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New biomarkers for checkpoint inhibitor therapy

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ABSTRACT

Immune checkpoint inhibitor blockade has vastly changed treatment paradigms and improved outcomes of many solid organ malignancies. The achievements of the last decade have transformed the outcomes of several tumour types, most notably metastatic melanoma. There are, however, still large numbers of patients who receive checkpoint inhibitor therapy and do not respond. In addition to potential lack of efficacy, checkpoint inhibitors also come with a unique and sometimes devastating side-effect profile. There exists a strong need for biomarkers to accurately predict response, improve treatment selection and avoid exposing patients to toxicity where there is minimal likelihood of response. There is a wide range of methodologies investigating predictive biomarkers in this space; in this review, we address the major putative biomarkers of interest. These include conventional serum tests such as lymphocyte indices and lactate dehydrogenase, and more novel research markers such as interleukin-6 and T receptor clonality. We discuss tumorous factors that may be of interest in certain tumour types, and finally gene expression profiling. Significant research continues into many of these potential predictive biomarkers in response to the emergent need to better select patients who will benefit from treatment.

Immune checkpoint inhibitor (ICI) blockade has vastly changed treatment paradigms and improved outcomes of many solid organ malignancies. The achievements of the last decade have transformed the outcomes of several tumour types, most notably metastatic melanoma. There are still large numbers of patients who receive ICI and do not respond, however, spending precious time receiving ineffective treatment. Even in a cancer, such as melanomathat is considered to be highly immunogenic, 24% of patients receiving combination checkpoint inhibitor as part of Checkmate 067 had progressive disease as their best response.[1] In addition to potential lack of efficacy, ICI also come with a unique and sometimes devastating side-effect profile. Few oncologists would not have experienced the disquieting situation of treating a patient, particularly in the adjuvant setting, only for their patient to experience severe or permanent autoimmune toxicity, which dramatically impairs their quality of life or even leads to death. There exists a strong need for biomarkers to accurately predict response, improve treatment selection and avoid exposing patients to toxicity where there is minimal likelihood of response. The ideal biomarker would be reliably reproducible and cost-effective, has minimal interobserver variation and correlates strongly with clinical outcomes. In practice, few tests have all of these attributes. Despite these challenges, the need to develop biomarkers to better stratify treatment approaches is more urgent than ever. There is a wide range of methodologies investigating predictive biomarkers in this space; here, we attempt to provide a comprehensive overview of those currently of interest.

ESTABLISHED BIOMARKERS

PD-L1 expression

PD-L1 testing has been and remains the focus of extensive research, due to the coupling of PD-L1 biologically to agents targeting this pathway and due to some of the intriguing clinical data that have been generated. PD-L1 immunohistochemistry (IHC) emerged as the companion diagnostic to PD-1 checkpoint blockade largely based on the results of the phase III KEYNOTE 024 trial, which demonstrated superior outcomes for patients with non-small cell lung cancer (NSCLC), with tumour PD-L1 expression ≥50% treated with pembrolizumab in the first-line setting.[2] The role of PD-L1 as a predictive biomarker unfortunately remains complex, with inconsistent data between studies. The key issues include disparity between biopsy specimens and resected tumour, varying significance of PDL1 between tumour types and variations in the assays themselves.[3–5] Disparities between assays are also of concern, though relatively good harmonisation of these has been demonstrated, with the exception of the SP142 assay.[6] Gibney *et al*[7] noted that the negative predictive value of PD-L1 status in metastatic melanoma may be as low as 58%, referencing data from the single-agent nivolumab arm from Checkmate 067, in PD-L1-negative patients.[8] Alternative methods of assessing

tumour PD-L1 status have been explored in attempts to improve accuracy. PD-L1 RNA sequencing was not more accurate than IHC in a study of 209 patients.[9] A systematic review of methods of PD-L1 expression analysis found that more sophisticated methods of PD-L1 assessment, such as multiplex immunofluorescence, have a greater positive predictive value (though comparable negative predictive value). Of course the advantage of IHC as a time-efficient and cost-efficient test is lost in this case, though the rapid evolution of technology may improve this.[10] While PD-L1 expression (IHC in particular) clearly has a place in guiding treatment, its combination with other biomarkers may ultimately be needed to improve its predictive value. Some of these combinations will be discussed later.

