Emerging biomarkers for cancer immunotherapy in melanoma

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Abstract

The treatment and prognosis of metastatic melanoma has changed substantially since the advent of novel immune checkpoint inhibitors (ICI), agents that enhance the anti-tumor immune response. Despite the success of these agents, clinically actionable biomarkers
to aid patient and regimen selection are lacking. Herein, we summarize and review the evidence for candidate biomarkers of response to ICIs in melanoma. Many of these candidates can be examined as parts of a known molecular pathway of immune response, while others are clinical in nature. Due to the ability of ICIs to illicit dramatic and durable responses, well-validated biomarkers that can be effectively implemented in the clinic will require strong negative predictive values that do not limit patients with who may benefit from ICI therapy.

Keywords: melanoma, biomarker, checkpoint inhibitor, PD-1, CTLA-4

Abbreviations: Immune checkpoint inhibition (ICI), immune related adverse event (irAE), tumor microenvironment (TME)

Introduction

The incidence of melanoma has been rising for the last 30 years, and it is estimated that over 87,000 new diagnoses of melanoma and over 9,700 melanoma-related deaths will occur in 2017\(^1\). The treatment and prognosis of melanoma, particularly in the metastatic setting, has changed significantly over the past decade. For instance, prior to 2011, the median survival for metastatic melanoma was 9 months, compared to greater than 2 years today\(^2\). These improvements in outcomes are largely due to the approval and clinical use of small molecule inhibitors of BRAF and MEK, as well as immune checkpoint inhibitors (ICIs). Although immune-modulating agents have a long history in the treatment of melanoma, the advent of ICIs, specifically those targeting the Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA-4) and Programed Death 1 (PD-1)/Ligand-1 (PD-L1) pathways have perhaps most drastically altered the treatment and prognosis of metastatic melanoma.

CTLA-4 is expressed by naïve T cells and binds to the B7 costimulatory protein on antigen-presenting cells (APCs) in the lymph node, leading to a robust inhibitory signal during T cell priming. CTLA-4 can also lead to decreased expression of B7, contributing to widespread inhibition of T cell priming. Another inhibitory receptor, PD-1, acts on antigen-experienced T cells and inhibits their proliferation and propagation of the immune response. PD-1 interacts with PD-L1 and programed death ligand-2 (PD-L2). Ipilimumab is an anti-CTLA-4 antibody which has demonstrated efficacy in several clinical trials with
objective response rates (ORR) of 11-15% and overall survival of 10.1-11.2 months\textsuperscript{2}. Ipilimumab monotherapy has also been compared at two different doses: 10 mg/kg versus 3 mg/kg. The higher dose produced a benefit in median overall survival (15.7 months versus 11.5 months), but with an additional burden of immune-related toxicity (34% grade 3-4 toxicities vs 19%). Clinical trials with anti-PD-1 agents, such as nivolumab and pembrolizumab, have shown ORR ranging from 20-45% with fewer toxicities (10-15% grade 3-4 adverse events)\textsuperscript{2}. Further, the anti-PD-1 antibodies nivolumab and pembrolizumab have both improved overall survival compared with ipilimumab in metastatic melanoma patients that are naïve to both agents\textsuperscript{3,4}. Combination therapy with anti-CTLA-4 and anti-PD-1 blockade has demonstrated additional clinical activity with ORR ranging from 50-60% and improved overall survival compared with ipilimumab alone. However, in some trials grade 3-4 toxicity rates were approximately 50%, and it remains unclear whether the combination confers overall survival benefits compared with anti-PD-1 monotherapy\textsuperscript{2,4}. These clinical trial data show clearly superior median and long-term survivals compared to any historical treatment options for metastatic melanoma; however, a substantial number of patients do not respond to immune checkpoint blockade. Currently, there are no clinically available biomarkers to aid in patient selection in melanoma.

Despite the successes of ICIs, immune related adverse events (irAEs) are common, sometimes serious, and difficult to predict. The most common irAEs are colitis, rash, vitiligo, hypothyroidism, hepatitis, pneumonitis, and hypophysitis. Most irAEs are relatively mild or manageable with corticosteroid treatment, but irAEs can occasionally be serious. In one randomized phase III trial, more serious, grade 3-4 toxicities occurred in 16%, 27%, and 55% of patients treated with nivolumab, ipilimumab, and both drugs respectively. Though thankfully rare, some fatal autoimmune toxicities have been reported, including encephalitis\textsuperscript{5}, fulminant myocarditis\textsuperscript{6} and others\textsuperscript{7}. Most patients recover completely with treatment, but some irAEs may persist after discontinuation of the offending agent, including endocrine toxicities, which can require permanent hormone replacement. Additionally, recent reports suggest that a small subset of patients may develop accelerated progression of their disease while on ICI, termed hyper-progression\textsuperscript{8,9}. These adverse events highlight the urgent need to develop suitable biomarkers for patient risk-benefit management.
Currently, there are no approved clinically-useful biomarkers of melanoma patient response to ICIs, optimal regimen selection, or development of irAEs. However, there are a number of candidate biomarkers in development and a body of work elucidating mechanisms of response and resistance to ICI. Many candidate biomarkers of ICI response fit within the known pathways of immune response and can be understood through that lens. In order for there to be an effective anti-tumor immune response, 1) T cells must be able to infiltrate the tumor microenvironment, 2) these T cells must be capable of being activating by ICI, and 3) there must be neoantigens capable of being presented to, and recognized by, T cells. Finally, T cells must be capable of mounting an effective cytotoxic response (Figure 1). In this review, we will discuss emerging molecular and clinical biomarkers that may be useful to predict patient outcome and possibly toxicity to ICIs in melanoma.

