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REVIEW ARTICLE

Biomarkers for immune checkpoint inhibitors in solid tumors

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Abstract
The use of immune checkpoint inhibitors in solid organ malignancies has become widespread in the last decade. Accumulating evidence shows broad survival benefit as compared to traditional chemotherapies. At the same time, a need has emerged to stratify these drugs in various patient populations and histologies. Consequently, various immune biomarkers have been proposed to help in selecting patients for these therapies. Here, we review the evidence pertaining to biomarkers including programmed death-ligand 1, defective mismatch repair, tumor mutational burden, tumor-infiltrating lymphocytes, gene expression profiles, circulating blood cells, circulating DNA and the gut microbiome. The value of PD-L1 testing in certain malignancies, such as lung and urothelial cancer is highlighted as well as emerging data from trials such as GARNET and CheckMate142.

Keywords Biomarkers · Immune · Checkpoint · Inhibitors · Tumors

Introduction

It is by now common knowledge among the medical community that immune checkpoint inhibitors (ICI) represent a sea change in the treatment of solid organ malignancies. Survival benefit from such therapies has been seen across a wide variety of histologies and patient populations [1]. However, only about twenty percent of tumors achieve a response [2]. Moreover, immune-related adverse events (irAE) can have ubiquitous organ involvement, prolonged courses and in some cases are even life threatening [3]. Thus, there remains a need for validated biomarkers. Doing so will allow for the identification of patients with the most potential for benefit. Conversely, biomarkers can be used to spare those who are unlikely to experience benefit or to better define subpopulations that are predisposed to toxicity. What's more, biomarkers can lead to trial enrichment strategies and minimize cost burden to patients and payers. The goal of this article is, therefore, to review the available biomarkers pertinent to ICI and evaluate their clinical utility in the prediction of anti-tumoral response. For more information related to biomarker signals in other types of immuno-modulating agents, the SITC cancer immunotherapy resource document is recommended [4].

Immune checkpoint inhibitors work by augmenting the tumor microenvironment (TME). Under ideal circumstances, tumor-infiltrating lymphocytes (TIL) are recruited to the site of malignancy, recognize tumor cells (TC) and then destroy them through the release of cytolytic granules [5]. To summarize this recognition process, oncogenic mutations give rise to neoantigens, which are then captured by dendritic cells or other antigen presenting cells (APC), whereupon these proteins are displayed on Major Histocompatibility Complex (MHC). Cytotoxic T-cells, characterized by their expression of the CD8 receptor, then read these neoantigens using the T-cell Receptor (TCR), but this priming is heavily regulated by multiple costimulatory and inhibitory receptors. The receptors of most relevance to ICI being the Programmed Death Ligand (PD-L1) and Cytotoxic T Lymphocyte protein 4 (CTLA-4). PD-L1 is a transmembrane immunoglobulin expressed on TC, although not exclusively, and inhibits lymphocytes via its binding with PD-1 present on the surface of these T-cells. Antibody blockade of either the PD-1 or PD-L1 results in increased lymphocyte activation and proliferation [6, 7]. Similarly, T-cells can express CTLA-4, alongside the costimulatory molecule CD28, and both can bind CD80/86 expressed on APC. Dominance of CTLA-4-binding results in the suppression of cytotoxic T-cells. Vice versa, blockade by anti-CTLA-4 drugs allows

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these TILs to destroy the tumor. Currently, there are 7 FDA approved ICI. These include monoclonal antibodies targeting PD-1: nivolumab, pembrolizumab, cemiplimab and dostarlimab. Additionally, there are those ICI targeting PD-L1: atezolizumab, avelumab and durvalumab. The CTLA-4 inhibitor, ipilimumab, is also FDA approved.