Tumour mutational burden

The predictive utility of a high number of somatic non-synonymous mutations, commonly referred to as tumour mutational burden (TMB), has also been demonstrated, both in the preclinical space and in clinical trial outcomes. The most notable of these is Checkmate 227, using a diagnostic limit of 10 mutations per mega base (mut/Mb) in patients in receiving combination ICI blockade for NSCLC.[11 12] Interestingly, in the subset analyses by PD-L1 expression, both of which were statistically significant, combination of TMB with PD-L1 status of ≥1% did not appear to confer additive progression-free survival (PFR) benefit in patients treated with ipilimumab and nivolumab (HR 0.62 in patients with PD-L1 ≥1% vs 0.48 for those PD-L1 negative). This result was consistent with the findings of Checkmate 568, where PFS according to TMB appeared to be independent of PD-L1 status.[13 14]

Like PD-L1 expression, TMB is not a binary marker, and its predictive capacity differs between tumour types.[15] It should be noted that patients with a low TMB have responded favourably to ICI, further confounding the predictive value to this individual test.

Many patients will have access to targeted molecular profiling of tumour-specific relevant mutations (eg, Epidermal Growth Factor Receptor (EGFR) in NSCLC), but few patients readily have access to more complex testing, such as whole exome sequencing, which is required for an accurate assessment of TMB. Commercial next-generation sequencing platforms are now well developed, some of which include algorithms to estimate TMB. These represent an attractive option given the scale of testing and ability to use archival formalin-fixed paraffin-embedded tissue, but come at a significant per-patient cost that may limit universal availability.

Mismatch repair deficiency

The third established biomarker is mismatch repair deficiency (dMMR). The results of five single-arm studies of pembrolizumab in MMR-deficient/microsatellite instability (MSI)-high cancers led to the first tumour agnostic regulatory approval for an anticancer drug worldwide.[16] These studies included 90 patients with dMMR/MSI-high colorectal cancer and 59 with other dMMR/MSI-high cancers, with an overall response rate (ORR) across tumour types of 39.6%. The most impressive outcomes for patients with dMMR colorectal cancer are perhaps from early results from Checkmate 142, where patients receiving ipilimumab and nivolumab had an objective response rate of 60% and a 12-month survival rate of 83%.[17] Despite these compelling results, a significant number of patients did not respond to ICI in these trials. Among multiple possible explanations for this, inaccurate interpretation of either MMR IHC or MSI PCR results may have actually contributed to the predictive nature of the 'test', especially where only one method of testing has been used.[18] MMR deficiency may in fact be a stronger predictor than currently thought, in the presence of accurate diagnostic processes.

NEW BIOMARKERS

Conventional serum indices

Lymphocyte indices

Serum indices are an attractive biomarker, given their non-invasive means of sampling. Absolute lymphocyte count has been postulated in several retrospective studies to be predictive of benefit; as small studies, some with no comparator arm, these do not clearly differentiate a predictive capacity over a prognostic role as yet.[19–21] For many years, the value of neutrophil-to-lymphocyte ratio (NLR) has been noted as a poor prognostic marker in various cancer types. More recently, it has also been suggested as a predictor of outcome from immunotherapy (see online supplementary table). Saravia *et al*[22] reported that NLR may complement the use of PD-L1 status in patients with NSCLC, based on the retrospective categorisation of cases by PD-L1 status and NLR using a cut-off ratio of 5. Patients were categorised into good (high PD-L1, low NLR), bad (low PD-L1, high NLR) or intermediate (either high PD-L1 or low NLR). The authors demonstrated that high PD-L1/low NLR predicted for better outcomes, while low PD-L1/high NLR was a predictor of lack of response.[22] Of note, they did not examine NLR independently of PD-L1. Another group demonstrated that the prognostic value of NLR was significant in patient with a TMB >10 mut/Mb, but not in patients with a low TMB, using an alternative NLR ratio cut-off of 2.5.[23] Other authors suggested a predictive capacity independent of other markers.[24–26] While these results are of interest, care should be taken not to overinterpret these retrospective studies until more robust data demonstrating a predictive, and not just prognostic, value are available.

