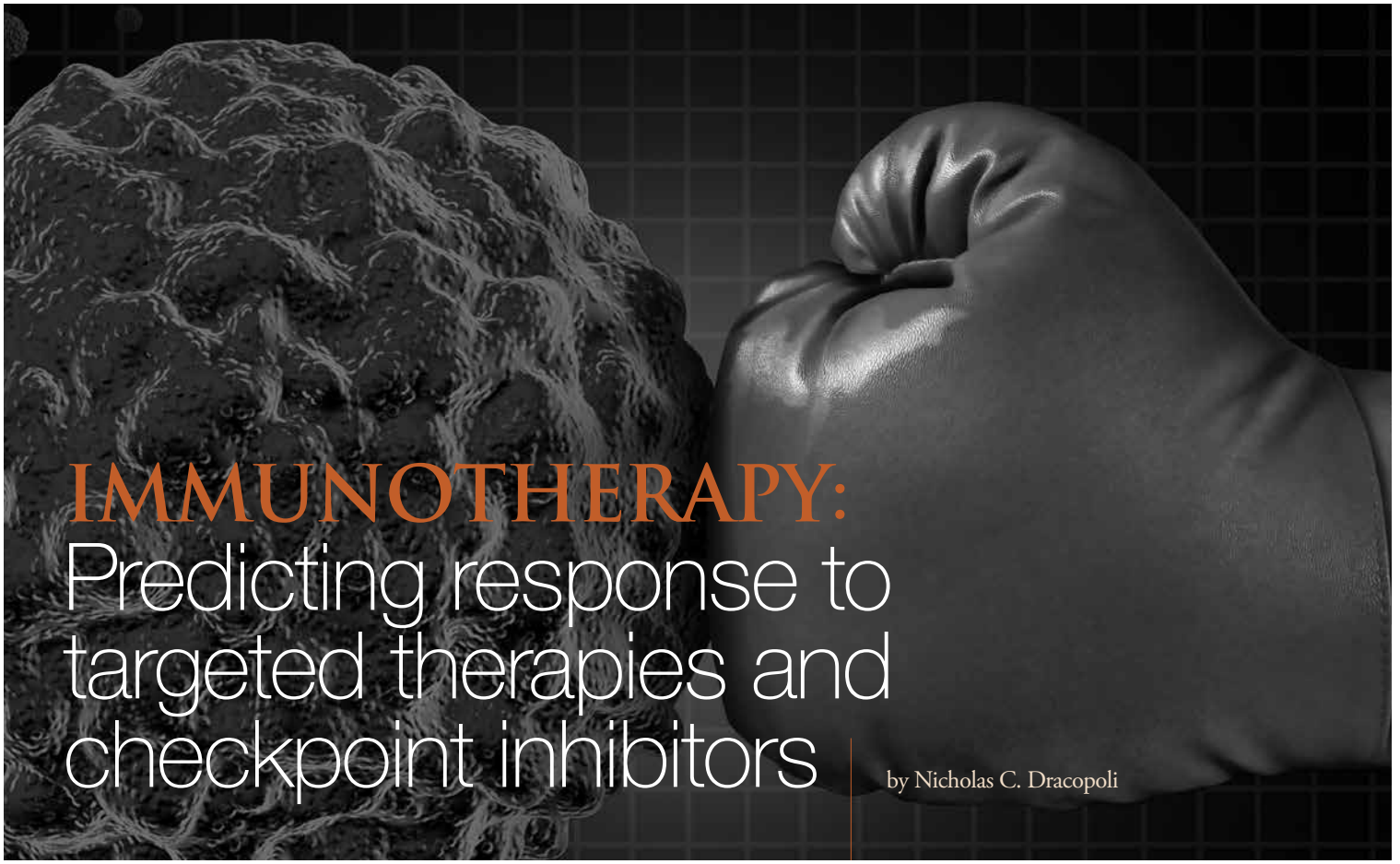


IMMUNOTHERAPY: Predicting response to targeted therapies and checkpoint inhibitors

