

Peer Review File

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Reviewer A: Major Revision*Comments:*

In this manuscript entitled “Endobronchial ultrasound bronchoscopy for 1 minimally invasive assessment of immunotherapy biomarkers in non-small cell lung cancer” by Dr. Steven Bozinovski et al., the authors reviewed the biomarkers (including candidates under investigations) for the efficacy of immune checkpoint inhibitors for patients with advanced NSCLC. They’re also introducing their original methods to differentiate the expression level of PD-L1 using multiplex ddPCR assay where absolute MMP9, TIMP3 and PD-L1 transcript copy numbers were determined within a single assay. I think that their methods are quite interesting and possibly informative for readers of this journal. However, in my opinion, extensive revisions are needed to consider this manuscript for publication. Please see my comments below. I hope my comments are useful for improvement of the article.

Major comments:

- 1. As a review article, the main subject of this manuscript seems to be unclear. It is especially unclear to what extent they want to focus their ddPCR assay or bronchoscopy modalities in this article. I felt that it was left half-done either as (1) review on clinical biomarker for ICI in general or (2) the one more focused on molecular mechanisms including their original methods. For (1), they should reconsider the order of explanations throughout the article. The explanations on novel techniques need to be introduced in the last by answering the following questions: What are the problems or weakness of the currently available biomarkers? How the novel techniques solve them? For (2), they should avoid redundant explanations on the apparently well-known (for thoracic oncologist) evidences on clinical efficacy of ICI or currently available biomarkers considering the readership of this journal. Although the topic itself the explanation on molecular interactions is interesting, the manuscript has a substantial room for improvement in the way of presentation.*

Response:

We agree with reviewer A and have made substantial changes to the review in line with these suggestions. The changes should now represent a review that focuses on 1) current and emerging ICI biomarkers, 2) the limitations of the current biomarkers and 3) a discussion of integrating EBUS bronchoscopy with novel molecular approaches to improve selection of patients that will benefit from ICI therapy.

We now provide the following sections in an order that is consistent with the above recommendation.

Background

Limitations of established and emerging immunotherapy biomarkers.

Bronchoscopic sampling may address some limitations of PD-L1 IHC testing.

Molecular analysis of PD-L1 levels may reduce heterogeneity in testing results.

Analysis of molecular regulators that control *PD-L1* expression

Conclusions.

We now also provide a more succinct discussion around well-known evidences on clinical efficacy of ICIs. We have also re-written and shortened sections that discuss molecular mechanisms as to emphasise pathways that specifically control PD-L1 expression levels. We feel that the review is now clearer in terms of its focus and presentation.

2. *The main title and subtitles seem to fail representing the contents of this article or each chapter.*

Response:

As suggested, we provide a new title “Integrating endobronchial ultrasound bronchoscopy with molecular testing of immunotherapy biomarkers in non-small cell lung cancer”.

We have also completely re-written the abstract to be consistent with the revised review.

Abstract

Immunotherapy has transformed treatment of advanced non-small-cell lung cancer (NSCLC) patients leading to remarkable long-term survival benefit. However, only about 20% of advanced NSCLC patients typically respond to immune checkpoint inhibitors (ICIs) that target the PD-1/PD-L1 pathway. The only validated biomarker for ICI therapy is the PD-L1 immunohistochemistry (IHC) test, which is considered an imperfect assay due to several variables including availability and integrity of tumour tissue, variability in staining/scoring techniques and heterogeneity in PD-L1 protein expression within and across tumour biopsies. Herein, we discuss integrating minimally invasive EBUS bronchoscopy procedures with novel molecular approaches to improve accuracy and sensitivity of PD-L1 testing. EBUS guided bronchoscopy facilitates repeated sampling of tumour tissue to increase the probability of detecting PD-L1 positive tumours. Since intra-tumoural PD-L1 (CD274) copy number is reported to be less heterogeneous than PD-L1 protein detection, quantifying PD-L1 transcript levels may increase detection of PD-L1 positive tumours. PD-L1 transcript levels show excellent concordance with PD-L1 IHC scoring and multiplex digital droplet PCR (ddPCR) assays that quantify absolute PD-L1

transcript copy number have been developed. ddPCR can also be automated for high throughput detection of low abundant variants with excellent sensitivity and accuracy to improve the broader application of diagnostic cut-off values. Optimizing diagnostic workflows that integrate optimal EBUS bronchoscopy procedures with emerging molecular ICI biomarker assays may improve the selection criteria for ICI therapy benefit.

