Clinical Chemistry 65:10
1228–1238 (2019)

Review

Biomarkers for Predicting Response to Immunotherapy with Immune Checkpoint Inhibitors in Cancer Patients

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BACKGROUND: Immunotherapy, especially the use of immune checkpoint inhibitors, has revolutionized the management of several different cancer types in recent years. However, for most types of cancer, only a minority of patients experience a durable response. Furthermore, administration of immunotherapy can result in serious adverse reactions. Thus, for the most efficient and effective use of immunotherapy, accurate predictive biomarkers that have undergone analytical and clinical validation are necessary.

CONTENT: Among the most widely investigated predictive biomarkers for immunotherapy are programmed death-ligand 1 (PD-L1), microsatellite instability/defective mismatch repair (MSI/dMMR), and tumor mutational burden (TMB). MSI/dMMR is approved for clinical use irrespective of the tumor type, whereas PD-L1 is approved only for use in certain cancer types (e.g., for predicting response to first-line pembrolizumab monotherapy in non-small cell lung cancer). Although not yet approved for clinical use, TMB has been shown to predict response to several different forms of immunotherapy and across multiple cancer types. Less widely investigated predictive biomarkers for immunotherapy include tumor-infiltrating $CD8^+$ lymphocytes and specific gene signatures. Despite being widely investigated, assays for MSI/dMMR, PD-L1, and TMB lack standardization and are still evolving. An urgent focus of future research should be the optimization and standardization of method for determining these biomarkers.

SUMMARY: Biomarkers for predicting response to immunotherapy are paving the way for personalized treatment for patients with diverse cancer types. However, standardization of the available biomarker assays is an urgent requirement.

Immunotherapy, which involves the administration of treatments to stimulate the immune system, has revolutionized the management of many different cancer types in recent years. In contrast to cytotoxic chemotherapy and driver gene targeted therapies, which are primarily directed against malignant cells, immunotherapy inhibits tumor cell growth indirectly, i.e., by stimulating the immune response to eliminate tumor cells. Several different forms of immunotherapy are currently available or are undergoing clinical trials *(1–3)* (Table 1). Of the therapies listed in Table 1, the most widely investigated and most widely used in the clinic involves treatment with immune checkpoint inhibitors (ICIs)[4] *(3, 4)*.

ICI treatment involves administration of monoclonal antibodies against negative regulators of T-cell function, i.e., against immune checkpoint regulators such as cytotoxic T-lymphocyte associated protein 4 (CTLA4), programmed cell death-1 (PD-1), or programmed death-ligand 1 (PD-L1) (Table 2). ICI has been approved for the management of several different cancer types, including melanoma (anti-PD1 and anti-CTLA4), non–small cell lung cancer (NSCLC) (anti-PD-1/PD-L1), renal cell carcinoma (anti-PD-1), Merkel cell carcinoma (anti-PD-L1), bladder cancer (anti-PD-L1), and head and neck squamous cell cancer (anti-PD-L1) *(5)*. In addition, specific anti-PD-l/PD-L1 antibodies can be used in patients with high microsatellite instability (MSI-H) tumors, irrespective of the tissue of origin.

Although ICI has revolutionized the management of specific cancer types, the majority of patients are intrinsically resistant and fail to respond. Overall, only approx-

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Received February 27, 2019; accepted May 23, 2019.
Previously published online at DOI: 10.1373/clinchem.2019.303644

[4] Nonstandard abbreviations: ICI, immune checkpoint inhibitor; CTLA4, cytotoxic T-lymphocyte associated protein 4; PD-1, programmed cell death-1; PD-L1, programmed death-ligand 1; NSCLC, non-small cell lung cancer; MSI-H, high microsatellite instability; HR, hazard ratio; FDA, Food and Drug Administration; EGFR, epidermal growth factor receptor; IHC, immunohistochemistry; MS, microsatellite; MSI, microsatellite instability; MMR, mismatch repair; dMMR, defective mismatch repair; CRC, colorectal cancer; NGS, next-generation DNA sequencing; TMB, tumor mutational burden; WES, whole exome sequencing; HER2, human epidermal growth factor receptor 2.

Table 1. Different forms of immunotherapy used to manage cancer.

