Current landscape and future directions of biomarkers for predicting responses to immune checkpoint inhibitors

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**Abstract:** Immune checkpoint inhibitors (ICIs), represented by anti-CTLA-4 or anti-PD-1/anti-PD-L1 pathway antibodies, have led to a revolution in cancer treatment modalities. ICIs have unique clinical benefits, such as effectiveness against a broad range of tumor types, strong overall impact on survival, and persistent responses after the cessation of therapy. However, only a subset of patients responds to these therapies, and a small proportion of patients even experience rapid progression or an increased risk of death. Therefore, it is imperative to optimize patient selection for treatment. This review focuses on the mechanisms of tumor escape from immune surveillance, the composition and activity of a preexisting immune infiltrate, the degree of tumor foreignness (as reflected by the mutational burden, expression of viral genes, and driver gene mutations), and host factors (including peripheral blood biomarkers, genetic polymorphisms, and gut microbiome) to summarize current evidence on the biomarkers of responses to ICIs and explore the future prospects in this field.

**Keywords:** immune checkpoint inhibitor, programmed death-1, programmed death ligand-1, cytotoxic T-lymphocyte-associated antigen-4, biomarker, efficacy

**Plain language summary**

The significant differences in patients’ responses to immune checkpoint inhibitors (ICIs) have generated intense interest in identifying biomarkers to guide patient selection.

We summarize current potential biomarkers for the prediction of ICI efficacy, focusing on four levels (the mechanisms of tumor immune escape, the composition and activity of the immune system in the tumor, the foreignness of the tumor, and host factors).

Multivariate analyses must consider a variety of variables, including the aforementioned four aspects to identify the combinations of factors that predict patients’ response to ICIs.

**Background**

Cancer immunotherapy has undergone revolutionary progress in recent years, mainly due to the breakthrough regarding the extraordinary clinical outcomes associated with immune checkpoint inhibitors (ICIs) targeting the cytotoxic T-lymphocyte-associated antigen (CTLA-4) and programmed death-1 (PD-1)/programmed death ligand-1 (PD-L1) pathway. Although the heterogeneity of somatic mutations in tumors raises challenges for the methods that target a single mutation, it also raises the possibility of using the large number of neoantigens to induce immune responses to kill tumor cells. However, the recognition and cytotoxicity functions of the innate and adaptive...
immune systems are inhibited by immune checkpoint pathways. Based on this theory, many ICIs, such as CTLA-4 and PD-1/PD-L1 pathway inhibitors, have emerged. CTLA-4 inhibitors mainly affect the early stage of the immune responses, during T-cell priming and activation, blocking the contact inhibition functions of regulatory T cells (Treg) on effector T cells (Teff) and thus enhancing Teff functions.\(^1\),\(^2\) PD-1/PD-L1 inhibitors mainly exert their effects primarily on immune responses within the tumor microenvironment (TME); they can reverse the status of Teff cell anergy and depletion to restore tumor cell killing functions and induce effective anti-tumor immune responses.\(^3\),\(^4\)

**Materials and methods**

To summarize the recent research on the biomarkers of ICIs, we searched the PubMed database, using the following search terms “((((checkpoint) OR PD-1) OR PD-L1) OR CTLA-4) AND ((inhibitor) OR blockade)) OR (((anti-PD-1) OR anti-PD-L1) OR anti-CTLA-4) AND (((biomarker) OR predictive) OR prediction) AND response”. PubMed was last searched in May 2018. A flow diagram of this review is presented in Figure 1. Eligible trials in [https://clinicaltrials.gov](https://clinicaltrials.gov/) were also included in the survey. Additionally, reports from annual meetings of the American Society of Clinical Oncology and the European Society for Medical Oncology were searched through these organizations’ official websites at [http://meetinglibrary.asco.org/](http://meetinglibrary.asco.org/) and [http://www.europeancancercongress.org](http://www.europeancancercongress.org).

**Biomarkers to predict responses to ICIs**

The advance of ICIs has revolutionized the approach of cancer treatment. The unique advantages of ICI therapy, such as crossing different histological types of tumors, significant elongation of the survival period, and persistent effectiveness after drug withdrawal, have generated widespread...
enthusiasm among patients, clinicians, and scientists. However, the heterogeneity of responses to ICIs has also generated new challenges. To date, anti-CTLA-4 therapy has shown reproducible activity only in patients with malignant melanoma (MM). In contrast, PD-1/PD-L1 inhibitors have a broad range of activity extending beyond MM to an expanding list of cancers, including non-small-cell lung cancer (NSCLC), renal cell cancer (RCC), head and neck squamous cancer (HNSCC), bladder cancer, and Hodgkin’s lymphoma. However, certain types of cancer, such as prostate cancer and pancreatic cancer, have proven to be much more resistant to PD-1/PD-L1 inhibitors. Champiat et al reported that a small group of patients (~10%) showed rapid progression after treatment with anti-PD-1/ PD-L1 drugs. The US Food and Drug Administration (FDA) recently issued a statement requiring the cessation of trials of pembrolizumab in combination with dexamethasone and an immunomodulatory agent (lenalidomide or pomalidomide) for the treatment of patients with multiple myeloma due to the increased risk of death to patients in two recently halted clinical trials. The above-mentioned facts underscore the need for biomarker development. Given the dynamic nature of the immune system and the complexity of immune responses, the identification of the biomarkers of ICIs is more challenging than the identification of the biomarkers of targeted therapy. Based on research performed to date, four prerequisites, namely, tumor antigen release, tumor antigen presentation, attenuated immune suppression, and tumor antigen-specific T-cell activation, need to be satisfied to achieve the optimal adaptive response. As such, we elucidate the current landscape and future directions of work on biomarkers for the prediction of ICI efficacy, focusing on the mechanisms of tumor immune escape, the composition and activity of the immune system in the tumor, the foreignness of the tumor, and host factors.

