

Beyond PD-L1 testing-emerging biomarkers for immunotherapy in non-small cell lung cancer

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Abstract: Recently, a firmer understanding of tumor immunology and tumor escape mechanisms has led to the development of immune checkpoint inhibitors, antibodies against programmed death-1 (PD-1) and its ligand (PD-L1). Nivolumab, pembrolizumab, and atezolizumab have dramatically altered the treatment paradigm in non-small cell lung cancer (NSCLC) and have each demonstrated improvements in outcomes and quality of life when compared to chemotherapy. Enrichment strategies to better select those patients more likely to respond have identified PD-L1 staining by immunohistochemistry (IHC) to be a predictive biomarker in both treatment naïve and refractory patients. Unfortunately, many challenges exist with this strategy and underscore the need for further exploration for more reliable biomarkers. Multiple tissue and plasma-based enrichment strategies have been identified in the hope of identifying patients more likely to benefit from checkpoint inhibitors. These include tumor mutational load; the “inflamed phenotype” including tumor infiltrating lymphocytes (TILs) and immunoscore; T-cell receptor clonality; gene signatures, and several plasma biomarkers. Several studies have revealed many of these biomarkers to be reliable predictors of response to immune checkpoint inhibitors across multiple tumor types. Given the small nature of these studies, additional prospective studies are warranted to formalize and validate each of these enrichment strategies.

Keywords: Biomarkers, immunotherapy, non-small cell lung cancer (NSCLC)

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Introduction

Greater understanding of tumor immunology and tumor escape mechanisms has recently led to a resurgent interest in various forms of immunotherapies for cancer. One of the greatest successes has been the development of immune checkpoint inhibitors, antibodies against programmed death-1 (PD-1) and its ligand (PD-L1). Nivolumab,

pembrolizumab, and atezolizumab have dramatically altered the treatment paradigm in non-small cell lung cancer (NSCLC) and have each demonstrated improvements in outcomes and quality of life when compared to chemotherapy (1-7). In addition, cytotoxic T-lymphocyte associated protein 4 (CTLA-4) blockade with ipilimumab in combination with PD-L1 agents has shown promising activity in both non-small cell and small cell lung cancer

(8,9). Enrichment strategies to select patients more likely to respond have identified PD-L1 staining by immunohistochemistry (IHC) to be a predictive biomarker in both treatment naïve and refractory patients. Specifically, a tumor proportion score (TPS) >50% is predictive of improved response rates (RR), progression free survival (PFS) and overall survival (OS) with pembrolizumab when compared to platinum chemotherapy in treatment-naïve, advanced stage NSCLC patients (4). While this study underscores the importance of PD-L1 testing in guiding therapeutic decisions, many challenges exist with this strategy including the use of different testing platforms, utilization of different antibodies, and varying definitions of PD-L1 positivity. In addition, PD-L1 expression within tumors can be both heterogeneous and dynamic as reflected by the discrepancies in staining between primary and metastatic sites as well as the clinical observation that many patients with PD-L1 low or negative tumors still respond to these agents. Given these hurdles, there remains an urgent need for further exploration for more reliable biomarkers. Multiple tissue and plasma-based enrichment strategies have emerged to identify patients more likely to benefit from immune checkpoint inhibitors. These include tumor mutational load, the “inflamed phenotype” including tumor infiltrating lymphocytes (TILs) and immunoscore, T-cell receptor clonality, gene signatures, and several plasma biomarkers (Tables 1,2). Herein we review the available literature addressing these biomarkers and discuss the clinical implications and strategies moving forward.

Tumor mutational burden (TMB)

Somatic mutations in the cancer genome are a consequence of multiple processes including deficiencies in DNA repair, endogenous and exogenous carcinogen exposure, and enzymatic alterations in DNA (10). The prevalence of somatic mutations among neoplasm ranges from 0.01 per megabase to more than 400 mutations per megabase, with lung and melanoma harboring high numbers of non-synonymous genetic alterations (10). Most of these mutations derive from passenger genes and lead to translation of novel peptide epitopes, or neoantigens, which are presented by the major histocompatibility complex (MHC) on the surface of malignant cells. Theoretically, the presence of neoantigens enhances the immunogenicity of the tumor by eliciting T cell repertoires that recognize these antigens as “foreign” and infiltrate the tumor microenvironment (TME). Recent advances in sequencing

technology allow for whole exome interrogation and qualitative calculation of the total number of mutations per coding region or total mutation burden (TMB). Several recent studies have evaluated TMB as a candidate biomarker for immunotherapeutic approaches in both melanoma and lung cancer. In addition, given the intricate biologic link between deficiencies in DNA repair and the accumulation of genomic errors, micro-satellite instability (MSI) has also been evaluated as a potential biomarker to checkpoint inhibitors in colon cancer.