by Nicholas C. Dracopoli

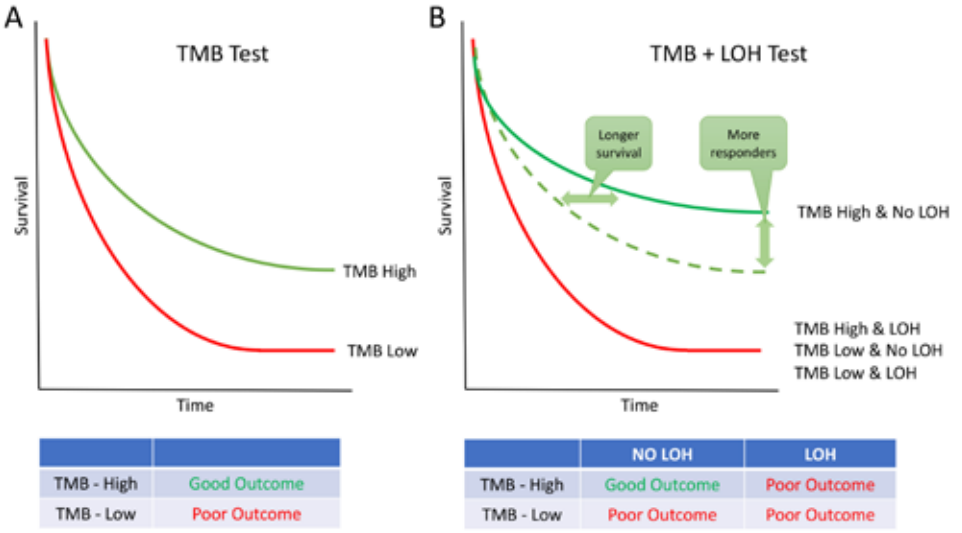
Predicting treatment response and long-term disease outcome for cancer therapies is difficult. Until recently most cancer patients were treated much the same way for each indication as their disease progressed. Patients were subjected to a common sequence of first, second and subsequent line of therapies according to the stage and grade of their disease without testing for genotypic or phenotypic biomarkers. The emergence of targeted therapies over the last two decades requires predicting which patients will benefit from a particular therapy using prespecified biomarkers. This strategy has been particularly successful for a single class of drugs – the Tyrosine Kinase inhibitors (TKi).[1,2]

Developing a CDx for a TKi is facilitated by the presence of driver mutations. These mutations effect the functional status of the tyrosine kinase receptor and are usually located in the ATP-binding pocket or transmembrane domain of the drug target. Hence, they are easy to find without the need for complex genomic association studies. Since the driver mutations are prespecified, they avoid the risk of overfitting data to obtain false positive associations. The effect size of the driver mutations is large, so only a small number of patients need to be enrolled in clinical trials to demonstrate statistical significance for the association of the mutation with response to therapy and eventual disease outcome. The driver mutations also lead to a simple clinical hypothesis (**Table 1**) that inhibiting signal transduction in the drug target receptor kinase will only delay or halt tumor proliferation in cells dependent on signaling through that particular pathway. Consequently, only patients carrying a driver mutation will derive any benefit from therapy as measured by overall response (OR), progression free survival (PFS) or

overall survival (OS) and patients that are not dependent on that particular signaling pathway will not be expected to obtain any benefit from targeted therapy.

Predicting response to immunotherapies is a different challenge. In this case, we are looking for patients with a prior, suppressed immune response (**Table 1**) instead of a driver mutation. Immunotherapies, such as the checkpoint inhibitors, work by releasing the suppression of an immune response through blocking the receptor-ligand interaction of the *PD-1* or *CTLA4* genes, thus mediating endogenous pathways normally engaged to limit uncontrolled exacerbation of the immune response. This results in the release of the suppressed immune response and destruction of the tumor by CD8+ cytotoxic T-cells. Consequently, we would expect only patients with a suppressed immune response to benefit from therapy with checkpoint inhibitors and that those without a suppressed immune response will require alternative options including targeted therapies,[1] adoptive cell therapy[3] or cancer vaccines.[4]

There are two types of biomarkers (**Table 2**) to predict a suppressed immune response in cancer patients. The first type does not actually measure any immune markers but predicts the likelihood of a suppressed immune response by estimating the tumor mutation burden (TMB) and the functional ability of the cell to present neoantigens by their HLA Class I complex. The second type measures different immune responses directly and estimates the likelihood of a suppressed immune response by evaluating the density of tumor infiltrating lymphocytes (TIL), the clonality of TILs by sequencing the T-cell receptor, the expression of the PD-L1 checkpoint inhibitor ligand, and levels of circulating inflammatory markers including cytokines and interferons.



