



# Integrating endobronchial ultrasound bronchoscopy with molecular testing of immunotherapy biomarkers in non-small cell lung cancer

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**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.

**Keywords:** Immunotherapy; biomarkers; programmed death Inhibitor 1 ligand (PD-L1); lung cancer; non-small cell lung cancer (NSCLC); bronchoscopy

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## Introduction

Immune checkpoint inhibitors (ICIs) are currently changing how advanced non-small cell lung cancer (NSCLC) patients that do not harbour actionable mutations are being treated. Programmed death inhibitor 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) are classic inhibitory immune checkpoint receptors that are critical in maintaining self-tolerance, as they will transmit inhibitory signals to prevent tissue pathology when engaged by their ligands. Tumour cells can exploit this process and evade cytotoxic T cell immuno-surveillance by expressing the PD-1 ligand (PD-L1). Engagement of PD-1 expressing tumour-infiltrating CD8<sup>+</sup> T cells with PD-L1 on tumour cells suppresses cytotoxic T cell cytokine production and proliferation in NSCLC (1). In the past five years, multiple checkpoint inhibitors targeting PD-1, PD-L1 and/or CTLA-4 have been approved and targeted for use in treating advanced, refractory, or relapsed solid tumours that have not responded to standard-of-care cancer treatments. Nivolumab is a fully human IgG4 anti-PD-1 antibody that improves overall survival compared to docetaxel monotherapy in non-squamous NSCLC patients (2) and response rates were even better in squamous cell carcinoma (SCC) patients (3). Pembrolizumab is an alternative humanized monoclonal antibody against PD-1, which significantly improved overall survival in NSCLC patients compared to platinum-based chemotherapy (4).

## Limitations of established and emerging immunotherapy biomarkers

About 20% of advanced NSCLC patients typically respond to biologics that target the PD-1/PD-L1 pathway and this response can be doubled by screening for PD-L1 tumour levels (4), however the majority of advanced NSCLC patients will not respond to ICI monotherapy. The assessment of PD-L1 tumour staining by immunohistochemistry (IHC) is currently the only validated predictive biomarker for ICI therapy in NSCLC (5). Pembrolizumab is now approved as a first line therapy for NSCLC patients with  $\geq 50\%$  PD-L1 tumour expression and a second line therapy for  $\geq 1\%$  PD-L1 tumour expression (6). The rationale for selecting ICI responders with predictive biomarkers extend beyond enriching for response or resistance. Toxicities including underlying autoimmune disorders represent a typical exclusion criterion in ICI therapy trials, as reactivation of primed lymphocytic

populations can be detrimental or fatal. Biologics such as monoclonal antibodies that suppress the PD-1/PD-L1 pathway are also very expensive, thereby limiting the broad application of this therapy. Hence, the relatively low response rate and potential toxicities relating to ICI therapy necessitate the need to identify predictive biomarkers to guide treatment.

PD-L1 IHC is established as the principal ICI response biomarker in NSCLC, as discussed above. However, a number of limitations of PD-L1 IHC have been recognized in both the clinic and the published literature where the evaluation of PD-L1 expression in advanced NSCLC patients can be technically challenging for pathologists and oncologists. Studies validating PD-L1 tumour expression included only histologic specimens, however in clinical practice diagnostic specimens are more frequently cytologic/small volume specimens. Hence, the availability and quality of fixed tissue for PD-L1 testing is variable in practice, as small bronchoscopy specimens can be crushed and unsuitable for IHC. In addition, the process of collecting, processing and staining tissue blocks is not necessarily standardised across different sites and this can collectively influence test performance and accuracy. Direct comparison between different PD-L1 IHC assays suggests that individual tests cannot be interchangeably used in clinical practice (7), however other groups have shown that 22C3, 28-8, SP263 but not SP142 can be used interchangeably (8).

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.

