



# Biomarker for personalized immunotherapy

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**Abstract:** Programmed cell death 1/programmed death ligand 1 (PD-1/PD-L1) interaction protects cancer cells from immune destruction. Blocking the pathway allows infiltrated T cells to kill tumor cells (TCs), in order to prevent tumor proliferation and distant migration. Immunotherapy with checkpoint blockade (CPB) has emerged as a powerful weapon conquering multiple cancer. However, PD-L1 expression and tumor mutation burden (TMB) are not perfect biomarkers for patients selection to treated with immunotherapy. Increasing evidence showed that other immune-related factored must be payed enough attention and combined biomarkers present promising prospects. Here, we give an overview on available biomarkers in clinic and potential biomarkers for future research.

**Keywords:** Immunotherapy; biomarker; programmed death ligand 1 (PD-L1); tumor mutation burden (TMB)

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## Introduction

In 2018, Nobel Prize in Physiology or Medicine was awarded to two famous scientists for their discovery of two checkpoints, cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death 1 (PD-1) (1). Immunotherapy has emerged as a powerful weapon in multiple cancer types, with checkpoint blockade (CPB) blocking the PD-1 and programmed death ligand 1 (PD-L1) pathway (2). By releasing the ability of cytotoxic T cells, the innate immune system could recognize and kill cancer cells and prevent cancer metastasis. Wide application in cancer disease, overall survival (OS) greatly prolonged. Anti-PD-1/PD-L1 therapy proceed by steady steps from second line to first line setting in advanced stage non-small cell lung cancer (NSCLC) patients (3-6). No matter as monotherapy or combined therapy with platinum-based doublets, CPB have brought promising results with durable responses, improved OS and favorable tolerability in published phase III studies

(7,8). Undoubtedly, one of the key points to the success is the biomarker design that should screen out suitable patients for CPB.

PD-L1 immunohistochemistry (IHC) testing with Dako 22C3 antibodies have been approved by Food and Drug Administration (FDA) as companion diagnostic for the treatment of pembrolizumab in NSCLC patients at first line and second line setting. Dako 28-8 and Ventana SP142 have been approved as complementary diagnostic for the treatment of nivolumab and atezolizumab in NSCLC patients at second line setting. Recently, FDA extend review period for frontline nivolumab plus ipilimumab in TMB-high NSCLC, because tumor mutation burden (TMB) is uncertain for the predictive value. Patients with driver gene variation are excluded from immunotherapy in multiple cancer guidelines. EGFR mutant or ALK rearranged patients would not be recommended CPB, but still clinical trials seek for possibility in progression with EGFR-TKIs

but T790M negative patients. An increasing number of researches are in an attempt to explore a more favorable predictor of CPB response than PD-L1 expression alone. Thus, combined biomarkers, such as PD-L1 with tumor infiltrating lymphocytes (TILs), KRAS mutation with TP53 mutation, and other immune-related gene profile emerged, which not only could be a potential biomarker to predict efficacy of CPB, but give an illustration to understand the background of tumor microenvironment (TME). Here, we give an overview on the available and potential biomarkers for personalized immunotherapy.

### PD-L1 expression

PD-1 is a receptor which expressed on the surface of cytotoxic T cells. Its ligands PD-L1 and PD-L2 express on the cell surface of macrophages, epithelial cells and other normal cells (9). The combination of PD1 and PD-L1 could protect normal cells from immune recognition and inhibiting the destruction by cytotoxic T cells. In recent years, increasing research indicated that tumors cells could evade immune surveillance by up-regulation of PD-L1 expression. When PD-L1 binds to the PD-1, activated T cells become inactivated and depleted, resulted in the prevention of tumor cells (TCs) from host immunity. So far, 2 PD-1 (nivolumab, pembrolizumab) and 2 PD-L1 blocking antibodies (durvalumab, atezolizumab) were approved by the FDA for the treatment of lung cancer (1).

