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Adequate tumour cellularity is essential for accurate PD-L1 immunohistochemistry assessment on cytology cell block specimens.

1. Introduction

Tumour cell expression of PD-L1 as assessed by immunohistochemistry (IHC) is established as a predictive biomarker for anti-PD-1 immune checkpoint inhibitor therapy in non-small cell lung cancer (NSCLC). Indeed, high expression of PD-L1 (tumour proportion score ≥50%) is required for access to first line pembrolizumab [1,2]. Many patients with advanced NSCLC may only have cytology specimens available[3], for example fine needle aspirations (FNAs) of primary or metastatic lesions, pleural effusions, or endobronchial samples such as brushings, washings or EBUS-guided FNAs. However, the pivotal immune checkpoint inhibitor trials in NSCLC have only included biopsy specimens in their assessment of biomarkers, not cytology specimens[4-6]. A number of small studies have examined the accuracy of PD-L1 IHC on cell blocks prepared from cytology specimens as compared to biopsies or resection specimens, with somewhat conflicting results. A major concern with the use of cytology specimens is the often-low cellularity of the cell blocks. In this study, we sought to compare PD-L1 IHC scores between matched cytology and histology specimens, and examine the effect of cell block cellularity on the accuracy of PD-L1 IHC results.

2. Materials and Methods

2.1 Case selection

Databases from the Royal Melbourne Hospital and the Royal Prince Alfred Hospital pathology departments were retrospectively searched for cases of NSCLC between 2013 and 2017 with matched cytology cell block and histology samples obtained either during the same operational procedure (n=33) or within a time frame of five to forty-one days (n=27). Final diagnoses were based on the biopsy or resection specimen according to WHO 2015 criteria[7].

2.2 Cell block preparation

Bronchial brush tips and FNA needles were either rinsed in Hank's solution or saline, while small clots and tissue fragments were preserved in formalin before being centrifuged in to cell pellets, as previously described[8,9]. Cell blocks were then produced using a standard thrombin clot or agar cytology preparation prior to fixation in 10% neutral buffered formalin and processed as formalin fixed paraffin embedded blocks. Transbronchial needle aspirates were placed in formalin prior to centrifugation into cell pellets, as previously described[10].

2.3 PD-L1 immunohistochemistry

PD-L1 IHC was performed at the Peter MacCallum Cancer Centre on freshly cut slides from the cell blocks and histology specimens, using the Ventana PD-L1 (SP263) CE-IVD assay kit on the Ventana Benchmark Ultra staining platform, as per the manufacturer's instructions. The SP263 assay was selected for this study as it is the PD-L1 IHC assay most commonly used in diagnostic laboratories in Australia, and has shown superior performance in previous studies[11,12]. To limit variation all matched samples were stained sequentially on identical automatic staining platforms and control slides consisting of human placenta and tonsil tissues were used to monitor staining across different runs. Staining examples can be seen in figure 1. PD-L1 scoring was performed by two experienced pathologists (MC, WC) trained in PD-L1 HC evaluation, according to manufacturer's guidelines. The tumour proportion score was determined by scoring the proportion of viable tumour cells showing any perceptible partial or complete membranous staining, with respect to the total number of viable tumour cells in the section. Scores were recorded as continuous variables, with retrospective classification into categories of 0%, 1-49%, and ≥50%, to reflect

the regulatory requirements for predictive PD-L1 IHC. The number of tumour cells present in the sections was also recorded into categories of <50, 50-99, or ≥100.

2.4 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics, version 26. The intraclass correlation coefficient (ICC) was calculated to assess concordance between matched cytology and histology specimen PD-L1 scores as a continuous variable, and Cohen's kappa (unweighted) was calculated to assess concordance when the PD-L1 scores were categorised into 0%, 1-49%, and ≥50% bins, as well as overall percent agreement (OPA), sensitivity and specificity. The chi-squared test was used to compare proportions between independent groups.

2.5 Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

3. Results

3.1 Sample description

Consecutive cases of NSCLC with both cytology cell blocks and histology specimens available were retrieved from the archives of the Royal Melbourne Hospital (Melbourne cohort, n=27) and the Royal Prince Alfred Hospital (Sydney cohort, n=33). Two cases from the Melbourne cohort were excluded as no tumour cells were present in the cell block on review. Final biopsy diagnoses included adenocarcinoma (n=48, 83%), squamous cell carcinoma (n=9, 15%) and NSCLC, NOS (n=1, 2%). Patient age ranged from 41 years to 91 years, with a mean of 71, and a male to female ratio of 1:1.

