


## LONG COURSE ARTICLE



# Biomarkers of response to checkpoint inhibitors beyond PD-L1 in lung cancer

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Immunotherapy, including use of checkpoint inhibitors against PD-1, PD-L1, and CTLA-4, forms the backbone of oncologic management for the majority of non-small cell lung carcinoma patients. However, response to these therapies varies widely, from patients who have complete resolution of metastatic disease and long-term remission, to those who rapidly progress and succumb to their cancer despite use of the newest checkpoint inhibitors. While PD-L1 protein expression by immunohistochemistry serves as the principle predictive biomarker for immunotherapy response, neither the sensitivity nor the specificity of this approach is optimal, and clinical PD-L1 testing is plagued by concerns around result reproducibility and confusion born from the proliferation of different companion diagnostic assays. At the same time, insights into tumor and host immune-specific factors that inform both prognosis and response prediction are beginning to define better immunotherapy biomarkers. Beyond immune checkpoint expression status, common themes in analyses of immunotherapy response prediction include cancer neoantigen production, the state of the antigen presentation pathway in both tumor and antigen presenting cells, the admixture of effector and suppressor immune cells in the tumor microenvironment, and the genomic drivers and comutations that can influence the all of these variables. This review will address the state of PD-L1 testing in lung cancer, the role for tumor mutation burden as a predictive biomarker, the evolving status of human leukocyte antigen/major histocompatibility complex expression as a marker of antigen presentation, approaches to tumor immune cell quantitation including by multiplex immunofluorescence, and the importance of tumor genomic profiling to ascertain oncogenic driver (*EGFR*, *ALK*, *KRAS*, *MET*, etc.) and co-mutation (*STK11*, *KEAP1*, *SMARCA4*) status.

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## INTRODUCTION

Lung cancer is a global source of morbidity and mortality<sup>1</sup>. Historically stigmatized as a “smoker’s disease”<sup>2</sup>, the medical and lay communities have come to acknowledge the diversity of patients affected by lung cancer only in the past one to two decades. Underpinning that patient-level diversity is a pathobiologic heterogeneity with major implications for disease prognosis and therapeutic options. Today, we recognize that lung cancers—specifically those with “non-small cell” histology, can be broadly divided into those with a defined oncogene addiction and those that display a more complex and potentially hypermutated genome. A significant proportion of the former group, including tumors with *EGFR*, *MET*, *BRAF*, *ALK*, *ROS1*, *RET*, *ERBB2*, and *NTRK1-3* alterations may respond to specific inhibitors of the oncogenically-activated protein<sup>3</sup>. The latter group, which is highly enriched in smokers, tends to show MAPK pathway dependency with frequent tumor suppressor gene alterations and a smoking mutational signature that manifests as elevated tumor mutational burden (TMB) and an increased likelihood of generating mutation-associated neoantigens. In theory, this milieu may be conducive to therapies that harness the anti-tumor properties of the host immune system<sup>4</sup>. However, only a subset of patients, even among those who fit this phenotype, actually respond to immunotherapies, including inhibitors of the immune checkpoints programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1) and cytotoxic

T lymphocyte associated protein-4 (CTLA-4). This review aims to address the current state of PD-L1 testing, specifically for patients with advanced stage non-small cell lung carcinoma (NSCLC), then examine other established and evolving biomarkers that may predict response to immune checkpoint blockade across lung cancers.

## PD-L1 TESTING

A variety of anti-PD-1, anti-PD-L1 or immune checkpoint inhibitor combinations are approved in the United States for use in the first line treatment of patients with advanced/metastatic non-small cell lung carcinoma (Table 1). Each of these immune checkpoint therapies has a paired companion diagnostic in the form of PD-L1 immunohistochemistry<sup>5</sup>. Each drug has a unique “approved” assay, although two anti-PD-1 drugs, pembrolizumab and ceplimimab, use the same 22C3 assay; in the United States specifically, use of the former drug as monotherapy requires that at least 1% of tumor cells show membranous expression, whereas the latter requires 50% tumor cell expression<sup>6</sup>. Nivolumab, another PD-1 inhibitor, may be used as monotherapy in the second line treatment setting and beyond, in which case the paired PD-L1 assay (using clone 28-8) is considered complementary in that positivity can inform likelihood of response but testing is considered optional. In the first line, nivolumab is approved in

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**Table 1.** FDA approved immunotherapies in advanced stage non-small cell lung carcinoma in 2021.