**Melanoma genomics**

There is a well-known repertoire of common driver mutations in melanoma, with the most prevalent being mutations in *BRAF* (50% of melanomas), *NRAS* (10-25%), and *NF1* (14%)\(^2,10\). With the approval of BRAF-mutant targeted inhibitors in melanoma, driver mutation status is a key determinant in clinical decision making. Thus, integrating ICI therapy into clinical practice raises obvious questions into how different genetic subtypes of melanoma respond to ICIs. Several studies have shown that *BRAF* mutational status does not appear to be associated with response to ipilimumab or combination ipilimumab plus nivolumab therapy\(^11,12\). *NRAS* mutations have historically been associated with inferior survival compared to other common melanoma subtypes\(^13,14\). In a retrospective study, *NRAS* mutated patients had superior response rates to first-line immune therapy with IL-2, ipilimumab or anti-PD-1/PD-L1 therapy compared to *NRAS* wild type patients (28% vs. 16%, \(P=0.04\))\(^13,14,14\). The *NRAS* mutated group had a striking 64% response rate to anti-PD-1 therapy, and these tumors demonstrated a non-significant trend toward increased PD-L1 expression\(^13,14\). *NF1* mutated melanomas are associated with ultraviolet light damage, a high mutational load and have been associated with high response rates to anti-PD-1 therapy\(^15\). Interestingly, *BRAF, NRAS, NF1* and triple wild type melanomas have been associated with widely different mutational loads (median 12.0 vs. 17.6 vs. 62.7 vs. 2.2 mutations/MB, respectively; \(P<0.001\))\(^15\), which appears to be an independent predictor of ICI outcome, discussed further below. It remains unclear whether any differential response rates to immunotherapeutics between different driver mutation
subgroups is related to the driver mutations themselves, associations with prior therapies (e.g. prior BRAF inhibitors), or associations with other biomarkers of response to immunotherapies such as mutational load or PD-L1 expression.

**Molecular biomarkers of anti-tumor immune response: Tumor microenvironment**

The tumor microenvironment (TME) consists of the surrounding blood vessels, immune cells, fibroblasts, and extracellular matrix that interact with and influence the growth of the tumor. An effective response to ICIs is dependent on T cells infiltrating the microenvironment and on expression and engagement of immune checkpoints within the TME. T cell infiltration and expression of PD-1 and PD-L1 may be potentially predictive molecular biomarkers of an anti-tumor immune response. In contrast, CTLA-4 appears to be most important in lymphoid structures during T cell priming, and thus has been less frequently studied in the TME.

Initial studies have evaluated the most basic component of an anti-tumor immune response: cytotoxic T cells. In particular, characterizing the location and immunophenotype of these tumor-infiltrating T cells has been correlated with anti-PD-1 responses. Several studies have demonstrated that density of CD8+ T cells at the tumor margin is associated with response to ICI\textsuperscript{16,17}. Using immunohistochemistry (IHC) to characterize the TME, pretreatment samples from patients who responded to treatment with pembrolizumab (anti-PD-1) showed greater degrees of preexisting CD8+ cells at the tumor margin\textsuperscript{17}. In contrast, CD8+ T cells in the tumor center did not correlate with response. Another technique for characterizing cells in the TME is multi-parameter flow cytometry, which permits study of a greater number of cells with more parameters per cell, but is unable to analyze tissue architecture or spatial relationships. Flow cytometry techniques reveal that the immunophenotype of tumor-infiltrating lymphocytes (TILs) is vital to the response to immunotherapy\textsuperscript{16}. For patients with greater than 20% CTLA-4\textsuperscript{hi}PD-1\textsuperscript{hi} cells within their CD8+ TILs prior to treatment with anti-PD-1 therapy, progression free survival (PFS) was 31.6 months compared to 9.6 months with 20% or fewer TILs being CTLA-4\textsuperscript{hi}PD-1\textsuperscript{hi} (P=0.017)\textsuperscript{16}. Further characterization of the CTLA-4\textsuperscript{hi}PD-1\textsuperscript{hi} TILs revealed a partially exhausted phenotype characterized by lack of production of TNF-\alpha and IL-2, while retaining the ability to produce IFN-\gamma\textsuperscript{16}. However, due to the complexities of flow
cytometry from solid tissue, these findings may be challenging to directly translate to a clinical assay for ICI response.

It remains largely unclear what molecular mechanisms govern T cell infiltration, and multiple factors likely mediate this process. As discussed further below, the presence of immunogenic antigens is thought to be a necessity for ICI response. Therefore, lack of immunogenic antigens may result in poor T cell recognition of tumor cells in the TME leading to immune-exclusion of T cells. However, bioinformatic analyses of hundreds of melanomas in The Cancer Genome Atlas (TCGA) suggested that neoantigen and tumor mutation burden were not directly associated with a T cell-inflamed gene expression signature in the TME\textsuperscript{18}. No statistically-significant differences were observed between the highly-inflamed and uninflamed melanomas with respect to a variety of relevant parameters including density of nonsynonymous somatic mutations, and shared differentiation or cancer-testis antigens. These results suggest that lack of antigens in the TME are unlikely to be the primary driver or rate-limiting step in anti-tumor immunity\textsuperscript{18}. Some have hypothesized instead, that particular cell signaling pathways (e.g. β-catenin, PTEN) may exclude T cells from the tumor, thus precluding an immune response (see “Molecular Markers” section below).

Many aspects of antigen presentation (discussed below) occur through dendritic cells (DCs) in both the lymph node and TME. Batf3 is a transcription factor that impacts the development of CD103+ tissue-resident DCs\textsuperscript{19}. Thus, these cells likely play a crucial role in major histocompatibility complex (MHC) class I and II-mediated antigen presentation specifically within the TME. It has been suggested that infiltration of tissue resident Batf3-lineage DCs may be the rate-limiting step in T cell inflammation of a tumor\textsuperscript{19}. Batf3-lineage DCs are strongly correlated with effector T cell markers in human melanoma and are required for trafficking of effector T cells following adoptive T cell therapy\textsuperscript{19}. DC markers were also strongly correlated with a T cell inflamed microenvironment\textsuperscript{18}.