Mechanisms of tumor immune escape
To date, the detection of PD-L1 expression by immunohistochemistry (IHC) has been the most widely used clinical approach to predicting the efficacy of PD-1/PD-L1 inhibitors. The FDA has approved the use of a relevant antibody (22c3) to quantify PD-L1 expression in tumor cells by IHC in NSCLC. An expression level ≥50% is required for using pembrolizumab in the first-line setting. Regarding the target of PD-1/PD-L1 inhibitors, patients with high PD-L1 expression are expected to be more responsive to these inhibitors. Many studies have shown that both the objective response rate (ORR) and the overall survival (OS) of PD-L1-positive patients after ICI therapy were higher than those of PD-L1-negative patients. Recently, atezolizumab was shown to result in a significant improvement in OS compared with docetaxel in stage IIIB or IV NSCLC (OAK trial), and patients with high levels of PD-L1 (≥50% on tumor cells or ≥10% on tumor-infiltrating lymphocytes [TILs]) derived the greatest benefit from atezolizumab. In particular, the comparison between the Keynote 024 and Checkmate 026 clinical trials further suggested the significance of high PD-L1 expression in predicting the efficacy of the first-line treatment of metastatic NSCLC.

However, there are many challenges related to using PD-L1 expression as a prediction biomarker. First, no definitive conclusion has been drawn regarding the association between PD-L1-positive tumors and ICI efficacy, and some contradictory results have even been obtained in some cancers, such as RCC, MM, and urothelium carcinoma. Chae et al performed a combined analysis of studies on ICI therapy biomarkers in NSCLC and concluded that there was still no consensus on the use of PD-L1 expression as an ideal marker for patient selection. Additionally, PD-L1-negative patients can still benefit from anti-PD-1/PD-L1 therapy. Taking the findings of the studies performed to date into consideration, it was shown that using only PD-L1 expression levels for the prediction of ICI efficacy is insufficient. Moreover, because of differences in the biological characteristics of tumors at different locations and the different types of antibodies used in IHC, it is more difficult to develop uniform IHC criteria for PD-L1 evaluation. Owing to the limitation presented by the semi-quantitative nature of IHC, some researchers used the Her-2 detection method in breast cancer to propose combining IHC and gene amplification to achieve qualitative and quantitative unification. In this regard, Inoue et al retrospectively analyzed 654 postoperative NSCLC patients and showed that the gene amplification number of PD-L1 could be used as a supplemental or alternative biomarker of PD-L1 expression. Additionally, PD-L1 expression in tumor cells and immune cells is a dynamic process. Thus, the detection of PD-L1 expression occurring at a particular point in time may be insufficient. Furthermore, the heterogeneity of PD-L1 expression in the same tumor tissue and between primary lesions and different metastatic tumors in the same patient also increases the difficulty of assessing PD-L1 expression levels. The details of PD-L1 detection in large Phase III trials performed to date are summarized in Table 1. However, the differences in their conclusions regarding PD-L1 expression and efficacy are probably related not only to the method of performing the PD-L1 assay but also to the complex interactions between tumors and the immune system.
**Table 1** Phase III clinical trials of ICIs with available efficacy results