	<b>Nivolumab/Ipilimumab</b>	<b>Pembrolizumab</b>	<b>Atezolizumab</b>	<b>Cemiplimab</b>
Type of therapy	Anti-PD-1/Anti-CTLA4	Anti-PD-1	Anti-PD-L1	Anti-PD-1
Company	Bristol-Myers Squibb	Merck	Roche	Regeneron/ Sanofi
Biomarker(s)	PD-L1 (28-8)	PD-L1 (22C3) & Tumor mutation burden	PD-L1 (SP142)	PD-L1 (22C3)
Diagnostic Designation	Complementary & Companion	Companion	Companion	Companion
Scoring and Interpretation	Complementary: ≥ 1%, ≥ 5%, ≥ 10% TC Companion: ≥ 1% for ipi/nivo in first line	Monotherapy in first and later lines: TPS ≥ 1% Following progression on prior therapies: TMB-H (≥ 10mut/Mb)	PD-L1 stained tumor-infiltrating ICs of any intensity covering ≥ 10% of tumor area or ≥ 50% TC	≥ 50% TC

TC tumor cells, TPS tumor proportion score, IC immune cell score.

combination with the CTLA-4 inhibitor ipilimumab, and 28-8 companion testing is required to show at least 1% tumor cell staining. In contrast to the anti-PD-1 therapies, the anti-PD-L1 therapy atezolizumab has been approved for use in the first line with a companion diagnostic that considers both tumor and immune cell staining. Using the companion diagnostic assay with the SP142 antibody, two potential staining patterns will qualify a patient for therapy with atezolizumab, either at least 50% tumor cell staining or the presence of PD-L1 positive immune cells involving at least 10% of the tumor area.

The diversity of immunohistochemical assays and cutpoints defining a positive result has been a source of confusion and frustration in the medical community and has driven a number of harmonization efforts. These efforts have been reviewed extensively in other publications<sup>5,7</sup>, but to briefly summarize, the performance characteristics of the 22C3 and 28-8 assays appear to be similar based on side-by-side evaluation in retrospective cohorts<sup>8,9</sup>. Some antibodies such as SP263 and E1L3N are in routine use although they have not been approved as companion diagnostics (and are therefore considered laboratory developed tests) but can show comparable patterns of staining to the approved assays when properly validated<sup>10,11</sup>. The one consistent outlier has been the SP142 assay<sup>9</sup>, which shows lower tumor cell staining, despite the SP142 antibody recognizing identical or nearly identical epitopes as SP263 and E1L3N<sup>12</sup>. The SP142 assay was reportedly optimized for both tumor cell (TC) and immune cell (IC) scoring<sup>13</sup>, however its performance as an immune cell marker is further confounded by poor interobserver agreement around interpretation of immune cell expression<sup>9,14</sup>.

Despite the overall evidence for comparable performance for many available PD-L1 assays, to date there has been limited demonstration of “clinical validity”—i.e., direct comparison of the predictive ability of different PD-L1 assays in well-controlled prospective clinical trials of checkpoint inhibitor therapies. However, in the clinical trial of atezolizumab in the first line setting (IMPower 110), investigators assessed the predictive power of multiple different PD-L1 antibodies, including SP142, SP263, and 22C3<sup>15</sup>. Consistent with prior retrospective analyses, the overlap in tumor cell staining between SP142–SP263 and SP142–22C3 was limited, with SP142 capturing a lower percentage of the tested population with high level (> 50%) tumor cell staining. As expected, SP263 and 22C3 captured highly overlapping populations of high expressers. Despite the discrepancies in tumor cell staining, application of the clinically-defined cutpoints for high level expression with the three antibodies (50% tumor cell staining for 22C3 and SP263; 50% tumor cells or

10% IC with SP142) identified populations with similar benefit from atezolizumab as compared to platinum chemotherapy. Notably, in IMPower 010, a phase 3 trial of adjuvant atezolizumab versus best supportive care for resected stage IB to IIIA NSCLC, patients were enrolled based on PD-L1 status using the SP142 assay but outcomes were stratified according to tumor cell PD-L1 expression of ≥ 1% using the SP263 assay; a disease free survival benefit was observed for patients receiving atezolizumab when tumors met the definition of PD-L1 positive according to the SP263 assay<sup>16</sup>. While these observations do not necessarily indicate that the IHC assays are “interchangeable” it does suggest that application of the clinically-defined cutpoints for certain assays can predict benefit from different but related immune checkpoint blockade therapies. Similar analyses within trials of other commonly used PD-1/PDL1 inhibitors would be informative.

The biologic heterogeneity of PD-L1 expression presents a further challenge to its use as a predictive biomarker, implying a risk of conflicting results depending on the sample(s) studied. Primary lung adenocarcinomas can show striking pattern-dependent heterogeneity of expression, with lepidic growth areas on average showing little to no PD-L1 expression and high-grade growth patterns (solid, micropapillary, pleomorphic) showing more extensive positivity<sup>17</sup>. It stands to reason, therefore, that a limited biopsy or fine needle aspiration (FNA) sampling of a larger primary mass may not accurately represent the PD-L1 status of the whole tumor. However, a number of studies have suggested that PD-L1 status for limited samples, including core biopsies and FNA, is overwhelmingly concordant with the results derived from paired resections<sup>14,18,19</sup>. PD-L1 expression status can change dramatically between sites of disease; an overall 19% incidence of PD-L1 status change is observed in NSCLC between primary and metastatic sites, more commonly from positive to negative<sup>20</sup>. Intervening therapy, including with immune checkpoint blockade or platinum chemotherapy may also influence the PD-L1 expression status<sup>21,22</sup>.