One somewhat intuitive biomarker of response to ICI is expression of immune checkpoints and their ligands: PD-1, PD-L1, PD-L2 or CTLA-4. Patients who responded to pembrolizumab (anti-PD-1) showed higher expression and closer proximity of PD-1 and PD-L1 expressing cells within pretreatment tumor biopsies examined with IHC\textsuperscript{17}. As mentioned above, high expression of CTLA-4 and PD-1 on CTLs was associated with better PFS after treatment with anti-PD-1 therapy\textsuperscript{16}. CTLA-4 and PD-L2 expression were
significantly higher in melanoma patients who responded to the anti-CTLA-4 antibody ipilimumab than those who did not respond (P=0.033 and P=0.041, respectively)\textsuperscript{20}. Further, pembrolizumab recently received FDA approval for treatment of first-line, PD-L1-expressing non-small cell lung cancer (NSCLC) based on phase III results demonstrating improved PFS and response rate compared with chemotherapy in tumors with >50% PD-L1 positivity, which is higher than cutoffs used in many melanoma trials\textsuperscript{21}.

Some clinicians now use PD-L1 as a biomarker to assist in immune therapy selection for melanoma, although this is very controversial. A phase III study demonstrated that melanomas with ≥5% of tumor cells expressing PD-L1 had equivalent outcomes when treated with ipilimumab and nivolumab or nivolumab (although still inferior response rates with monotherapy)\textsuperscript{4}. However, patients with lower PD-L1 expression had improved PFS with the combination. The limitation of the data (discordant effect on PFS and response rate, lack of overall survival benefits), and technical aspects listed below have constrained the widespread adoption of PD-L1 expression as a biomarker. Further, despite the biologic sense of PD-L1 as a biomarker of response to anti-PD-1 therapy, there are a substantial number of PD-L1 negative tumors which respond to anti-PD-1 therapy, limiting the ability to exclude patients from potentially lifesaving therapy. An alternative means of separating patients may be “capability” to express PD-L1, by studying nuclear expression of a PD-L1 transcription factor, Interferon Regulatory Factor-1 (IRF-1). In a study of melanoma patients treated with nivolumab (anti-PD-1), pembrolizumab (anti-PD-1) or combination ipilimumab (anti-CTLA-4) and nivolumab, nuclear expression of IRF-1 was significantly higher in patients with a partial or complete response, compared to those with stable or progressive disease (P=0.044)\textsuperscript{22}.

An important barrier to the utilization of PD-1 and PD-L1 IHC staining is the variety of non-standardized assays available, with each anti-PD-1/PD-L1 agent utilizing its own companion assay. Several recent efforts have evaluated the degree of concordance between methods, often showing good agreement\textsuperscript{23, 24, 25}. A comparison of four different immunohistochemical assays for PD-L1 expression in resected NSCLC found that three of the four assays performed nearly equivalently, but that the fourth only identified about half of the patients who were PD-L1 positive by the other methods \textsuperscript{25}. In this study, the scores of the 13 participating pathologists were generally concordant for PD-L1 tumor cell scoring, which is the measure that has been used in most clinical trials, regardless of assay. However, concordance was poor for immune cell scoring, suggesting that inter-
observer variability currently limits the reliability of measuring immune cell specific PD-L1 expression by IHC, independent of the assay.

Limitations of examining characteristics of the tumor microenvironment as a biomarker of response include relying on biopsy specimens, which are invasive to obtain and represent only a small portion of the tumor, failing to account for inter- and intra-tumor heterogeneity. IHC techniques have the advantage of retaining information about tissue architecture and spatial relationships while multi-parameter flow cytometry has the advantage of analyzing multiple markers on each cell and using a larger number of cells. In particular, the use of PD-L1 IHC is complicated by different thresholds and binding antibodies used by different assays, and an only modest correlation between expression levels and response. However, tissue requirements, methodologic complexity, and lack of routine use in solid tumor clinical practice are clear disadvantages for flow cytometry. Due to utility of IHC in clinical practice from biopsies and resected tumor tissue, there are clear advantages to development of predictive biomarkers that can be assessed by IHC as opposed to flow cytometry.

**Molecular biomarkers of anti-tumor immune response: Tumor cell signaling**

As tumors develop to evade the immune system, they frequently activate oncogenic cell signaling programs that can suppress antitumor immunity, and thus response to ICIs. Multiple pathways and aberrations in cell signaling such as the Ras/MAPK pathway\(^26\), WNT/β-catenin\(^{27,19}\) and PTEN/PI3K pathways\(^28\) have been associated with absence of a T cell infiltrate. Activation of the Ras/MAPK pathway in tumor cells suppresses antigen presentation in multiple tumor types, and combinations of MEK inhibitors with ICIs have shown synergy in animal models as well as clinically in immune-refractory tumor types\(^{26,29,30}\). Additionally, other pathways may suppress anti-tumor immunity through alternative mechanisms. Loss of PTEN correlates with decreased tumor T cell infiltration in melanoma patients and worse responses to anti-PD-1 therapy, possibly through PI3K-mediated expression of immunosuppressive cytokines\(^28\). Likewise, activation of WNT/β-catenin signaling has been correlated with the absence of a T cell gene expression signature in melanoma patients\(^{27,19}\). Finally, a transcriptional program termed IPRES (innate PD-1 resistance) was significantly enriched in patients with de novo resistance. This profile was associated with wound-healing, angiogenesis, and epithelial-mesenchymal transition, suggesting that these tumor cell intrinsic pathways influenced
anti-tumor immune responses\textsuperscript{31}. Ultimately, these mutational and transcriptional alterations have more value as a roadmap to tumor-immune interactions rather than as true predictive biomarkers.