Undoubtedly, PD-L1 expression is the only selected biomarker used for clinical practice. Tumor proportion score (TPS) should be calculated by IHC analysis with PD-L1 antibodies. In first line setting, NSCLC patients with PD-L1 expression (TPS  $\geq 1\%$ ) could be treated with pembrolizumab monotherapy, and so as patients with positive PD-L1 expression (TPS  $\geq 1\%$ ) in second line (5,6). Nivolumab and atezolizumab choosing unselected patients also achieved great success and are approved by FDA in second line setting (3,4,10).

Recent years, combined immunotherapy made great progress with astonishing speed, having stepped into first line treatment for unselected NSCLC patients. The series of Keynote clinical trials announced their results in *New England Journal Medicine* (11,12). Regardless of PD-L1 expression, pembrolizumab plus chemotherapy favored in longer survival than chemotherapy alone. Although the incidence of adverse events is comparable in two groups, toxicity in pembrolizumab-combination group is well tolerated. Nevertheless, in the sub-analysis, the survival

differences in PD-L1 high group (TPS  $\geq 50\%$ ) is bigger than PD-L1 low group (50% > TPS  $\geq 1\%$ ) and PD-L1 negative group (TPS < 1%). Altogether, these data indicate a new therapeutic strategy: (I) in the first line setting, TPS of PD-L1 expression is more than 50%. Pembrolizumab is recommended for NSCLC patients. When TPS is less than 50%, pembrolizumab plus platinum-based chemotherapy will be recommended. (II) In the second line setting, TPS is more than 1%. Pembrolizumab is recommended. When PD-L1 expression unknown or negative, nivolumab and atezolizumab is recommended (13).

However, as an imperfect biomarker, IHC analysis of PD-L1 expression remains a few questions to be settled on:

- (I) Four IHC antibodies (Dako, 28-8; Dako, 22C3; Ventana, SP142; Ventana, SP263) are used for assessment of the positivity of PD-L1 expression in clinical trials evaluating clinical efficacy of different CPIs. Previous studies showed that the assessment of PD-L1 expression on TCs was comparable with 22C3, 28-8 and SP263 assays, while SP142 assay exhibiting lower positive expression. Immune cells (ICs) staining across four assays appears to be consistent, but more variable than for TC staining (14). Meanwhile, different cutoff values are applied for the evaluation of PD-L1 expression. Thus, standardization and harmonization of PD-L1 testing assays is urgently needed to be work on. Different international and national initiatives have been conducted for PD-L1 IHC harmonization and validation.
- (II) Although at least two pathologists completed evaluation on PD-L1 expression on TCs by IHC independently, the proportion of inconsistent outcomes should be given more attention. After all, the assessment is indeed too subjective and empirical. In phase II results of the project Blueprint, strong reliability among pathologists in TC PD-L1 scoring with all five assays of PD-L1 IHC (22C3, 28-8, SP142, SP263, and 73-10), but poor reliability in IC PD-L1 scoring (15).
- (III) Objective response rate (ORR) is not as high as expected. Only PD-L1 expression could not screen out most potential benefited patients, since low or negative stained tumor could also response to CPB. Comprehensive genomic signature is urgently needed to be explored.
- (IV) IHC staining need to be performed on tumor sample through invasive operations at the baseline

of immunotherapy. Dynamic monitoring of clinical efficacy or prediction of primary resistance is impossible for IHC analysis of PD-L1 staining on tissue sections. However, relevant research has been initiated with exosomal PD-L1 expression or CTC PD-L1 expression obtaining from peripheral blood (16,17).

Altogether, PD-L1 expression has been accepted for clinic practice to identify if patients could be treated with CPB but it is still not enough. Combined with other biomarkers, such as TILs, tumor neoantigen, other checkpoints, such as Lag3, TIMs and TIGIT is the future direction (18,19).