Programmed death ligand

It was noted that in early clinical trials of ICI the duration of response (DOR) often exceeded overall survival (OS) thus suggesting that there was a subpopulation of individuals more likely to benefit from treatment [8]. PD-L1 expression, either by the tumor itself or the corresponding immune cell milieu, was seen as a potential biomarker. As such, companion immunohistochemical (IHC) tests were developed with many immune checkpoint agents, the details of which are summarized below (Table 1). The Blueprint Study was a project comparing these 22C3, 28–8, SP142 and SP263 assays [9]. This demonstrated that PD-L1 staining for TC was comparable between tests when the 22C3, 28–8 and SP263 assays were utilized, however, when SP142 was employed, fewer stained TC were observed. All the IHC assays demonstrated greater variability with IC staining than with TC. It is, therefore, recommended that pathologists be trained in the proper interpretations of these tests and that clinicians be aware of each IHC test as it relates to prognostic and predictive evidence in disease-specific clinical trials. Another industry-sponsored effort also found the SP142 test to be the outlier with significantly less PD-L1 detection in both TC and IC as compared with 22C3 or 28–8 [10]. Once more, pathology concordance was stronger for TC scoring than it was for IC.

A summary of predictive biomarker signals for PD-L1 expression in solid tumor malignancies from clinical trials leading to FDA approval of ICI is provided below (Supplemental Table 1). Melanoma was the first malignancy whereby PD-(L)1 blockade was shown to improve clinical outcomes. Interestingly, across a broad spectrum of melanoma trials, PD-L1 expression has not proven to reliably differentiate ICI responders from non-responders. The lone exception to this is Keynote-006 where PD-L1 $\geq$ 1% did demonstrate superior OS for pembrolizumab treatment over ipilimumab. Non-small cell lung cancer (NSCLC) has the preponderance of both clinical trials and FDA approved indications for ICI. Initial studies (Keynote-024, Keynote-042) had seemed to suggest some predictive value for PD-L1, and the FDA approved indications adhere to these. However, the majority of subsequent studies have shown no benefit for PD-L1 testing. As for NSCLC histology, non-squamous may have more use for PD-L1 testing than squamous, if such a benefit does exist, as Keynote-189 suggested progression-free survival (PFS) benefit from PD-L1 $\geq$ 1% while Keynote-407 did not. Furthermore, in early-stage NSCLC, there may be disease-free survival (DFS) benefit but this is reserved to higher PD-L1 expression levels as seen with TC expression $\geq$ 50% in IMpower010 and $\geq$ 50% in CheckMate 189. To date, OS benefit has not been shown in early-stage NSCLC treated with immunotherapy. Conflicting results have been observed for Head and neck squamous cell carcinoma (HNSCC) with CheckMate 141 showing overall survival (OS) benefit from PD-L1 $\geq$ 1%, while Keynote-048 showed none. For urothelial carcinoma (UC), most studies have demonstrated predictive value for either TC or IC expression of PD-L1, although this benefit has ranged from only DFS all the way up to OS superiority. The trend appears to be that PD-L1 is less useful in the early muscle-invasive stage, as compared with unresectable and metastatic disease. Keynote-826 suggests that cervical cancer (CC) with PD-L1 $\geq$ 1% have improved survival with pembrolizumab. In renal cell cancer (RCC), variable results have been observed with some studies showing that PD-L1 is predictive, including in the post-nephrectomy setting (Keynote-564), whilst other studies have offered evidence to the contrary. No obvious differences are seen between those studies excluding non-clear-cell histology and those that do not. CheckMate 040 suggests that PD-L1 testing is not useful for hepatocellular carcinoma (HCC). Most studies in esophageal, gastroesophageal (GEJ) and gastric cancer have shown that high combined positive scores (CPS) predict improved OS from ICI therapy, but in Keynote-181 and Keynote-590 this finding was only statistically significant with expression greater than 10%. Results in triple-negative breast cancer (TNBC) have consistently identified high PD-L1 expressors as having benefit but this has ranged from pathological complete response (pCR) (Keynote-522) to OS advantage (IMpassion 130). Most recently, CheckMate 743 has shown that PD-L1 $\geq$ 1% predicts OS benefit from nivolumab plus ipilumumab in pleural mesothelioma.

A meta-analysis encompassing 6664 cancer patients across 41 ICI trials found that PD-L1 was predictive of favorable overall response rate (ORR) (OR 2.26, $p < 0.001$) [11]. These results include statistically significant benefit in NSCLC, UC, RCC, gastroesophageal, HNSCC and even melanoma, although the high proportion of PD-L1-negative patients with melanoma responding to treatment is duly noted. Other meta-analysis have largely affirmed these findings with the exception of breast cancer specific meta-analysis which have had conflicting results on the benefit of PD-L1 [12, 13].