**Molecular biomarkers of anti-tumor immune response: Mutation burden and neoantigens**

Another essential component of an immune response and, by extension, response to ICI, is the presence of a unique antigen that can stimulate T cell recognition and anti-tumor immunity. Cancer is driven by genetic alterations which often create novel protein products (neoantigens; point mutations, insertions and deletions) or overexpress inappropriate tissue-restricted antigens (amplifications or epigenetic alterations). In order to generate an effective anti-tumor immune response, the tumor must present an aberrant antigen or neoantigen that can be recognized by the immune system. Total mutational burden, the number of mutations in a tumor, is highly correlated with the number of immunologically-significant neoantigens\textsuperscript{18,20,32}. Thus, increasing the number of mutations increases the probability of having an immunologically significant neoantigen. One reason melanoma is thought to be immunologically active is the high rate of mutations associated with ultraviolet sun damage. Both mutational burden and immunologically-significant antigens have been investigated as possible biomarkers of response to ICI\textsuperscript{20,32,33}.

In melanoma patients treated with anti-CTLA-4 antibodies, mutational load as detected by whole exome sequencing (WES) was significantly associated with clinical benefit, but there were still tumors with high mutational loads that did not benefit, suggesting that mutational load alone is not a sufficient predictor of response\textsuperscript{33}. However, the sequence of resulting mutated peptides predicted binding to MHC class-I molecules, models of T cell receptor (TCR) binding, patient-specific human leukocyte antigen (HLA) type, and epitope-homology analysis showed a greater association with clinical benefit to anti-CTLA-4. Sequences were identified which were shared by patients who experienced long-term clinical benefit but completely absent from patients who did not, regardless of mutational load\textsuperscript{33}.

Interestingly, candidate neoepitopes from patients who derived long-term clinical benefit were homologous to more viral and bacterial antigens than candidate neoepitopes from patients who derived minimal or no clinical benefit\textsuperscript{33}. However, other studies have
failed to replicate the tetrapeptide signature seen in this analysis. Along these lines, another intriguing mutational event associated with response to anti-CTLA-4 therapies in melanoma are mutations in \textit{SERPINB3} and \textit{SERPINB4}. Interestingly, serpin proteins are homologs of the ovalbumin antigen and are associated with autoimmunity, suggesting a pre-existing ability to be recognized by the immune system. Thus, a high mutational burden makes it more likely, but does not guarantee, the formation of immunologically-significant neoepitopes that may resemble known pathogens and trigger a T cell response. Studies in NSCLC and mismatch repair deficient tumors have recapitulated these results in patients treated with anti-PD-1, consistently demonstrating a strong correlation with response and high mutational load. It is important to consider that tumors are genetically heterogeneous with respect to distribution of neoantigens. Clonal neoantigens are present in all tumor cells, while subclonal neoantigens are present in only a subset. In melanoma patients treated with anti-CTLA-4 antibodies, a low degree of neoantigen intra-tumor heterogeneity and a high number of clonal neoantigens were significantly associated with improved overall survival. Thus, these factors need also to be considered in the utility of NGS-driven approaches, as discussed below, to predictive biomarkers of ICI efficacy.

Using WES to identify every mutation within a tumor in every patient would be expensive and impractical. An alternative is to use smaller next generation sequencing (NGS) panels that are already utilized clinically to extrapolate or estimate total mutation burden. Using archival samples of anti-PD-1 and anti-PD-L1 treated melanoma patients, NGS was performed on 236-315 genes on initial and validation cohorts. Patients who responded had higher mutational loads than non-responders (median 45.6 versus 3.9 mutations/MB in the initial cohort with \( P=0.003 \) and median 37.1 versus 12.8 mutations/MB in the validation cohort with \( P=0.002 \)). In this analysis, the authors noted frequent mutations in the gene \textit{LRP1B} among responders. In TCGA samples melanomas with a mutation in \textit{LRP1B} harbored significantly more mutations than those with wild type \textit{LRP1B} (median 542 vs. 219 mutations, \( P<0.001 \)), suggesting that \textit{LRP1B} mutations may serve as a single gene surrogate of mutational load. In another NGS panel of 170 genes, predicted mutational load was correlated with response to ipilimumab and adoptive T cell therapy in cutaneous melanoma. Finally, this concept was validated further in urothelial bladder cancer, as high mutation load in 315 genes associated with response to atezolizumab.
Some subtypes of melanoma display substantially different mutation profiles, which may impact ICI response. Uveal melanoma is a relatively uncommon subtype driven by GNAQ and GNA11 mutations and has a very low mutation burden. Interestingly, some non-uveal melanomas also harbor GNAQ and GNA11 mutations and these tumors are also associated with a low mutational load compared to all other melanomas. Of 11 patients with GNAQ/GNA11 mutations treated with immunotherapy, only one responded to treatment, suggesting that GNAQ/GNA11 mutations may be a surrogate marker for low mutational load and poor response to immunotherapy in non-uveal as well as uveal melanoma39. Conversely, BRCA2 and NF1 has been reported to be mutated more frequently in responders to anti-PD-1 therapy and mutations are associated with a higher mutational load15,40. However, many of these findings require substantial validation in larger numbers of patients. It is important to note that there is controversy in the field on whether total mutation burden, or presence of a high neoantigen load are likely to develop useful predictors of ICI outcome.