Given that immunotherapy was first approved in melanoma, TMB was initially explored as a potential biomarker to checkpoint inhibitors in this tumor type. Using massive parallel sequencing from 64 malignant melanoma patients treated with CTLA-4 blockade with either ipilimumab or tremelimumab, Snyder *et al.* were able to demonstrate that a mutational load of more than 100 non-synonymous mutations was associated with a longer OS than those patients with a lower TMB (P=0.04 in the discovery set, n=25; P=0.10 in the validation set, n=39) (11). Van Allen *et al.* carried whole exome sequencing from pretreatment biopsies in 110 patients with malignant melanoma treated with ipilimumab and demonstrated that elevated non-synonymous mutational load and neoantigen load (>100 non-synonymous somatic mutations or neoantigens) were associated with clinical benefit from ipilimumab (n=27) (P=0.0076 and 0.027, respectively) (12). Finally, utilizing hybrid capture-based next generation sequencing (NGS) on archival biopsies from advanced stage melanoma patients, Johnson *et al.* demonstrated that patients who responded to anti-PD-1/PD-L1 therapies had higher mutational loads in both an initial cohort (n=32; median 45.6 vs. 3.9 mutations/MB; P=0.003) and a validation cohort (n=33; 37.1 vs. 12.8 mutations/MB; P=0.002) (35). Response rate, PFS and OS were superior in the high mutational burden group compared with intermediate and low mutational load groups.

Similar to TMB in melanoma, mismatch repair deficiency has been explored, as potential biomarker for checkpoint inhibition in advanced colorectal cancer. De *et al.* recently carried out a phase II study evaluating 41 patients with progressive metastatic carcinoma treated with pembrolizumab and through selected microsatellite sequencing of tissue, identified a cohort of mismatch repair-deficient (n=11) and repair-proficient (n=21) colorectal cancers. Objective RR (40% vs. 0%), median PFS (NR vs. 2.2 months) and OS (NR vs. 5.0 months) were superior in mismatch repair-deficient patients versus those in repair-

Table 1 Emerging tissue-based biomarkers of immune checkpoint inhibitor response

Candidate biomarker	Assay	Literature support
TMB	Total number of mutations per DNA coding region	Melanoma: higher TMB was associated with longer overall survival, clinical benefit with anti-CTLA-4, and response to anti-PD-1/PD-L1 therapies (10-12) NSCLC: higher TMB associated with improved clinical response, clinical benefit, and progression free survival to anti-PD-1/PD-L1 therapy (13-16)
Cytotoxic CD8+ T cells/TILS	Presence of CD8+ T cells within the tumor	Melanoma: higher baseline CD8+ T cell density at the tumor IM was noted in patients with anti-PD-1 response (17); baseline CD8+ TILs density was a better predictor of response than PD-L1+ cell density (17). Increase in CD8+ T cell density after anti-PD-1 therapy was associated with treatment response (17) NSCLC: increase in post-treatment TILS was associated with anti-CTLA-4 response (18); CD8+ TILs in early stage lung cancer was prognostic for disease free survival and overall survival (19)
Immunoscore	A composite biomarker that integrates a combination of immune features (four IHC parameters: density of CD8+ T cells, density of CD3+ T cells, and their locations in the core of tumor or tumor infiltrating margin) into a validated scoring system	Colorectal cancer: immunoscore colon predicts the time to tumor relapse and survival better than the tumor-node-metastasis staging system (20)
Multiplex IHC	An advanced protein-labeling methodology that simultaneously assesses multiple proteins of interest on one FFPE slide	Melanoma: a larger PD-1/PD-L1 co-localized area was associated with anti-PD-1 response (17) Pancreatic adenocarcinoma: multiplex IHC showed an association between PD-L1 up-regulation on myeloid derived cells and activated CD8+ T cells in the tumor post vaccine therapy (21) NSCLC: multiplex IHC showed temporal influx of CD8+ T cells, with PD-1 co-expression, in the tumor after checkpoint inhibition (22)
T cell receptor clonality	Quantification of unique CDR3 region in the TCR, which determines the TCR's antigenic recognition	Melanoma: higher TCR clonality at baseline and post-treatment was associated with anti-CTLA response (23). Ten-fold increase in expanded T cell clones was associated with anti-PD-1 response (17) Breast: increase in T cell clones was shown after anti-CTLA-4 +/- cryoablation (24)
Immune gene signature	Microarray technology to evaluate multiple genes and their expression at the same time	Melanoma: high expression of immune related genes at baseline responded better to ipilimumab (25); immune gene signature appeared to predict response to combination of MAGE-3 vaccine plus immunostimulant Lung: a nine gene "immune gene signature" was conserved across varying tumor types, including lung; 3 of these 9 genes were prognostic of survival in NSCLC on univariate analysis. Analysis of PRECOG showed that FOXM1 and KLRB1 co-expression were prognostic of survival on multivariable analysis

TMB, tumor mutational burden; TILS, tumor infiltrating lymphocytes; IHC, immunohistochemistry; FFPE, formalin-fixed paraffin-embedded; CDR3, complementarity-determining region 3; TCR, T cell receptor; IM, infiltrating margin.

proficient patients (36). Whole-exome sequencing identified a mean of 1,782 somatic mutations per tumor in mismatch repair-deficient tumors as compared to only 73 in mismatch repair-proficient tumors ($P=0.007$).