These findings, while encouraging, indicate that detection of PD-L1 expression is not always associated with response and even patients with no PD-L1 detected on IHC have been found to achieve durable responses from ICI. Several reasons are postulated for the poor reliability of PD-L1 IHC as a biomarker for anti-PD-1 or anti-PD-L1

Table 1 Immunohistochemistry assays for PD-L1 expression

PD-L1 assay	Companion immune checkpoint inhibitor (target)	Predefined expression levels	Companion diagnostic indications (threshold)	Notes
22C3 pharmDx	Pembrolizumab (PD-1)	TPS ≥ 50%	NSCLC (TPS ≥ 1%)	TPS defined as defined as the percentage of PD-L1 + TC divided by the total number of TC, wherein PD-L1 positivity is based on at least weak intensity (≥ 1 +), partial or complete membrane staining for the PD-L1 antibody. Cytoplastic PD-L1 staining by itself is excluded from TPS scoring as are non-viable cells and infiltrating immune cells
		TPS ≥ 1–49%	Gastric or GEJ (CPS ≥ 1)	CPS defined as the number of PD-L1 positive cells, including TC and IC, divided by the total number of TC and multiplied by 100
		TPS < 1	CC (CPS ≥ 1) UC (CPS ≥ 10) HNSCC (CPS ≥ 1)	
28–8 pharmDx	Nivolumab (PD-1)	≥ 10% ≥ 5%	NSCLC (PD-L1 ≥ 1%)	Expression determined by percentage of viable TC which exhibit complete circumferential or partial linear membrane staining of any intensity
	Ipilimumab (CTLA-4)	≥ 1% < 1%		
SP142 Ventana	Atezolizumab (PD-L1)	Various	NSCLC (TC ≥ 50, IC ≥ 10%) TNBC (IC ≥ 1%) UC (≥ 5%)	Unlike 22C3 and 28–8, SP142 scores expression independently for both TC and IC. TC scoring is based on percentage of viable TC showing membranous staining of any intensity. IC type or staining localization is not taken into account for TC scoring. IC scoring is based on the proportion of tumor area that is occupied by PD-L1 + IC of any intensity. Tumor area in this respect is defined by viable TC, associated peritumoral stroma, but not necrosis
SP263 Ventana	Atezolizumab	TC ≥ 25% ICP > 1% and IC + 25% ICP = 1% and IC + = 100%	NSCLC (PD-L1: ≥ 1%)	Expression based upon the percentage of TC with any membrane staining above background or by the percentage of tumor-associated IC with staining at any intensity. The percentage of tumor-associated IC staining positive for PD-L1 (IC +) is itself determined by the percentage of ICP in a given tumor area

TPS tumor proportion score, *TC* tumor cells, *IC* immune cells, *CPS* combined positive score, *ICP* immune cells present

therapies. As an example, in breast cancer, the heterogeneity between hormone status, histological subtypes and metastasis make it difficult to assign a holistic PD-L1 status [14]. Discordant PD-L1 expression is also seen between primary and metastatic sites in melanoma and NSCLC [15, 16]. Finally, careful consideration must be made with regards to sampling time points and interventions as chemotherapy and radiation can increase both PD-L1 expression and TIL density [17].

Microsatellite instability and defective mismatch repair

Microsatellites are short nucleotide sequences with repeating motifs. These sequences usually range from 1 to 6 base pairs in unit length with anywhere from 10 to 60 repetitions per sequence. Together, they account for 3% of the entire genome [18]. Microsatellites by their very nature are prone to slippage and mispairing events [19]. Microsatellite

instability (MSI) occurs when there are gains or losses to one or more of such repeats but researchers differ on the exact number of tandem repeats that constitute a microsatellite. Related to this concept is the mismatch repair (MMR) system, a conglomerate of DNA repair mechanisms that is responsible for correcting such errors. The key proteins involved in MMR are the gene products of MLH1, MSH2, PMS2, and MSH6 [20]. A defect in any one of these genes results in a defective mismatch repair (dMMR) mechanism, which in turn results in high microsatellite instability (MSI-H). The MSI-H phenotype has been associated with carcinogenicity, most notably that pertaining to Lynch Syndrome [21]. Evidence of MSI-H has been shown in several cancers including gastric, adrenocortical, CC, endometrial, CRC, mesothelioma, esophageal, breast, RCC and cholangiocarcinoma [22]. Furthermore, MSI-H status correlates with mutational burden, TIL presence and the expression of inhibitory immune checkpoint markers [23–25]. Because of this, it was theorized that these patients would be more likely to respond to PD-(L)1 blockade.