| Trials                      | Drug(s)                  | Setting                        | Line of treatment              | Primary end point | PD-L1 cut-off value | ORR (%) Median  OS (m) | Median PFS (m) | Biomarker of survival benefit |
|-----------------------------|--------------------------|--------------------------------|--------------------------------|-------------------|---------------------|-------------------------|-----------------|--------------------------------|
| RCC                         | Checkmate 025 Nivo vs everolimus | Advanced                      | Second or higher OS           | 1% and 5%         | 25.1 vs 5.4         | 25 vs 19.55            | 4.60 vs 4.44    | Independent of TC PD-L1 level |
| NSCLC                       | Checkmate 017 Nivo vs Docet | Stage IIIB/IV, squamous        | Second OS                    | 1%, 5%, and 10%   | 20 vs 9             | 9.2 vs 6.0              | 3.5 vs 2.8      | Independent of TC PD-L1 level |
|                             | Checkmate 057 Nivo vs Docet | Stage IIIB/IV/recurrent, non-squamous | Second OS | 1%, 5%, and 10% | 19 vs 12          | 12.2 vs 9.4             | 2.3 vs 4.2      | TC PD-L1                       |
|                             | Checkmate 026 Nivo vs IC PT-DC | Stage IV/recurrent, TC PD-L1 ≥1%, untreated | First PFS | 1% and 5%       | 26 vs 33           | 14.4 vs 13.2           | 4.2 vs 5.9      | High TMB and TC PD-L1 ≥50%    |
|                             | Keynote 010 Pembro 2 mg/kg vs Pembro 10 mg/kg vs Docet | Advanced, TC PD-L1 ≥50%, untreated | First PFS | 50%             | 30 vs 29 vs 8       | 14.9 vs 17.3 vs 8.2 | 5.0 vs 5.2 vs 4.1 | TC PD-L1 ≥50%                     |
|                             | Keynote 024 Pembro vs IC PT-DC | Stage III                     | Consolidation therapy PFS, OS | NR               | 28.4 vs 16.0        | NR                     | 16.8 vs 5.6     | Independent of baseline TC PD-L1 level |
|                             | OAK Atezo vs Docet | Stage IIIIB/IV               | Second or higher OS | IC: 1%, 5%, and 10%; TC: 1%, 5%, and 50% | 14 vs 13 | 13.8 vs 9.6 | 2.8 vs 4.0 | TC PD-L1 ≥50% or IC PD-L1 ≥50% |
|                             | PACIFIC Durva vs placebo | Stage III                   | Consolidation therapy PFS, OS | NR               | 28.4 vs 16.0        | NR                     | 16.8 vs 5.6     | Independent of baseline TC PD-L1 level |
|                             | Checkmate 227 Nivo + Ipi vs Chemo vs Nivo | Stage IV or recurrent | First PFS, OS¹ | 1%             | 45.3 vs 26.9 vs NR | NR                     | 4.9 vs 5.5 vs 4.2 |                                |
| HNSCC                       | Checkmate 141 Nivo vs SAST | Recurrent                    | Second OS | 1%             | 13.3 vs 5.8          | 7.5 vs 5.1              | 2.0 vs 2.3      | TC PD-L1 ≥1% or p16-positive |
| Melanoma                    | Checkmate 066 Nivo vs Dacar | Metastatic without Braf mutation, untreated | First OS | 5%             | 40 vs 13.9           | NR vs 10.8             | 5.1 vs 2.2      | Independent of TC PD-L1 level |
|                             | Checkmate 067 Nivo + Ipi vs Nivo vs Ipi | Stage III/IV, unresectable, untreated | First PFS, OS | 5%          | 72.1 vs 57.5 vs 21.3 | NR vs 37.6 vs 19.9 | 11.5 vs 6.9 | Independent of TC PD-L1 level |
|                             | Checkmate 037 Nivo vs IC CT¹ | Unresectable/metastatic, progressed after Ipi | Stage IIIIV, unresectable | Second or more ORR, OS | 27 vs 10 | 16 vs 14 | 3.1 vs 3.7 | NS                             |
|                             | Keynote 006 Pembro q2w vs Pembro q3w vs Ipi | Stage IIIIV | Second OS | 1%          | 37 vs 36 vs 13 | NR vs NR vs 16.0 | 5.6 vs 4.1 vs 2.8 | NS                             |
|                             | CheckMate 238 Nivo vs Ipi | Stage IIIB/III/C/IV after complete resection | Adjuvant RFS | 5% | NR | NR | 12 mRFS: 70.5% vs 60.8% | 26.1 vs 17.1 | Regards regardless of PD-L1 and Braf status |
|                             | EOR TC 18071 Ipi vs Placebo | Stage III complete resection, high risk of recurrence | Adjuvant RFS | NR | NR | NR | 2.8 | NS |
| Urothelial cancer           | Keynote 045 Pembro vs IC CT² | Advanced                       | Second OS, PFS | 10% | 22.1 vs 11.4 | 10.3 vs 7.4 | 2.1 vs 3.3 | Independent of TC and IC PD-L1 level |

**Notes:** PFS, OS¹: PFS in populations selected on the basis of TMB; OS in populations selected on the basis of PD-L1 expression.

**Abbreviations:** Atezo, atezolizumab; Chemo, chemotherapy; Dacar, dacarbazine; Docet, docetaxel; Durva, durvalumab; HNSCC, head and neck squamous cancer; IC, immune cell; IC-CT, investigator’s choice of chemotherapy; IC CT¹, dacarbazine alone or carboplatin plus paclitaxel; IC CT², paclitaxel, docetaxel, or vinflunine; IC PT-DC, investigator’s choice of platinum-based doublet chemotherapy; ICI, immune checkpoint inhibitor; Ipi, ipilimumab; m, months; Nivo, nivolumab; NR, not reached; NS, no specific biomarker mentioned; NSCLC, non-small-cell lung cancer; ORR, objective response rate; OS, overall survival; PD-L1, programmed death receptor-ligand I; Pembro, pembrolizumab; PFS, progression-free survival; RCC, renal cell cancer; RFS, relapse-free survival; SAST, single-agent systemic therapy; TC, tumor cell; TMB, tumor mutational burden; q2W, every 2 weeks; q3W, every 3 weeks.
system, which along with tumor mutation burden (TMB) have been revealed as other potential biomarkers.