## TMB

TMB varies greatly between different cancer types, among which melanoma, lung adenocarcinoma and lung squamous carcinoma are the top three (20,21). Till now, four CPIs were approved by FDA for the treatment of melanoma and NSCLC in different stages, with PD-L1 expression as patients selected biomarker (1). After the failure of Checkmate 026, BMS used TMB as biomarker to conduct a retrospective analysis (22). The results showed that compared with PD-L1 expression, TMB could better identify benefited NSCLC patients for the treatment of nivolumab plus ipilimumab. TMB  $\geq 10$  Mut/Mb is selected as the cutoff value to definite TMB high population. Clinical efficacy was indeed more outstanding in patients with TMB  $\geq 10$  Mut/Mb, regardless of PD-L1 expression. Another study by Ready *et al.* (23) showed when PD-L1  $\geq 1\%$ , the proportion of TMB  $\geq 10$  Mut/Mb and TMB  $< 10$  Mut/Mb is 70% versus 30%; when PD-L1  $< 1\%$ , the proportion of two subgroups is 90% versus 10%. In previous study, patients with TMB results was less overlapped with PD-L1 expression (24). It seems that these two biomarkers could identify independent and unique population that could get benefit from CPB (25).

In 2018 AACR, the initial outcomes of CheckMate 227 indicated that median PFS was 7.2 months in TMB high patients who were treated with nivolumab plus ipilimumab at first line setting, compared with 5.4 months in chemo cohort (HR 0.75, 95% CI: 0.53–1.07). The results were consistent in PD-L1  $< 1\%$  subgroups. Last year, FDA announced that review period will be extended for another 3 months for frontline nivolumab combined with ipilimumab in TMB-high NSCLC. Additional survival data was required in TMB-low patients. The final results

showed that in TMB  $\geq 10$  Mut/Mb group, median OS is 23.0 versus 16.7 months (HR 0.77, 95% CI: 0.56 to 1.06); in TMB  $< 10$  Mut/Mb group, median OS is 16.2 months versus 12.4 months (HR 0.78, 95% CI: 0.61 to 1.00). In 2018, Devarakonda *et al.* reported the result of the Lung Adjuvant Cisplatin Evaluation (LACE)-Bio-II study and found that high nonsynonymous TMB appears to be a favorable prognostic biomarker for patients with resected NSCLC (26). A recent research by Samstein *et al.* demonstrated that higher TMB (top 20%) was associated with better OS after immunotherapy across multiple cancer types. The HR in NSCLC was 2.08 (95% CI: 1.61–2.68;  $P < 0.001$ ) (27). TMB cut-points associated with improved survival varied markedly, with 13.8 Mut/Mb in NSCLC. Regarding the predictive or prognostic role of TMB, available evidence is inconsistent (27,28). We need to wait and see the outcomes of other ongoing trials and research, avoiding to jump to an uncertain conclusion. Nevertheless, progress has been achieved by blood-based TMB (bTMB) as biomarker to predict PFS in patients receiving atezolizumab in NSCLC (24). The correlation between tumor tissue-based TMB and bTMB is positive. The proportion of shared variants is 59%, and the tissue only and blood only variants are 25% and 26%, respectively. It was confirmed in two clinical trial POPLAR and OAK that bTMB  $\geq 16$  Mut/Mb is a clinically meaningful cut-point in NSCLC patients. Another study published on the journal of *JAMA Oncology*, identified bTMB of 6 or higher correlated with favorable PFS in patients treated with CPIs (29). Meanwhile, 150 genes panel was used to illustrate that NGS has high consistence with WES for TMB evaluation. This is promising results and we hope that in the future, tissue biopsy for guiding the immunological therapeutic strategy is less needed.

TMB has a few limitations, although FDA has authorized FoundationOne CDx and MSK-IMPAC for clinical use. As is known, whole exome sequencing (WES) could better calculate TMB theoretically. However, owing to its great cost, long time consuming and complicated process, WES is only applied in scientific research (30). Available data showed that the consistence of comprehensive genomic profiling by next generation sequencing (NGS) and WES is strong and both could reflect the predictive role of TMB for CPIs (21). However, various platforms of NGS results has no standardization of TMB testing and no unified cutoff value, ranging from 7.4 to 10.0 Mut/Mb. In addition, analytical and preanalytical factors could affect the quantity and quality of DNA and thus influence the TMB values,

which include sequencing depth, sample collection, input material quality and quantity and so on (30).