As previously stated, MSI status can be inferred by IHC staining for dMMR gene loss, providing a quick and relatively inexpensive means of assessing this. The sensitivity of IHC for MSI is more than 90% when all four dMMR proteins are tested together, but immunostaining is not without its own perils [26]. Missense mutations have been identified in these genes that can result in altered proteins that are non-functional but still recognizable by antibody staining [27]. This circumstance may then result in false negatives. Additionally, extensive loss of MSH6 immunoexpression is common in CRC following neoadjuvant chemoradiation [28]. MSI/dMMR status can also be detected by Polymerase Chain Reaction (PCR). The Bethesda panel which included 2 mononucleotide repeats and 3 dinucleotide repeats was initially proposed for the uniform analysis of MSI status [29]. Later, a Pentaplex panel consisting of 5 mononucleotide repeats was developed which did not require germline testing [30]. In a NCI workshop, this Pentaplex panel was recommended for the evaluation of MSI-H [31]. Alternatively, Next Generation Sequencing (NGS) affords a highly accurate and increasingly available way of evaluating for MSI/dMMR but false negatives can still occur due to tumor DNA dilution [32]. Currently, utilizing any of the methods described above to determine the MSI status of tumors is appropriate.

In a phase 2 study evaluating pembrolizumab in 41 patients with metastatic carcinoma, superior PFS was observed in CRC tumors that were dMMR (78% at 20 weeks vs 11% with proficient MMR) [33]. In that same trial, no prognostic value for dMMR was observed with non-CRC tumors. A supplemental biologics licensing application was put forth containing data from 149 patients with MSI-H/dMMR cancers treated on 5 Keynote clinical trials (012, 028, 016, 158 and 164) [34]. While the majority of these patients were CRC, there were 59 patients that were not, these representing 14 other solid cancers. The responses from this heavily pretreated population was favorable (ORR 39%) with 78% having a DOR ≥ 6 months. Consequently, site-agnostic FDA approval was granted to pembrolizumab for the treatment of adult and pediatric patients with unresectable or metastatic solid tumors that are MSI-H or dMMR and who have progressed following prior treatment with no satisfactory alternative options. For colorectal malignancy specifically, the FDA granted further approval, this time in the frontline setting, after Keynote 177 showed that in 307 patients with metastatic CRC and MSI-H/dMMR status, treatment with pembrolizumab improved PFS [35]. Recent non-randomized data from cohort D of KEYNOTE-158 led to further approval of pembrolizumab in advanced endometrial carcinoma with MSI-H/dMMR. Likewise, Checkmate 142, a phase 2 study of nivolumab in patients with recurrent or metastatic CRC harboring MSI-H/dMMR, confirmed similar improvements in disease control and response [36]. This in turn led to the approval of nivolumab on a site agonist basis. An update from CheckMate 142 showed further benefit with the addition of ipilimumab, which resulted in expanded FDA approval in 2020 for MSI-H/dMMR metastatic CRC that had progressed on traditional chemotherapy [37]. More recently, results from the GARNET study, a phase 1 trial of dMMR or polymerase ὲ (POLE) mutant solid tumors, demonstrated a favorable ORR (38%) from treatment with dostarlimab [38]. From this, dostarlimab received FDA approval in August 2021 for the treatment of recurrent or advanced solid tumors harboring dMMR and having progressed on prior treatment with no satisfactory alternative options.