**CTLA-4 and PD-L2**

Associations of other immunosuppressive molecules with the rate of response to ICI treatment have also been reported. It has been shown that the CTLA-4 mRNA expression level before treatment is correlated with the efficacy of both the anti-CTLA-4 antibody and the anti-PD-L1 antibody, which might be associated with the promotion of the inhibitory function by Tregs on Teffs via CTLA-4 in TME; however, this inhibitory function was weaker than that of PD-L1.\(^{1,39,40}\) Moreover, Yearley et al\(^{41}\) reported that PD-L2 status was also a significant predictor of progression-free survival (PFS) with pembrolizumab and that it operated independently of PD-L1 status in HNSCC. Although there are some limitations, tumor immune escape clearly plays a critical role in the mechanism of immune action and in the prediction of the biomarkers of ICIs.

**Immune composition and activity in tumors**

**Tumor immunophenotypes**

Chen et al\(^{42}\) identified three tumor immunophenotypes: immune-inflamed, immune-excluded, and immune-desert phenotypes. Tumors with the immune-inflamed phenotype show immune cell infiltration at the tumor edge or in the tumor stroma, which is regarded as reflecting an inflammatory tumor. In this type of tumor, immune responses can be suppressed by the expression of immune checkpoints.\(^{42}\) Therefore, ICIs can unleash the suppressed immunity and have better efficacy. The latter two types are non-inflammatory tumors. Owing to steric hindrance, effective immune responses are lacking inside these tumors; therefore, the function of ICIs is very limited in such cases. The classification of the above-mentioned immunophenotypes is based on the differences in the infiltration patterns of immune cells inside tumors. The proposed immunophenotypes provide a basis for personalized tumor immunotherapy. However, some immune-inflamed tumors may also not respond to ICIs, partly because the early Treg recruitment inhibits an effective anti-tumor immune response.\(^{45}\) Additionally, several factors that influence immunophenotypes, such as TMB and the tumor microbial spectrum, might become biomarkers for the prediction of ICI efficacy.\(^{42}\) Page et al\(^{44}\) proposed that T-cell receptor (TCR) sequencing can provide additional information on TIL number and clonal diversity. The combination of TCR sequencing and IHC can assess TILs more comprehensively and accurately. However, these immunophenotypes focus on the numbers and aggregation patterns of TILs and ignore TIL functions. The use of a multi-parameter flow cytometer for the analysis of markers of TIL activation and depletion can compensate for this deficiency. Daud et al\(^{45}\) analyzed 40 MM patients at the progressive stage treated with nivolumab or pembrolizumab and found that patients with CTLA-4\(^{+}\)PD-1\(^{+}\) expression in more than 20% of CD8+TILs had a better prognosis. Interestingly, the improved prognosis linked to ICI therapy was associated only with the CTLA-4\(^{+}\)PD-1\(^{+}\) double-positive population and was not associated with the single-positive one.\(^{45}\) Other important biomarkers of exhaustion, including TIM-3, LAG-3, and VISTA, are usually co-expressed with PD-1 in excessively exhausted Teff cells.\(^{52,46}\) T cells that express many types of exhaustion/activation markers usually show a poor response to anti-PD-1/PD-L1 treatment.\(^{42}\) The effects of the TIL infiltration patterns and exhaustion/activation markers on ICI efficacy require further studies with large sample sizes.

**Immunosuppressive factors in TME**

Some studies have shown that immunosuppressive factors, particularly Tregs in TME, are potentially involved in the lack of response to ICIs in specific subtypes of cancer that are heavily infiltrated with adaptive immune cells.\(^{43,47,48}\) Enhancing the immune response to these tumors by depleting Tregs in addition to immune checkpoint inhibition impaired tumor growth and prolonged survival.\(^ {45}\) As Lowther et al\(^{49}\) showed that PD-1-high Tregs in the TME and circulating blood were an exhausted type, it is reasonable to speculate that the function of ICIs may be impaired if PD-1 was preferentially expressed on these cells or if these Tregs were activated in the presence of ICIs.\(^ {53}\) In contrast, in an earlier Phase II trial of melanoma patients treated with ipilimumab, higher infiltration of Foxp3+Tregs at baseline was significantly positively associated with clinical outcome.\(^ {50}\) More research on baseline Treg infiltration and the role of immune checkpoints on Tregs, such as CTLA-4 and PD-L1, is warranted. Some studies also showed that the depletion of Tregs during ICI treatment may be associated with ICI efficacy.\(^ {51,52}\) Although some studies showed that eradicating or reprogramming other immunosuppressive factors, such as myeloid-derived suppressor cells (MDSCs), γδT cells, and macrophages, could enhance clinical responses to ICI treatment, few studies have demonstrated whether they can be a biomarker for predicting its efficacy.\(^ {48}\)
Inflammatory gene signature