Form of immunotherapy	Example(s)	Tumor type(s) where used
Cytokines	IL2, interferon-α	Melanoma, renal
Vaccines	Sipuleucel-T	Prostate
	BCG[a]	Noninvasive bladder
Antibodies	Rituximab	B-cell lymphoma
	Trastuzumab[b]	Breast, stomach
Adoptive cell transfer	Tisagenlecleucel	Acute lymphoblastic leukemia
ICIs	Ipilimumab, pembrolizumab, nivolumab	Melanoma, lung, kidney, urothelial, Merkel cell carcinoma, Hodgkin lymphoma

[a] BCG, Bacillus Calmette-Guerin.
[b] Acts in part via an immune mechanism, i.e., via antibody-dependent cell-mediated cytotoxicity.

imately 20% to 30% of patients treated show objective regression, although this varies depending on the tumor type. Furthermore, administration of immunotherapy can result in severe side effects, especially immune-mediated reactions against healthy organs *(6)*. In addition, immunotherapy is particularly expensive, with most therapies costing in excess of $100 000 annually *(7)*. Clearly, therefore, for the most effective, efficient, and cost-effective use of ICI, it is important to have validated biomarkers that upfront predict whether a patient is likely to respond to these therapies.

The aim of this article is, therefore, to discuss the most widely investigated predictive biomarkers for immunotherapy involving ICI. Although the topic of predictive biomarkers for immunotherapy has previously been discussed *(8–10)*, this is a highly active area of research with new developments being frequently reported. Furthermore, in contrast to previous reviews, a major focus in the current article is the measurement of the most widely investigated predictive biomarkers.

Predictive Biomarkers for Immune Checkpoint Inhibitors

PD-L1

PD-L1 (CD274, B7-H1) was 1 of the first and remains the most widely investigated biomarker for predicting response to ICI *(5, 8)*. PD-L1 is present on a wide variety of cell types, including cancer cells, dendritic cells, activated T and B lymphocytes, as well as macrophages. PD-L1 normally plays a role in protecting tissues from excessive inflammation and autoimmune reactions. However, the ability to produce PD-L1 can be coopted by some tumors, leading to their escape from immune

Table 2. ICIs in clinical use or undergoing clinical trials together with their targets.[a]

Inhibitor	Type of antibody	Target	Cancer(s) where indicated
Ipilimumab	IgG1	CTLA4	Melanoma
Tremelimumab	IgG2	CTLA4	Mesothelioma[b]
Pembrolizumab	IgG4	PD-1	NSCLC, HNSCC[c], Hodgkin lymphoma, MSI-defective, gastric, urothelial cancer
Nivolumab	IgG4	PD-1	Melanoma, kidney, NSCLC, HNSCC, HCC, Hodgkin lymphoma, urothelial cancer, MSI-defective tumors
Atezolizumab	IgG1	PD-L1	NSCLC, urothelial cancer,
Atezolizumab plus nab-paclitaxel	IgG1	PD-L1	Triple-negative breast cancer
Durvalumab	IgG1	PD-L1	NSCLC, urothelial cancer
Avelumab	IgG1	PD-L1	Merkel cell carcinoma, urothelial cancer

[a] Data reviewed from *(1–3)*.
[b] Orphan approval only.
[c] HNSCC, head and neck squamous cell cancer; HCC, hepatocellular cancer.

response. Escape from immune response occurs when PD-L1 produced by malignant cells binds to PD-1 on T cells. Binding of PD-L1 to T cells leads to attenuation or inhibition of their activity, which allows tumors to avoid immune surveillance, potentially resulting in malignant growth and progression. Therefore, blocking the PD-L1/PD-1 interaction with monoclonal antibodies might be expected to reactivate T cells, enabling the immune cells to exert their anticancer activity. Several antibodies, dubbed ICIs, have now been shown to block this interaction and inhibit tumor growth *(4, 5)* (Table 2).

Because PD-L1 plays a role in suppressing immunogenicity and is a direct or indirect target of PD-L/PD-L1 antibodies, it was investigated as a potential predictive biomarker for these therapies. Overall, patients with PD-L1-positive tumors derive more benefit from PD-l/PD-L1 antibodies than those with PD-L1-negative tumors *(4, 5)*. However, as a predictive biomarker for PD-l/PD-L1 antibodies, PD-L1 lacks diagnostic accuracy in differentiating between patients who are likely or unlikely to benefit. In particular, PD-L1 has a relatively low negative predictive value, as up to 20% of patients with apparently negative PD-L1 tumors have been reported to benefit from PD-1/PD-L1 antibodies *(8)*. Furthermore, the value of PD-L1 as a predictive biomarker appears to depend on the tumor type being treated, as well as the specific ICI administered *(4, 5, 9)*.

One of the most comprehensive studies to have addressed the value of PD-L1 measurement for predicting benefit from PD-1/PD-L1 involved a meta-analysis of 8 prospective randomized clinical trials that included 4174 patients with 5 different types of advanced or metastatic cancers *(11)*. Combined analysis of these trials showed that compared with conventional chemotherapy, administration of PD-1/PD-L1 inhibitors was associated with significantly increased overall survival in both patients who were PD-L1 positive [hazard ratio (HR), 0.66; 95% CI, 0.59–0.74] and PD-L1 negative (HR, 0.80; 95% CI, 0.71–0.90). However, the efficacies of the PD-1/PD-L1 antibodies were significantly superior in patients who were PD-L1 positive than in those who were PD-L1 negative ($P = 0.02$). In this meta-analysis, the cutoff point used for defining PD-L1 positivity was 1% tumor cell staining.