One study of particular interest used prospectively collected data obtained from an ipilimumab access programme, with 720 patients with metastatic melanoma, and categorised them according to high neutrophil count and elevated NLR. In a multivariate analysis, they reported a HR of 2.29 (CI 1.86 to 2.82) for risk of death, and 2.03 (CI 1.66 to 2.47) for progression, in

patients with a NLR of ≥3 (p<0.0001). Patients with both an elevated absolute neutrophil count (ANC) (7500 or greater) and an elevated NLR had a 1-year survival rate of 2% compared with 43% for those without an elevated neutrophil count or NLR.[26] Although it remains unclear whether this represents a predictive in addition to a prognostic benefit, these results warrant further prospective investigation.

Lactate dehydrogenase

Elevated lactate dehydrogenase (LDH) is well-documented as an adverse prognostic marker in a number of cancers, most notably in melanoma.[27] It has also been evaluated in the same setting as a predictive marker. Kelderman *et al*[28] demonstrated an inverse relationship between survival and elevated LDH in patients treated with ipilimumab. Nosrati *et al*[29] reported an OR of 0.48 (CI 0.25 to 0.90) for elevated LDH and radiological response to ipilimumab, using a ratio derived by dividing the patient's value by the institutional upper limit of normal. They incorporated this into a predictive scale for response to PD-1 checkpoint blockade, which they subsequently validated.[29] In the prospective study by Ferrucci described earlier, the authors performed a subgroup analysis in patients where LDH was available. When patient had all three stratification factors of elevated ANC, NLR and LDH, the HR for death rose to 13.24 (8.10–21.66<0.0001).[26] Mezquita *et al*[30] reported similar data using a combination of pretreatment NLR and LDH in patients with NSCLC. Although we already know these factors to be prognostic, these results perhaps also suggest that we should factor these indices into our assessment and counselling of a patient who is considering starting ICI therapies.

Tumorous factors

Epstein-Barr virus: gastric cancer

Cancers driven by oncogenic viruses possess a high neoantigen load, higher rate of immune signatures and high response rates to ICI therapy.[31 32] In cancers with an extremely high prevalence of an oncogenic virus, it may not be relevant to test for this. In cancers only a subset is virally driven; however, this may be a useful predictor of response. For example, a subset of gastric cancers has been shown to respond favourably to ICI blockade. Molecular characterisation of gastric cancers via the Cancer Genome Atlas revealed a subset of Epstein-Barr virus (EBV)-positive gastric cancers, which are characterised by immune cell infiltration. They commonly display genomic amplification of the genes encoding for the T cell signalling ligands PD-L1 and PD-L2.[33] A small but robust Korean study of 61 patients with gastric cancer treated with pembrolizumab highlighted the relevance of EBV positivity using EBV DNA sequence profiling. Six patients were EBV positive, and they were mutually exclusive with the MSI-high cohort. All six patients had a partial response to pembrolizumab, with a median duration of response of 8.5 months.[34] Based on these data, this approach is being formally tested in a prospective clinical trial specifically targeting EBV-positive gastric cancer, irrespective of PD-L1 status (NCT03755440).

Tumour infiltrating lymphocytes

The prognostic value of tumorous lymphocytic infiltration, under various names, has been observed for some years across a variety of tumour types.[35–37] Biologically it seems intuitive that the presence of lymphocytes either at the tumour bed or within the tumour itself is indicative of immune recognition and trafficking, and that this would therefore increase the likelihood of a favourable response to ICI. Tumeh *et al*[38] demonstrated a correlation between pretreatment CD8+ cell infiltrate and radiological response in patients with metastatic melanoma, using subset data from the KEYNOTE 001 study. They further validated their findings using a small, separate blinded data set. Daud *et al*[39] contributed further to this picture, reporting a statistically significant correlation between an increased fraction of CD8+ lymphocytes expressing CTLA-4 and PD-1 in pretreatment samples, and response to single-agent PD-1 inhibitor therapy. This was achieved using flow cytometry of immune cells rather than a morphological approach using IHC, which they concluded may be difficult to discern on small biopsy specimens, particularly if relation to tumorous margin is required.[39] These studies, both in melanoma, add credence to the hypothesis that the number and location of lymphocytes could accurately predict for response, perhaps regardless of method of immune infiltrate analysis. Tumour infiltrating lymphocytes (TILs) may in fact be localised to the stroma, tumour or lymph nodes, and they require careful, standardised assessment. Other immune cells may also be present, and their significance and therefore reporting remain undefined.[40] Of course, H&E staining does not provide more nuanced information about functional status or T cell subsets. TILs require further validation but hold promise as a reliable biomarker of immunotherapy response.