Somatic mutation burden has been studied as a predictive

biomarker in advanced stage lung cancer, following the clinical observation that a heavy smoking history can predict response to immunotherapy. Several small studies have identified a relationship between TMB and benefit to immunotherapy. Rizvi *et al.* performed whole exome

Table 2 Emerging blood-based biomarkers of immune checkpoint inhibitor response, prognosis, or toxicity

Candidate biomarker	Assay	Literature support
TCR clonality	Quantification of unique CDR3 region in TCR, which determines the TCR's antigenic recognition	Melanoma and prostate: increase in peripheral blood TCR diversity in patients who received anti-CTLA4 therapy (26)
Eosinophil count	Absolute eosinophil count calculated from peripheral blood	Melanoma: high AEC associated with improved survival at two years in patients who received ipilimumab or pembrolizumab (27); Relative eosinophil count <1.5 was associated with higher risk of death in patients who received pembrolizumab (28); baseline eosinophils may be associated with toxicity in patients who get atezolizumab, pembrolizumab, or nivolumab (29)
Neutrophils and lymphocytes	ALC, ANC, or NLR calculated from peripheral blood	Melanoma: pretreatment ALC and ANC appear prognostic in patients who received immunotherapy (30); NLR >3 associated with progression and death in patients who received ipilimumab (30) Lung: pretreatment NLR ≥ 5 associated with inferior OS, PFS in patients who got nivolumab (31)
Peripheral blood cytokines	Plasma IL-6, cytoscore	Lung: plasma IL-6 and IDO elevated in tumors that expressed an inflammatory tumor microenvironment (32); a low cytoscore associated with worse survival in patients who received nivolumab or docetaxel (1)
Peripheral T cells	FOXP3+ Tregs, CD4 and CD8 T cells	Melanoma: frequency of FOXP3+ regulatory T cells after ipilimumab associated with worse survival (27); higher FOXP3+ Treg at baseline may be associated with survival (33); increased % CD4 and CD8 T cells after ipilimumab associated with survival (34)

TCR, T cell receptor; CDR3, complementarity-determining region 3; ALC, absolute lymphocyte count; ANC, absolute neutrophil count; NLR, neutrophil lymphocyte ratio.

sequencing on two independent cohorts of NSCLC patients (n=16 and 18, respectively) treated with pembrolizumab and demonstrated that higher nonsynonymous mutation burden was associated with improved objective response, durable clinical benefit, and PFS (13). More recently, Spigel *et al.* correlated TMB from NGS performed on more than 11,000 samples from a Foundation One database with the clinical outcomes of 64 advanced NSCLC patients from this data set who had received immunotherapy with either pembrolizumab, atezolizumab or avelumab. The amount of time on drug was used as surrogate for clinical response and was longer in those patients with high TMB versus those with low TMB (64 weeks for >15 mutations/MB *vs.* 17 weeks for <15 mutations/MB; P=0.010) (14). Kowanetz *et al.* carried out targeted genetic sequencing to calculate TMB on pretreatment tumor samples from 102 treatment naive patients (1L) and 465 treatment refractory patients (2L+) enrolled in three phase II atezolizumab monotherapy trials for advanced NSCLC (POPLAR, second/third line; BIRCH and FIR, single arm first line/second line in PD-L1+ selected patients). Atezolizumab clinical benefit correlated with higher TMB in 1L and 2L PD-L1 positive

patients and was enhanced in unselected 2L+ patients from POPLAR when compared to docetaxel (15). Finally, Peters *et al.* recently calculated TMB scores by whole exome sequencing in a subset of patients from a randomized phase III trial comparing nivolumab to platinum chemotherapy in treatment naive, advanced stage NSCLC (Checkmate 026). In patients with high TMB, PFS was improved (median PFS of 9.7 *vs.* 5.8 months; HR: 0.62; 95% CI, 0.38–1.00) and objective response rate was higher with nivolumab versus chemotherapy (46.8% *vs.* 28.3%) (16).

In sum, strong evidence across multiple tumor types suggest TMB to be a potential biomarker for clinical benefit to checkpoint inhibitors. Given the retrospective nature of many of these trials, prospective studies will be needed for TMB validation. These trials will be aided by the breadth and depth of newer sequencing platforms that are less cost prohibitive.

Specific genotypes

In contrast to high mutational burden, patients with specific genotypes including EGFR mutations and

ALK rearrangements have consistently demonstrated lack of response to checkpoint inhibitors. A recently published retrospective analysis of 28 advanced adenocarcinoma patients with EGFR mutations (n=22) or ALK rearrangements (n=6) treated with PD-L1 inhibitors demonstrated an RR of only 5% and 0%, respectively (37). In addition, a meta-analysis evaluating advanced stage patients from three randomized trials comparing checkpoint inhibitors (nivolumab, pembrolizumab) to docetaxel as second line therapy showed no OS benefit in the EGFR-mutant subgroup (n=186, HR: 1.05, $P < 0.81$; treatment mutation interaction $P = 0.03$) (38). Whether the lack of benefit witnessed is due to low PD-L1 expression or low TMB identified in these molecular cohorts remains unknown. Nevertheless, checkpoint inhibitors are most likely not the optimal therapy for patients with EGFR mutations or ALK rearrangements although ongoing prospective trials will hopefully address their role in combination with other novel agents in these specific genotypes.

Potential in-situ biomarkers to identify tumors with an inflamed phenotype

Within the TME, tumors are often infiltrated by a spectrum of lymphocytic and inflammatory cells—T cells, B cells, antigen presenting cells including dendritic cells, myeloid cells including macrophages and myeloid derived suppressor cells (MDSCs), and natural killer (NK) cells (39). The characterization of the specific immune cell population within in the TME have been associated with outcomes in multiple tumor types (40,41). Baseline “hot” tumors are associated with high T cell infiltration, presence of chemokine profiles that support effector CD8+ T cell influx, and type I interferon signals (42). “Hot” tumors thus have a T-cell inflamed phenotype due to immune recognition of these tumors and may be open to further immune activation and immune mediated tumor kill. Identifying *in situ* immune biomarkers from tissue biopsies can categorize tumors as “hot” or “cold” and may help select patients more likely to respond to immune checkpoint inhibition. Multiple studies have demonstrated that presence of CD8+ TILs, immunoscore, and multiplex IHC may identify an inflamed phenotype and thus be predictive of checkpoint inhibitor response.