As a predictive biomarker for PD-L/PD-L1 antibodies, PD-L1 has perhaps been best validated in patients with NSCLC (adenocarcinoma and squamous cell carcinoma). Thus, in a phase I trial, Garon et al. *(12)* reported that patients with advanced NSCLC expressing high concentrations of PD-L1 (≥50% tumor cell staining) achieved an overall response rate of 45.2%. In a subsequent phase III trial comparing pembrolizumab with docetaxel in patients with PD-L1-positive advanced NSCLC (≥1% of tumor cells positive), the median survival with pembrolizumab was 10.4 months compared with 8.5 months for those receiving docetaxel *(13)*. However, in the subgroup of patients with increased concentrations of PD-L1 (≥50% of tumor cells positive), median survival in patients treated with pembrolizumab (2 mg/kg) was 14.9 months vis-à-vis 8.2 months in those receiving docetaxel (HR, 0.54; 95% CI, 0.38–0.77; $P = 0.0002$). Similarly, patients with untreated advanced NSCLC expressing increased levels of PD-L1 (≥50% of tumor cells positive) were found to have a significantly longer progression-free and overall survival when treated with pembrolizumab than in those receiving platinum-based chemotherapy *(14)*. More recently, pembrolizumab was shown to be superior to platinum-based chemotherapy in extending overall survival in patients with locally advanced or metastatic NSCLC with PD-L1 concentrations of ≥1% cell staining *(15)*.

Based on these findings, pembrolizumab monotherapy was initially approved by the US Food and Drug Administration (FDA) for the first-line treatment of patients with metastatic NSCLC whose tumors contain high concentrations of PD-L1 (≥50% of tumor cell staining) but lack epidermal growth factor receptor (EGFR) mutations or anaplastic lymphoma kinase translocations and who have received no previous systemic chemotherapy for metastatic NSCLC. Approval was based on tumors having high expression of PD-L1 as measured with the FDA-approved assay PD-L1 IHC 22C3 pharmDx (Dako). Subsequently, pembrolizumab received FDA approval for the first-line treatment of patients with stage III NSCLC who are not candidates for surgical resection or definitive chemoradiation. In this situation, PD-L1 staining must be present in ≥1% of cells and be determined by an FDA-approved test. In addition to receiving approval for the first-line treatment, pembrolizumab has also been approved for second-line treatment of NSCLC patients expressing PD-L1 (i.e., in ≥1% of tumor cells). Approval in this situation was also based on tumors expressing PD-L1, as determined with the PD-L1 IHC 22C3 pharmDx assay. In both these situations, PD-L1 is referred to as a companion biomarker, i.e., provides information that is essential for the safe and effective use of pembrolizumab.

As in NSCLC, high expression of tumor cell PD-L1 has also been associated with benefit from pembrolizumab in patients with head and neck squamous cell cancer *(16)* and with benefit from avelumab in patients with urothelial cancer *(17)*. Response to atezolizumab in patients with urothelial cancer and response to the same antibody plus chemotherapy in patients with triple-negative breast cancer, however, correlated with PD-L1 expression in infiltrating immune cells *(18, 19)*.

In other situations, the value of PD-L1 in predicting response to PD-1/PD-L1 antibodies is less clear. Thus, in contrast to the above situations, PD-L1 concentrations did not appear to be a reliable biomarker in predicting

Table 3. Assays for PD-L1, together with the situations in which they are approved by the US FDA for clinical use.

Test	Company	FDA approval	ICI therapy	Cancer[a]
PD-L1 IHC 22C3 pharma Dx	Dako/Agilent Technologies	Companion[b]	Pembrolizumab in patients with untreated and previously treated NSCLC patients	NSCLC
PD-L1 IHC 28–8 pharma Dx assay	Dako/Agilent Technologies	Complementary[c]	Nivolumab in second-line treatment of NSCL patients	NSCLC
PD-L1 IHC SP 142	Ventana	Complementary	Atezolizumab in patients with progressive NSCLC, also in patients with urothelial cancer	NSCLC, urothelial
PD-L1 IHC SP263	Ventana	Complementary	Durvalumab in patients with urothelial cancer	Urothelial

[a] Refers to advanced or metastatic cancers.
[b] A companion diagnostic provides information that is essential for the safe and effective use of a corresponding therapeutic product.
[c] A complementary diagnostic can be used to assist but not determine treatment decision-making.

benefit from nivolumab in patients with melanoma *(20)*, renal cell cancer *(21)*, or squamous-type lung cancer *(22–24)*. Similarly, PD-L1 concentrations were not found to be associated with benefit from combined treatment with nivolumab and ipilimumab in patients with melanoma *(25)* or from combined treatment with pembrolizumab plus chemotherapy in patients with NSCLC *(26)*. In summary, the presence of PD-L1 neither guarantees nor precludes response to anti-PD-1/PD-L1 antibodies. However, overall, its expression in tumors can enrich for a likely response to these antibodies *(9)*.