Immunotherapy has provided only modest survival benefits in patients with small cell lung carcinoma (SCLC), a highly aggressive form of lung cancer that is strongly associated with smoking and tends to show a high tumor mutation burden. Immune evasion appears to be a hallmark of SCLC<sup>23</sup>, thus it may not come as a surprise that this therapeutic strategy has shown limited efficacy. Both PD-1 and PD-L1 inhibitors have been approved for extensive stage SCLC, but PD-L1 expression status does not appear to be a useful biomarker in this setting. Indeed, PD-L1 expression tends to be very low, with 98% of patients showing PD-L1 expression level of < 5% in SCLC tumor cells<sup>24</sup>. A greater proportion of SCLC

patients are considered PD-L1 positive when applying a combined positive score (CPS)<sup>25</sup>. Although the median gain in survival is only 1–2 months with the addition of immunotherapy, a small group of select patients do enjoy more sustained response rates<sup>26</sup>. PD-L1 status does not appear predictive of survival benefit; rather it is possible that certain subtypes of “inflamed” small cell lung carcinoma - defined by the presence of immune checkpoint and human leukocyte antigen expression- may be more likely to respond to immunotherapy<sup>27,28</sup>. This will be discussed in more detail below.

### TUMOR MUTATIONAL BURDEN

Tumor mutational burden is defined by the number of small mutation calls (typically nonsynonymous missense mutations, but definitions vary- more on this later) per megabase (Mb) of sequenced genome. TMB serves as a surrogate for neoantigen generation within the tumor cells, and thereby anticipates the likelihood that the host immune system will recognize a peptide as foreign and initiate a cytotoxic response<sup>29,30</sup>. The gold standard for TMB estimation is whole exome sequencing, but it can be derived with some reliability from most panel next generation sequencing assays that cover at least 1.5 Mb of genome<sup>31</sup>. In 2020, the FDA approved the use of pembrolizumab for any advanced, microsatellite-stable, TMB-high solid tumor that has progressed on standard of care therapy. (<https://www.fda.gov/drugs/drug-approvals-and-databases/fda-approves-pembrolizumab-adults-and-children-tmb-h-solid-tumors>) The definition of TMB-high was established based on higher response rates among a cohort of 102 patients representing 9 different tumor types with a TMB of at least 10 mutations (mut) per Mb using a commercial assay<sup>32</sup>. This represents one of the few tumor-agnostic FDA approvals to date, however it is important to note that most common carcinomas, including NSCLC, were not studied in this trial. For patients with advanced NSCLC who have the option of receiving a PD-1 or PD-L1 inhibitor in the first or second line based on diagnosis alone, this particular approval may be less clinically relevant. However, it does throw TMB into the spotlight as a viable biomarker for selecting patients for immunotherapy and lends it greater credence as a variable that predicts response to therapy.

Overall, lung cancers tend to straddle this basket trial-defined cutpoint for TMB-high, ranging from a median of about 8 mut/Mb for non-squamous NSCLC to about 10 mut/Mb for small cell carcinoma<sup>33</sup>. In contrast, pulmonary carcinoids show an average 0.4 mut/Mb<sup>34</sup>, begging the question of whether a single TMB-high cutpoint is actually clinically relevant as a pan-cancer biomarker<sup>35</sup>. Indeed, multiple studies in NSCLC suggest that TMB score is an inconsistent predictor of immunotherapy response around the median, but a better predictor at TMB cutoffs in the 80th to 90th percentiles for the tested tumor populations<sup>36,37</sup>. The 90th percentile for NSCLC equates to about 18 mut/Mb with the commercial next generation sequencing assay used in the pembrolizumab approval<sup>37</sup>. One can justifiably argue that such a high cutpoint will miss some patients who stand to benefit from immunotherapy. In NSCLC, however, lack of a significant association between PD-L1 levels and TMB argues that these may be complementary biomarkers. For instance, there may be a role for TMB in guiding the choice of immunotherapy alone versus combined immunotherapy plus chemotherapy in patients with low positive PD-L1 status<sup>38</sup>.

The large number of technical variables that go into TMB calculation further complicate the clinical implementation of TMB as a biomarker. One important pre-analytic variable is the actual tumor content within the tested sample; *in silico* analyses showed a correlation between estimated tumor purity and TMB for those samples with fewer than 50% tumor cells in the tested sample; in other words, the greater a sample's tumor purity, the higher the TMB, and vice-versa<sup>39</sup>. Given the acknowledged challenge of

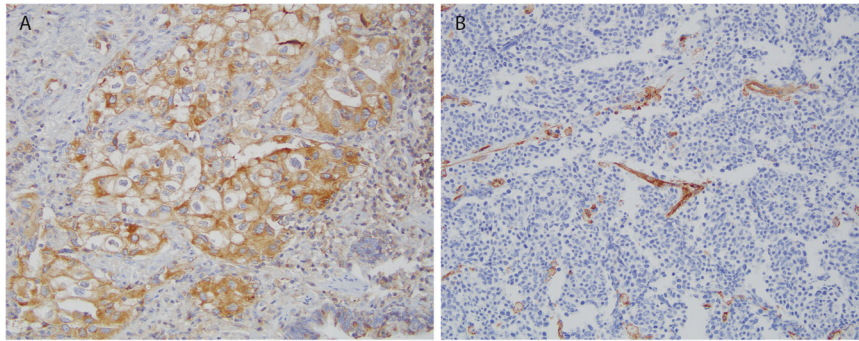
accurately estimating the tumor content based on pathology review<sup>40</sup>, this observation has significant implications for interpretation of the TMB, particularly for lung cancer where samples are often highly infiltrated by stroma and inflammatory cells. Bioinformatic approaches that enable more accurate and reproducible tumor content estimation<sup>41</sup> may be employed to confirm the pathologic impression of fractional tumor content and may aid in highlighting cases with potentially underestimated TMB calls.