CD8+ TILS

TILs found within the TME reflect the dynamic elimination phase of immune-editing in which the innate and adaptive arms of the immune system attempt to suppress tumor growth. Clinically, an extensive TIL presence has been associated with improved survival in multiple solid tumor types including melanoma (43), NSCLC (44-46), and colorectal cancer (47). Within TILs, cytotoxic (CD8+) T lymphocytes are the effectors cells of the innate immune system and their infiltration within the TME may be a surrogate for checkpoint inhibitor efficacy. Several studies have evaluated baseline or changes in intratumoral CD8+ TIL alone or in combination with PD-L1 expression as a predictive biomarker of immunotherapeutic approaches in multiple malignancies.

As with development of checkpoint inhibitors, evaluation of CD8+ TILs as predictive checkpoint biomarkers were first studied in advanced melanoma. A secondary analysis of 46 patients with metastatic melanoma treated with pembrolizumab showed a higher baseline CD8+ TIL density at the tumor invasive margin in responders versus non-responders (17). Importantly in this cohort, CD8+ TIL density determined by IHC was a better predictor of response than PD-L1+ cell density. Additionally, pembrolizumab responders had a parallel increase in CD8+ TIL density in serial post-treatment tumor biopsies, both in the tumor margin and within the tumor parenchyma, when compared to non-responders (Spearman's $r = 0.71$, $P < 0.001$). An increase in serial CD8+ TIL density correlated with decrease tumor size (Spearman's $r = 0.75$, $P = 0.0002$). CD8+ TIL density has also been evaluated in early stage lung cancer. In a retrospective study of 797 pathologic stage I-IIIa NSCLC, stromal CD8+ TIL density was independently prognostic of disease free survival, disease specific survival, and OS (all $P < 0.001$) (19).

TILs have also been evaluated as a biomarker. In a phase II prospective biomarker trial including 82 melanoma patients treated with ipilimumab, an increase in post-treatment TILs was associated with response (18). Recently, investigators correlated PD-L1 expression both on tumor cells and TILs with response to atezolizumab in 277 patients with advanced solid tumors (48). In the cohort of NSCLC patients (n=53), PD-L1 expression on TILs ($P = 0.015$) but not on tumor cells ($P = 0.920$) correlated with response to treatment response highlighting that location of PD-L1 expression may be an important distinction in

predicting response to immunotherapy.

In sum, PD-L1 expressing tumors lacking an appropriate immune infiltrate may explain the failure of checkpoint inhibition in a subset of patients. Immune infiltration parameters at baseline and after initiation of checkpoint blockade, specifically CD8+ TIL presence, present an opportunity to better select patients who would benefit from checkpoint inhibition and provide insight into the immune-editing process that may occur. Establishment of TILs, CD8+ TILs, its association with PD-L1, and clinical cutoffs of these levels in NSCLC is needed.

Immunoscore

Immunoscore is a standardized immune-based assay that measures intra- and peritumoral T cell infiltration in formalin-fixed paraffin-embedded (FFPE) tissue sections (49). Unlike single immune cell markers within the TME like CD8+ TILs, the immunoscore assay is a composite biomarker that integrates a combination of immune features into a validated scoring system that may prove to be a better predictive biomarker of checkpoint inhibition response.

Immunoscore[®] Colon provides a risk of relapse score from 0 to 4 based on average percentages of 4 IHC parameters: density of CD8+ T cells, density of CD3+ T cells, and their location in the core of tumor (CT) or tumor infiltrating margin (IM). Immunoscore's ability to detect decreased time to recurrence was validated using 3,800 resected colorectal specimens from an international cohort of stage I-III colorectal cancers without neoadjuvant treatment from 17 countries. Specifically in the validation set, time to relapse was shorter among 303 patients with a "low" immunoscore versus 327 patients with "high" immunoscore (HR: 0.54; 95% CI, 0.34–0.84; P=0.006). Impressively, this assay was found to prognosticate outcomes more accurately than tumor-node-metastasis (TNM) staging system and microsatellite instability status (50). Immunoscore demonstrates that the presence of a localized immune reaction in the TME can prognosticate survival in colorectal cancer. The development of an Immunoscore is currently under-investigation in melanoma (20), and more recently in NSCLC (51,52).

Multiplex immunochemistry

Multiplex IHC is an advanced antibody-protein labeling

methodology that allows simultaneous assessment of multiple proteins of interest on one FFPE slide (Figure 1). It requires a one step process or successive cycles of a sequential IHC antibody staining and stripping process on one FFPE slide (53).

Antibody-protein complexes are then linked to fluorescent dyes, to chromagens that produce color when precipitated, or to heavy-metals that can be analyzed by mass cytometry to allow visualization. Multispectral cameras or computational image analysis software are used to visualize multiplex IHC stains or to co-register and output images with overlaid IHC stains (54). Finally, analytic dedicated software such as image cytometry can be used to sort and analyze cell populations based on IHC staining within section of the slide (55). Multiplex IHC has advantages of optimizing the evaluation of the TME of limited tumor biopsy samples, mapping out the spatial/temporal resolution of immune cells and its corresponding phenotype or function in the TME, and visualizing co-expression of functional proteins on immune cells.