Currently, there are a number of technical hurdles associated with further development of mutational burden and neoantigen load as routinely-utilizable biomarkers. WES is currently expensive and bioinformatically complex to interpret and validate. The use of targeted NGS panels, which are already routinely used for other prognostic information, may be more feasible to develop for this purpose. Predicting neoantigens adds further technical hurdles including the necessity of high resolution HLA haplotyping in each patient and simultaneously examining expression of neoantigens by RNA sequencing. Most studies evaluating neoantigen burden impute HLA haplotype from non-dedicated NGS data, and the expression, clonality, and functionality of putative neoantigens are rarely validated. Additionally, predicted neoantigen load correlates strongly with total mutational burden20, suggesting that, at present, the additional methodologic complexity needed to assess possible neoantigens may not yield any additional information. Until these technical hurdles can be resolved, the use of NGS panels to predict mutational burden is the most practical approach to develop as a biomarker in this area.

Notably the Food and Drug Administration (FDA) recently granted its first tissue/site-agnostic approval to pembrolizumab (anti-PD-1) for unresectable or metastatic, microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) solid tumors that have progressed on prior treatment and who have no satisfactory alternate treatment
options. MMR is a process by which cells identify and correct mismatched DNA bases, an error that is common in microsatellites due to polymerase slippage. Tumors which are dMMR consequently have an increased mutational burden and can be identified by the presence of MSI41. MSI has been incompletely studied in melanoma, but estimates of the frequency of MSI in primary melanoma range from 16% to 32% and up to 70% for metastatic melanoma42. These numbers represent all MSI positive melanomas and do not distinguish between MSI-low and MSI-high, an important clarification given that the FDA approval is for MSI-high only. It is thought that most melanomas with MSI belong to the MSI-low category and do not harbor mutations in mismatch repair genes42,43; however, this conclusion is limited by small sample sizes and not all reports are in agreement44. It is possible that evidence for MSI that is observed in melanoma is due to DNA damage from UV exposure, rather than through defects in the MMR pathway. Though MSI and dMMR have established prognostic implications in colorectal cancer, their significance is unclear in melanoma and other tumors. As a biomarker of response to immunotherapy in melanoma, it is uncertain whether MSI would add any predictive capability beyond that of mutational and neoantigen loads.

Molecular biomarkers of anti-tumor immune response: Antigen presentation

Presentation of an antigen to the immune system by MHC molecules is another critical step in anti-tumor immunity. MHC class I expression is ubiquitous in melanoma cell lines, and it is unclear whether MHC-I is required for response to anti-PD-145. Interestingly, a subset of melanoma cell lines express MHC class II molecules at basal or IFN-γ stimulated conditions. In cell lines, these MHC-II positive melanomas were associated with pro-inflammatory gene signatures, such as “PD-1 pathway”, “allograft rejection” and “viral myocarditis.” In melanoma patients treated with anti-PD-1 antibodies, expression of MHC-II was associated with improved response and improved overall survival45. Mechanistic studies are ongoing to determine whether MHC-II, as an interferon-γ responsive molecule, simply represents a surrogate of an inflamed TME, or whether it is a marker of enhanced antigen expression. Systematic evaluation of the predictive power of MHC-II/IDO1-positive melanomas, in concert with a proximity assay of PD-1 and PD-L1 was recently confirmed in a large dataset of over 120 patients using multiplexed quantitative immunofluorescence. In this study, melanoma patients passing the pre-defined biomarker cutoff experienced a three-fold increase in progression free survival (hazards ratio (HR) = 0.33; p = 0.003) and overall survival (HR = 0.34; p = 0.004)46. Although patients negative
for the assay still benefited, this assay may be useful for stratifying patients to single-agent anti-PD-1 therapy versus combination anti-CTLA-4 and anti-PD-1 therapy.

Loss of antigen presentation appears to be a key driver of both innate and acquired resistance to anti-PD-1\textsuperscript{47,48}. In two elegant studies, pre-existing or acquired mutations in \textit{JAK1} or \textit{JAK2} resulted in loss of JAK-STAT mediated IFN-γ signaling, leading to antigen-presentation defects, loss of PD-L1 expression, and therapeutic resistance\textsuperscript{47,48}. Similarly, loss of β-2 microglobulin (\textit{B2M}), a key assembly chaperone in the formation of viable MHC-I oligomers at the cell surface, resulted deregulation of MHC-I expression and was associated with acquired resistance to anti-PD-1\textsuperscript{48}.

**Molecular biomarkers of anti-tumor immune response: Recognition of antigens by T cell receptors**

Antigens that are successfully presented on MHC molecules must be recognized by their cognate T cell receptors (TCRs). A specific immune response to tumor antigens may therefore be reflected by a clonal repertoire of TCR sequences. In a study of response to pembrolizumab, a more clonal TCR population, particularly with clonal expansion after treatment, was associated with response\textsuperscript{17}. However, other studies have failed to replicate the association of TCR clonality with response to PD-1 blockade\textsuperscript{15}. Another group looked at CD8\textsuperscript{+} T cells in two patients with melanoma who responded to CTLA-4 blockade and was able to identify CD8\textsuperscript{+} populations which were specific for tumor neoantigens\textsuperscript{32}. TCR clonality and T cell recognition of antigens have been inconsistent as biomarkers in prior studies and are difficult to measure, limiting their possible utility thus far.

**Molecular biomarkers of anti-tumor immune response: Cytotoxic T cell response**

As a final step in the pathway of an anti-tumor immune response, cytotoxic T cells must be able to mount an effective cell-killing response. Perforin and granzyme transcripts, two components of a cytotoxic response, were enriched in ipilimumab responders compared to non-responders. These two markers are also elevated in long-term survivors\textsuperscript{20}. Although not specific to cytotoxic T cells, a critical effector molecule in potentiating an anti-tumor immune response is the cytokine interferon-gamma (IFN-γ). Loss of IFN-γ pathway genes has been associated with innate resistance to anti-CTLA-4 therapy and worse overall survival\textsuperscript{49}. Conversely, melanoma patients with higher pretreatment expression of IFN-γ and IFN-γ inducible genes were more likely to respond
to anti-PD-L1 therapy. Similarly, other studies have looked at the consequences of aberrations in downstream IFN-γ signaling, such as JAK-STAT. Loss-of-function mutations in JAK1/2 genes have been associated with both primary and acquired resistance to anti-PD-1 therapy, suggesting a functional connection between IFN-γ release, tumor cell response, and ICI therapy outcomes. As such, a number of gene expression signatures aimed at measuring IFN-γ activity are currently being employed in ongoing clinical trials.