Tumor mutational burden

Tumor mutational burden (TMB) is the number of somatic mutations in a genomic region, but the precise calculation for this varies with both the sequence location, as well as the mutation's characteristics, for instance if non-coding or silenced genes are included therein [39]. Preclinical studies have demonstrated that the strong immunogenicity of early tumors is a function of neoantigen expression [40]. More so, immunoediting gives rise to a population of tumors without an easily recognizable antigenic repertoire [41]. To that point, TMB by itself is of negative prognostic value [42]. As expected, higher TMB correlates with CD8-mediated cytotoxicity and these findings suggest that TC with an elevated TMB will be more likely to be impacted by lymphocyte-modulating therapy [43]. It is hypothesized that two factors determine a neoantigens ability to induce tumoral response from checkpoint inhibitors. First, the likelihood of presentation on MHC and second, recognition by the lymphocytes

themselves [44]. Exome analysis from melanoma patients treated with CTLA-4 blockade has affirmed that TMB correlates with survival [45]. These missense mutations were found, using in silico translation, to bind MHC1 and, in vitro, to elicit a polyfunctional T-cell response. However, it is important to remember that not all tumors, nor epitopes, are created equally. For instance, in pancreatic cancer, it appears that neoantigen homology to peptides derived from certain infectious diseases may play a greater role than just the frequency of neoantigens alone [46].

TMB can be measured from tumor-derived tissue using NGS and involves testing of either specific gene panels or whole exome sequencing (WES). Originally considered the gold standard, WES has many limitations, including the specialized platforms, expertise, turnaround time and the need for germline comparisons [47]. Disparity between WES data from different vendors has been attributed to tumor heterogeneity, proprietary mutation cutoffs and sequencing artifacts from formalin fixation but pre-analytic microdissection can ameliorate this to some extent [48]. For these reasons and more, targeted gene panels were developed which differ from WES in terms of input requirements, covered region and bioinformatic algorithms. Because of these, variation in TMB can arise but tends to be low with one study showing only 9% of mutations being missed with comprehensive testing [49]. Unfortunately, panel TMB can overestimate, particularly at higher values and standardization between these two modalities is needed [50].

In addition to tissue sampling (tTMB), tumor mutational burden can also be established from peripheral blood (bTMB). This method of analyzing tumor DNA from serum is less invasive and expensive than tissue biopsy. Although not specific to TMB, cohort NGS studies have shown a 98% concordance between blood and tissue sampling in the identification of alterations [51]. A study evaluating data from two NSCLC studies, POPLAR and OAK, found clinically significant predictive value from bTMB [52]. For the phase 2 POPLAR study, OS benefit was observed for bTMB using a cutoff of ≥ 16 mutations per megabase (mut/Mb), however, a ≥ 20 mut/Mb cutoff for OS was statistically significant. Data from the subsequent phase 3, however, showed OS benefit with all cutoffs. Furthermore, bTMB and PD-L1 expression as biomarkers had little overlap, suggesting that these represent mostly divergent subgroups receiving benefit from immunotherapy.

Indeed, in a retrospective cohort analysis of 1662 cancer patients analyzed by NGS (MSK-IMPACT), the majority of whom had metastatic disease and all of whom had received prior ICI, higher TMB (TMB-H) was associated with improved OS (HR 0.52, $p = 1.6 \times 10^{-6}$) [53]. This was true across a broad distribution of histologies, the association being strongest in NSCLC, HNSCC, CRC, UC. However, it is also important to note that the predictive value of TMB was greatest when taken as a top percentage cutoff for each histology individually. As such, there does not appear to be a universal cutoff value for TMB but rather a disease-specific range at which this biomarker is most predictive. More recent cohort evidence has supported this hypothesis [54]. It is also worth noting that glioma failed to show even a trend toward significance with this approach. This result may portend a lack of efficacy on the part of immunotherapy, reflect the lower incidence of dMMR, or showcase the deleterious effects of hypermutation caused by alkylating therapies. While on the subject of tumor heterozygosity with respect to TMB, it is worth mentioning that in gastrointestinal cancers TMB and MSI appear to coincide, as opposed to melanoma, skin SCC and lung cancer where they do not [55].