Host factors

Gut microbiome

Several seminal papers examining the influence of gut microbiota on response to checkpoint inhibitor therapy were concurrently published in 2018. This work indicated that a greater diversity of faecal microbiota, as well as specific composition may be associated with response to ICI. Matson *et al*[41] demonstrated significant differences in the composition of commensal organisms in the faecal microbiota of patients with metastatic melanoma in responders versus non-responders. Specifically, they noted that an abundance of certain species was associated with a favourable clinical response. Gopalakrishnan *et al*[42] examined a population of patients with metastatic melanoma, finding that responders were characterised by increased commensal diversity. Notably, there are two bodies of work contrast in that the Matson *et al*[41] reported an abundance of *Bifidobacterium longum*, *Collinsella aerofaciens* and *Enterococcus faecium* in the microbiota of responders, and

Rumincoccus as abundant in non-responders, whereas Gopalakrishnan *et al*[42] reported *Ruminococcacae* species as associated with response. There are methodological differences that likely account for this, but this serves as a caution for unqualified overinterpretation of significance of certain species. Other groups have described differing predominant species in responders versus non-responders, but generally it seems that microbiome diversity is positively associated with a clinical response.[43 44] At this point, the use of this as a predictive biomarker is impractical in both sampling analysis and application to clinical practice; but the evidence base supporting microbiota's composition in modulating the immune response continues to grow, and at least in these authors' opinions it appears compelling.

Genetic polymorphisms

There is a growing body of research into various non-modifiable host factors. For example, HLA class I genotyping has been examined, suggesting improved survival in patient with maximal heterozygosity at HLA class I loci.[45] Other work has examined single nucleotide polymorphisms; for example, a more favourable response to CTLA-4 therapy was observed in patients with inflamed melanoma, with the $CD16A^{V158F}$ polymorphism in the gene encoding for the FcγR, which results in increased binding affinity of immunoglobulin G.[46] Ongoing investigation of the complex interplay of immune signalling may equip us to better understand its biological function, but it appears unlikely to be applicable for individual patient-centred predictive markers.

Research markers

Interleukin-6

There is an increasing appreciation of the significance of the cytokine milieu within the tumour microenvironment. Interleukin-6 (IL-6) plays a role in both T cell trafficking and priming, and it has been considered a barrier to effective tumour killing.[47] Preclinical studies suggest an augmented response to PD-1 inhibition in a murine model deficient in IL-6.[48] Using the clinical data set from Checkmate 064, Weber *et al*[49] described a prognostic value of IL-6, with patients with elevated levels at baseline having a shorter survival. They also described a correlation between baseline IL-6 level and response in patients who received ipilimumab and nivolumab, and additionally noted a correlation with C-reactive protein (CRP) in some but not all of their data set. The authors are careful to make the distinction that they demonstrated a prognostic relationship, but that the predictive capacity requires further delineation.[49]

T cell receptor clonality

The diversity of T cell receptor (TCR) populations may alter the response to ICI. Further to their work on TILs described earlier, Tumeh *et al*[38] found that a more restricted repertoire of TCR beta chain regions in the peritumorous lymphocytes of patients responding to PD-1 inhibitor therapy versus those who did not. Patients who did respond had a greater than 10-fold clonal expansion of their existing TCR sequences, which suggests a stronger tumour-antigen-specific T cell response. Interestingly, this did not correlate well with density, perhaps suggesting that patients without TILs may still have a strong, tumour-specific response. Postow *et al*[50] also examined TCR diversity in a pilot study of 12 patients with metastatic melanoma who were treated with CTLA-4 inhibitor therapy. Instead of peritumorous lymphocytes, however, they analysed peripheral blood TCR diversity. Their results were markedly different, finding that higher TCR evenness (indicative of clonality) correlated positively with PFS (though not overall survival).[50] Whether this difference reflects the different roles of lymphocytes in the periphery versus the tumour, or simply studies of small numbers and different methodologies is unclear. It seems biologically plausible that increased TCR clonality, particularly at the tumour bed, might generate a stronger immunological response, but larger confirmatory studies correlated with clinical outcomes will be required to answer this question.