Tumour mutational burden (TMB) is emerging as an independent biomarker for ICI benefit. It is broadly defined as the total number of somatic mutations in the tumour exome (15) and is associated with neoantigen burden which can activate T lymphocytes to kill cancer cells (16). In the context of clinical trials, a high TMB has been shown to have greater predictive capacity than PD-L1 IHC in advanced NSCLC (17). This concept was validated in the CheckMate 227 study, the first randomised phase III trial evaluating ICI outcome in NSCLC patients predefined in relation to TMB. In this open label trial, the combination of nivolumab plus ipilimumab (anti-CTLA-4) was particularly effective in increasing one year progression-free survival in patients with a high TMB (at least 10 mutations per megabase) relative to chemotherapy, irrespective of PD-L1 expression level (17). Furthermore, determining TMB has become increasingly feasible with the availability of sequencing technology. However, challenges arise with regards to tumour tissue quantity and quality; lack of standardisation in TMB assessment methods; and variability in definition of high TMB. Efforts are being made to harmonise TMB distributions across different cohorts (18), and this may help to determine TMB threshold and validate the clinical value of TMB as a predictive biomarker. Recent clinical studies have also defined tumour immunophenotypes that correlate with response to ICI (19). Inflamed (“hot”) tumours contain a high density of infiltrating cytotoxic CD3+ lymphocytes with an “inflamed” gene expression profile (20), providing a more favourable environment for T-cell activation and expansion. “Hot” tumours, where the suppressive phenotype is driven by upregulation of immune checkpoints such as PD-1 and CTLA-4, demonstrate a high rate of response to ICIs. Non-inflamed (“cold”) tumours associated with immune suppression or tolerance (19) exhibit lower rates of response to ICIs.

### **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). Specimens can be processed as a cell block for morphologic and immunohistochemical assessment (27). While initial studies regarding the predictive value of PD-L1 were limited to histologic specimens, recent studies have confirmed that, with adequate tumour cellularity, PD-L1 IHC on cytology cell-block specimens (including EBUS-directed specimens) is an acceptable alternative to histological specimens in NSCLC (28). Sampling of pulmonary parenchymal lesions is undertaken using radial EBUS, a different method which includes both cytologic and histologic sampling. Cytologic samples may be assessed by IHC (29), as well as next-generation sequencing (including kRas) (23).

Bronchoscopic cryobiopsy can achieve larger parenchymal lung specimens than possible through standard EBUS-forceps biopsy (30). Hence, radial EBUS-directed sampling may be augmented by use of cryobiopsy (31,32) and our group has used this for assessment of tumour-infiltrating lymphocyte density (unpublished data). In addition, EBUS-guided transbronchial needle aspiration (EBUS-TBNA) is used for diagnosis of mediastinal/hilar lymphadenopathy (25). While initially designed for diagnostic sampling of pathologic lymphadenopathy, EBUS-TBNA is increasingly used for systematic staging of normal-size lymph nodes (22,33). Samples yield significant amounts of highly intact DNA (34), and our group has used fresh tissue acquired by EBUS-TBNA to complete whole genome sequencing to map genomic heterogeneity in inoperable lung cancer (35). From this dataset we have been able to establish TMB scores (unpublished data). In addition, EBUS-TBNA sampling has recently been used to complete immunophenotypic characterization of tumour-draining lymph nodes in NSCLC (36).

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

Elevated PD-L1 levels in the tumour microenvironment

can be driven by amplification of the *PD-L1* (*CD274*) gene in certain cases. A study comparing PD-L1 IHC scores with *PD-L1* gene amplification in pulmonary carcinoma patients detected *PD-L1* gene amplification in about 9% cases and 12/14 of the amplified cases demonstrating higher PD-L1 IHC scores (37). Furthermore, whilst *PD-L1* copy number was moderately concordant with PD-L1 IHC scores in EBUS-TBNA biopsies, intra-tumoural *PD-L1* copy number was less heterogeneous than PD-L1 IHC staining in resected tumour tissue (14). There are also other biological processes within the tumour microenvironment that will drive an increase in PD-L1 levels, which will be discussed later. Since they all converge of increasing *PD-L1* transcript levels, there are biological and practical reasons for redirecting PD-L1 testing towards a molecular approach. The comparison of *PD-L1* transcript levels with PD-L1 IHC tumour cell scoring has shown excellent agreement in NSCLC biopsies, and supports the quantitative determination of *PD-L1* transcript levels as an alternative molecular approach that minimises interobserver variability (38,39). We have also developed a novel approach to detect PD-L1 status and multiple tumour mutations using a single EBUS bronchoscopy specimen. In this study, *PD-L1* transcript expression determined by RT-qPCR was highly concordant with PD-L1 IHC tumour cell staining in NSCLC, as determined using the Ventana (SP263) PD-L1 Assay (40). In contrast to tumour staining, there was a weaker association between *PD-L1* transcript levels and PD-L1 immune cell staining (40).

