression and molecular characteristics

	TPS < 1%		TPS ≥ 1%		p value	TPS 1–49%		TPS ≥ 50%		p value
EGFR mutation status					0.000					0.000
Wild type	1219 (41.1)	39.6–42.6	1747 (58.9)	57.4–60.4		703 (23.7)	22.4–25.0	1044 (35.2)	33.8–36.7	
Mutated	147 (48.5)	43.7–53.4	156 (51.5)	46.6–56.3		79 (26.1)	21.9–30.6	77 (25.4)	21.3–29.9	
Individual EGFR mutations					0.483					0.735
Exon 19 deletions	59 (50.0)	42.1–57.9	59 (50.0)	42.1–57.9		30 (25.4)	18.9–32.9	29 (24.6)	18.2–32.0	
L858R	50 (54.3)	45.3–63.2	42 (45.7)	36.8–54.7		20 (21.7)	14.9–30.0	22 (23.9)	16.8–32.4	
G719X	14 (51.9)	34.7–68.7	13 (48.1)	31.3–65.3		4 (14.8)	5.2–30.8	9 (33.3)	18.6–50.9	
L861Q	4 (30.8)	11.3–57.3	9 (69.2)	42.7–88.7		5 (38.5)	16.6–64.5	4 (30.8)	11.3–57.3	
Exon 20 insertions	7 (38.9)	19.9–60.8	11 (61.1)	39.2–80.1		6 (33.3)	15.6–55.4	5 (27.8)	11.6–49.8	
S768I	2 (40.0)	7.6–81.1	3 (60.0)	18.9–92.4		2 (40.0)	7.6–81.1	1 (20.0)	1.0–65.7	
EGFR mutation type					0.037					0.163
Classical singlet mutations	109 (51.9)	46.0–57.8	101 (48.1)	42.2–54.0		50 (23.8)	17.9–29.7	51 (24.3)	19.5–29.6	
Rare singlet mutations	27 (42.9)	32.2–54.0	36 (57.1)	46.0–67.8		17 (27.0)	16.4–38.1	19 (30.2)	20.7–41.0	
Compound mutations	11 (37.9)	22.9–54.9	18 (62.1)	45.1–77.1		12 (41.4)	26.4–58.3	6 (20.7)	9.4–36.8	
ALK translocation status					0.000					0.000
Negative	1426 (43.0)	41.5–44.4	1894 (57.0)	55.6–58.5		765 (23.0)	21.8–24.3	1129 (34.0)	32.6–35.4	
Positive	12 (21.4)	12.9–32.4	44 (78.6)	67.6–87.1		20 (35.7)	25.1–47.5	24 (42.9)	31.6–54.7	
KRAS mutation status					0.853					0.765
Wild type	32 (50.8)	39.8–61.7	31 (49.2)	38.3–60.2		13 (20.6)	12.7–30.8	18 (28.6)	19.4–39.4	
Mutated	13 (44.8)	28.9–61.6	16 (55.2)	38.4–71.1		9 (31.0)	17.2–47.9	7 (24.1)	11.9–40.6	

had unambiguously indicated a single predominant growth pattern. Nonetheless, we identified a highly significant relationship between growth patterns at both cut-offs: the more poorly-differentiated solid and micropapillary patterns were much more likely to express PD-L1, and the better-differentiated lepidic and mucinous patterns were much less likely. This finding, which has been conspicuously uniform throughout the published literature [7, 13, 16, 19, 22, 24–28], lends weight to the previously-reported finding that poor differentiation in general is associated with higher PD-L1 expression [7, 10, 20, 22, 25, 30, 32, 36, 38].

Given that PD-L1 expression is known to demonstrate both spatial and temporal heterogeneity, we hypothesised that there might be systematic differences in the rates of expression amongst different sampled tissues, and in the reported rates of expression between different sampling modalities.

It has previously been reported that substantial differences exist in PD-L1 expression between primary and metastatic tumour deposits [25, 37]. We found that tumour samples taken from lung showed significantly lower rates of PD-L1 expression than those from the pleura or from lymph nodes, with this difference being driven by very high rates of high positivity in pleural and lymph node samples. Interestingly, other investigators have reported similar trends, although owing to smaller sample sizes, statistical significance was not reached [11, 21]. Given that we had no paired primary-metastatic doublet samples in our series, it is not possible to conclude that this means that pleural and nodal metastases more frequently express PD-L1 than their corresponding primary tumours; another plausible explanation is that patients with early-stage, lung-limited disease have lower PD-L1 expression rates than those with metastatic disease, but we did not have available data to test this hypothesis. Nonetheless, it is at least conceivable that exposure to the leukocyte-rich environment of a lymph node or to inflamed pleural fluid might alter PD-L1 expression in metastatic tumour deposits. Whatever the reason, this finding is clinically important because it suggests that very different treatment decisions might be made on the grounds of the type of tissue tested for PD-L1 expression, with primary lung biopsies being less likely to lead to institution of immunotherapy than pleural aspirates or lymph node samples. Further work is needed to corroborate this finding and to establish whether samples from different tissues are equivalent in predicting response to anti-PD-1 therapy.