A number of other sample-specific and sequencing assay-specific analytic variables can influence TMB. These include: cytosine deamination artifact in archival FFPE tissues, sample cross contamination during histology or laboratory handling, involvement of tumor tissue with multiple clonal processes (especially clonal hematopoiesis of indeterminate potential (CHIP)), polymerase chain reaction artifacts, germline variants, and sequence alignment errors. All of these factors can lead to an inflated TMB in practice<sup>38</sup>. Approaches to mitigating these sources of “TMB artifact” include bioinformatic error suppression, defining a threshold for inclusion of “subclonal” mutations, and use of a paired blood or buffy coat sample to subtract out germline and CHIP variants. In the post-analytic phase, variation in the definitions of TMB-eligible DNA changes (non-synonymous missense mutations only versus inclusion of synonymous missense and indel mutations; inclusion of variants in coding versus non-coding regions) can also influence the final count. Even for laboratories using the same NGS platform and variant calling criteria, imprecision driven by chance will lead to error in the TMB value, particularly for lower quality samples<sup>42</sup>. Fortunately, there are several international efforts ongoing to harmonize approaches to TMB calling<sup>43,44</sup>; in the meantime, absolute TMB values should be interpreted with caution and with the acknowledgement that a TMB-high result may not be reproducible from one lab to the next.

### Major histocompatibility complex proteins

Major histocompatibility complexes I and II (MHC-I and MHC-II) play crucial roles in antigen presentation and immune cell activation in health and disease. MHC-I is encoded by HLA-A, -B, and -C genes and is ubiquitously expressed on nucleated cells where it presents endogenous 8–12 amino acid peptides that are subject to surveillance by CD8 + T-cells. Presentation of foreign peptides, such as in virally-infected cells or tumor cells, will induce a cytotoxic T lymphocyte response. MHC-I expression at the same time inhibits natural killer cell activity<sup>45</sup>. MHC-I requires Beta-2 microglobulin (B2M) to be expressed at the cell surface. Although MHC-I is usually constitutively expressed, expression levels vary between cell types and it can be induced by inflammatory pathways such as interferon-gamma (IFN-g) and NF-kB. Tumor cells have a host of mechanisms for down regulating MHC-I expression, including mutations or deletion of the HLA genes, loss of B2M, and defects in transport to the cell surface, among others<sup>45</sup>. Mutations in HLA genes or genes encoding MHC-I transport proteins appear to be rare in lung cancers<sup>46</sup>; rather, HLA gene loss of heterozygosity is common, reported in about 40% of NSCLC. Interestingly, HLA LOH can be detected as a subclonal event in early lung tumors and may be enriched in advanced stage/metastatic tumors, suggesting it undergoes positive selection and bolsters tumor fitness later in cancer evolution<sup>47</sup>.

MHC-II, in contrast, is expressed principally on professional antigen presenting cells such as dendritic cells, macrophages, and B lymphocytes, where it acts to present exogenously-derived 13+ amino acid peptides to CD4 + T cells. The polymorphous nature of MHC-II isotypes (HLA-DR, HLA-DP, HLA-DQ) and the greater length and complexity of the peptides accommodated in its binding pocket drive higher diversity of presented peptides as compared to MHC-I<sup>48</sup>. Notably, MHC-II expression on tumor cells (tumor-specific MHC-II), typically occurs as a result of interferon (IFN)-gamma pathway activity. MHC-II expression then depends on



**Fig. 1 MHC-I expression is variable in lung cancers.** **A** Major histocompatibility complex-I (MHC-I) expression by immunohistochemistry (clone EMR8-5, Abcam, Cambridge, UK) showing diffuse cytoplasmic and membranous expression on lung adenocarcinoma cells. Expression is also visible on stromal leukocytes as well as benign respiratory epithelium (bottom right). **B** MHC-I is largely absent on the surface of this small cell lung carcinoma, with strong staining visible on associated stromal cells and occasional infiltrating leukocytes (image courtesy of Dr. Navin Mahadevan, Dana Farber Cancer Institute and Brigham and Women's Hospital).

downstream activation of the JAK/STAT pathway, which upregulates expression of IFN-response genes including class II transactivator (CIITA), a transcriptional master regulator. CIITA is necessary and sufficient to drive expression of MHC-II alpha and beta chains and its essential molecular chaperones CD74 (Ii), HLA-DO, and HLA-DM<sup>49</sup>. Mutations in JAK/STAT pathway members have been described as mechanisms of immunotherapy resistance due to depressed IFN-gamma signaling<sup>50</sup>. Certain hematologic neoplasms are characterized by genomic alterations in CIITA, such as deleterious mutations, deletions, or structural variants that blunt expression of MHC-II<sup>51</sup>.