The question remains whether molecular approaches can be adapted to the clinical diagnostic setting and improve the application of predictive biomarkers for ICI therapies. The rapid advancement of molecular technologies is providing an unparalleled opportunity to transform the clinical diagnostics field in lung cancer. It is increasingly feasible to isolate tumour DNA and screen for mutations from primary and metastatic sites. The sensitivity and accuracy of molecular technology is also facilitating the detection of low abundance circulating tumour DNA in blood to screen for genetic mutations in a non-invasive and dynamic manner in advanced patients. An example of such technology is digital droplet PCR (ddPCR), which is now established for plasma genotyping of venous blood samples. This procedure allows for the rapid detection of common EGFR mutations including the EGFR p.T790M acquired resistance mutation in patients with multiple metastatic site (41). ddPCR is particularly adept at detecting low abundant variants and reported to be more sensitive

than amplification-refractory mutation system (ARMS) for detection of EGFR variants, and may be more useful in monitoring disease progression (42). In addition, ddPCR can address some limitations associated with RT-qPCR as it allows for absolute quantification of mRNA copy number to generate diagnostic cut-off values and is more suitable for higher order multiplexing of gene targets.

We have developed a multiplex ddPCR assay where absolute MMP9, TIMP3 and PD-L1 transcript copy numbers were determined within a single assay using Taqman primers and the QX200 Droplet Digital PCR System (43). The MMP9:TIMP3 ratio was significantly elevated in NSCLC biopsies and was highly sensitive (99%) for discriminating malignant biopsies. Analysis of the PD-L1:TIMP3 ratio in the same assay revealed that *PD-L1* transcript levels are significantly increased in biopsies with  $\geq 50\%$  PD-L1 IHC tumour cell score ( $P < 0.0001$ ) (43). Hence, our novel ddPCR approach can accurately quantify PD-L1 tumour levels and provide sufficient nucleic acid for additional genetic tests including EGFR mutations or ALK and ROS1 fusions from a single small biopsy, thereby potentially avoiding the need for re-biopsy. ddPCR technology also has the capacity to be automated, performed within 24 hours of specimen collection and quantitative analysis removes potential inter-assay and inter-observer variation in the scoring of PD-L1 levels. Whilst our findings found that PD-L1 tumour expression is the dominant driver of increased PD-L1 transcript levels in NSCLC biopsies, our approach does not differentiate between tumour and immune cell PD-L1 expression. What is becoming increasingly apparent is that PD-L1 expression on tumour and infiltrating immune cells has a non-redundant role in regulating immunity to cancer, where immune cell expression is equally important in predicting benefit to atezolizumab in NSCLC (44). Future studies need to evaluate the utility of our ddPCR test as a predictive independent biomarker, as the assessment of clinical response is the 'gold standard' to compare different ICI therapy biomarker strategies.

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

An alternative approach to developing ICI biomarkers may be to quantify the molecular regulators that control expression of inhibitory receptor ligands such as PD-L1. We now understand that the increase in PD-L1 expression in solid tumours can be regulated by multiple

mechanisms. *PD-L1* gene expression can be controlled in an interferon-gamma-inducible manner, whereby IRF1 transcription factor binds to the CD274/*PD-L1* promoter region via a JAK/STAT dependent mechanism (45). The inflammatory nature of the solid tumour microenvironment includes activated innate and adaptive immune cells that can produce interferons, which can control the spatial and temporal expression of *PD-L1* in tumour and immune cells. Retrospective analysis of interferon regulated genes in advanced melanoma patients developed a 28-gene signature score, which showed a significant correlation with overall response and progression-free survival to ICI therapy (46). Whilst the importance of interferons in regulating *PD-L1* in NSCLC requires further investigation, it may not be a uniform mechanism as the association between ICI response and interferon gene signatures appears to be weaker in NSCLC when compared to melanoma (47).