MEASUREMENT OF PD-L1

Although detection of PD-L1 by immunohistochemistry (IHC) is widely used for predicting response to anti-PD-1/PD-L1 therapy in patients with cancer, several problems exist with the measurement of this biomarker *(27–29)*. One of these is the absence of a standardized assay for all tumor types and for all approved anti-PD-1/PD-L1 antibodies. This lack of standardization is at least partly because of the previous practice of using different assays in the various clinical trials that have evaluated the different PD-1/PD-L1 antibodies.

Currently, at least 4 different commercial assays are available for measuring PD-L1 *(30)* (Table 3). Based on recent studies attempting to harmonize these assays, 3 assays, i.e., 22C3, 28–8, and SP263, gave comparable results in detecting of PD-L1 in tumor cells *(31–33)*. Thus, the currently available comparative data are promising, suggesting that in the future it may be possible to use specific tests interchangeably. Further research, however, is necessary to establish this in clinical practice, as well as to address preanalytical factors that may affect PD-L1 staining.

In addition to the lack of assay standardization, several other specific issues must be addressed for optimization of PD-L1 assays. These include:

- Establishing an optimum cutoff point for defining biomarker positivity.
- Establishing whether the optimum cutoff point is tumor-type or PD-1/PD-L1 antibody-type dependent.
- Testing the possibility of using PD-L1 concentrations as a continuum.
- Investigating the impact of tumor heterogeneity of PD-L1 expression on its predictive ability.
- Determining how best to report PD-L1 expression, i.e., should it be its presence in tumor cell, in stromal cell expression, or combined expression in both types of cells.
- Determining whether the detection of PD-L1 on metastatic tumors better predicts response than measurement at the corresponding primary site.
- Establishing whether PD-L1 concentrations vary over time and, if so, identify factors that contribute to the change (e.g., treatment, local production of cytokines, oncogene activation).

MICROSATELLITE INSTABILITY/DEFECTIVE MISMATCH REPAIR

Microsatellites (MSs) are short stretches of DNA (usually 1–6 nucleotides long) tandemly repeated throughout the genome. These sequences are located in both gene and intergene areas, being frequently present in introns, promoter regions, untranslated terminal regions, and coding exons *(34, 35)*. MS instability (MSI) occurs when the genome gains or loses ≥1 repeats. The DNA repair mechanism responsible for correcting these errors is known as the mismatch repair (MMR) system. The key

proteins involved in MMR are MLH1, MSH2, PMS2, MSH6, or epithelial cellular adhesion molecule *(34, 35)*. Germline or somatic mutations in any of these genes or hypermethylation in the promoter of the *MLH1*[5] gene result in defective MMR (dMMR) and, thus, an inability to repair errors that occur during DNA replication. As these errors tend to occur predominantly in MS regions, tumors with such errors are regarded as having MSI-H.

As a result of dMMR, both the number of mutations and predicted number of neoantigens are higher in tumors with this defect than in those with intact or proficient MMR. Thus, in 1 report, the mean number of mutations in dMMR colorectal cancers (CRCs) was 1782 compared with 73 mutations in tumors with intact MMR, while the predicted numbers of neoantigens were 578 and 21, respectively *(36)*. The increased number of neoantigens might be expected to render tumors more immunogenic and, thus, more likely to respond to immunotherapy.

In an early study to test this hypothesis, Le et al. *(36)* evaluated response to pembrolizumab in 41 patients with advanced metastatic carcinoma with or without dMMR. Objective response was found in 4 of 10 (40%) patients with dMMR CRC, in 7 of 9 (78%) patients with dMMR non-CRC, but in none of 18 patients with intact MMR. Subsequent studies across a broad range of different cancer types confirmed these findings *(37, 38)*. Thus, an overview study using combined data from MSI-H/dMMR tumors across 15 different cancer types enrolled in single-arm studies showed that pembrolizumab induced a complete or partial response in 40% of 149 patients *(38)*. Based on these findings, the FDA granted accelerated approval for the use of pembrolizumab in patients with pediatric or adult MSI-H/dMMR solid tumors, irrespective of tumor type. This use was for patients with unresectable or metastatic tumors that have progressed after previous treatments. The approval of MSI-H/dMMR for predicting response to ICI is the first example of the US FDA clearing a cancer test based on the presence of a biomarker, irrespective of the tumor location. Thus, MSI-H/dMMR can be regarded as a pan-cancer biomarker for predicting response to specific ICI.