Inflammatory cells and proteins can participate in tumor metastasis, tumor growth, and angiogenesis. Moreover, in some tumors, PD-L1 is not constitutively expressed but rather is induced in response to inflammatory signals produced by an active anti-tumor immune response, with expression induced on most tumor cells in response to IFN-γ. This interactive function allows inflammatory gene signatures to be used as ICI biomarkers to select appropriate patient populations. Ribas et al indicated that IFN-γ signaling-related genes may allow the improved selection of patients likely to respond to anti-PD-1 therapy with pembrolizumab. In the exploratory analysis of the POPLAR study, patients with high Teff-IFN-γ-associated gene expression had improved OS with atezolizumab. Additionally, several studies showed that the loss of IFN-γ signaling in tumor cells may represent a common mechanism for tumor resistance to ICIs. These studies indicated that consideration of the characteristics of IFN-γ-related genes in tumors would be useful in the ICI prognosis model.

Tumor foreignness

Tumor mutation spectrum and mutation burden

TMB refers to the number of somatic cell mutations in the tumor genome after removing germline mutations. Many studies have explored the association between TMB and ICI efficacy (Table 2). Patients with a high TMB had significantly higher response rates, and longer PFS and OS than those with a lower TMB. Notably, most of these studies were retrospective and tested old biopsy specimens, which may not accurately reflect the current mutational burden of a tumor. Recently, Checkmate 227 showed that, in patients with advanced NSCLC and a tumor mutational burden of ≥10 per megabase, first-line treatment with nivolumab plus ipilimumab was associated with longer PFS than chemotherapy. These results indicate that TMB is an important and independent biomarker in advanced NSCLC. Some other studies may indirectly support the use of TMB as a biomarker of ICI efficacy. For example, in studies about NSCLC and urothelial cancer, higher response rates were seen in current and former smokers than in non-smokers, which may be suggestive of the role played by a high mutational load. A comparison among different types of tumors showed that tumors with higher TMB, such as MM, HNSCC, and bladder cancer, have a good effect on ICI therapy, with a response rate of more than 15%. Tumors with low TMB, such as pancreatic cancer and prostate cancer, have a poor response to ICI therapy. TMB can thus be used for cross-sectional analyses across many types of tumor to identify the patient population that can benefit from immunotherapy. However, TMB also has its limits. First, cancers are not static and can acquire mutations as they evolve. Issues related to the need for the dynamic monitoring of TMB and the timing required to detect TMB warrant further exploration. Second, immunogenic antigen expression is a necessary – but not a sufficient – condition for immune responses. Therefore, TMB can predict only the effectiveness of ICIs to some extent, and not all patients with high TMB can obtain obvious benefits after ICI therapy (immune tolerance might be caused by mechanisms other than PD-1/PD-L1 and CTLA-4). Moreover, the effect of ICIs on some patients with a low mutation burden is not poor (the recognition of DNA damage-induced neoantigens by T cells is a relatively random process, and low mutation burden sometimes also produces strong neoantigens). Furthermore, a recent study suggested that not all neoantigens are positively correlated with prognosis. McGranahan et al showed that the percentage of clonal neoantigens was positively correlated with ICI efficacy in lung adenocarcinoma, whereas the percentage of subclonal neoantigens was negatively correlated with efficacy. Therefore, if the majority of mutations were subclonal mutations, the presence of high TMB may not predict treatment efficacy. Thus, further classification of neoantigens might be necessary. TMB also has some problems, such as an unclear cut-off value, tumor heterogeneity, high cost of next-generation sequencing, and complicated data analysis. Nevertheless, a number of studies on the use of TMB as a biomarker for the prediction of ICI efficacy are now underway. The findings obtained thus far suggest the potential for including TMB analysis in the stratification of ICI clinical trials.

Mismatch repair deficiency (dMMR)

As with TMB, dMMR has recently become a marker for the prediction of ICI efficacy. Beyond the context of colorectal cancer, Le et al expanded the application of dMMR across 12 different tumor types; in this study, 53% of patients showed an objective response, and 21% achieved a complete response. In May 2017, the FDA has approved pembrolizumab for the treatment of adult and pediatric cancers that progressed after prior treatment, which are dMMR or microsatellite instability high, irrespective of tumor type. DNA mismatch repair (MMR) is a critical mechanism in DNA repair. Its major function is to proofread mismatched bases in a timely manner to maintain genome stability. dMMR results in many mutations that enhance tumor immunogenicity and induce more active immune responses. Additionally,
## Table 2: Studies utilizing TMB as a predictor of response to treatment with ICIs