Peripheral blood PD-1/PD-L1

The idea of peripheral assessment of PD-1 by various methods as a type of 'liquid biopsy' is very attractive, however published data is limited. Arrieta *et al*[51] examined the peripheral blood lymphocyte subsets in 70 treatment-naïve patients with NSCLC and found that higher PD-1, PD-L1 and PD-L2 expression on lymphocytes was negatively associated with prognosis. Gros *et al*[52] were able to demonstrate neoantigen-specific PD-1+ CD8 T cells in the peripheral blood of patients with melanoma. There is inadequate data to date to conclude that PD-L1 on peripheral lymphocytes is reliably associated with response to ICI. Given the interest and value provided by peripheral blood-based biomarker testing, the evidence base and utility of PDL1 will undoubtedly evolve quickly; but at this stage data remain limited and focus is still needed on developing the assays to provide consistency in interpretation.

Cell-free DNA

As discussed earlier, the difficulty in accessing tissue biopsy for some patients has compelled exploration of established biomarker testing using peripheral blood. Gandara *et al*[53] reported a positive correlation of a cell-free DNA (cfDNA) next-generation sequencing method compared with tissue TMB, using a novel assay. While there appeared to be some variations depending on the tissue TMB assays used, they were able to demonstrate a positive predictive value of cfDNA (compared with tissue TMB) of 93.5%.[53] Using data from the POPLAR and OAK studies they were able to demonstrate that blood TMB may have utility as a predictive marker in their validation cohort, using a cut-off of ≥16 mut/Mb. Wang *et al*[54] have demonstrated superior outcomes (ORR and PFS) using a targeted cancer gene panel with a TMB cut-off of 6

mut/Mb in a NSCLC cohort. In contrast, however, early results of the NEPTUNE trial did not indicate a predictive capacity, and this was using a higher cut-off of 20 mut/Mb. Whether this is due to the utility of the therapies themselves, the platforms or methodology used to calculate TMB is unclear.[55] These results are interesting and warrant further investigation, but methodology is varied and a lack of consensus on definitions limits immediate clinical applicability. Incorporating cfDNA collection into clinical trials may allow further validation of this, as well as ascertainment of optimal cut-offs as is already being done.[56]

Gene expression profiling

PTEN inactivation

Loss of function of the tumour suppressor gene *PTEN* can occur by several genomic mechanisms, and it results in increased activation of the PI3K-AKT pathway, which is involved in cellular proliferation and survival processes. *PTEN* inactivation results in increased VEGF and other immune suppressive cytokines, leading to recruitment of immune suppressing cells, particularly T regulatory cells, to the tumour microenvironment.[57 58] Miao *et al*[59] demonstrated the relevance of inactivating *PTEN* mutations in their study of whole exome sequencing of 249 solid organ tumours. They observed clustering of patients with inactivating *PTEN* mutations in patients with primary resistance to ICI, confirming prior preclinical observations.[58–60] It seems premature to consider targeted sequencing for *PTEN* alterations alone as a negative predictor of immunotherapy response, however in patients where sequencing has occurred either within research projects or via self-funded commercial testing, this finding might influence the recommendation for ICI therapy.