In addition to predicting response to pembrolizumab, MSI-H/dMMR has also been associated with benefit from nivolumab and from dual ICI therapy with nivolumab and ipilimumab in patients with advanced CRC *(39, 40)*. Based on these findings, nivolumab and a nivolumab–ipilimumab combination were approved for the treatment of patients with MSI-H or dMMR metastatic CRC that had progressed after chemotherapy.

Although MSI-H can be present in most or all solid tumor types, its prevalence is variable across the different tumor types. To estimate the number of patients with MSI-H/dMMR tumors, Le et al. *(35)* determined the prevalence of these defects in approximately 12 000 tumors from patients with 32 different cancer types. Overall, the prevalence of the defect was found in only about 5% of the patients investigated. Tumors with the higher prevalence (>10%) included CRC and endometrial cancers. On the other hand, glioblastomas, esophageal cancer, breast cancer, and NSCLC had low levels (<2%) *(35)*. This low prevalence of MSI in human tumors limits its use as a broad-based predictive biomarker for immunotherapy.

MEASUREMENT OF MSI/dMMR

Three different methods are available for determining MSI-H/dMMR status. Two of these are currently in clinical use, i.e., PCR for detecting MSI-H and IHC for detecting dMMR *(34, 35)*. These tests are currently widely used in screening for Lynch syndrome and assessing prognosis in patients with CRC. The third method, which involves next-generation DNA sequencing (NGS), is still experimental.

The PCR method involves amplification of a panel of MS sequences. Over the years, several different biomarker panels have been proposed for determining MSI status *(35, 41)*. In 1997, an international consensus group proposed the use of 5 MS biomarkers referred to as the Bethesda or National Cancer Institute panel *(42)* for testing for MSI. Two of these were mononucleotides (BAT 25 and BAT 26) and 3 were dinucleotides (D2S123, D5S346, and D17S250). It was proposed that these biomarkers be measured in both tumor and corresponding normal tissue. If ≥2 of these 5 biomarkers were found to be unstable, the tumor was referred to as having MSI-H. On the other hand, if 1 marker was unstable, the tumor was regarded as having low MSI, whereas tumors lacking alterations in any of these 5 markers were regarded as being MS stable.

A follow-up workshop was held in 2002 to review the above proposal for the detection of MSI status *(43)*. At this second workshop, the participants noted caveats in the use of biomarker panels that included dinucleotide repeats. Consequently, 3 new suggestions regarding the measurement of MSI-H were published. These were:

- If only dinucleotide repeats are mutated, it was suggested to test a secondary panel of MS biomarkers with mononucleotide repeats (e.g., BAT40 and/or MYCL) to exclude low MSI.
- It was also stated that dinucleotide repeats were less sensitive than mononucleotide repeats for determining MSI-H. Dinucleotides, however, were regarded to be useful, as they provided an internal control for minimizing of sample mix-up.

[5] Human gene: *MLH1*, mutL homolog 1.

- It was stated that a pentaplex panel of 5 quasimonomorphic mononucleotide repeats was more sensitive for detecting MSI-H tumors than other microsatellite biomarkers, that the use on mononucleotide repeats may obviate the need for normal tissue for comparison, and that the use of mononucleotides required ≥3 mutant alleles to indicate MSI-H.

The recommendation to use a 5 or pentaplex panel of mononucleotides was largely based on the report by Suraweera et al. *(44)*, who showed that the measurement of 5 mononucleotide biomarkers, i.e., BAT26, BAT25, NR21, NR22, and NR24, detected the MSI status of 124 colon and 50 gastric tumors with 100% diagnostic sensitivity and specificity without the need for matching normal cell DNA. In a subsequent large population-based prospective study, this biomarker panel was shown to be diagnostically more sensitive than the original Bethesda panel *(41)* for detecting MMR-H CRC (95.8% vs 76.5%) *(45)*. A related 5-mononucleotide biomarker panel, i.e., NR-21, BAT-25, MONO-27, NR-24, and BAT-26, is currently available as a commercial kit (MSI Analysis System, version 1.2, Promega) for detecting MSI status.