Another plausible mechanism by which *PD-L1* expression is increased in NSCLC is the oncogene-driven upregulation of *PD-L1* gene expression at a transcriptional level. *kRas* is a commonly mutated oncogene in western countries that is present in about 30% of adenocarcinomas, where it is more commonly detected in smokers. *kRas* has been considered undruggable due to its high affinity for GTP and lack of clear binding pocket, however new generation inhibitors have now been developed and are showing more rapid and durable tumour regression in mice (48). The RAF/MEK/ERK MAPK signalling pathway is the classic *kRas* effector pathway that controls cellular proliferation, differentiation and survival. Activation of the MAPK kinase pathway also converges on the c-Jun and STAT3 transcription factors, which can increase expression of *PD-L1* transcript levels (49). In addition, increased oncogenic *kRas* signalling can stabilize *PD-L1* mRNA, which drives tumour cell-intrinsic *PD-L1* expression and immunoresistance (50).

There is emerging data to suggest that *kRas* status can influence survival outcomes in response to ICI therapies. As an independent biomarker, *kRas* status does not appear to influence nivolumab efficacy, where patients were treated irrespective of *PD-L1* expression (51). However, in a separate study that factored in *PD-L1* expression, the efficacy of ICIs was consistently higher in *kRas*<sup>mutant</sup>*PD-L1*<sup>high</sup> patients compared to *kRas*<sup>wildtype</sup>*PD-L1*<sup>high</sup> patients (52). In a recent meta-analysis of five trials involving over 3000 patients with advanced NSCLC, patients receiving ICIs displayed prolonged overall survival in the *kRas* mutant subgroup but not in the *kRas* wild-type subgroup compared

to docetaxel (53). A similar finding was observed in a smaller meta-analysis of three trials where ICIs significantly improved overall survival in previously treated *kRas*<sup>mutant</sup> patients, but not in *kRas*<sup>wildtype</sup> patients (54).

Activating EGFR mutations can also stimulate *PD-L1* expression in tumour and immune cells (55). However, unlike *kRas* mutant tumours, harboring an activating EGFR mutation does not appear to confer improved response to ICI therapy in NSCLC (56). This likely reflects the observation that EGFR wild-type tumours are more likely to express higher *PD-L1* than mutated tumours (57). In contrast to EGFR mutations, the presence of ALK fusions were positively associated with increased *PD-L1* expression (57). NSCLC cell lines that are positive for ALK-fusions also display increased expression of *PD-L1* levels that was dependent on MAPK and AKT signaling and sensitive to the ALK inhibitor alectinib (58). Gene deletion of Phosphatase and tensin homolog (PTEN) tumour suppressor commonly occurs in NSCLC and is associated with more aggressive tumours due to activation of the AKT/mTOR signaling pathways in cancer cells (59-63). The genetic loss and inactivation of PTEN and Lkb1 in mice recapitulated pathological features of SCC and resulted in a marked increase in *PD-L1* tumour expression (64). Furthermore, inhibition of the mTOR pathway effectively reduced *PD-L1* levels in lung tumors from A/J mice exposed to the tobacco-carcinogen NNK and in *kRas*<sup>LA2</sup> mice (64).

## Conclusions

The screening of *PD-L1* levels by IHC is relatively straightforward and cost-effective in the experienced diagnostic pathology setting, but it is not without its limitations. The availability of good quality tumour tissue, non-standardised tissue collection, processing and staining protocols and intra-tumoural heterogeneity collectively contribute to variability in *PD-L1* IHC biomarker performance. Nonetheless, *PD-L1* expression analysis remains the only validated approach to guiding ICI therapies. Since *PD-L1* transcript levels show excellent concordance with *PD-L1* tumour levels determined by IHC, there is an opportunity to track *PD-L1* levels using new molecular technology that is highly sensitive and readily automated. Multiplex ddPCR assays can accurately quantify *PD-L1* absolute copy number in EBUS bronchoscopy specimens (43). The integration of advanced tumour sampling procedures such as radial EBUS-directed

sampling by cryobiopsy with ddPCR technology may offer an unparalleled level of sensitivity and accuracy to generate reliable prognostic cut-off values for PD-L1 expression. In summary, a diagnostic workflow that integrates the collection of designated molecular biospecimens by EBUS bronchoscopy will facilitate evaluation of multiple ICI molecular biomarkers including ddPCR based PD-L1, TMB or kRas status. The sensitivity of such techniques now makes it feasible to simultaneously screen for multiple molecular biomarkers within a single EBUS sample, whereby a signature of ICI response combining two or more markers may prove to be optimal. This approach requires further clinical validation and can be integrated in the multitude of immunotherapy trials. Further studies are needed to validate and benchmark molecular ICI biomarkers in a coordinated approach, which ultimately strive to improve selection criteria for immunotherapy.

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