At the protein level, variable levels of MHC-I and MHC-II protein expression are observed in human tumor samples (Fig. 1). Loss of MHC-I expression is reported in a third to a half of NSCLC, where it correlates with decreased CD8 + TILs, increased M2-polarized macrophage infiltration, and worse prognosis relative to tumors with intact MHC-I expression<sup>52,53</sup>. MHC-II expression is reported on the tumor surface in a minority of lung cancer cell lines, in a quarter to a third of NSCLC patient samples, and essentially never in SCLC<sup>54</sup>. MHC-II expression on tumor immune cells, in contrast, may be seen more commonly, including in a minor subset of SCLC<sup>54</sup>. The significance of MHC-II expression on tumor cells remains under study, but current evidence suggests that it permits for direct presentation of tumor neoantigens to CD4+ T helper cells and confers an improved prognosis in resected lung adenocarcinomas<sup>55</sup>.

The association between HLA-high gene expression and a "hot" immune phenotype defined by infiltrating CD8+ T cells and NK cells has been confirmed in larger datasets derived from bulk RNA sequencing; in keeping with this correlation, signatures of IFN-gamma signaling and upregulated immune checkpoints are observed in HLA-high tumors. A modest but significant association between higher average HLA gene expression and response to immunotherapy is observed in pan-cancer datasets, but data specific to lung cancer is limited<sup>56</sup>. Much work remains to be done to correlate HLA status at the DNA and transcriptome level with MHC-I and MHC-II protein expression by immunohistochemistry or other in situ methods. Further studies are needed examining the role of MHC expression as a predictive biomarker for immunotherapy, particularly in the context of the broader immune cell and checkpoint milieu.

Small cell lung carcinoma generally shows absent to minimal MHC-I and MHC-II protein expression. MHC-I loss of expression is mediated by epigenetic silencing of members of its antigen presenting pathway via the polycomb repressive complex-2<sup>57</sup>. Overexpression of Myc family members or human achaete-scute complex homolog-1 and loss of Rb function, common features of small cell lung carcinoma, lead to downregulation of CIITA and

MHC-II co-chaperone HLA-DR, respectively<sup>48</sup>. However, recent studies have identified a subset of small cell carcinomas with high levels of MHC-I expression<sup>28</sup>. MHC-I expression in this tumor type corresponded with the presence of increased cytoplasm, more prominent nucleoli, and a shift away from an organoid growth pattern, possibly reminiscent of the tumor previously called "small cell carcinoma of intermediate type"<sup>58</sup>, but nonetheless reflecting the recognized morphologic heterogeneity of small cell carcinomas. MHC-I high expression correlated with increased PD-L1 positive tumor infiltrating lymphocytes (TILs) as well as increased intratumoral cytotoxic T lymphocytes. Most strikingly, a cohort of small cell lung carcinoma patients showing durable responses to immunotherapy was significantly enriched with MHC-I positive tumors, suggesting that this may be a predictive biomarker of immunotherapy response in this rapidly fatal disease<sup>28</sup>. Validation in larger data sets is needed.

#### Tumor microenvironment

Infiltrating CD8 + cytotoxic T lymphocytes appear to be a consistent variable in the so-called "hot" immune phenotype that reflects an IFN-gamma driven adaptive immune resistance phenotype and increased likelihood of tumor response to immune checkpoint blockade therapies<sup>59-61</sup>. It is logical, therefore, to consider straightforward morphologic and immunohistochemical strategies for quantitating these immune effector cells and leveraging these numbers as predictors of immunotherapy response. In practice, however, it has proven to be challenging to establish a predictive metric based on CD8 + cell number that holds up across studies as an independent variable<sup>62</sup>. This is driven no doubt in part by the heterogeneity and small size of the studies themselves, both in terms of patient populations and variable approaches to scoring. (Table 2) It also seems likely that CD8 + enumeration alone is not completely predictive and that it will be necessary to understand the physiologic state of the infiltrating cytotoxic T cells. Some studies have shown that PD-1 coexpression status is an important predictor of immunotherapy outcomes, with absence of PD-1 expression (thereby defining naive or resting T cells) predicting improved outcomes following checkpoint blockade therapy<sup>63</sup>. The data in this space is conflicting, however, with other studies suggesting that a T cell exhaustion phenotype (CD39+ CD8+) indicates a potential role for PD-1/PD-L1 blockade in reversing this state of exhaustion and triggering cytotoxic activity. It is possible that the efficacy of immune checkpoint blockade in tumors with T cell exhaustion depends on the genomic context<sup>63</sup>. Other studies have highlighted the variability within this exhausted T cell population, identifying in particular a dysfunctional "burned out" population of highly proliferative CD8+ T cells that express multiple co-

inhibitor receptors (including PD-1) and that confer primary resistance to immune checkpoint blockade by crowding out functional effector cells<sup>64</sup>.