### Tumor infiltrating cells

The existence of TILs is necessary for anti-PD-1/PD-L1 antibodies to restore the immunity of host to destroy TCs (31). Thus, the density of TILs by IHC analysis is another important predictor for clinical efficacy with CPB. Based on the infiltrating T cells and PD-L1 expression, TME could be divided into four groups: TILs+/PD-L1+; TILs-/PD-L1+; TILs+/PD-L1-; TILs-/PD-L1- (32,33). Another method for clustering the context of the immune TME is inclusion with many immune-related factors as immune profiles to predict responses to immunotherapy. One subtype with presence of multiple TILs subpopulations and positive PD-L1 expression, regarded as adaptive immune resistance or inflamed tumor, is the most likely to respond to immunotherapy (34). In fact, the effects of the immune infiltrate on the prognosis of patients with cancer is much different. CD8+ T cells, tertiary lymphoid structures and M1 macrophages have a positive prognostic association with OS (35,36).

Not only the density but also the location of TILs plays a predictive role on clinical efficacy of CPB. High infiltration of cytotoxic T cells, within a certain distance to cancer cells, significantly correlated with prolonged patient survival. It seems that the cytotoxic T cells near to cancer cells has important immune biological function (37). Accompanied by the emergence of single-cell sequencing, profiling single ICs in tumor samples of patients treated with CPB is no longer an impossible thing (38).

Defining certain phenotypes of ICs associated with response to CPB could be achieved by bioinformatic analysis on clustering infiltrated T cells (39-41). In melanoma, researchers found that the presence of TCF7 protein on CD8+ T cells was related to clinical response to CPB. Although CD8+ T cells is regarded as immune effector cells with the function to recognize and kill TCs, diverse phenotypes of CD8+ T cells are undefined. Last year, Simoni and his colleges identified a subtype of CD8+ TILs with the absence of CD39, named as bystander CD8+ T cells, which are not specific for tumor antigens (42). It greatly broadens our knowledge of immune TME and the ability of TILs subpopulations. Further study still needs to find something else about the function of T cells and unknown T cells phenotypes.

### Genetic variations

Gene testing gives us much information to guide clinic decision. In addition to TMB, somatic mutations assistant to classify tumors into different molecular subtypes. Clinical trials with CPB mostly exclude NSCLC patients with EGFR mutation and ALK rearrangement. In addition, patients with ROS1 rearrangement and BRAF mutations should also be excluded, owing to better clinical efficacy of targeted therapy. It has been furtherly proved that these genetic variation lead to unfavorable efficacy to immunotherapy (43). Less PD-L1 expression and fewer CD8+ TILs in patients with driver gene mutations could be the underlying mechanism of the lower response rate to CPB (44). Dong *et al.* brought an insight into the background of immune context and PD-L1 expression in patients with EGFR mutation. Uninflamed phenotype and weak immunogenicity could interpret the impaired response to PD-1 blockade. Targeted therapy with EGFR-TKIs is the standard treatment for EGFR mutation with advanced stage NSCLC. However, 7% patients with primary resistance could not get clinical benefit from TKIs. An interesting finding from Su *et al.* demonstrated that PD-L1 expression would give an interpretation of the poor response and *de novo* resistance to EGFR-TKIs (45). Almost half of the patients with *de novo* resistance have PD-L1 and CD8 co-expression. These patients may have potential therapeutic sensitivity to PD-1 blockade therapy. It seems that when EGFR-TKIs is not suitable for EGFR mutant patients, PD-L1 and CD8 IHC analysis maybe recommended to give guidance for the treatment of CPB.