In melanoma, Tumeh *et al.* used multiplexed immunofluorescence to demonstrate that the proximity of PD-1 expressing cells and PD-L1 cells in pre-treatment samples correlated to pembrolizumab response. In analyzing 22 patients treated with pembrolizumab (n=11 responders; n=11 non-responders), a higher PD-1/PD-L1 area was found in responders (P=0.0005) (17). Multiplex immunofluorescence was also able to demonstrate colocalization of CD8 and PD-1 on a single individual cell in the TME to prove that CD8+ T cells were the primary cellular source of PD-1 receptors in this study.

Tsujikawa *et al.* used multiplex IHC to perform a secondary retrospective analysis of patients with pancreatic adenocarcinoma enrolled on trial receiving neoadjuvant granulocyte-macrophage-colony-stimulating tumor vaccine (GVAX therapy) prior to resection (21). The investigators showed that activated CD8+ T cells in the TME co-expressed with granzyme B, was associated with an improved OS after GVAX. An increased percentage of granzyme B+ CD8+ T cells to all T CD8+ cells present was associated with improved 2 year survival (P<0.05). Interestingly, PD-L1 status was mostly found in the CD45+ CD68+ tumor associated macrophages (TAMs) and CD45+ MHCII+ dendritic cells, and significantly correlated in TME location to activated granzyme B+ CD8+ T cells (P=0.0008) demonstrating the potential association between up-regulation of PD-L1 expression on myeloid derived cells

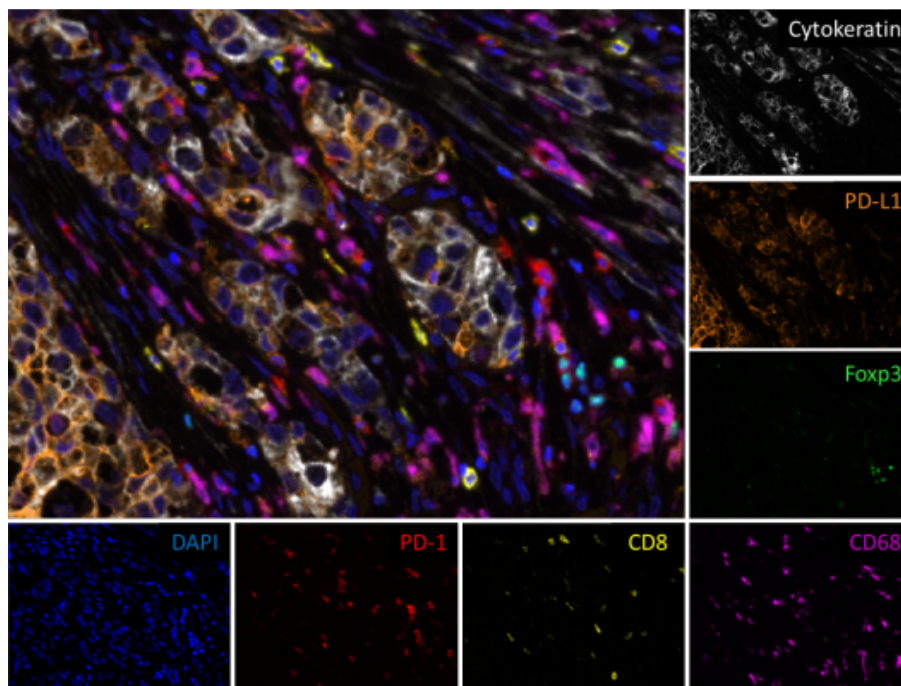


Figure 1 Multiplex immunofluorescence ($\times 20$) of a pulmonary adenocarcinoma shows tumor cells (white) surrounded by stromal and immune cells, including CD8+ T cells (yellow), regulatory T cells (green), and macrophages (magenta). PD-L1 (orange) is predominantly expressed on tumor cells while PD-1 (red) is seen on lymphocytes. Cell nuclei are shown in blue (DAPI). Image provided by Tricia R. Cottrell and Janis M. Taube.

and CD8+ T cell activation.

In NSCLC, Forde *et al.* presented preliminary results of a phase II trial evaluating the use of neoadjuvant nivolumab in high risk stage IB-IIIa NSCLC prior to resection at ESMO 2016 (22). In the 18 patients treated, seven patients (38%) had less than 10% residual tumor on resection and one had a complete response after neoadjuvant checkpoint inhibition. Multiplex immunofluorescence was used to demonstrate a temporal influx of CD8+ T cells, many of which expressed PD-1, in the TME after receipt of checkpoint inhibition when compared to pre-treatment tumor samples.

In sum, multiplex IHC allows for in-situ immune monitoring prior to and serially during immune checkpoint blockade using precious tumor samples. Using multiple data point captures, multiplex IHC may allow for elucidation of the plasticity and functionality of immune cells in the TME—by means of understanding complex immune cell surface protein expression profiles, cell type/location of expression, and its evolution with immune checkpoint inhibition response or resistance. Its utility in NSCLC will need to be validated in larger prospective studies.