TMB - High	Good Outcome
TMB - Low	Poor Outcome

	NO LOH	LOH
TMB - High	Good Outcome	Poor Outcome
TMB - Low	Poor Outcome	Poor Outcome

Figure 1: Comparison of potential survival curves high and low TMB (A) and the combination of the TMB test with a LOH sequence-based test for functional antigen presentation (B). Adding the antigen presentation test will split the high TMB group into good outcome (High TMB and No LOH) and poor outcome (High TMB and LOH). This will result in elevating the curve for the good outcome group that will increase the positive predictive value (PPV) of the test, while the poor outcome group (High TMB and LOH) will have a similar survival curve as the low TMB group.

Table 1: Diagnostic markers for targeted and immunotherapies

	Targeted Therapy	Checkpoint Inhibitor
CDx hypothesis	Differential response between tumors with and without a driver mutation	Differential response between tumors with and without a prior, suppressed immune response
Expected clinical benefit	Inhibiting activated receptor tyrosine kinase pathway will reduce tumor proliferation, delay disease progression and improve overall survival	Releasing the suppressed immune response will permit CD8+ T-cell driven destruction of tumor cells, delay disease progression and improve overall survival
CDx test	Presence of a driver mutation	Evidence for a suppressed immune response
Biomarkers	Mutations in the ATP-binding pocket or transmembrane domain of the drug target	Tumor mutation burden (TMB) HLA Class I antigen presentation CD8+ T-cell infiltration & clonality PD-L1 expression Cytokine and interferon levels

Tumor mutation burden

Today, the TMB test appears to be the most predictive test of response to checkpoint inhibitors.[5-8] This test measures the numbers of mutations per exome by whole exome sequencing (WES), or sequencing a representative portion of the exome. Consequently, this test does not directly measure the immune response, but the probability of an immune response to the observed mutational burden. In essence, this is a stochastic test that simply assumes that the chance of an immunogenic neoantigen required to enable CD8+ T-cell mediated cell destruction increase proportionally with the number of mutations in the tumor. Although overly simplistic, high TMB scores

have been associated with improved OR, PFS and OS in melanoma,[6] non-small cell lung cancer (NSLCC),[8] and several other indications[9] (**Figure 1A**). While TMB clearly shows promise as a useful predictive biomarker, much is still to be done to optimize its use. This includes developing better predictors of immunogenicity to add to raw mutation score as, for example, frame-shift mutations are about 3-fold more immunogenic than single nucleotide substitutions.[10] Developing appropriate cutoffs has also been difficult as the numbers of mutations varies greatly in different indications, suggesting the need for different TMB cutoffs for therapeutic intervention in different indications. The TMB also tends to have a continuous distribution, so a binary cutoff may not be the best way to optimize therapy and it may be necessary to use TMB quintiles or other distributions to optimize therapeutic response[9] as more data emerges.

Antigen Presentation

Loss of Heterozygosity at the HLA Class I Genes

A high TMB will not matter if none of the mutated peptides are presented to the T-cell receptor on the cytotoxic CD8+ T-cells (**Figure 1B**). Indeed, restricted antigen presentation is a well-known mechanism of resistance to the immune system, as cells not presenting neoantigens will be, in effect, invisible to CD8+ T-cell mediated destruction.[5,11-13] Cytosolic proteins are digested into 9-12 amino acid peptides and presented on the cell surface by the HLA Class I genes. Each antigen presenting complex consists of one of the three HLA class I genes (*HLA-A*, *HLA-B* and *HLA-C*) non-covalently bound to the β-2-microglobulin gene (*B2M*). Since these three HLA Class I genes are highly polymorphic, each cell will have a maximum of six antigen presenting complexes derived from the three ubiquitously expressed HLA Class I genes and the two haplotypes inherited from each parent. These six complexes represent the full antigen presentation repertoire. Loss of heterozygosity (LOH) of the region of chromosome 6p21 containing the HLA Class I genes is common in many solid tumors[14,15] and eliminates either the maternal or paternal repertoire. The LOH results from a chromosomal non-disjunction or loss of the entire short arm (6p) during the unstable mitosis of rapidly dividing aneuploid cancer cells. Since the antigen presentation is co-dominant, the loss of the HLA Class I haplotype carrying the immunogenic neoepitope will result from a single mitotic event and lead to antigen presentation restriction (**Figure 1B**). Similar results have also been seen at the *B2M* gene. This requires two hits, often a mutation of the remaining *B2M* gene and LOH of the normal allele. This double hit of *B2M*, although less common, has been shown to have similar

clinical outcome as a single hit on the HLA Class I genes.[16]