POLE mutations

Certain genomic mutations are uncommon but are highly significant for the small cohort of patients in whom these are present. Mutations within the DNA proofreading polymerase ε gene, *POLE*, most notably found in endometrial cancer, are one such example. Tumours with mutated *POLE* exhibit an ultramutated phenotype (232×10^{-6} mut/Mb) with a high neoantigen load. The number of mutations far exceed those found even in other hypermutated cancers, including microsatellite instable tumours (18×10^{-6} mut/Mb). There is accumulating evidence that they appear to have a more favourable prognosis.[61–63] Limited available data also suggest a favourable response to ICI.[64 65] They are rarer in other tumour types, but have been observed in NSCLC and colorectal cancer, with similar favourable outcomes.[11 66–68] While the biological rationale is compelling for the predictive value of *POLE* mutations, robust clinical data are lacking at present to conclusively affirm this.

KRAS/STK11 co-mutation

Another example of rare but significant genomic mutations are the specific subset of patients with lung adenocarcinoma whose tumour harbours both a *KRAS* mutation and an *STK11/LKB1* mutation, the latter being a tumour suppressor gene that modulates the mTOR pathway. Using a cohort of 174 patients with *KRAS* mutant lung adenocarcinoma, most treated with PD-1 inhibitor monotherapy, Skoulidis *et al*[69] described a poor ORR in patients with co-mutations of just 7.4%, compared with those co-mutated with *p53* or alone (35.7% and 28.6%, respectively), as well as superior survival. A study monitoring cfDNA on checkpoint inhibitor therapy also noted a poorer response in patients with STK11 mutations without specifically looking at the co-mutation, though these observations are not confirmed to be meaningfully predictive in isolation.[70 71]

Mutational signatures

Whole exome sequencing may reveal patterns of somatic mutations causing base changes, which generate a characteristic transcriptional signature. These signatures have been validated in large reference libraries and they are observed to occur in response to certain carcinogenic processes such as environmental exposures. In some cases, they may also provide information about likelihood of response to treatment.[72 73]

The search for unique gene signatures that are highly predictive for response to immunotherapy has been published by multiple researchers. Ock *et al*[32] described a unique immune signature, using 105 genes that were significantly associated with treatment response in a phase II immunotherapy trial, and demonstrated that this signature could differentiate responders versus non-responders to CTLA-4 inhibitor therapy in melanoma specimens. This also appeared to be correlated with PFS and overall survival.[32 74]

Interferon-gamma (IFN-ɣ) is an important mediator of the tumour microenvironment, as a determinant of PD-L1 expression and other immune suppressive molecules. It appears that IFN-related gene expression may be a robust marker of immunotherapy response. Ayers *et al*[75] identified and validated an IFN-ɣ gene expression profile across nine cancer types, which was necessary but not always sufficient for response to PD-1 inhibitor therapy. Clinical proof of this concept is demonstrated in the phase II POPLAR trial, where patients with an elevated T-cell effector–IFN-ɣ signature had superior overall survival (HR of 0.43 (CI 0·24 to 0·77)).[76]

CONCLUSIONS

Due to the rapid pace of discovery and wide breadth of medical literature, covering all putative biomarkers is not possible. A diverse selection of potential biomarkers are being studied which have not been covered here, from widely available tests such as CRP, to research markers such as B7-H4 IHC and to extremely novel factors such as CT-assessed macroangiopathy and morphomics.[49 77–79] It should be acknowledged that many of the suggested biomarkers are factors that correlate indirectly with levels

of inflammation and performance status, which have stood the test of time as robust markers both determining prognosis and predicting for response to any anticancer therapy, not just ICI.

While significant research effort continues into predictors of response, the majority of newer biomarkers are far from validation in prospective cohorts, let alone appropriate for use in clinical practice to determine whether to use ICI in an individual patient. Our understanding of the incredible complexity of the dynamic processes mediating the immune response to cancer remains limited, at least as far as application of simple assessments of individual biomarkers in clinical practice. That said, the translational interface between laboratory and clinical medicine is closer than ever before, due to the exponential pace of technological discovery carefully applied to more coordinated and strategic approaches. Inclusion of translational substudies within prospective clinical trials to validate suspected biomarkers will also add greatly to our current knowledge.

In the meantime, judicious selection of patients for ICI based on our current knowledge remains appropriate. Established biomarkers, such as PD-L1 status, TMB and MMR status, along with patient factors, and consideration to some of the exploratory biomarkers discussed here, should inform patient selection until we have more refined predictors to guide practice.

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