The data published to date increasingly makes clear that single protein markers are unlikely to provide the nuanced insights into the complex tumor-immune interaction required to improve our ability to predict the efficacy of immune checkpoint blockade therapies. Multiplexed imaging strategies are essential to more accurately describe these cellular interactions but need not be restricted to the research and development space. Indeed, the availability of technically standardized staining and imaging platforms can enable more widespread clinical adoption of multiplex immunohistochemical and immunofluorescence (mIF) platforms. (Fig. 2) Multiplex staining permits for analysis of coexpression and cell-cell interactions; coupled with digital imaging analysis these complex variables can be rapidly quantified and assigned to specific compartments (intratumoral, tumor-stromal interface)<sup>65</sup>. Computational power and storage limitations currently place restrictions on use of this technology in routine practice, however. File sizes for whole slide multiplexed immunofluorescence analysis (on the order of terabytes)<sup>65</sup> may be prohibitive, particularly within the computing infrastructure of an average hospital or academic medical center. Focusing the analysis to regions of interest can save on computing power but are at risk of bias or undersampling. Multiplex immunohistochemistry with digital quantitation may be less data-intensive but is limited to 3–5 markers per slide<sup>66</sup>. Accurate marker quantitation using conventional mIF approaches can also suffer from sample-intrinsic variables such as background autofluorescence or from technical challenges like nonspecific antibodies, inappropriate cell segmentation, and cross-channel signal bleed-through<sup>66</sup>. Assay optimization and increased access to computing power can overcome many of these hurdles<sup>65</sup>. The Society for Immunotherapy in Cancer (SITC) has developed a set of recommendations for development and validation of multiplexed imaging techniques, with an emphasis on the need to cross validate multiplexed markers against the current gold standard of single-plex chromogenic immunohistochemistry<sup>66</sup>. Published validation reports of mIF panels including PD-L1 have demonstrated excellent concordance between immunofluorescence and manual IHC for categorical and continuous scoring, however one study showed poor concordance at the 1% cutpoint<sup>67–69</sup>. Scoring discrepancies are driven largely by interpretative challenges with IHC, including very weak staining that is detected by mIF but is not discernable by IHC and misclassification of cell type (in particular macrophages mistaken for tumor cells)<sup>67</sup>. Well-optimized, automated mIF analyses may help to overcome many of the pitfalls inherent to PD-L1 IHC scoring by pathologists that drive interobserver variability, although broader logistical hurdles such as high capital and labor expenses and lack of interoperability of vendor-specific image analysis software are likely to present barriers to widespread adoption of mIF technologies.

### Tumor genomic variables

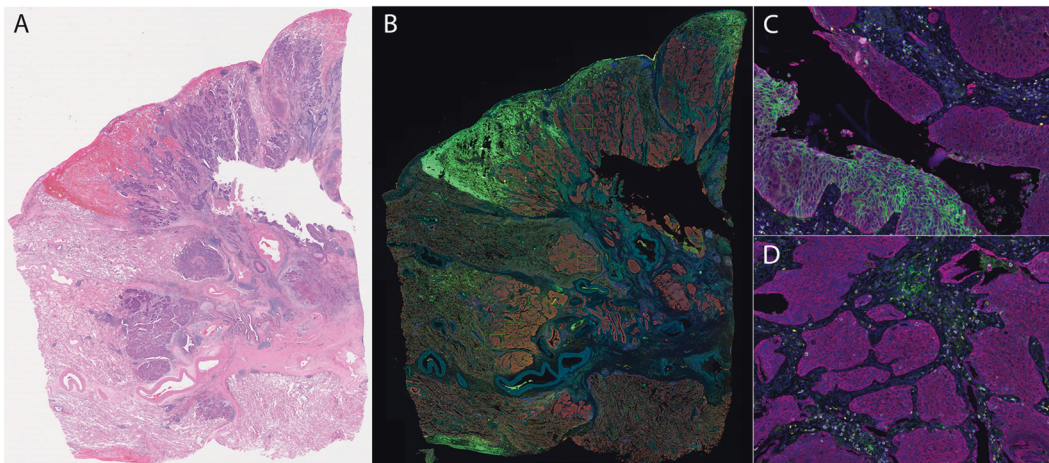
In NSCLC, the driver mutation profile is a key predictor of response to targeted biomarker therapy and/or immunotherapy. *EGFR* mutations and *ALK* fusions, which are both significantly more common in light and never smokers, predict a lack of response to immunotherapy<sup>70</sup>, likely as a result of their low mutational burden and therefore lower likelihood of immunogenicity relative to tumors arising in patients with a more substantial smoking history. *ROS1* fusion-positive tumors are predicted to respond poorly to immunotherapy for similar reasons, however their rarity precludes more systematic evaluation<sup>71</sup>. Tumors with *BRAF* V600E and *MET* exon 14 skipping mutations, which are found in both smokers and non-smokers, show a variable response to immunotherapy<sup>71</sup>, and *MET*-mutated tumors in particular can be associated with high PD-L1 expression, specifically in areas with sarcomatoid features<sup>72</sup>.

**Table 2.** Published studies examining the predictive role of CD8+ T cells for immunotherapy in non-small cell lung carcinoma.