The cytology specimens included image-guided FNA of lung lesions (n=34, 58%), bronchial brushings (n=19, 33%) and transbronchial needle aspiration (TBNA) (n=5, 9%), and the histology specimens included both core biopsies (n=33, 57%) and surgical resections (n=25, 43%).

3.2 Cell block cellularity

The cell blocks showed a range of tumour cellularity, with 17 (29.3%) of specimens containing less than 50 tumour cells, 12 (20.7%) containing 50-99 tumour cells, and 29 (50.0%) containing ≥100 tumour cells. These proportions were similar between the Melbourne and Sydney cohorts (p = 0.708), across diagnoses (p = 0.151), and specimen types (p = 0.444). All core biopsy and resection specimens contained 100 tumour cells or more.

3.3 PD-L1 expression

Overall, PD-L1 expression was absent (0%) in 43 (74.1%) of the cytology cell block specimens, present in 1-49% of tumour cells in 12 (20.7%) and present in ≥50% tumour cells in 3 (5.2%). All three cases with PD-L1 expression ≥50% had ≥100 tumour cells in the cell block, and the extent of PD-L1 expression varied significantly across the cell block cellularity groups (p<0.001). There was no significant difference in PD-L1 expression across diagnosis (p = 0.095) or specimen type (p= 0.063) groups.

The core biopsy specimens showed no tumour cell PD-L1 expression (0%) in 32 (55.2%) cases, 1-49% in 20 (34.5%) and ≥50% of in 6 (10.3%).

3.4 Agreement between paired cytology and histology specimens

Agreement between the paired cytology and histology specimens was substantial when assessing PD-L1 expression as a continuous variable, with an ICC of 0.653 (95% CI 0.468 - 0.782). Using a three-tiered classification of PD-L1 expression in 0%, 1-49% and ≥50% tumour cells, 40 (69.0%) cases showed agreement between the cytology and histology specimens with a kappa of 0.397.

The ICC increased to 0.826 (95% CI 0.697 - 0.904) when only samples with ≥50 tumour cells in the cell block were included (n=41), and increased further when the analysis was restricted to cases with ≥100 tumour cells present in the cell block (n=29), to 0.957 (95% CI 0.908 - 0.980). When categorising PD-L1 expression into three tiers as above, agreement increased substantially when only cases with ≥50 tumour cells were included, and marginally increased further when the analysis was restricted to cases with ≥100 tumour cells. At both the 1% and 50% cut-offs,

sensitivity increased with increasing cell block cellularity, while specificity remained high (Table 1).

Interestingly, agreement between cytology and histology PD-L1 results was substantially higher for adenocarcinomas (ICC = 0.819) than squamous cell carcinomas (ICC = 0.019). The number of squamous cell carcinomas included in the cohort was low, which may preclude meaningful comparison between diagnoses. Agreement between concurrently obtained cytology and core biopsy samples was only fair (ICC = 0.394), whereas agreement between cytology samples and surgical resection specimens obtained at a later time point was excellent (ICC = 0.979).

4. Discussion

In this study we have demonstrated substantial agreement between PD-L1 IHC performed on cytology cell blocks and the matched biopsy/resection specimens, with an overall ICC of 0.653, and excellent agreement with an ICC of 0.957 when the analysis is restricted to cell blocks containing ≥100 tumour cells. While specificity of PD-L1 IHC remains high regardless of specimen cellularity, the improved ICC with increasing cellularity of cytologic specimens appears to be due to the improved sensitivity of high cell content (>100 cells) cytologic specimens.

The landscape of predictive biomarkers for anti-PD-1 immunotherapy is complex, and often challenging to translate into real-life practice. A number of previous studies have investigated the potential usefulness of cytology specimens in PD-L1 IHC assessment in NSCLC, reviewed in Hernandez et al[13], however the range of different assays, staining platforms, scoring systems and reported statistics makes comparison across studies difficult. In general, studies of paired histology and cell block specimens have shown acceptable concordance[13-18], while direct IHC performed on smears or liquid based cytology (LBC) slides appears less concordant with subsequent biopsy specimens[15,19-21]. Bulk comparisons (i.e. non-paired samples) appear to show slightly higher PD-L1 expression in cytology specimens, of uncertain significance[22,23].