T cell receptor (TCR) repertoire and clonality

An immune system's TCR repertoire reflects the sum of prior unique antigen exposures to the host. Through antigen-presenting cell's MHC-antigen complex interaction with the TCR, a naive T cell is activated and clonally expands. DNA sequencing of the TCR complementarity-determining region 3 (CDR3) domain, which uniquely encodes and determines the TCR's antigenic recognition, allows for quantification of the T cell clonal expansion that occurs after immune stimulation (56). Expansion in TCR clonality after immune checkpoint inhibition can be detected by an increase in the frequency of a unique CDR3 variable region sequence, and has been evaluated as a potential biomarker in multiple solid tumors.

A longitudinal cohort of 56 melanoma patients who received anti-CTLA-4 blockade followed by anti-PD-1 inhibition at the time of progression showed that higher TCR clonality at baseline ($P=0.041$) and on-treatment ($P=0.032$) was associated with response to PD-1 blockade (23). Furthermore, of the 8 patients with matched longitudinal pre-CTLA-4 and pre-PD-1 tumor samples, an increase in

TCR clonality after initial CTLA-4 checkpoint inhibition was associated a response to subsequent PD-L1 blockade (n=3 of 3 anti-PD-1 responders had increase in TCR clonality versus n=1 of 5 anti-PD-1 non-responders). In another retrospective analysis of 46 patients with metastatic melanoma receiving pembrolizumab from KEYNOTE-001, sequencing of TCR beta chain CDR3 variable region in matched pre- and post-treatment tumor samples showed that responders had a ten-fold increase in expanded T cell clones compared with non-responders (17). Another small pilot study of ipilimumab, cryoablation, or both in 18 early stage breast cancer patients showed increase intra-tumoral T cells clones detected by TCR sequencing after ipilimumab +/- cryoablation (24).

Interesting, TCR clonality increase has also been shown to be a potential biomarker of ipilimumab immune toxicity (57). Investigators reviewed the peripheral blood samples of 27 advanced prostate cancer patients treated with androgen deprivation therapy plus ipilimumab in a phase II trial. An increase of ≥ 55 CD8+ T cell clones in the blood was associated with future development of any immune-related adverse event (irAE) on study (OR: 1.2, 95% CI, 1.01–1.04; $P=0.01$).

Diversification or increase in TCR repertoire may also be a biomarker of checkpoint inhibition response. In a retrospective analysis of 25 melanoma and prostate cancer patients treated with ipilimumab on a phase I protocol, responders had an increase in the number of unique TCR clones found in peripheral blood mononuclear cells (PBMCs) 1 month after treatment. In responders, at least a 2-fold increase in TCR diversity was seen in 47% of anti-CTLA-4 therapy paired samples compared to no increase in those paired samples from nine untreated patients. A higher fold (10 \times) increase was found in melanoma patients (43%) with paired pre- and post-ipilimumab samples (26).

An increase in intra-tumor and peripheral blood TCR clonality or diversity may be a potential biomarker of response, and even irAE, in NSCLC. Further evaluation in NSCLC will need to be carried out in prospective studies.

Immune gene signature

Gene signatures using microarray technologies allow for multiple genes to be evaluated at the same time and can be correlated with clinical outcomes (58). Prior to the widespread use of immunotherapy agents for NSCLC, various gene expression signatures were found to have prognostic value in NSCLC (59–63). Furthermore, there is

growing evidence that gene signatures may be predictive of outcome to adjuvant chemotherapy in patients with resected NSCLC (63–65). With the integration of immunotherapy agents into the armamentarium for NSCLC, interest has emerged to further study the role of immune gene signatures (3–6). Investigators are beginning to explore whether unique gene signatures could potentially be prognostic or predictive of both toxicity and response to immunotherapy in various malignancies, including NSCLC (66–68).

Immune gene signatures may be prognostic in many malignancies, including lung cancer. Chifman *et al.* utilized microarray expression datasets of 5,295 breast, colon, ovarian, prostate, and lung tumors and hypothesized that they would be able to identify unifying gene signatures across tumor types that were enriched for immune biological functions (69). They were able to identify nine immune-gene (“metagene”) signatures (T/NK1, T/NK2, T/NK3, B/P/T/NK, B/P, B/M/D, M/D/N1, M/D/N2, DLPS) that were conserved across all of the tumor types. Furthermore, they demonstrated on univariate analysis that the immune /NKian, prostate2 ($P\leq 0.05$), T/NK3 ($P\leq 0.01$), B/P/T/NK ($P\leq 0.05$), and B/P ($P\leq 0.01$) were prognostic of improved survival in lung cancer.

Gentles *et al.* also evaluated the prognostic role of immune gene signatures in multiple cancers, including lung cancer (70). The investigators compiled cancer gene expression and clinical outcome data from 18,000 patients with 39 different malignancies from the public domain into a resource for the PREdiction of Clinical Outcomes from Genomic profiles (PRECOG). They demonstrated that expression of a proto-oncogene, Forkhead box M1 (FOXM1) was an adverse prognostic factor, and KLRB1, expressed on multiple T cell subsets, was prognostic of improved survival on multivariate analysis (HR: 1.5; 95% CI, 1.3–1.8, $P<0.0001$) (70). The investigators demonstrated the feasibility in identifying candidate genes and tumor associated leukocyte subsets that were potentially prognostic or predictive across multiple malignancies.