### Clinical trials

| Clinical trials | Study drug | Tumor type and stage | Calculation methodology for TMB | Cut-off | Results |
|-----------------|------------|----------------------|---------------------------------|---------|---------|
| Prespecified analysis | Checkmate 227 | Nivo + Ipi | Stage IV or recurrent NSCLC | CGP (Foundation Medicine) | 10 per Mb | In patients with high TMB (≥10 per Mb), median PFS: 7.2 m vs 5.5 m (Nivo + Ipi vs Chemo) |
| Exploratory analysis | IMvigor 210 | Atezo | Locally advanced and metastatic UC | CGP (Foundation Medicine) | Median TMB: 12.4 vs 6.4 per Mb (responders vs non-responders) |
| Retrospective study | Checkmate 026 | Nivo | Stage IV or recurrent NSCLC | WES | Low TMB: 0–100 mutations, Medium TMB: 100–242 mutations, High TMB: ≥243 mutations |

| Retrospective study | Author | Drug | Tumor type and stage | Calculation methodology for TMB | Results |
|---------------------|--------|------|----------------------|---------------------------------|---------|
| Campesato et al | Pembro | NSCLC | CGP (Foundation Medicine) | TMB was calculated using just mutated genes present in the cancer gene panel |
| Rizvi et al | Pembro | NSCLC | WES | Higher somatic nonsynonymous mutation burden was associated with the clinical efficacy of Pembro |
| Johnson et al | Nivo or Pembro or Atezo | Melanoma | CGP (Foundation Medicine) | Mutational load effectively stratified patients by likelihood of response |
| Yaghmour et al | Ipi or Pembro or Nivo | Any solid tumor | Not mentioned | Higher TMB was associated with improved OS |
| Kowanetz et al | Atezo | NSCLC | CGP (Foundation Medicine) | OS, PFS, and RR were improved in patients with increased TMB treated with Atezo in both unselected and selected patients |
| Goodman et al | anti-PD-1/ PD-L1, anti-CTLA-4, anti-CTLA-4 + anti-PD-1/PD-L1, high-dose IL-2, and other agents | Melanoma, NSCLC, and other types | CGP (Foundation Medicine) | Higher TMB was independently associated with better outcome parameters |

**Notes:** 1. Other agents: OX40, anti-CD73, talimogene laherparepvec, OX40 + anti-PD-L1, and IDO + anti-PD-1. 2. Tumors included the following: adrenal carcinoma, appendix adenocarcinoma, basal cell carcinoma, bladder transitional cell carcinoma, breast cancer, cervical cancer, colon adenocarcinoma, cutaneous squamous cell carcinoma, hepatocellular carcinoma, head and neck, Merkel cell carcinoma, ovarian carcinoma, pleural mesothelioma, prostate cancer, renal cell carcinoma, sarcoma, thyroid cancer, unknown primary squamous cell carcinoma, and urethral squamous cell carcinoma

**Abbreviations:** Atezo, atezolizumab; Chemo, chemotherapy; CTLA-4, cytotoxic T-lymphocyte-associated antigen-4; CGP, comprehensive genomic profiling; ICI, immune checkpoint inhibitor; Ipi, ipilimumab; m, months; Mb, megabase; Nivo, nivolumab; NSCLC, non-small-cell lung cancer; Pembro, pembrolizumab; OR, odds ratio; OS, overall survival; PD-1, programmed death receptor-1; PD-L1, programmed death receptor-ligand 1; PFS, progression-free survival; RR, response rate; SCLC, small-cell lung cancer; TMB, tumor mutational burden; UC, urothelial carcinoma; WES, whole-exome sequencing.
some studies have also confirmed that mutations in other
genes involved in the DNA replication repair process (e.g.,
the POLE gene) are associated with ICI prognosis.79 However,
individuals with dMMR account for only a small percentage
of patients. Some patients with a proficient MMR system can
still benefit from ICI therapy.80

Expression of viral genes
Recently, the association between the PD-1-PD-L1 pathway
and virus infection in certain tumors, such as HPV-induced
cervical cancer and HNSCC, and EBV-induced gastric
cancer and nasopharyngeal carcinoma, has elicited consid-
erable attention. First, PD-L1 expression is thought to play
a role in the initiation and persistence of HPV infection by
providing an immune-privileged site where T-cell activity is
downregulated.81–83 Second, viral antigens that will generally
not be lost or downregulated can trigger an immune response
due to their exogenous nature. Moreover, virally mediated
tumors develop in the context of chronic infection in which
immune checkpoints may be activated over time. Many stud-
ies have demonstrated the positive correlation between PD-L1
expression and virus infection in various cancers, including
HNSCC, cervical cancer, and EBV-induced malignant
tumors.81,84–87 Additionally, recent studies have shown that
more T-cell infiltration was observed in virus-positive tumors
than in the same type of virus-negative ones.88