Reference	Drug	N	Method	Outcome	Comments
Fumet et al. <sup>92</sup>	Nivolumab 2 + L	85	CD8 high by IHC and mRNA	Improved RR + PFS	PD-L1 status did not improve prediction
Niemeijer et al. <sup>93</sup>	Nivolumab 2 + L	139	CD8 high <sup>a</sup> by IHC in stroma and tumor	Improved DCB and OS	Timing of biopsy influences relevance of PD-L1 and IC status
Hu-Lieskovan et al. <sup>62</sup>	Pembrolizumab 2 + L	32	CD8 infiltration % relative to all nucleated cells	CD8 higher in responders and associated with improved PFS and OS	In multivariable model, PD-L1 expression was best predictor
Hashemi et al. <sup>94</sup>	Nivolumab 2 + L; Pembrolizumab 1 + L	141	Stromal CD8 > median	Improved PFS and OS	In multivariable model, albumin also predicts PFS and albumin + LDH predict OS
Althammer et al. <sup>95</sup>	Durvalumab 1 + L	163	CD8 TIL density of ≥ 297 cells/mm2 using digital image analysis	Improved PFS and OS	Combination CD8 + PD-L1 (SP263) scores outperform CD8 or PD-L1 alone
Mazzaschi et al. <sup>63</sup>	Nivolumab	26	Ratio of CD8 T cells to CD3 cells by multiplexed IF	Improved PFS	CD8 + PD-1 low infiltrates better predictors of response
Yeong et al. <sup>96</sup>	Nivolumab, Pembrolizumab, Atezolizumab 1 + L	35	CD8 proportion of total cells by multiplexed IF	CD8 + T cell proportion not predictive of response	CD39 + CD8 + T cell proportion an independent predictor of response

L line of therapy, RR response rate, PFS progression free survival, DCB durable clinical benefit, OS overall survival.

<sup>a</sup>Based in Donnem et al. (97) Clin Cancer Res. 2015.



**Fig. 2** Multiplexed immunofluorescence for immunoprofiling in lung cancer. **A** Whole slide hematoxylin and eosin stained slide of a resected primary lung squamous cell carcinoma; **(B)** whole slide multiplexed immunofluorescence with regions of interest for quantitative immunoprofiling indicated by the green rectangles. **C** Area of probable carcinoma in situ showing focally intense PD-L1 expression (green) on tumor cells (magenta). Peritumoral stroma shows frequent CD8+ T cells (white), scattered FoxP3+ cells (yellow), and rare PD-1 positive cells (orange). **D** Areas of invasive carcinoma show no discernible PD-L1 expression on tumor cells but do show expression on stromal immune cells. (Images courtesy of Brigham and Women's Hospital/Dana Farber Cancer Center ImmunoProfile Initiative).

*ERBB2*-mutated and *RET*-fusion positive tumors show rates of response to immunotherapy that are similar to that in genomically-unselected populations, however for many of these oncogene-addicted tumors it is important to keep in mind that the response rates to the appropriate targeted therapy outpace that of immunotherapy, particularly in the first line setting<sup>73</sup>. Thus, it is critical to identify potential driver mutations before deciding on the initial systemic therapy whenever possible.

*KRAS* mutations are associated with the best response rates to immunotherapy among genomic driver-positive NSCLC<sup>71,74</sup>. However, there is a strong suggestion that the co-mutation status has a significant influence on immunotherapy outcomes<sup>75</sup>. Common comutations that appear to alter the immune milieu or metabolic state of lung tumors such as in *STK11* (encoding LKB1) and *KEAP1* may be negative prognostic factors overall, but may specifically predict lack of immunotherapy response in *KRAS*-mutated tumors. In genetically-engineered mouse models (GEMM) of lung carcinomas, deletion of *Stk11/Lkb1* leads to neutrophil infiltration with concomitant increase in markers of T-cell exhaustion<sup>76</sup>. Interestingly, *Lkb1* mutation also appears to directly contribute to increased TMB in the GEMM lung tumors; this increased mutational rate may be due in part to homologous recombination deficiency mediated by a failure of Rad51 recruitment to double strand breaks in the *Kras/Lkb1*-mutant cells. At the same time, these cells appear to have deficient mRNA expression of antigen presenting pathway members and compromised antigen presentation via the MHC-1 pathway<sup>77</sup>. This may help to explain the paradox of a dampened immune response despite a higher TMB. In retrospective analyses of PD-1 inhibitor monotherapy-treated patient cohorts, tumors with *KRAS* and *STK11* comutations showed shorter progression free and overall survival relative to *KRAS* mutant/*STK11* wild type tumors<sup>78</sup>. *STK11* mutations are detectable using many commercially and academically available panel NGS assays, however the spectrum of events that can knock out Lkb1 function is broad and it is likely that *STK11* may be defective in a subset of tumors that appear wild type using standard exome sequencing. Immunohistochemistry can also be used to detect Lkb1 loss of expression in tumor tissues, however loss may be heterogeneous, including in the context of premature truncating mutations;<sup>79</sup> the significance of this finding is unclear. Further validation of IHC as a predictor of *STK11* inactivation is required. Moreover, the negative predictive value of *KRAS/STK11* comutation has not yet been confirmed in prospective analyses; as

a result, the *STK11* mutation status is not routinely used to inform choice of therapy<sup>75</sup>.