Peripheral Blood Biomarkers

A number of peripheral blood markers have shown promise as both pre-treatment predictors of response and early on therapy monitoring. Biomarkers associated with early treatment changes could be clinically useful in deciding when to continue or switch treatments. Due to the ease of accessibility as well as potential for longitudinal monitoring during therapy, peripheral blood biomarkers of ICI response are highly sought-after.

Serum lactate dehydrogenase (LDH) is an established prognostic marker in melanoma, with elevated levels portending a worse prognosis. In one study of response to ipilimumab, baseline LDH less than 1.2 fold higher than the upper limit of normal (ULN) median overall survival (OS) was 10 months, compared to 5 months for >1.2 fold higher than ULN and 2 months for >2.3 fold (P=1.54x10^{-12}). Another study confirmed the association of elevated baseline LDH with worse OS in anti-PD-1 treated patients and further showed that change in LDH during treatment was significantly associated with response. In patients with an elevated baseline LDH those with a partial response had a mean reduction of 27.3% in their serum LDH compared to those with progressive disease who had a mean increase of 39% in serum LDH. Elevated LDH is also correlated with poor prognosis in untreated patients and those receiving other therapies.

Other peripheral blood cell markers have been studied as pretreatment biomarkers. In patients treated with ipilimumab, a relative lymphocyte count (RLC) of <10.5% was associated with a 1-year survival probability of 5% compared to 40.8% for those with a RLC >10.5% (P=3.30x10^{-12}). In this analysis a low frequency of myeloid-derived suppressor cells (MDSCs), defined by flow cytometry as Lin^CD14^HLA-DR^low^, was associated with the highest probability of long term survival; 2-year OS was 34.5% for patients with baseline MDSCs <5.1% compared to 0% of 65 patients with >5.1% MDSCs (P=6.73x10^{-11}). Other markers associated with a favorable outcome included
absolute monocyte count <650/microliter, CD14+ monocytes <28%, low absolute eosinophil count, low relative eosinophil count and CD4+CD25+Foxp3+ T-regulatory cells >1.5% \( (P=1.35\times10^{-8}, P=6.58\times10^{-7}, P=5.06\times10^{-5}, P=2.14\times10^{-4}, \text{and } P=8.7\times10^{-5}, \text{respectively}) \)\textsuperscript{51}.

Martens \textit{et.al.} also evaluated changes in peripheral blood markers as indicators of response to ipilimumab. Increases in absolute lymphocyte counts at 2 to 8 weeks and increases in percentages of CD4+ and CD8+ T cells at 8 to 14 weeks were associated with improved survival \( (P= 0.001, P=0.001, \text{and } P=0.02 \text{ respectively}) \)\textsuperscript{53}. An increase in absolute eosinophil count at 8 to 14 weeks was also correlated with improved OS \( (P=0.047) \)\textsuperscript{53}.

Another potential serum biomarker for both baseline and early treatment changes is angiopoietin-2, a well-established pro-tumor, pro-angiogenesis molecule involved in resistance to anti-VEGF therapies. Angiopoietin-2 has also been associated with recruitment of pro-tumor M2 polarized macrophages to the TME and up-regulation of PD-L1 on tumor-associated macrophages, which contributes to immunosuppression. High baseline angiopoietin-2 is associated with worse OS in patients treated with ipilimumab alone or ipilimumab plus the anti-VEGF agent bevacizumab (10.9 vs. 19.3 months, \( P=0.0125 \))\textsuperscript{54}. Angiopoietin-2 increases 3 months after treatment initiation with ipilimumab were also associated with worse OS \( (P=0.019) \). Similarly, in patients treated with PD-1 blockade, high pretreatment angiopoietin-2 was associated with worse overall survival \( (P=0.004) \) and a rise in angiopoietin-2 at 3 months was associated with a lower response rate \( (P=0.002) \)\textsuperscript{54}.

Other peripheral blood biomarkers are in development. Soluble PD-L1 (sPD-L1) has shown mixed results as a prognostic marker; highly elevated pretreatment levels were associated with a trend toward rapid progression on CTLA-4 blockade, but a rise in sPD-L1 five months after treatment initiation was associated with partial responses to anti-CTLA-4 or anti-PD-1 therapy\textsuperscript{55}. It is unclear whether levels of sPD-L1 may be associated with an anti-tumor immune response, a pro-tumor inflammatory response or both, depending on the tumor context and levels of sPD-L1\textsuperscript{55}.

Circulating tumor DNA (ctDNA) is another candidate biomarker which can be measured in peripheral blood and may be a useful indicator for baseline prognosis and
early treatment response. In patients treated with PD-1 blockade, clinical response was observed in 72% of those with undetectable ctDNA at baseline, 77% of those with elevated ctDNA at baseline but undetectable within 12 weeks of therapy, and 6% of those with elevated ctDNA at baseline that remained elevated during treatment. Overall survival was not reached in the first two groups and was 9.2 months for the group with persistently elevated ctDNA (P<0.001)\textsuperscript{56}. In this analysis, ctDNA was a better predictor of response and prognosis than LDH, disease burden, or performance status. An important caveat is that ctDNA profiles were not accurate in patients with brain metastases\textsuperscript{56}. A small prospective pilot study evaluated patients with multiple tumor types (non-small cell lung cancer, uveal melanoma, and microsatellite-instable colorectal cancer) treated with the anti-PD-1 antibodies nivolumab or pembrolizumab, assessed ctDNA at baseline and at 8 weeks, and measured response using the immune-related response criteria. At 8 weeks, there was a significant correlation between changes in ctDNA levels and tumor size (R=0.86; P=0.002)\textsuperscript{57}. These studies suggest that ctDNA may be a useful predictor of response early in treatment.