At present, study reports about ICI efficacy are limited to
HNSCC. Both Keynote 012 and Checkmate 141 showed that
HPV-positive tumors obtained more benefits from ICIs than
HPV-negative ones.15,89 Data were insufficient in other types
of virus-infected tumors, such as HPV-infected cervical
cancer and EBV-induced malignant tumors. Keynote 028 showed
the antitumor activity of pembrolizumab in PD-L1-positive
cervical cancer, but it did not evaluate the association between
the efficacy of pembrolizumab and HPV infection.83 On
the other hand, the preliminary results of Checkmate 358
showed that a response to nivolumab was observed regard-
less of PD-L1 or HPV status.90 However, Checkmate 358 is
a Phase I/II study including only 24 patients, the final results
of which are yet to be published.90 Further evaluation of the
role of virus infection in ICI efficacy should be performed.

Driver gene mutation
Not all kinds of tumor cell gene mutations can enhance TIL-
mediated immune responses. Recent studies have shown
that tumor-associated driver gene mutations not only fail to
enhance but also actually attenuate immune responses. The
subgroup analysis in the Checkmate 057 trial showed that
NSCLC patients with EGFR mutations or ALK rearrange-
ments obtained relatively minor benefits from ICI therapy.10
Currently, the mechanism underlying the effects of driver
gene mutations on tumor local immunity and ICI efficacy
is still unclear. It is speculated that tumors with driver gene
mutations might have lower total mutation levels due to the
lower mutation heterogeneity. A retrospective study showed
that fewer NSCLC patients with EGFR mutations or ALK
rearrangements exhibited both positive PD-L1 expression
and high CD8+TIL infiltration.91 Moreover, individuals
with EGFR mutations with non-T790M-acquired drug
resistance might benefit more from PD-1 inhibitors than
patients with T790M-acquired drug resistance.82 Based on
these observations, recent studies on EGFR mutations have
mainly adopted therapy of ICIs combined with tyrosine
kinase inhibitors.83 Although the Checkmate 142 trial showed
that KRAS or BRAF mutations did not affect the efficacy
of PD-1 inhibitors, some studies showed that KRAS and
BRAF mutations or other mutations in the MAPK pathway
attenuated immunity by reducing the transcription of major
histocompatibility complex class I (MHC I) molecules.94–96
Additionally, β-catenin pathway activation and the direct or
indirect loss of PTEN resulted in the reduction of CD8+TILs
infiltration in melanoma.97,98 The effects of driver gene muta-
tions on the immune microenvironment and on the efficacy
of immunotherapy still require further research.

In summary, the T-cell immune response is closely asso-
ciated with the increase of neoantigens that results from
DNA damage, or repair system defects, and foreign antigens
expressed by viral genes. DNA and RNA sequencing plays
an important role in the evaluation of the tumor foreignness
and can optimize the selection of patients for ICI therapy.
However, the presence of immunogenic antigens is only
one of the necessary conditions of immune responses in
tumors. Furthermore, the effects of driver gene mutations
on the immune microenvironment and the efficacy of immu-
notherapy are more complicated. Most studies have shown
that, in patients with driver gene mutations, ICIs have poor
efficacy. The use of ICIs combined with corresponding tar-
geted therapy is a promising direction of future research for
the treatment of these patients.

Host factors
Peripheral blood markers
Several studies have reported that the absolute counts of
certain cell populations in peripheral blood (e.g., lympho-
cytes, monocytes, and neutrophils) were associated with ICI
efficacy.99–106 However, some other studies cast doubt on this.
Sun et al\textsuperscript{107} reviewed all consecutive patients treated with anti-PD-1/PD-L1 monotherapy in Phase I trials performed at our institution between December 2011 and January 2014 and found that baseline absolute lymphocyte count (ALC) was not associated with response to anti-PD-1/PD-L1; thus, patients should not be excluded from early-phase clinical trials testing immune checkpoint blockers because of ALC. Additionally, a study by Subrahmanyam et al\textsuperscript{108} also did not find that lymphocyte and monocyte frequencies had predictive value for ICI efficacy. However, they found differences in CD4+ and CD8+ memory T-cell subsets between responders and non-responders to anti-CTLA-4 and differences in specific NK cell subsets (CD69+ and MIP1β+ NK cell populations) in responders and non-responders to anti-PD-1. The distinct sets of candidate biomarkers for anti-CTLA-4 and anti-PD-1 therapies may be attributable to the different sites at which they function.\textsuperscript{4} Moreover, some other subsets in peripheral blood, such as circulating MDSCs and CD14+CD16-HLA-DRhi monocytes, were reported as predictors of ICI efficacy.\textsuperscript{109,110} At present, the evidence that subsets of circulating blood cells can be used as predictors of ICI efficacy remains insufficient and this issue warrants further research.