Recent data has demonstrated immune gene signatures to be predictive of response to checkpoint inhibitors in multiple tumor types. In a phase II study evaluating ipilimumab in melanoma patients, gene expression profiling was performed on tumor biopsies prior to treatment and at three weeks (25). Patients with high expression levels of immune related genes responded to ipilimumab better than those patients with lower baseline expression. Similarly an immune gene signature that encompasses CCL11, CCL5, IFN- γ , inducible T-cell costimulatory

(ICOS), and CD20 predicted response to vaccine therapy (71-73). Specifically, analyzing gene expression signatures by mRNA microanalysis from pretreatment tumor specimens of 54 malignant melanoma patients enrolled in a phase II trial evaluating a combination of MAGE-3 vaccine plus an immune-stimulant, investigators identified a gene signature that discriminated patients with clinical benefit (GS-positive) from those without clinical benefit (GS-negative). OS was significantly higher in GS-positive (29 mo; 95% CI, 20.5–40.2 mo) patients versus GS-negative patients (16.2 mo; 95% CI, 9.0–20 mo) (73). Furthermore, when the classifier was applied to samples from early stage NSCLC patients enrolled in a randomized trial evaluating MAGE-A3 with an immune-stimulant, AS02B (n=182), those who were identified as GS-positive demonstrated improved response and survival compared to those who were GS-negative (HR: 0.39; 95% CI, 0.19–0.81; P=0.01). Finally, utilizing a Fluidigm-based expression platform, Fehrenbacher *et al.* analyzed immune gene expression patterns in pretreatment samples from the randomized phase II POPLAR trial evaluating second line atezolizumab versus docetaxel. Improved survival was witnessed in those patients with tumors characterized by high expression of T-effector-associated and interferon—gamma associated genes treated with atezolizumab (HR: 0.43; 95% CI, 0.24–0.77) (7).

Given the rare, yet fatal adverse events related to immunotherapy, predicting immune-related toxicity to checkpoint inhibitors will be valuable in optimizing therapeutic decisions. Immune gene signatures may play a role in this area but published data is restricted to other malignancies (74). Shahabi *et al.* evaluated candidate biomarkers from 162 advanced melanoma patients on two phase II clinical trials that could potentially predict for immune related gastrointestinal (GI) toxicities after the use of ipilimumab (74). The mean cell cycle and immune related gene expression was significantly higher in patients who experienced grade 2 or higher GI toxicity compared to those who did not experience GI toxicity after ipilimumab (n patients who experienced g Additionally, they identified higher pre-treatment to post treatment increases in immunoglobulin related gene expression in patients who experienced GI toxicity compared to those who did not.

Serum/plasma markers

The accessibility and convenience of serum and blood makes plasma-based biomarkers an attractive enrichment strategy for patients receiving checkpoint inhibitors (Table 2).

Peripheral blood counts are readily available and utilization of eosinophil, lymphocyte, and neutrophil counts for prognostic or predictive purposes is a growing area of study. Relative eosinophil counts, for example, have been evaluated in patients with melanoma who have been treated with either ipilimumab or pembrolizumab (27,28). Martens performed an analysis on prospectively collected blood from 209 melanoma patients who received ipilimumab and identified that a high absolute eosinophil count (AEC) $\geq 50/\mu\text{L}$ was associated with improved survival compared to patients with AEC $< 50/\mu\text{L}$ at 2 years (27.2% *vs.* 6.7%, $P=5.1 \times 10^{-5}$) (34). Similarly, in a retrospective analysis of 616 melanoma patients who received pembrolizumab, relative eosinophil count (REC) $< 1.5\%$ was independently associated with increased risk of death (HR: 2.0, $P < 0.001$) (28). Finally, a preliminary analysis of 44 patients treated with either atezolizumab, nivolumab, or pembrolizumab, suggests that high levels of baseline eosinophils is associated with increased risk of toxicity (OR: 6, $P=0.014$) (29).

There is also great interest in serum neutrophils and lymphocytes as a potential biomarker for immunotherapy. Absolute neutrophil or lymphocyte count (ANC, ALC), or the neutrophil lymphocyte ratio (NLR) calculated by dividing ANC by ALC are prognostic in many malignancies and at various stages of disease, including lung cancer. In a retrospective analysis of 98 consecutive advanced melanoma patients, pre-treatment ALC $\geq 1,000/\mu\text{L}$ (HR: 0.40, $P=0.004$) and ANC $< 4,000/\mu\text{L}$ (HR 0.46, $P=0.014$) were significantly associated with OS on multivariate analysis (33). Ferrucci *et al.* found that an NLR > 3 was associated with increased risk of progression (HR: 2.03; 95% CI, 1.66–2.47; $P < 0.0001$) and death (HR: 2.29; 95% CI, 1.86–2.82; $P < 0.0001$) in 720 melanoma patients treated with ipilimumab (30).

In lung cancer, NLR has been studied in the patients receiving immune checkpoint inhibitors. A retrospective study of 175 patients with advanced NSCLC treated with nivolumab demonstrated that a pre-treatment NLR ≥ 5 was independently associated with inferior OS and PFS compared to a pre-treatment NLR < 5 (OS: 5.5 *vs.* 8.4 mo; HR: 2.07; 95% CI, 1.3–3.3; $P=0.002$; PFS: 1.9 *vs.* 2.8 mo, HR: 1.43; 95% CI, 1.02–2.0; $P=0.04$) (31). Multiple other retrospective analyses have started to evaluate and demonstrate a prognostic and predictive role of NLR in lung cancer patients receiving immune checkpoint inhibitors. However, these studies have all been small and retrospective and further prospective evaluation is warranted (75-79).