Other investigators have previously reported that the method of tissue sampling may have a bearing on PD-L1 expression rates [9, 15, 23], with the suggestion that cytological and small biopsy specimens may underestimate PD-L1 expression [18, 39]. Overall, we found that cytological specimens were significantly more likely to show positivity than biopsy specimens, which in turn were significantly more likely to show positivity than resection specimens. This is a worrying finding: the Keynote trials which established the utility of PD-L1 in predicting response to pembrolizumab did so on the basis of

Table 5 The rates of PD-L1 expression in other series based on the 22C3 antibody. * Baseline characteristics refer only to a subset of the tested patients

Reference	N	Population	Male	Age	Modality	Histology	TPS <1%	TPS 1–49%	TPS ≥ 50%
Present study	10,005	UK	54.1%	Mean 68.4 Median 69.0	64.1% biopsy 25.4% cytology 10.5% resection	Adenocarcinoma 54.8% Squamous cell carcinoma 26.7%	44.4%	25.0%	30.6%
Keynote-001 [3]*	824	Global	52.7%	Median 64–68.5	Biopsies only	Non-squamous carcinomas 81.0% Squamous cell carcinoma 17.2%	39.2%	37.6%	23.2%
Keynote-010 [2]*	2222	Global	61.4%	Median 62.0–63.0	Not reported	Non-squamous carcinomas 70.0% Squamous cell carcinoma 21.5%	33.7%	37.9%	28.3%
Keynote-024 [1]*	1653	Global	61.3%	Median 64.5–66.0	Biopsy or resection	Non-squamous carcinomas 81.7% Squamous cell carcinoma 18.4%	30.7%	39.1%	30.2%
[4]	319	EGFR-mutated NSCLC South Korea	39%	Median 62	Resection	97% adenocarcinoma	48%	44%	8%
[5]	378	Argentina	61%	Not reported	Not reported	90% adenocarcinoma 10% squamous cell carcinoma	45.5%	33.1%	21.4%
[6]	204	Denmark	45%	Median 65	Biopsies only	72% adenocarcinoma	25.0%	50.0%	25.0%
[7]	329	China	Not stated	Median 60.5–61	Resections	67% adenocarcinoma	86.0%	9.1%	4.9%
[8]	1070	South Korea	67.1%	Median 63	Resections	62% adenocarcinoma 28% squamous cell carcinoma	56%	38%	6%
[9]	190	China	70.5%	Not reported	Resections	50.5% adenocarcinoma 38.9% squamous cell carcinoma	63.1%	25.8%	11.1%
[10]	678	Early-stage NSCLC Australia	70.4%	Median 65.5–69	Resections	40.7% adenocarcinoma 40.0% squamous cell carcinoma	71.8%	20.8%	7.4%
[11]	71	Adenocarcinoma US	Not reporter	Not reporter	Biopsy, cytology and resection	100% adenocarcinoma	42.3%	28.2%	29.6%
[12]	30	US	46.7%	Median 61.5	Not reported	86.7% adenocarcinoma 10.0% squamous cell carcinoma	20.0%	46.7%	33.3%

biopsy and resection specimens only; our data suggest that the use of cytological material might lead to ineffective over-treatment with anti-PD-1 therapy. To explore this further, we compared the rates of PD-L1 expression between cytology, biopsies and resections within particular tissue types. We found that all three modalities were associated with higher positivity rates in both pleural and lymph node specimens, with no significant difference between them. Surprisingly, we found that lung resections were actually associated with slightly lower positivity rates than lung biopsies, and that bronchial fluids were associated with slightly lower rates than bronchial biopsies. Taken together, our findings suggest that cytological sampling (when fixed in formalin and embedded in paraffin) is a reasonable means of assessing PD-L1 expression and can be used to expand anti-PD-1/PD-L1 therapy to a much wider range of patients who may not be able to undertake biopsy [11, 21]; however, in routine practice, should more tissue later become available, it would be prudent to repeat PD-L1 expression assessment [18]. Given that PD-L1 expression also shows temporal heterogeneity and may be affected by therapy, this approach may have additional utility [40].