Apart from these circulating immune cells, peripheral blood TCR diversity also plays an important role in CTLA-4 inhibitor therapy. CTLA-4 inhibitors can promote reconstruction of the TCR repertoire and increase its diversity.\textsuperscript{111–113} Cha et al\textsuperscript{111} showed that the maintenance of high-frequency TCR clonotypes was associated with longer OS in patients following ipilimumab therapy; however, patients who lost more high-frequency clonotypes usually had shorter OS. These high-frequency TCR clonotypes might represent high-affinity T cells associated with anti-tumor responses.\textsuperscript{111} Notably, Huang et al\textsuperscript{114} recently developed a “reinvigoration score” by relating changes in circulating exhausted-phenotype CD8+ T cells to tumor burden to predict anti-PD-1 response. They found that these responding exhausted-phenotype CD8+ T cells in the blood contained TCR clonotypes shared with TILs, which may be the factor underlying this phenomenon. However, immune cell functions in TME clearly differ markedly from those in peripheral blood.

Genotypes of patients

Genotype may affect ICI efficacy; however, current evidence is limited to studies with small samples. Queirolo et al\textsuperscript{115} analyzed 14 MM patients and found that the rate of response to ipilimumab was higher in patients with CTLA-4-11577G/A and CT60G/A heterozygous genotypes. Another earlier study on the treatment of melanoma using ipilimumab showed that three types of CTLA-4 single-nucleotide polymorphisms (SNPs) (rs4553808, rs11571327, and missense SNP rs231775) were associated with the response to anti-CTLA-4-specific antibodies.\textsuperscript{116} However, a Phase II clinical trial of MM did not reveal an association between CTLA-4 SNPs and treatment response.\textsuperscript{50} Therefore, the association between SNPs and ICI efficacy still requires further verification.

Microbial spectrum

Several studies have demonstrated that manipulation of the microbiota may modulate the effect of cancer immunotherapy.\textsuperscript{117–119} For example, the transplantation of fecal microbiota from cancer patients who responded to ICI into germ-free or antibiotic-treated mice was reported to ameliorate the anti-tumor effects of ICIs.\textsuperscript{117–119} Moreover, Matson et al\textsuperscript{120} recently analyzed baseline stool samples from MM patients before immunotherapy treatment and observed a significant association between commensal microbial composition and clinical response. Bacterial species that were more abundant in responders included \textit{Bifidobacterium longum}, \textit{Collinsella aerofaciens}, and \textit{Enterococcus faecium}.\textsuperscript{120} Similar to the previously mentioned results, Chaput et al\textsuperscript{121} suggested that baseline gut microbiota enriched with \textit{Faecal} \textit{bacteria} and other \textit{Firmicutes} is associated with a beneficial clinical response to ipilimumab. The search is underway for components of the microbiota that enhance the action of other immunotherapies. Discovery of the effect of gut microbiota on ICI efficacy has clearly opened up another direction for ICI biomarker discovery.

Overall, although some inspiring results have been obtained, few studies on host factors such as peripheral blood markers, gene polymorphisms, and gut microbiota have been performed thus far, and this work is still at the exploratory stage. It is challenging to identify the factors that actually predict treatment response and to separate them from the confounding factors.

Conclusion

Our analyses showed that the main functions of ICIs are to unleash immune tolerance, which results from the activation of immune checkpoint pathways. The effectiveness of these therapies requires cooperation with all other aspects of the immune system. First, the expression of immunogenic antigens on tumor cells is an essential condition for the induction of anti-tumor immune responses. Therefore, evaluation of the tumor foreignness using methods such as gene analysis is necessary. Second, immune activities in the TME include the distribution and function of TILs and inflammatory gene expression and are also associated with ICI efficacy. Third,
the specific mechanisms of tumor escape also play important roles in the effectiveness of ICIs. The detection of PD-L1 might require the use of combined measures. Furthermore, studies on peripheral blood markers, gene polymorphisms, and gut microbiota are still at an initial stage. These four classification methods provide a framework for our studies on ICI biomarkers (Figure 2).

It is worth noting that the majority of the aforementioned factors were used as solitary subjects of study in most previous studies, especially in large Phase III trials (Table 1). The fact that most of them focused only on PD-L1 expression may have been due to the early stage at which these studies were performed. Few studies on their association and weights have been performed. The cancer immunogram proposed by Blank et al122 is an approach involving the use of the above-mentioned methods, including many types of prediction markers, to predict ICI efficacy. It is imperative to perform multivariate predictive analyses that include tumor foreignness, immune composition, immune activity, tumor escape mechanisms, and some host factors. Additionally, many measures, including quantitative genetic analysis, IHC to determine the density and location of immune cell types, and flow cytometry for various cell surface markers, can be combined with some conventional laboratory examinations. With the implementation of large-scale ICI clinical studies and the emergence of some promising results, multivariate analyses can help us to optimize patient selection and possibly personalize cancer treatment using ICIs.

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**Author contributions**
YZ and ZL were responsible for the conception and design of the study. JY and FZ provided useful suggestions. All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work. All authors read and approved the final manuscript.
Disclosure
The authors report no conflicts of interest in this work.

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