HLA Haplotypes

Emerging data suggests that the inherited haplotypes for the three HLA Class I genes have a significant impact on the risk of PFS and OS for patients being treated with checkpoint inhibitors.[5,11,12] Each of the three HLA Class I genes are highly polymorphic in their α1 and α2 domains that form the polymorphic cleft presenting the 9-12 peptide antigens. Consequently, the chance of any one mutant peptide being presented by the HLA Class I complex varies according to the inherited HLA haplotypes. Evaluation of NSCLC has shown 4-fold hazard ratio differences for different haplotypes in risk of disease progression on patients being treated with a PD-1 checkpoint inhibitor.[5] Given the large numbers of mutations observed in many tumors, it would seem likely that this risk would average out over many cancer neoantigens and that these high hazard ratios are unexpected. However, it is evident that only a very few of these tumor mutations become immunogenic. A recent study of multiple myeloma showed an average of 65 mutations per tumor, but estimated that only 9 of these were potential neoantigens based on their occurring in an expressed gene and on the highly divergent binding strengths of the wild-type and mutant peptides in one of the HLA Class I genes.[17] This suggests that HLA Class I haplotypes should be taken into account when predicting response to checkpoint inhibitors, although our ability to predict peptide binding on the rare HLA Class I haplotypes is still limited today.

T-cell infiltration and clonality

The immunoscore[18] was developed to supplement staging and grading of human tumors because patients with the same stage and grade of tumor often had completely different outcomes to therapy. The immunoscore measures the amount and distribution of CD8+ and CD3+ T-cells in formalin fixed, paraffin embedded tissue (FFPET). Typically, tumors with high infiltration of CD8+ cytotoxic T-cells had better prognosis than those without evidence of immune cell infiltration in the tumor. The immunoscore has largely been developed by retrospective analyses of colon cancer and is showing promise in prospective analyses of 3,500 colon carcinoma patients where the immunoscore was shown to be an independent marker of time to recurrence and to be independent of patient age, sex, tumor stage, microsatellite instability and other prognostic factors.[19] However, the immunoscore has still to be widely tested as a predictive biomarker in other solid tumors and has not yet developed sufficient sensitivity and specificity to justify widespread use as a clinical biomarker.

Every person has billions of T-cells, each of which is unique and can be unequivocally identified by sequencing the V-D-J segments of the T-cell receptor.[7,20,21] If a T-cell recognizes a foreign antigen (either a viral or mutated human peptide) presented by the MHC Class I complex, it will expand with each derivative cell having the same V-D-J sequence. The clonal expansion of a single, or small number, of T-cells is evidence of an immune response and can be seen in the tumor sample by a non-random distribution of T-cell receptor sequences. Consequently, the combination of high, clonal T-cell infiltration in the tumor is good evidence of an immune response to the tumor and is associated with a higher chance of responding to a checkpoint inhibitor.[7,20,21]

Table 2: Biomarkers of an immune response

	Assay	Evidence for an immune response
Tumor mutation burden	DNA sequencing for number of somatic mutations/Mb	Probability of a neoantigen being recognized by the immune system is proportional to the total number of somatic mutations
Antigen presentation	DNA sequencing of the HLA Class I and *B2M* genes	Screen for LOH of the HLA Class I genes and mutations and LOH of the *B2M* gene
T-cell infiltration	CD8 IHC test	High CD8+ T-cell infiltration is indicative of an immune response to the tumor
T-cell clonality	TCR sequence	Increased clonality of invading T-cells is related to immune expansion of T-cell clone recognizing a specific neoantigen
PD-L1 expression	IHC test for PD-L1 expression	Checkpoint ligand expression is unlikely to be high in the absence of an immune response
Interferon, cytokine levels	Circulating levels of inflammatory markers	High levels of inflammatory markers are symptomatic of an immune response