Peripheral cytokines are another potential biomarker

that has been evaluated in lung cancer patients receiving immune checkpoint inhibitors. In an analysis evaluating gene expression profiles from lung samples from the Cancer Genome Atlas (TCGA) and two MD Anderson datasets, investigators found that plasma IL-6 and indoleamine 2,3-dioxygenase (IDO) were elevated in tumors that expressed an inflammatory TME (32). Borghaei *et al.* has also presented early data from two phase III clinical trials evaluating advanced lung cancer patients who received either nivolumab or docetaxel and developed a “cytoscore” in attempts to quantify the effect of various cytokines on OS (1). In this exploratory analysis, squamous cell lung cancer patients with a high cytoscore had improved survival relative to low cytoscore patients in both patients who received nivolumab (15.6 *vs.* 5.3 mo; HR 0.48; 95% CI, 0.36–0.64; $P < 0.0001$) and docetaxel (9.1 *vs.* 4.9 mo, HR 0.39; 95% CI, 0.27–0.56; $P < 0.0001$). Additionally, those with non-squamous histology and a high cytoscore demonstrated superior survival than low cytoscore in both patients receiving nivolumab (17.9 *vs.* 5.9 mo; HR: 0.52; 95% CI, 0.39–0.71; $P < 0.0001$) and docetaxel (11.5 *vs.* 8.5 mo, HR: 0.60; 95% CI, 0.45–0.79; $P = 0.0001$).

Another biomarker of interest in the context of immune checkpoint inhibitors is peripheral T cells (80). High frequency of FoxP3+ regulatory T cells ($\geq 1.5\%$) has been associated with improved PFS and OS in patients receiving ipilimumab for melanoma (27,33,34). In an analysis of 95 patients with advanced melanoma treated with ipilimumab, increased levels of FoxP3+ regulatory T cells after treatment with ipilimumab was significantly associated with an inferior survival compared to stable or decreased FoxP3+ cells (5.3 *vs.* 15.8 mo, $P = 0.03$) (33). A larger analysis of 209 patients receiving ipilimumab for advanced melanoma demonstrated that higher FoxP3+ Treg frequency ($\geq 1.5\%$) at baseline was also associated with improved 1-year survival rate on univariate analysis (43.3% *vs.* 7.1%, $P = 8.7 \times 10^{-5}$) but this was no longer significant in a multivariate analysis (27). Similarly, in another exploratory analysis of 82 patients with advanced melanoma receiving ipilimumab, increases in the percentage of CD4 T and CD8 T cells at 14 weeks after ipilimumab was associated with a superior 2-year OS (31.1% *vs.* 8.3%; $P = 0.020$ and 41.4% *vs.* 5.3%; $P = 0.020$) (34).

Limited emerging data in lung cancer also suggests that peripheral T cells may be a useful biomarker. In a small analysis presented in abstract form, investigators observed that following two cycles of neoadjuvant ipilimumab with carboplatin and paclitaxel, CD4+ and CD8+ T cells were highly activated in NSCLC patients, demonstrated by

increased frequency of Tregs, defined by CD4+, CD25+, and FoxP3 expression (81). Another small analysis was performed using next-generation sequencing for TCR alpha/beta chain in 54 NSCLC patients receiving anti-PD1 therapy and suggested that early clonal T cell expansion within tumor and PBMC correlated with response to therapy (82). Further study of peripheral T cells in NSCLC with the use of immune checkpoint inhibitors is warranted.

Discussion

As we advance outcomes for patients with lung cancer, the success of therapies will continue to be predicated on the identification of reliable predictive biomarkers. While delivery to genotype driven agents to patients with actionable mutations has been emblematic of precision oncology, there remains many outstanding issues related to the optimal biomarkers for checkpoint inhibitors. Given the cost and selective toxicities of these agents along with the challenges of PD-L1 testing, enhanced enrichment strategies are needed.

Ongoing efforts have identified several tissue and plasma based-biomarkers predictive of checkpoint inhibitors in solid tumors including NSCLC. While many of the studies have been small and retrospective, they have provided further insights into how these agents can be best optimized. Amongst the candidate biomarkers reviewed here, TMB seems promising as its predictive utility has been demonstrated across multiple tumor types although further validation will be needed in larger studies. Plasma based markers also remain attractive and are aided by the convenience and the capability to evaluate longitudinal samples with relative ease. Given the complex interplay between the adaptive immune system and the TME, further enrichment efforts may employ combination strategies in which two or more methods are combined as a composite biomarker. This has been recently demonstrated in a phase I/II trial evaluating the anti-PD-L1 agent durvalumab in advanced NSCLC patients in which pretreatment biopsies were analyzed for PD-L1 by IHC and immune gene expression, including interferon gamma. Patients who were both PD-L1 positive ($>25\%$) and expressed interferon gamma had a response rate of 46% which was greater than those patients who were only PD-L1 positive (RR: 27%) or only expressed interferon gamma (RR: 33%) (83). We look forward future biomarker enrichment efforts rooted in scientific rationale that will help better define those patients more likely to respond and exclude patients that otherwise receive no benefit with added potential toxicity

from these agents.

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Footnote

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