Minor comments:

1. *PD1/PDL1* → *PD-1/PD-L1*
2. *PD-L1 >50%* → *PD-L1 ≥50%*

Response: This has been corrected throughout the manuscript.

Reviewer B: Minor Revision

Comments:

1. *Page 4, line 95: Interchangeability among four kinds of clone antibodies (22C3, 28-8, SP142, and SP263).*

To my knowledge, some research groups made a proof of interchangeability among 22C3, 28-8, and SP263 (ref. J Cancer. 2020 Jan 1;11(4):974-982. doi: 10.7150/jca.34793. eCollection 2020). So far, SP142 could not be alternative antibody. The authors mentioned “Direct comparison between different PD-L1 IHC assays including 22C3, 28-8, SP142 and SP263 revealed that the indivisual tests cannot be interchangeably used in clinical practice.” I recommend they reconsider this sentence.

Response:

We have revised this statement as below:

Page 6:

Direct comparison between different PD-L1 IHC assays suggests that individual tests cannot be interchangeably used in clinical practice²⁰, however other groups have shown that 22C3, 28-8, SP263 but not SP142 can be used interchangeably²¹.

2. *Page 12, line 273: Bronchoscopic assessment of ICI biomarkers*

Inter-tumor heterogeneity should be considered when endoscopists decide biopsy site. Some researchers pointed out discrepancy of PD-L1 staining between primary tumor and metastatic lymph node (ref. Clin Lung Cancer. 2019 Sep;20(5):391-396. doi: 10.1016/j.clcc.2019.05.013. Epub 2019 Jun 5. J Thorac Dis. 2019 Dec;11(12):4982-4991. doi: 10.21037/jtd.2019.12.24.). I recommend you should better mention the issue of inter-tumor heterogeneity of PD-L1 staining.

We agree this is a clinically relevant issue and have added extensive discussion on this to the revised manuscript including citation of the above-mentioned articles.

Page 4-5:

In addition to the technical challenges of scoring PD-L1 using small bronchoscopy specimens, heterogeneity of PD-L1 expression remains an unresolved question and variability according to metastatic site is recognized (9, 10). Temporal heterogeneity has been reported following adjuvant therapy (10, 11), emphasizing the importance of re-biopsy in patients who experience recurrent/progressive disease. Intra-patient heterogeneity has been reported following multi-site biopsy (9, 12) with sufficient variance to result in alteration in clinical management. Heterogeneity may be attributable in part to variation in NSCLC histotype (13), and presents significant implications for its accuracy as a predictive biomarker. Use of more advanced molecular assessment, such as copy number alterations, may improve concordance in PD-L1 assessment compared to IHC (14), but this remains to be confirmed. Hence, PD-L1 IHC is considered an imperfect test, where there are NSCLC patients that are negative for PD-L1 expression, but still respond to ICI therapy. Nonetheless, screening PD-L1 levels still represents an important strategy for selecting NSCLC patients for ICI therapy, but there are opportunities to improve the workflow and evaluation of PD-L1 expression to minimise technical challenges and subjective interpretation of results.

Page 6:

Bronchoscopic sampling may address some limitations of PD-L1 IHC testing.

Heterogeneity of PD-L1 expression within and across tumour specimens from an individual creates significant challenges in utilising the PD-L1 IHC test as an accurate and predictive biomarker. To address this, extensive intra-tumoural and inter-tumoural sampling may increase the probability of detecting PD-L1 positive tumour cells. Endobronchial ultrasound (EBUS) sampling of tumours routinely involves multiple samples (21) taken from multiple sites (22), which provides a greater breadth of assessment to potentially overcome intra-lesional and inter-lesional heterogeneity. EBUS guided bronchoscopy is a minimally invasive technique that facilitates repeated sampling of tumour tissue. EBUS guided sampling when combined with rapid-on-site examination (ROSE) can diagnose malignancy in real time with high sensitivity and specificity (23-26).