**Clinical biomarkers associated with immunotherapy response.**

Clinical characteristics and covariates may also be useful and easily-accessible predictors of response to immunotherapy. An intriguing pharmacodynamic biomarker of increased survival is the presence of immune related adverse events (irAEs). Presence of an irAE indicates that ICI has caused its intended effect: immune activation. In melanoma patients treated with nivolumab those who experienced any irAE had a significantly longer OS than those who did not (P<0.001), with an additional benefit in OS for those with 3 or more irAEs compared with patients with none or 1 irAE (P<0.001)\textsuperscript{58}. Of the different types of irAEs, cutaneous irAEs, such as rash and vitiligo, were statistically significantly associated with improved OS (P=0.001 and P=0.012, respectively)\textsuperscript{58}. Likewise, a meta-analysis of patients with stage III-IV melanoma treated with immunotherapy showed that the incidence of vitiligo was 3.4% and that those patients with vitiligo had significantly longer progression free survival and overall survival (P<0.005 and P<0.003)\textsuperscript{59}. irAEs are an intriguing biomarker because they are clinically evident without any additional invasive studies and can provide valuable prognostic information. One potential caveat to these data is that irAEs may be time-dependent (e.g. patients who remain on therapy due to benefit have higher exposure and thus higher rates of toxicities).
There are some data to suggest that prior therapy can influence immunotherapy response. The biology is somewhat conflicting as cytotoxic chemotherapy and radiation may increase mutational load, or promote tumor cell destruction, enhanced antigen uptake by dendritic cells in the lymph node, and ultimately enhanced T cell priming. However, chemotherapy and radiation are also promote lymphopenia, and may contribute to a general functional decline and when ineffective, allow increased tumor bulk. Numerous studies have demonstrated lower response rates to anti-PD-1 in previously treated patients, for example one large phase I study demonstrated a 45% response rate to pembrolizumab in untreated patients, 29% for those with prior ipilimumab, and 33% for those with other prior therapies (including BRAF/MEK inhibitors).60

Presence of metastases and immune microenvironment at the metastatic site may also influence response to ICI. Liver metastases have been associated with inferior responses to anti-PD-1, and with reduced marginal CD8+ T cell infiltration in melanoma.61 Pretreatment samples from patients with liver metastases had lower CD8+ T cell densities than patients without liver metastases or patients with non-liver metastases. Comparatively, a nonsignificant trend toward improved progression free survival was observed with lung metastases. The systemic immune effects of liver metastasis may be related to the well-known but poorly understood phenomenon of liver-induced peripheral tolerance. Unlike other organs, liver allografts are occasionally spontaneously accepted and can induce tolerance to other transplanted organs from the same host. Further study is required to understand the immune mechanisms underlying these findings and investigate the immune environment at other common metastatic sites.

Along these same lines, visceral involvement (particularly liver metastases) has been associated with inferior responses to anti-PD-1, while tumor burden or bulk appears to be another possible clinical biomarker of anti-PD-1 resistance. A recent study showed that most patients are able to induce an immune response, as measured by an on-therapy increase in the proliferation (Ki67+) of CD8+ T cells to the anti-PD-1 antibody pembrolizumab, regardless of clinical response. However, it was the magnitude of reinvigoration of circulating exhausted T cells in relation to pretreatment tumor mass that was predictive of response. This study suggests that candidate biomarkers should be considered in relation to disease burden, and that activation of anti-tumor immunity may not be sufficient to override a high tumor burden. Finally, rare subtypes of melanoma not subjected to ultraviolet induced carcinogenesis (uveal, mucosal) have a low mutation
burden and low response rate to anti-PD-1, as discussed above, although some responses do occur, particularly in mucosal melanoma\textsuperscript{63,64}.

**Microbiome biomarkers**

Finally, one unique and intriguing clinical parameter that may influence response to ICI is composition of intestinal microbiota. Differences in melanoma growth were observed in mice with distinct microbiota that were eliminated after cohousing or fecal transfer. Bifidobacterium were associated with antitumor effects and oral administration of Bifidobacterium alone to mice produced tumor control comparable to anti-PD-L1 therapy\textsuperscript{65}. Thus, in addition to a biomarker response, gut microbiota may be a modifiable or interventional factor that could improve response to ICIs, and is currently being explored as such. Another intriguing study has suggested that pretreatment microbiota may be a biomarker of susceptibility to immune-mediated colitis following anti-CTLA-4 therapy. Increased representation of Bacteroidetes species was associated with resistance to immune-mediated colitis\textsuperscript{66}.

**Future challenges and new directions**

Development of clinically actionable biomarkers for response to immune checkpoint inhibition remains challenging. Despite numerous candidate biomarkers (Figure 2 and Table 1), which have demonstrated correlations with response in preclinical and clinical studies, no biomarker currently exists which is sufficiently robust as to aid in patient selection. A major challenge is identifying a biomarker with sufficient negative predictive value to only exclude patients who are very unlikely to benefit, without depriving patients who may respond a chance at a potentially life-extending therapy when there are few other options. Many current candidate biomarkers have good positive predictive value, which is not as useful in the clinical context of metastatic melanoma. An area where a strong positive predictive value may be more useful is for identifying potential responders to single agent anti-PD-1 therapy. Those patients who do not possess such a biomarker of response to single agent anti-PD-1 may be better candidates for combination therapy.