Checkpoint inhibitor ligand expression

High levels of the PD-1 ligand (PD-L1) are often reported in many advanced cancers.[22] The high levels of PD-L1 expression are presumed to be in response to immune recognition of the tumor, and the subsequent increase of tumor clones that blocked CD8+ mediated T-cell destruction by increasing expression of PD-1 ligands. While single agent treatment with PD-1 or PD-L1 inhibitor showed a correspondence of PD-L1 levels and response to therapy, a large study of pembrolizumab in

combination with platinum therapy showed benefits for all levels of PD-L1 expression from 1% to 50%.[23] On the whole, high levels of PD-L1 suggest the presence of a prior, suppressed immune response, and an improved outcome after treatment with a checkpoint inhibitor. However, the sensitivity and specificity of the IHC test for PD-L1 has proven problematic because of the use of multiple different antibodies and different cutoffs in many clinical studies.[22] Furthermore, the clinical utility of this test is challenged by the observation of strong clinical response in some patients with low or absent PD-L1 expression. So, today, PD-L1 testing is still used in some clinical studies but has not given rise to a well validated clinical biomarker.

Interferon and cytokine release

Circulating cytokine profiles have been developed for many solid tumors and hematologic malignancies and have been shown to be prognostic for many of these cancers.[24] Cytokines are abundant in the tumor microenvironment and along with other inflammatory mediators have an important role in immunosuppression, regulating tumor cell proliferation and angiogenesis. Increased levels of circulating cytokines have been reported in patients with breast, pancreatic, gastric, kidney, head and neck and prostate cancer, sarcoma as well as hematologic cancers including Hodgkin's lymphoma and acute myeloid leukemia.[24] Circulating cytokine levels tend to increase with disease stage and high levels of circulating cytokines, such as IL-6, are prognostic indicators of poor outcome in many solid tumor and hematologic cancers. These observations of high levels of circulating cytokines being associated with poor outcome are supported by genetic analyses of mutations in cytokine genes that are associated with disease outcome, as well as other side effects derived from the tumor including cachexia, anorexia, fatigue and pain. Consequently, circulating cytokines such as IL-6 are both drug targets (Siltuximab, for example, is used to treat IL-6 dependent multicentric Castleman's disease[25]) as well as prognostic markers for cancer progression and disease outcome.

Summary

The huge variability of clinical response is characteristic of immunotherapies. Some patients have a rapid response to treatment, delayed disease progression and prolonged survival measured in years. Some patients may have very slow response to treatment and their tumors may continue to expand due to pseudo-progression for a time, while others do not respond to treatment and do not gain any benefit from treatment with immunotherapies. This divergence makes the identification of predictive biomarkers so important as it is critical to know which

patients may benefit from existing therapeutic options, and which may need to enter clinical trials to test alternative targeted and immunotherapeutic options.

Understanding which patients have a prior, suppressed immune response is essential. Those patients whose tumors have a prior, suppressed immune response will be eligible for treatment with checkpoint inhibitors to release the immune blockade and enable CD8+ T-cell mediated destruction of their tumor. Those patients whose tumors have no evidence of a prior, suppressed immune response would presumably not benefit from checkpoint inhibitors and would require alternative approaches such as cancer vaccines[4] or adoptive T-cell therapy[3] to initiate a new immune response to the tumor.

A predictive biomarker to identify tumors with a prior, suppressed immune response is absolutely essential for the beneficial use of immunotherapies including checkpoint inhibitors. This test will need to have high predictive values, highly reproducible and cost effective to allow repeated monitoring of patients during the course of their disease. Ideally, the test would be able to use blood samples and not require invasive procedures to collect tissue biopsies, but it is likely that the test will be first implemented in FFPET samples, and subsequently evolve into a blood-based test as more sensitive testing methods are developed.

The TMB test is emerging as the best predictor of response to immunotherapies even though it is not a direct test of the presence of a prior, immune response. This test shows hazard ratio differences of 1.6 between the high and low TMB classes[5] and is already a useful biomarker for the selection of patients most likely to respond to checkpoint inhibitors (**Figure 1**). Emerging data supports the hypothesis that a fully intact HLA Class I antigen presentation complex in the tumor cell is required to present novel immunogenic neoepitopes generated by the high TMB patients. The combination of high mutation burden and intact antigen presentation complex test may prove to be the most accurate predictor of response to immunotherapies in the near future. ■

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