In June 2020, the FDA granted accelerated to pembrolizumab for treatment-refractory cancers with a high TMB, defined as greater than 10 mut/Mb. Companion diagnostic approval for FoundationOne CDx was also granted for this purpose. This approval was based on Keynote-158, a phase 2 study of 1073 patients with advanced solid tumors and progression on prior therapy [56]. A prespecified cutoff of ≥ 10 mut/Mb was used to define TMB-H. Amongst the 13% of patients with TMB-H, treatment with pembrolizumab resulted in an ORR of 29%. For comparison, the non-TMB-H group had an ORR of only 6%. There have been other signals of predictive value for TMB with most benefit observed in melanoma, NSCLC and UC [57]. CheckMate 227 was a phase 3 trial of 2876 patients with stage IV or recurrent NSCLC who were prospectively treated with nivolumab plus ipilimumab [58]. Of the 44% of patients with TMB-H tumors ($\geq$ 10mut/Mb), ICI therapy resulted in a modest, but statistically significant, advantage in PFS and overall survival readouts from this study are much anticipated [59]. Similarly, in MYSTIC, a phase 3 of 1118 patients with advanced NSCLC treated with durvalumab plus tremelimumab, TMB with a cutoff of ≥ 20 mut/Mb was found to be most predictive of clinical benefit, this finding made even more interesting by the fact that bTMB was superior to tTMB [60, 61]. However, the predictive value of TMB, and its recent FDA approval, is not without controversy. An analysis of 137 patients with advanced CRC treated with checkpoint inhibitors shows that any apparent benefit from TMB-H is abrogated once patients are stratified by MSI or POLE [62]. Many prospective trials, such as IM power 110, have shown no advantage from TMB and a TCGA analysis from over 10,000 patient tumors failed to associate this biomarker with immunotherapy outcomes [63]. Finally, it is important to consider if a high TMB reflects early or late branching events during the course of oncogenesis as clonal heterozygosity may contribute to resistance, the immunoresistance of this event overcoming any positive effects from more robust neoantigen presentation [64].

Tumor infiltrating lymphocytes

As mentioned, TILS play a critical role in the recognition and suppression of tumors by the immune system. Their presence in the TME heralds a better prognosis across a wide variety of tumors such as melanoma, NSCLC and TNBC but negative associations have been observed in CRC [65–68]. Accordingly, the International Immuno-Oncology Biomarker Working Group (IIOBWG) has proposed standardized methods of TIL quantification by IHC, differentiating between stromal and tumor compartments as well as the 1 mm invasive margin that is frequently seen on histology, and excluding necrosis. These IIOBWG guidelines are specific to malignancy type, accounting for unique differences in each microenvironment [69].

In addition to their prognostic value, TILs have also been shown to predict benefit from immunotherapy. In a meta-analysis of 14,395 patients with NSCLC who were treated with immunotherapy including ICI, tumor vaccines and cellular therapy, CD8 TIL scores were found to improve the combined predictive utility of PD-L1 and TMB [70]. The optimal time for sampling of these TILS, however, remains an open question. Analysis from patients with metastatic melanoma has shown that those who respond to ICI are more likely to have preexisting CD8 TILs involving the invasive margin [71]. These TILS displayed proximity between PD-1 and PD-L1 expressing cells and had a more clonal, meaning less diverse, TCR repertoire. In contrast, other cohort studies have suggested that early on-treatment samples are more predictive of future immune response than preexisting TILS [72].

Circulating blood cells

White blood cell (WBC) differentials are routinely used in clinical practice and offer the potential of being a non-invasive and rapid predictor for benefit from immune checkpoint modulation. Indeed, it could be argued that circulating blood cells represent the first biomarker used in oncology [73]. Staging criteria, such as the Rai stage in chronic lymphocytic leukemia (CLL), rely on lymphocytosis as do prognostic indicators such as the International Prognostic Score for non-Hodgkin's lymphoma. Circulating blood cells also have predictive value in hematological malignancies. For instance, high absolute lymphocyte counts ($\geq 25 \times 10^9$/L) are at high risk for tumor lysis syndrome in CLL patients treated with venetoclax [74].