The second established method for determining dMMR involves IHC detection of the MMR proteins hMLH1, hMSH2, hMSH6, and hPMS2. Loss of expression of ≥1 of these proteins in malignant cells in the presence of staining in internal positive control cells, such as in stromal, inflammatory, or nonneoplastic epithelial cells, usually correlates with dMMR and, thus, with MSI-H. Compared with PCR, IHC is simpler, cheaper, can be automated, and is more widely available. In different studies, the reported analytical sensitivity of IHC for detecting MSI varied from 92% to 94% *(41)*. A downside of ICH, however, is that 5% to 10% of MSI-positive tumors do not exhibit loss of MMR proteins, as missense mutation in the MMR genes can result in functional inactivation of protein without affecting its antigenicity and expression levels. Furthermore, reduced staining of MSH6 has been found in rectal cancers following neoadjuvant treatment with chemotherapy and radiation *(46)*. In general, however, good agreement has been found between the molecular determination of MSI status by PCR and measurement of MMR proteins by IHC, especially in CRC *(34, 35)*. Indeed, CRC and, to a lesser extent, endometrial cancer are the tumor types in which PCR and IHC have been best validated for determining MSI-H/dMMR.

The most recent method proposed for determining MSI-H/dMMR involves NGS of specific gene panels or the whole exome sequencing. Although expensive and not widely available, NGS is potentially more analytically specific and sensitive than PCR or IHC for determining MSI status *(47, 48)*. Overall, however, good correlation has been reported between NGS and IHC or MSI testing in determining MSI status in patients with CRC *(47, 48)*. As NGS is also being used to detect tumor mutational burden (TMB), it is discussed further below.

Although the US FDA has granted approval for the use of specific ICI in advanced cancers that are MSI-H or dMMR, it did not specify which assay should be used to measure these biomarkers. Indeed, the best assay for determining MSI-H/dMMR is currently unknown. Therefore, the College of American Pathologists is currently preparing guidelines that are aimed at addressing the optimum assay for these measurements.

TMB

As mentioned above, a high TMB is likely to increase the capacity of a tumor to generate neoantigens. The increased productions of neoantigens would be expected to render a tumor more immunogenic, thus increasing its likelihood of responding to immunotherapy. Consistent with this hypothesis, a high TMB has been shown to predict response to ICI across a diverse range of cancer types, including NSCLC, melanoma, and bladder cancers [for review, see *(49, 50)*]. Furthermore, a high TMB was shown to be associated with an enhanced benefit from a diverse form of ICI including anti-PD-1, anti-PD-L1, and anti-CTLA4 antibodies, as well as combined anti-PD1–anti-CTLA4 therapy, compared with patients with low TMB *(49–52)*.

Although the overall TMB is predictive of response to multiple forms of ICI therapy, not all types of mutations are equally predictive. Two factors appear to be critical in determining the type of mutation and, thus, the type of neoantigen that predicts response to ICI. First, the mutated peptide should have a greater binding affinity for class I MHC proteins than its wild-type counterpart. Second, T-cell receptors must then identify the neoantigen as foreign and initiate an immune response *(53)*. Thus, neoantigen binding to MHC1 and T-cell receptor recognition of neoantigen–MHC1 complexes is the main determinant of immune response.

Among the most cancer-associated immunogenic neoantigens identified to date are peptides with homology to bacterial or viral antigens *(54, 55)*. This finding may explain the higher than expected response (based on TMB) rate to anti-PD-L1 therapy found in patients with Merkel cell carcinoma, a virally associated cancer *(49)*. The enhanced immunogenicity of neoantigens related to viral or bacterial antigens may be explained by their foreignness or lack of similarity to self-antigens. Consequently, neoantigens have an increased ability to generate T-cell activation and, thus, response to immunotherapy.

The type of mutations also appears to determine response. Thus, tumors rich in frameshift indels (insertion or deletion of bases) were reported to be more im-

munogenic than those harboring nonsynonymous mutations *(56)*. The likely reason for this enhanced immunogenicity is that frameshift indels alter the reading frame of proteins and, thus, generate a greater number of neoantigens that bind with high affinity to HLA molecules *(56)*.

As well as frameshift indels and homology to microbial antigens, the clonality of tumor mutations appears to determine response to ICI. Thus, in cases of melanoma and NSCLC, patients with clonal mutations were shown to derive more benefit from PD-1 or CTLA-4 blockade than those with branching or subclonal mutations *(57)*. Finally, patients with NSCLC containing high levels of transversions were found to exhibit higher response rates to pembrolizumab than those with transitions *(58)*.

As with PD-L1 concentrations and MSI/dMMR, high TMB alone does not guarantee response to ICI. There are several examples of patients with low TMB who responded to ICI, as well as cases of patients with high TMB who failed to respond *(59)*. Thus, current research is focusing on different types of mutation and the neoantigen originating from these different types of mutation for predicting response to ICI.

MEASUREMENT OF TMB

As mentioned above, NGS for determining TMB involves mutation testing of panels of specific genes or the use of whole exome sequencing (WES). For clinical use, sequencing of genes panels (300–400 cancer-associated genes) has several practical advantages over WES, including relatively faster turnaround time, lower costs, potentially greater sensitivity than either PCR or IHC, and requiring smaller amounts of tissue. Several commercial gene panels are available for determining TMB *(49, 60)*. However, these panels differ in their methodology, number and type of genes tested, bioinformatic approaches to data analysis, and how results are reported *(60)*. Two gene panels, Foundation One CDx and MSK-IMPACT, are approved/authorized by the US FDA for general clinical gene analysis (e.g., testing for actionable mutations). Although these tests can determine TMB, they are not approved for this specific use.