*KEAP1* is a regulator of the NRF2 transcription factor that regulates antioxidant defense and is mutated in about 20% of lung adenocarcinomas. *Keap1* loss leads to NRF2 upregulation and glutaminolysis-dependent promotion of *Kras*-mutated GEMM lung cancers<sup>80</sup>. *KEAP1* and *STK11* mutations frequently co-occur; mechanistically it appears that an increase in NRF2 activity can counteract the redox stress induced by *STK11* loss<sup>81</sup>. In lung adenocarcinomas, *KEAP1* mutation may not be a predictor of poor outcomes following immunotherapy independent of its adverse prognostic effects. However, it does appear to behave as a negative predictive biomarker in lung squamous cell carcinomas<sup>81</sup>. As with *STK11*, it appears that *KEAP1* may be subject to a variety of inactivating mechanisms, not all of which are detected using standard DNA sequencing approaches; thus an Nrf2 expression signature has been proposed as an alternative biomarker of *KEAP1* loss/Nrf2 upregulation<sup>81</sup>. The role of *KEAP1* mutation/Nrf2 activation as a prognostic versus predictive biomarker in different histologic and genomic contexts is a subject of ongoing investigation<sup>82</sup>. As for *STK11*, the *KEAP1* status is not routinely used to inform choice of immunotherapy regimen in clinical practice<sup>75</sup>.

Alterations in genes within the SWI-SNF chromatin remodeling complex (ARID family members, *SMARCA4*) have also been proposed to influence response to checkpoint inhibitor therapy, however their reported effects on outcomes following immunotherapy are inconsistent. *SMARCA4* mutations in particular appear to confer an adverse prognosis, but one study reported improved benefit from immunotherapy<sup>83</sup>. In contrast, another study reported that *KRAS/SMARCA4* comutations predicted a dismal outcome following immunotherapy<sup>84</sup>. In interpreting these studies, it is important to keep in mind that a number of confounding factors may be at play. For one, SWI/SNF complex alterations are commonly seen in lung tumors arising in smokers with high TMB<sup>85</sup>, two features that may predict better outcomes with immunotherapy. At the same time, SWI/SNF complex mutations frequently co-occur with *STK11* and *KEAP1* loss of function<sup>84,86</sup>, which might be expected to serve as negative counterweight. Finally, many SWI/SNF complex alterations, particularly those occurring in the ARID family of genes, occur in minor tumor subclones, thus their influence on tumor biology may be uneven within a tumor cell population<sup>85</sup>.

## Challenges

Proof of concept studies that incorporate multiple biomarkers (TMB, CD8, PD-L1, MHC-I, mIF, etc.) suggest that a combinatorial strategy may be more powerful than any single biomarker<sup>87,88</sup>. Multimodality approaches hold significant academic appeal but may be difficult to implement in practice. Recognizing that up to a third of lung cancer patients already lack sufficient tissue for genomic profiling for routine biomarker testing<sup>89</sup>, it may be difficult to routinely obtain not just biomarker testing and tumor mutation burden, but also multiplex in situ profiling. Routine diagnostic use of cytologic preparations also confounds analysis of spatial relationships and heterogeneity in the tumor microenvironment; indeed, cytologic preparations have been systematically avoided in studies of multiplex immunofluorescence panels and are unreliable for evaluation of PD-L1 on immune cells<sup>14</sup>. Gene expression profiling has been examined extensively in clinical trials of immunotherapy agents, often as an exploratory biomarker, but appears to fall short of other strategies as a predictor of response, likely due to its inherently bulk analysis of an often poorly-defined admixture of tumor, immune, and stromal cells<sup>88</sup>. For patients where tumor tissue in any form is simply not available or accessible, plasma-based cell free DNA analysis may play a role in defining the tumor genomic profile, determining a TMB, and potentially serving as dynamic biomarker of response following checkpoint blockade therapy, however the latter two applications are not yet validated for clinical use<sup>74,90</sup>.

## CONCLUSIONS

Immunotherapy has had a profound effect on cancer management and by extension on pathologists and laboratory scientists in the business of biomarker discovery, development and clinical validation. It has forced practitioners in all aspects of oncology to brush up on basic immunology and has dramatically highlighted the relevance of the immune environment that is often overlooked—and not readily described using standard morphologic approaches—in routine diagnostic tissue specimens. For diagnosticians, it is helpful to understand that PD-L1 expression, while still the backbone of immunotherapy selection in NSCLC, has its limitations. Other potentially interacting and complementary biomarkers that feed into an “adaptive immune response” phenotype may serve to refine our ability to select patients most likely to benefit from immunotherapy, or to define those who may require some form of tumor-immune priming before checkpoint inhibitor therapy can be effective<sup>28,91</sup>. An embrace of more quantitative scoring methods for immune cell and immune checkpoint evaluation, assisted by digital algorithms, will likely become essential for any practice aiming to optimize patient selection for cancer immunotherapy. This data, integrated with information derived both from tumor genome and host response to neoantigens, is likely to define the future precision cancer medicine.

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## AUTHOR CONTRIBUTIONS

L.M.S. conceived, wrote, and edited the manuscript.

## COMPETING INTERESTS

L.M.S. reports consulting fees and research grants paid to her institution from Genentech, consulting fees paid to her institution from Lilly, Inc, and equity in Moderna.

## ADDITIONAL INFORMATION

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