More recently, the utility of circulating blood cells in predicting response in solid tumors has been examined. In a study of 616 patients with melanoma treated with pembrolizumab, elevated baseline counts in eosinophils ($\geq 1.5\%$) and lymphocytes ($\geq 17.5\%$) were found to be independent predictors of favorable survival [75]. Similar findings have been observed with anti-CTLA-4 [76]. Immunoprofiling with flow cytometry further suggests that baseline peripheral CD8 counts play more of a role after treatment with CTLA-4 blockade than after PD-1 blockade [77]. Lending credence to this is a meta-analysis of 4647 patients with advanced stage cancer, where elevated pretreatment neutrophil to leukocyte ratio (NLR), as measured by peripheral blood, was associated with inferior OS after treatment with ICI (HR 2.16, $p < 0.001$) [78]. Subgroup analysis shows that NLR predicts worse survival in melanoma, NSCLC, RCC, sarcoma, UC, HNSCC, CRC, hepatobiliary, esophageal and mesothelioma [79]. Lastly, erythrocyte sedimentation rate (ESR) has also been associated with improved OS after immune checkpoint modulation [80].

Lactate dehydrogenase

Lactate dehydrogenase (LDH) catalyzes the conversion of lactate to pyruvate and serves as a general marker for tissue damage. Regarding LDH as assessed by peripheral blood, some studies have shown that elevations are associated with favorable OS after ICI therapy whilst others have shown correlations in the opposite direction [75, 80, 81]. Worth mentioning is the fact that neither CheckMate 067 nor CheckMate 069 showed that baseline LDH exclusively predicted OS benefit from ICI in melanoma. Validation of this enzyme as a biomarker will require larger prospective studies in the future with attention to whether it is baseline values or trends during treatment that are most predictive [82].

TCR sequencing

In a retrospective pilot study of 12 patients with metastatic melanoma treated with anti-CTLA-4, TCR sequencing was performed on peripheral blood at baseline [83]. TCR diversity was graded based on 'richness', defined as the ratio between observed V-J rearrangements divided by theoretical, as well as 'evenness', reflecting the similarity between the frequencies of specific V-J rearrangements that were observed. Both high richness and high evenness were predictive of clinical benefit.

Gene expression profiles

Recently, a variety of gene expression profiles (GEP) relating to the TME have been identified as predictive for response to ICI. Early work focused on immune gene signatures inducible by interferon gamma (IFN- γ), including MHC-II. In two melanoma cohorts, MHC-II expression on TC was associated with improved response and OS after PD-1 blockade [84]. A T-cell inflamed GEP consisting of 18 genes was developed and validated with data from a

200-patient cohort accounting for 9 different malignancies to correlate with benefit from pembrolizumab [85]. Receiver operating characteristics (ROC) measure the ability for a test to differentiate between two groups, such as responders and non-responders, with area under curve (AUC) values of ≥ 0.8 being suggestive of most benefit. This T-cell inflamed GEP was comparable to PD-L1 IHC (AUC 0.75 vs 0.65, respectively). Another 8-gene panel defining effector T-cells (Teff) was developed for use with atezolizumab. Unfortunately, in the phase 3 IMpower150, Teff signatures were not found to be superior to PD-L1 expression at identifying NSCLC beneficiaries [86]. Along the same vein as this a companion GEP for durvalumab, consisting of IFN- γ, CD274, LAG3 and CXCL9 is currently under development [87]. Cross-modality meta-analysis shows that GEP is of greatest value when combined with other biomarkers. In comparison of 8135 patients representing 10 solid malignancies, multiplex IHC combined with immunofluorescence (IF) was found to have a superior AUC (0.79) to PD-L1 IHC alone (AUC 0.65) [88]. However, combining PD-L1 expression with GEP resulted in predictive value (AUC 0.74) which rivaled even that of multiplex IHC/IF.

Circulating tumor DNA

Circulating tumor DNA (ctDNA), not to be confused with cell-free DNA (cfDNA), has been proposed as a biomarker not only for initial response to ICI but also longitudinally for molecular relapse. An exploratory analysis from IMvigor010 found that ctDNA predicted OS benefit from atezolizumab versus observation in UC (HR 0.59, $p = 0.0024$) [89]. Transcriptomic analysis from ctDNA-positive patients benefiting from immunotherapy highlighted immune response signatures and basal-squamous genes. Analogous signals have been seen for OS in melanoma and pCR after neoadjuvant ICI in breast cancer [90] [91]. In prospective analysis of 94 patients with advanced solid tumors, ctDNA at baseline was correlated with OS benefit from pembrolizumab, and this association only became stronger once ctDNA kinetics were taken into account [92]. Among the 12 patients with ctDNA clearance, all were alive at 25 month follow-up.