In contrast to gene panel testing, WES provides a comprehensive profile of alterations in protein-coding genes. However, WES has limitations for routine practice because of its high costs, slow turnaround time for results, data storage requirements, and interpretation of results. Despite the large differences in the number of genes sequenced, good agreement has been found between WES and specific gene panels (Foundation One, Foundation One CDx, and MSK-IMPACT) in determining TMB *(61, 62)*.

Although TMB is a promising predictive biomarker for response to ICI, it has several problems. As with PD-L1, these include lack of assay standardization and lack of a validated optimum cutoff point. Indeed, the optimum cutoff point may vary with tumor type *(50)*. To address these problems, a working group is currently attempting to standardize assays for determining TMB. Furthermore, compared with PD-L1 and MSI-H/dMMR, measurement of TMB is expensive, is not routinely available, and takes considerably longer to perform. However, in contrast to PD-L1 and MSI-H/dMMR measurements, TMB can potentially be determined in blood, which may be an advantage when tumor tissue is not available or cannot be obtained *(63)*. A further advantage of TMB over PD-L1 is that the former appears to be capable of predicting response to multiple forms of immunotherapy, including PD-1/PD-L1 inhibitors, anti-CTLA4 antibodies such as ipilimumab, and adoptive T-cell transfer therapy *(49–52, 64)*. PD-LI, on the other hand, is apparently only predictive of benefit from PD-1/PD-L1 antibodies.

OTHER BIOMARKERS FOR OPTIMIZING THE USE OF IMMUNOTHERAPY

In addition to PD-L1, MSI, and TMB, several other biomarkers have been reported for predicting response to ICI-based immunotherapy (Table 4). Of these emerging biomarkers, 1 of the best validated is a multigene gene test known as the immunopredictive score *(65)*. This test has been validated in 10 different databases for predicting response to diverse forms of ICI in melanoma. Overall, immunopredictive score was reported to capture almost all true responders while misclassifying fewer than half of the nonresponders.

As well as the positive predictive biomarkers discussed, several biomarkers associated with resistance to ICI have been described (Table 4). Although none of these have been sufficiently validated for clinical use, they are potentially useful in complementing positive predictive biomarkers in guiding decision-making with respect to treatment with ICI.

As mentioned in the Introduction above, some patients treated with immunotherapy develop severe immune-related toxicity. It is important to be able to anticipate the development of such toxicity. An emerging biomarker test for predicting toxicity to ICI is the CYTOX score, which measures the concentration of 11 circulating cytokines (G-CSF, GM-CSF, fractalkine, FGF-2, IFNα2, IL12p70, IL1a, IL1B, IL1RA, IL2, and IL13) *(66)*. This test was found to be predictive of severe immune-related toxicity in patients with melanoma treated with combined anti-CTLA-4 and anti-PD-1 therapy *(66)*. In the discovery phase of this study, the area under the curve for the CYTOX score to discriminate severe toxicity was 0.78 at baseline and 0.77 during therapy ($P = 0.0009$). Similar predictive values were observed in the valida-

Table 4. Emerging biomarkers for predicting response, resistance, toxicity, and hyperprogression associated with administration of checkpoint inhibitors.

Biomarker	End point	Cancer	Reference number
CD8+ T cells[a]	Response	Melanoma	*(69)*
Specific gene signatures	Response	Melanoma, NSCLC	*(65, 70)*[b]
Interferon-γ	Response	Melanoma	*(71)*
PD-L1 amplification	Response	Multiple	*(72)*
Gut microbiome	Response or resistance[c]	Melanoma	*(73)*
IDO1[d]	Resistance	NSCLC, melanoma	*(74)*
JAK mutations	Resistance	Multiple	*(75)*
Cytox score[e]	Toxicity	Melanoma	*(66)*
MDM2/MDM4, EGFR mutations	Hyperprogression	Multiple	*(67)*

[a] Located at invasive tumor margin.
[b] Reference 70 relates to an IFN-γ-related mRNA profile, whereas reference 65 relates to immunopredictive score (IMPRES).
[c] Increased abundance of bacteria of the *Ruminococcaceae* family was associated with response, whereas a high relative abundance of the Bacteroidales order correlated with resistance.
[d] IDO1, indoleamine 2, 3-dioxygenase.
[e] Measures the concentration of 11 circulating cytokines (G-CSF, GM-CSF, fractalkine, FGF-2, IFNα2, IL12p70, IL1a, IL1B, IL1RA, IL2, and IL13).

tion set, i.e., an area under the curve of 0.68 at presentation and 0.70 during therapy ($P = 0$ 0.017).