PD-L1 IHC on histology specimens is acknowledged to be an imperfect biomarker and ultimately the ability of a test to predict responses to anti-PD-1 immunotherapy is the most appropriate standard against which to compare new tests or test conditions. Importantly, Torous et al showed no difference in clinical responses to pembrolizumab in NSCLC cases showing high PD-L1 expression whether that was determined on a cytology or histology specimen[24]. The lack of clinical data is a limitation to our study, however PD-L1 IHC on histology specimens, using an approved and validated assay, scored by trained pathologists, is the currently accepted standard in this field. Clinical response data will be an important component of future studies.

The Blueprint phase 2 study investigated the inter-observer agreement amongst a large group of pathologists and across different assays and specimen types[25]. Inter-observer agreement for cytology specimens was good (ICC = 0.78 – 0.85), however this was slightly lower than for the histology samples (ICC = 0.89 – 0.93). As part of the Blueprint phase 2B study, fine needle aspiration and small biopsy samples were taken from 31 primary resected NSCLC for comparison of PD-L1 in the triplicate samples. As in our study, there was good agreement between the specimens (most Kappa-Fleiss scores >0.7) with no significant difference in PD-L1 scores between cytology and histology samples. However, they also found cytology samples unevaluable in a significant proportion of cases (14%)[26].

In our study, concordance increased substantially when the analysis was restricted to cell blocks with ≥50 tumour cells present, and increased further when only cell blocks with ≥100 tumour cells were included. Hernandez et al described similar findings, with a marked improvement in agreement between cytology and histology specimens when cell blocks with less than 100 tumour cells were excluded from the analysis[13]. In contrast, Skov and Skov found no significant change when low cellularity cell blocks were excluded[18]. The high specificity demonstrated in our study regardless of cell block cellularity, suggests positive results on cytology samples can be relied upon even if 100 tumour cells are not present. This is a common occurrence in clinical practice, and obtaining an additional sample can be challenging and lead to additional

cost and morbidity. The low sensitivity when cellularity drops below 100 tumour cells suggests a negative result would require a repeat sample to avoid a false negative.

PD-L1 expression in tumours can be heterogeneous, often with enhanced expression at the tumour-stroma interface and sometimes with striking variation between different areas of the tumour. This heterogeneity may introduce discrepancies between PD-L1 scores in small biopsy and resection specimens, which have shown varying levels of concordance in reported studies[3]. Our study showed better agreement between cytology samples and surgical resection specimens than between concurrently obtained cytology samples and core biopsies, reflecting the ability of FNAs to potentially sample a wider area of the tumour than core biopsies, and reduce under-sampling. However, adequate cellularity is required to reduce the potential impact of this spatial heterogeneity. Recent studies have reduced concerns regarding temporal heterogeneity of PD-L1 expression[27], thus the time delay of 5 to 41 days between collection of the cytology samples and the surgical resection specimens in our study is unlikely to have had significant impact on the results.

5. Conclusion

Cellularity of cytologic specimens significantly impacts the sensitivity of PD-L1 assessment in NSCLC specimens. Our results support the manufacturer guidelines requiring at least 100 tumour cells to be present for assessment when scoring PD-L1 in NSCLC using the Ventana SP263 assay and the Dako 22C3 and 28-8 assays. The increase in agreement with increasing cell block cellularity is reflected in increased sensitivity at both 1% and 50% cut-offs when including only samples with >50 or >100 tumour cells, while specificity remained high. Clinicians should be mindful of the risk of false negatives seen with low cellularity cell blocks, to ensure patients would not be excluded from potentially beneficial treatment, while false positive results are unlikely even at low cellularity.

In conclusion, cell blocks prepared from cytology specimens are acceptable for PD-L1 IHC in NSCLC, provided at least 100 tumour cells are present for assessment. Due to the lack of inclusion in the pivotal clinical trials, adequate intra-laboratory validation of cell block preparation, staining procedures and pathologist scoring of cell block specimens is highly recommended to ensure concordance with histology

specimens and accurate PD-L1 IHC scores. Despite the likely need for a repeat invasive procedure in inadequate samples, pathologists must avoid the temptation to report PD-L1 IHC on samples with insufficient tumour cells to avoid false negative PD-L1 results.