Mature tertiary lymphoid

Much interest has also arisen in tertiary lymphoid structures (TLS) as predictive biomarkers for immune checkpoint blockade. These structures have a T-cell zones populated by mature APC and fibroblastic reticular cells. Interspersed within these are B-cell zones with germinal centers that house memory B cells and plasma cells [93]. In melanoma patients receiving neoadjuvant ICI, both the presence of TLS and its ratio to tumor area, as assessed by histology, correlates with response in early on-treatment specimens. However, these same findings were not significant for preexisting samples, this suggesting that biopsy after treatment initiation is of highest predictive value [94]. In line with this, gene signatures derived from TLS and taken from pretreatment samples are also associated with improved OS in patients treated with immune checkpoint blockade [95]. Another study has identified TLS gene signatures as predicative for response to PD-1 blockade in sarcoma populations [96].

Gut Microbiome

The microbiota has long been suspected to play a role in oncogenesis but recent evidence in the era of immunotherapy paints a much clearer picture of these interactions [97]. Preclinical studies in mice originally suggested an association between the gut microbiome and a host's response to immunotherapy. In one such study using two syngeneic models of melanoma (JAX or TAC), differences in tumor groups were shown to be due to commensal microbiota and extinguishable both by cohousing as well as through unidirectional (JAX to TAC) fecal material transfer (FMT) [98]. Combination FMT with ICI was found to be synergistic with increased tumor control and IFNγ production. Using16s ribosomal RNA sequencing, Bifidobacterium was identified as the most likely causative species and these previous results could then be recapitulated with oral Bifidobacterim plus checkpoint blockade. Further study confirmed that antibiotic therapy impairs both anti-PD-1 and anti-CTLA-4 therapy in mouse models of sarcoma and melanoma [99].

Clinical studies have corroborated the role of the gut microbiome with respect to immunotherapy in humans. Decreased survival after immunotherapy has been observed with antibiotic use in patients with NSCLC, RCC and UC even after multivariate adjustment [99]. Akkermansia muciniphilia, a prodigious member of the ileum microbiota, was found to be most enriched in responders. In 26 patients with metastatic melanoma prospectively treated with ipilimumab, fecal microbiota composition was assessed using 16S rRNA sequencing at baseline and before administration. Patients with Bacteroides predominant microbiota had improved OS compared to those with Firmicutes such as Faecalibacterium [100]. In another study of 112 patients with metastatic melanoma undergoing PD-1 blockade, 16S sequencing revealed Clostridiales, most notably Ruminococcaceae, and Faecalibacterium as enriched in responders [101]. Contrarily, Bacteroides was preeminent in non-responders. Again, fecal microbiome transplantation improved ICI responses in mice with a statistically significant abundance of Faecalibacterium seen in responding animals.

Conclusion

The age of immunotherapy comes with much promise but also several challenges, one of the greatest being identifying which patients are most likely to benefit from ICI and which can be spared potential toxicity. Accomplishing this will require biomarkers that are well validated, easily implemented and ideally capable of longitudinal monitoring to assess for recurrence. Several potential biomarkers predicting benefit from immune checkpoint blockade have been described. To date, no individual biomarker has been proven "best". Yet, there is much that has been discovered. PD-L1, expression appears to be of much greater value in NSCLC, UC than in melanoma. MSI/dMMR appears to be gaining traction with trials such as GARNET and CheckMate 142 showcasing these alterations as a potential enrichment strategy. As for TMB, this remains a controversial biomarker despite recent accelerated approval by the FDA, in part because the perceived benefit may actually be due to confounding variables like MSI. TILS appear quintessential for NSCLC but several questions, such as optimal time for sampling, and challenges, specifically the standardization of IHC methods, remain. Circulating blood cells, peripheral TCR sequencing and ctDNA offer a cheap, non-invasive biomarkers whose predictive value may rival tissue-based assessment. Unfortunately, the data on LDH are too conflicted to offer much current value. GEP hold great potential, particularly when combined with other biomarkers such as PD-L1 expression. Finally, an improved understanding of the gut microbiome will likely yield insights into which patients are potential responders, but Faecalibacterium seems an ideal candidate at this time.

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