So far, no effective inhibitors targeting KRAS mutation is available in the clinic. Previous studies found that KRAS and TP53 mutation have potential predictive value for response to PD-1 blockade therapy (46-48). Dong *et al.* illustrated that positive PD-L1 expression, CD8+T cell infiltration and high mutation burden maybe the reason of favorable clinical benefit from CPB, comparing with other genetic mutation. Mutations in the LKB1 (STK11) are the third most frequent genetic variation in NSCLC, which often presents with KRAS mutations. STK11 is a tumor suppressor gene that could exerts a negative regulation on the mTOR pathway. Recent studies showed that KRAS G12D, combined with STK11 mutations could result in inferior prognosis in NSCLC patients (48). However, combined with STK11, KRAS mutation would be resulted in immune invasion and primary resistance to anti-PD-1 therapy (49,50). Additionally, patients with JAK1/2

mutations may not respond to CPB. Because this genetic alteration would inhibit the downstream pathway of IFN-gamma and the favorable effects of anti-tumor immunity (2). Other mutations would lead to primary or adaptive resistant mechanism, such as B2M truncating mutation and loss of PTEN (51,52). However, these claims indeed make sense theoretically, but need to be confirmed by clinical data. Thus, resistance mechanisms and relevant biomarkers are under intensive investigation and development.

### TCR repertoire

T lymphocytes play a vital role in immune-protection mechanism. The diversity of T-cell antigen receptors (TCR) is dependent on the randomization of VDJ rearrangement, which furtherly results in the diversity of CDR3 region (53). With the advent of TCR sequencing, the internal immunity triggered by tumor neoantigens could be better understood. This new technology, combined with single-cell sequencing, could comprehensively analyze the antigen-specific T cells response. TCR repertoire reflect adaptive immune system in cancer patients after the treatment of immunotherapy, which could be a potential predictive biomarker for immune responses (54).

In recent years, TCR sequencing has already conquered a few challenges and make it possible not only to track the T cell subtypes that infiltrating in tumor microenvironment, but also to identify the dominant TCR clonotypes in tumor lesion and peripheral blood (55). TCR repertoire analysis could give two dimensions of information, namely T cells diversity and clonality. However, relevant research regarding lung cancer is scarce. Previous studies showed that the dominant T lymphocytes clones could be greatly different from primary tumor lesions to normal margins, and even to peripheral blood (56-58). This could be illustrated by the heterogeneity of TILs in tumor microenvironment and the dynamic changes in peripheral circulation. In addition, another important data is that if the diversity of T cells in tumor lesions or blood could be predictive to the response to immunotherapy, such CPIs. Similarly, limited data indicated that conclusions of available findings regarding prediction of response or survival to CPB are inconsistent (58-60). Altogether, TCR sequencing still have a long way to go.

### Predictive biomarkers for HPD

A proportion of patients will present primary resistance

to CPB therapy, which origins from lack of antigenic mutations, de-differentiation with loss of tumor antigen expression, constitutive PD-L1 expression and loss of HLA expression (52,61). Recent studies told us that immune CPB sometimes could make things worse.

Hyperprogressive disease (HPD) defined as double tumor growth rates between baseline and treatment. A small subtype of NSCLC patients could develop HPD, which likely emerges in older patients with a shorter median OS (62). This new pattern of disease progression in cancer patients treated with PD-1/PD-L1 blockade also happens on head and neck squamous cell carcinoma and other cancer types (63). Previous study performed analysis on genomic alteration associated with CPB treatment. Patients with MDM2 amplification and EGFR aberrations maybe the potential reasons of HPD patterns (64). Recently, Lo Russo *et al.* found that enrichment of tumor-associated macrophages (TAM) could be one mechanism for HPD in NSCLC patients followed by PD-1 blockade (65). Due to the limited evidence, further investigation is urgently needed.

### CTC PD-L1 expression

Circulating TCs (CTCs) is derived from primary or metastatic lesions, which could reflect the genetic or epigenetic variations in tumor tissue. Non-invasive liquid biopsy of circulating tumor DNA (ctDNA) for gene testing is achieved by NGS. Since PD-L1 expression in tumor tissue is an imperfect biomarker for CPB prediction, efforts have been made to investigate the performance of CTCs as an alternative biomarker to evaluated PD-L1 expression.