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Figure legend

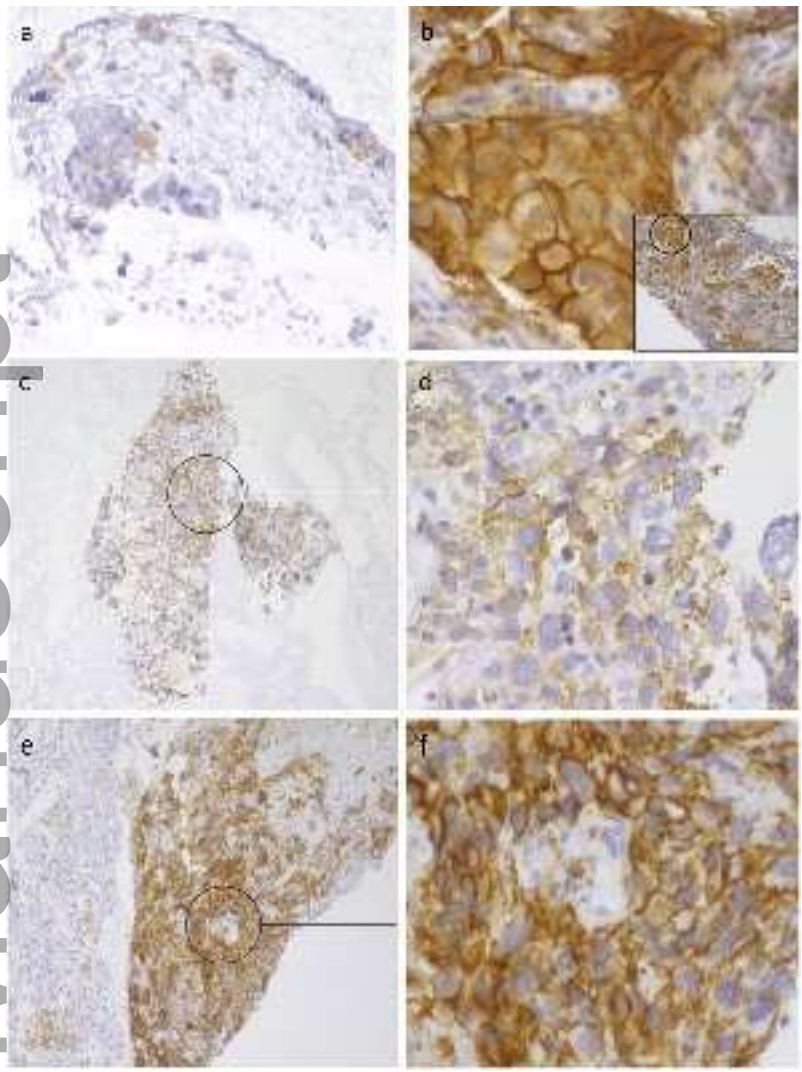
Figure 1: PD-L1 immunohistochemical staining of paired FNA and tissue biopsy samples from the same lung lesions in patients with NSCLC. a-b, matched FNA and core biopsy. a, FNA negative for PD-L1 staining (100x) b, core biopsy demonstrating strong PD-L1 membrane staining with TPS ≥ 70% (400x inset at 100x) . c-f matched FNA and core biopsy. c, FNA with ≥70% PD-L1 staining (100x black line marks

location of d). d, 400x image. e, core biopsy sample with ≥90% positive PD-L1 staining (100x black line marks location of f). f, 400x image.

Table

	ICC	Overall percent agreement (0%, 1-49%, ≥50%)	Kappa (0%, 1-49%, ≥50%)	Specificity at 1% cut-off	Sensitivity at 1% cut-off	Specificity at 50% cut-off	Sensitivity at 50% cut-off
All cases	0.653	69.0%	0.397	90.6%	46.1%	100%	50.0%
CB ≥50 tumour cells	0.826	75.6%	0.533	87.5%	64.7%	100%	75.0%
CB ≥100 tumour cells	0.957	75.8%	0.557	87.5%	61.5%	100%	100.0%

Table 1. Agreement between cytology cell block and histology specimens assessing PD-L1 expression using the SP263 IHC assay.



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