Although a number of biomarkers described to date clearly have preliminary potential for a place in therapy, validation in systematically-collected and uniformly-treated prospective studies have largely not been performed. Ideally, multiple candidate biomarkers should be assessed in the same dataset simultaneously to perform
redundancy and complimentary predictive values, and weigh the utility of each marker against one another. However, due to the proprietary nature of companion diagnostics, and the cost of performing many of the analyses (particularly next-generation analyses), such studies will likely have to be championed by academic scientists in an objective and non-conflicted approach. Furthermore, a careful pharmaco-economic analysis of the biomarker(s), costs and burden to the healthcare system, balanced against the cost of therapy, and impact of false-positives and negatives will be needed.

Another major challenge in the field of tumor immunology as a whole is accounting for the complexity of the anti-tumor immune response. A recent study utilized mass cytometry (CyTOF) to evaluate a wide array of immune parameters from multiple sites at multiple time points during effective treatment with immunotherapy in a mouse model of breast cancer. This study revealed the systemic nature of immune activation during tumor rejection and highlighted the dependence of such a response on peripheral immune cells\textsuperscript{67}. Based on these results, it is possible that future biomarkers, such as multi-parameter models, which take into account the capability for systemic immune activation will be more useful than those which focus solely on the local tumor environment. Multivariate analyses can also help determine if any current possible biomarkers are providing redundant information and which provide unique insights. It is likely that multi-parameter models may be able to better capture the likelihood of response to immune therapy than any single parameter.

In addition to developing biomarkers of response to ICI, biomarkers of toxicities are needed. Though ICI is often better tolerated than traditional chemotherapies, these drugs are not benign, with high rates of immune-related side-effects and rare but unpredictable serious, sometimes fatal, adverse events.

Many of the current candidate predictors of response to ICI help to elucidate the biology of the anti-tumor immune response. Molecular factors associated with response to ICI include pre-existing infiltrate of T cells into the TME, expression of immune checkpoints within the TME, high mutational burden/immunologically significant neoantigens, effective antigen presentation and recognition, intact IFN-\(\gamma\) signaling, and effective cytotoxic T cell response. Other predictors of response to ICI can be seen in the peripheral blood, such as circulating immune cells, LDH, ctDNA, and angiopoietin-2.
Further predictors include clinical characteristics, such as previous therapies, presence of irAEs, and tumor bulk. Some biomarkers such as LDH, driver mutation, and PD-L1 expression, have been well-studied and further studies are unlikely to yield new information about their usefulness as single-parameter prognostic indicators. Others, such as mutational burden, neoantigen load, ctDNA, and immune signaling have been less completely characterized and are more likely to provide insights into the biology of tumor immunity. However, these newer parameters are generally more expensive to measure, complex to interpret, and therefore less widely accessible to all patients who could benefit. Future studies should investigate both the potential of these biomarkers to be translated to clinical utility and contribute to current knowledge of tumor immunology as a whole. Altogether these candidate biomarkers represent a wealth of knowledge about the basic biology of response to immune checkpoint inhibition, despite a lack of actionable biomarkers to aid clinical decision-making as of yet.
References Cited:


Figure legends:

*Figure 1: The process of T cell mediated anti-tumor immunity and associated biomarkers.* The process of T cell mediated anti-tumor immunity is outlined. Sources of potential molecular or biological biomarkers along this process are identified.

*Figure 2: The spectrum of biomarkers for response and/or resistance to immune checkpoint inhibition.* Biological or clinical sources of reported biomarkers are designated and color coded according to positive association with response (green), positive association with resistance (red), or mixed interpretation (blue).
Figure 1:
Figure 2:

The spectrum of biomarkers for clinical response or resistance to checkpoint inhibition

**Tumor**
- CD8+ T cells
- Bafil3+ DCs
- PD-L1 expression
- TCR clonality
- MHC-I/II expression
- Interferon γ signals
- NRAS mutations
- Tumor mutation burden
- Neoantigen burden
- JAK1/2 or B2M loss-of-function mutations
- WNT/β-catenin signaling
- MEK activation
- PTEN loss

**Peripheral**
- Serum LDH
- Lymphocyte counts
- TCR clonality
- Angiopoietin-2 levels
- sPD-L1 levels
- Circulating tumor DNA levels

**Clinical Picture**
- Presence of immune-related adverse events
- Microbiome
- Prior therapies
- Visceral involvement of metastatic disease
- Total tumor burden
- Uveal subtype
### Tables:

Table 1: Implication of potential biomarkers in response to immunotherapy

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Checkpoint(s)/Therapies</th>
<th>Effect on response</th>
<th>Reference(s)</th>
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<tr>
<td><strong>Molecular</strong></td>
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<td></td>
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<tr>
<td>NRAS mutations</td>
<td>CTLA-4, PD-1</td>
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<td>NF1 mutations</td>
<td>PD-1</td>
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<td>CD8+ T cells at tumor margin</td>
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<td>DC infiltration</td>
<td>T cell transfer therapy</td>
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<td>PD-1 expression</td>
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<td>Positive</td>
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<td>CTLA-4 expression</td>
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<td>PD-L1 expression</td>
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<td>WNT/β-catenin activation</td>
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<td>PD-1</td>
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<td>Mutational burden/neoantigens</td>
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<td>SERPIN family mutations</td>
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<td>IPRES transcriptional signature</td>
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<td>GNAQ/GNA11 mutations</td>
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<td>Cytotoxic response</td>
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<td>IFN-γ response</td>
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<td><strong>Peripheral Blood</strong></td>
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<td>Elevated LDH</td>
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<td>Angiopoietin-2</td>
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<td>irAEs</td>
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<td>Prior treatment</td>
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<tr>
<td>Visceral involvement/ tumor bulk</td>
<td>PD-1</td>
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<td>Bifidobacterium in microbiome</td>
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