Finally, 1 of the features of immunotherapy is that it may accelerate tumor progression in a subset of patients. Potential biomarkers for identifying patients at risk of hyperprogression are MDM2 amplification, MDM4 amplification, and mutations in EGFR *(67)*.

Conclusion

Although considerable progress has been made in the identification and validation of predictive biomarkers for ICI, several challenges remain. Only 2 biomarkers are currently approved for clinical use: PD-L1, in specific tumor types, and MSI-H/dMMR, for all types of solid

Table 5. Some advantages and disadvantages of the most widely investigated biomarkers for predicting response to ICI.

Assay	Advantages	Disadvantages
PD-L1	Easy and cheap to assay, widely available, can be automated	Multiple assays exist, different assays used in different settings, lack of assay standardization, optimum cutoff point is unknown and may vary depending on type of therapy and tumor type being treated, relative importance of tumor cell vs stromal staining unclear and may vary depending on tumor type, accuracy for predicting response to ICI appears to depend on tumor type
MSI-H/dMMR	Can be used in all solid tumor types. Two types of assay already in clinical use (PCR for determining MSI status and IHC for determining dMMR)	Overall, MSI-H/dMMR is relatively rare in tumors (≤5%). It is especially rare in cancers such as melanoma, breast, and NSCLCs. Best method for determining MSI status is unclear
TMB	Applicable to most solid tumors and multiple ICIs, potentially can be measured in blood, allows the simultaneous detection of other potential predictive biomarkers (e.g., KRAS for predicting lack of benefit from anti-EGFR antibodies in CRC)	Expensive and time-consuming (especially WES), slow turnaround time for results, optimum cutoff point not established and may vary depending on tumor type, optimum panel of genes to be tested is unknown, requires high quality DNA, which may not always be possible

tumors. Compared with established predictive biomarkers for targeted therapy, such as estrogen receptors for endocrine therapy and human epidermal growth factor receptor 2 (HER2) for anti-HER2 therapy, PD-L1 especially has limited positive and negative predictive values. Of course, in contrast to PD-L1, both estrogen receptors and HER2 are long-established biomarkers with extensively validated and standardized assays that are supported by evidence-based guidelines. It is hoped the path followed for validation and standardization of these assays can be a model for similar studies on PD-L1, as well as for other emerging immunotherapy predictive biomarkers. A further limitation of PD-L1 is that its predictive impact appears to depend on the specific PD-1/PD-L1 antibody administered and/or tumor type being treated. A major limitation of MSI-H/dMMR is the low prevalence of this biomarker in metastatic cancers (<5%). Other advantages and disadvantages of these different assays are summarized in Table 5.

One of the most promising of the emerging predictive biomarkers for ICI is TMB. Although TMB is likely to be a predictive biomarker for multiple ICIs across different cancer types, its determination for clinical use is expensive, technically demanding, and not widely available in clinical pathology laboratories. Furthermore, additional research is necessary to identify the type(s) of mutations generating the most potent neoantigen for recognition by antitumor T cells. In this context, it should be mentioned that Danilova et al. *(68)* recently devised an assay for detecting functional mutation-associated neoantigen-specific T cells. Future developments with this assay are eagerly awaited.

Going forward, it is likely that predicting response to ICI will involve a combination of different biomarkers that are present not only in tumor cells but also in tumor-infiltrating immune cells. Although determination of multiple biomarkers will increase laboratory costs, it should ultimately be an overall cost saving, as it will reduce the unnecessary administration of expensive ICI to patients who are unlikely to benefit. Finally, although biomarkers are currently available for predictive response to ICI-related immunotherapy, such biomarkers are currently lacking for other forms of immunotherapy, like adoptive T-cell transfer. Clearly, the identification of validation of additional predictive biomarkers for immunotherapy will be an active area of research for several years to come.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: J. Crown, Pfizer, Seattle Genetics, Puma Biotechnology, Eisai, Astrazeneca.
Stock Ownership: J. Crown, OncoMark.
Honoraria: J. Crown, Pfizer, Boehringer Ingelheim, Eisai, Genomic Health, Roche, MSD.
Research Funding: The BREAST-PREDICT (CCRC13GAL) program of the Irish Cancer Society and the Clinical Cancer Research Trust.
Expert Testimony: None declared.
Patents: None declared.
Other Remuneration: J. Crown, Roche, Pfizer, MSD, Astrazeneca, AbbVie.

Acknowledgments: The authors thank the BREAST-PREDICT (CCRC13GAL) program of the Irish Cancer Society and the Clinical Cancer Research Trust for funding this work.

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