Unlike the driver gene alterations in NSCLC, it is known that PD-L1 expression can frequently coexist with other actionable molecular alterations. With combinations of classical chemotherapy, targeted agents and immunotherapy being actively investigated [14], there is a pressing need to establish how PD-L1 expression relates to EGFR mutation, ALK translocation and KRAS mutation.

Epidemiological studies have provided conflicting results: some showing that PD-L1 expression is commoner in EGFR wild-type tumours [7, 9, 16, 19, 27, 30], others in mutant tumours [11, 14, 33, 34, 41, 42], and others still showing no significant association [3, 10, 20, 22, 24, 26, 33, 37, 43]. We found that EGFR mutations as a whole are associated with lower rates of PD-L1 expression at both cut-offs; however, the magnitude of this difference was small and so unlikely to be of clinical significance. There is evidence that EGFR-mutated tumours are characterised, in general, by lower tumour mutation burden (TMB) [44] and there is evidence that this is associated with low PD-L1 expression [45]; in contrast, however, there are preclinical data showing that activating mutations in EGFR drive PD-L1 overexpression [14, 31, 38, 46–48]. We hypothesise that the conflicting results in the literature might reflect the complex opposition of these two factors.

We were able to take advantage of the size of our dataset to explore the relationship between EGFR and PD-L1 in more detail. We failed to identify significant differences in PD-L1 expression rates between any of the individual EGFR mutations, but we noted a tendency for the non-classical EGFR mutations to be associated with higher rates of PD-L1 expression, which has also previously been suggested [29]. By grouping the mutation types, we showed that classical EGFR mutations were associated with the lowest rates of PD-L1 expression, with non-classical EGFR mutations being associated with higher rates, and compound EGFR mutations being associated with higher rates still. We hypothesise that the presence of non-classical EGFR mutations and, especially, of compound mutations, may be a proxy for higher TMB overall; this may act synergistically with the tendency of EGFR mutations to drive PD-L1 expression, leading to high expression. This has clinical significance: it is known that exon 20 insertions convey primary resistance to tyrosine kinase inhibitor therapy, and that other non-classical EGFR mutations generally have less favourable responses than exon 19 deletions and L858R [49]; in these groups of patients, therefore, anti-PD-1 therapy may be a more appropriate first-line treatment than TKIs. Again, clinical studies are required.

We found a strong relationship between the presence of ALK translocations and PD-L1 expression, which was driven by high rates of both low-positive and high-positive expression in ALK-translocated tumours. Preclinical studies have demonstrated that ALK translocation can drive PD-L1 expression [31, 41, 48], and in general, clinical series show either no or positive associations between PD-L1 expression and ALK translocation [3, 10, 11, 16, 19, 22, 24, 29–31, 33–35, 42, 43]. The question remains, however, whether ALK-driven PD-L1 expression has the same clinical significance as that seen in non-translocated tumours, and whether TKI or immunotherapy in these patients yields better outcomes.

Previous studies have suggested that KRAS-mutated tumours show higher rates of PD-L1 expression, both in preclinical [48] and clinical investigations [3, 10, 16, 17, 19–22, 24, 26, 30, 33–35, 42, 43]. We identified no significant association between KRAS mutation and PD-L1 expression status, likely owing to the very small number of specimens tested for KRAS mutation.

The most significant limitation of our study was the fact that it relied, for the most part, on clinicopathological data provided by external centres; as a result of our decision to prioritise data accuracy over completeness by excluding any data which were of uncertain veracity, this meant that data were missing for large numbers of cases. Furthermore, we were not able to account for differing policies of PD-L1 testing amongst different referring centres, and so we cannot exclude the possibility of selection bias in our series.

Conclusion

PD-L1 represents a powerful tool in predicting response to much-needed novel therapies in NSCLC. However, it must be borne in mind that it is an imperfect one, with studies repeatedly demonstrating that PD-L1 expression fails to predict response in a substantial minority of patients. The reasons for this remain unclear. It is highly likely that spatial

heterogeneity – as demonstrated by our data – contributes to this discrepancy, at least in part. However, it is also likely that interactions between PD-L1 expression and other molecular alterations influence in complex ways the extent to which tumours respond to immunotherapy. There is evidence that TMB is important in predicting the subset of PD-L1-positive tumours which will respond to immunotherapy [50], and we have demonstrated that non-random associations exist between PD-L1 expression, EGFR mutation and ALK translocation. The implication of all this is that optimal management of NSCLC may well require sophisticated integration of the results of a variety of molecular markers assessed using a range of platforms, perhaps assessed at multiple timepoints, and leading to the prescription of multi-modal therapies.

Compliance with Ethical Standards

Conflicts of Interest All Authors Declare that they Have no Conflict of Interest

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