Cell Search CXC Kit, Vortex HT chip, or digital PCR detection following microfluidic enrichment process are applied for capture CTCs from blood when patients under treatment (17,66-68). Detectable rate, which means the proportion of peripheral blood contains at least one CTC, is around 83-97%, depending on the sensitivity of capture methods. On the basis of limited data, PD-L1 expression on CTCs and concordance rate of PD-L1 expression on tumor tissue and CTCs vary markedly in different studies (17,69). Nevertheless, the prognostic trending of PD-L1 on CTCs in NSCLC patients has been verified by use of various PD-L1 primary antibodies, and various thresholds. And a few case reports showed dynamic PD-L1 + CTC count may help to identify changes in response to treatment, and these cells could be a readily obtainable source to understand tumor evolution (17,67).

However, current studies on association of CTCs with PD-L1 positive expression and response to CPB is not enough for blood-based monitoring. Hong *et al.* combined microfluidic enrichment for CTCs together with RNA based droplet digital PCR quantitation. Molecular signatures of CTCs score in melanoma was used to predict early response to CPB. Outcomes presented that decrease in CTC score made a good correlation with marked improvement in PFS (HR 0.17; P=0.008) and OS (HR 0.12; P=0.04) (70).

Although there are several advantages of the PD-L1 expression on CTCs, such as minimally invasive inspection method without high risk of complications; dynamic monitor at different points during the CPB treatment; providing important complementary information on therapeutic responses; reflection of heterogeneity of tumor tissue. Uncertain questions remain: (I) the stability and specificity of CTCs capture methods vary greatly; (II) which IHC antibodies should be applied for PD-L1 staining; (III) how to define PD-L1 positive expression; (IV) what is the optimal cutoff points of PD-L1 to correlate with clinical efficacy of immunotherapy with CPB.

### Comprehensive signature

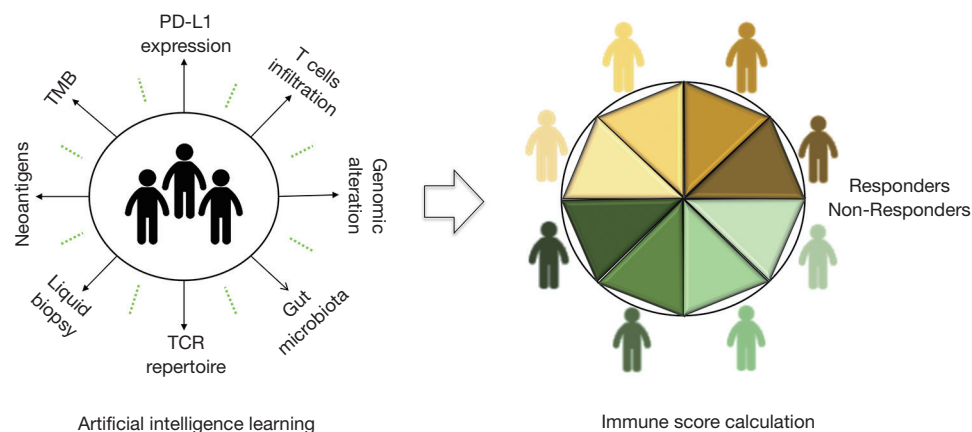
Tremendous progress has been made in checkpoints blockade. Current potential predictive markers have been mentioned above separately. However, the background of immune TME and the tumor-IC interaction are very complicated (35). To identify the determinants of tumor immunogenicity, increasing efforts have been made by incorporating all the immune-related biomarkers in TME and peripheral blood. Comprehensive analysis is increasingly performed, using genomic data and bioinformatics tools (71). Charoentong and his colleges analyzed the data from TCGA database and clinical trials with CPIs in 20 solid cancers and named the great project as The Cancer Immunome Atlas (TCIA). Combining the TILs, cancer antigenomes, tumor heterogeneity and immunophenotypes, the immunophenoscore was developed that could be a predictor of response to CPIs in melanoma (AUC =1.00, 95% CI: 0.99–1.00). In addition, they open the database to researchers for further data mining from the immunological insights, having successfully developed web-accessible resource (72). Using immuno-gram or immunoscore, researchers aim to better understand why some patients respond to immunotherapy and why some not,

ultimately aiming at improving anticancer therapy (73,74).

Karasaki *et al.* performed WES and RNA sequencing in 20 resected NSCLC patients. Three immune-gram patterns characterized by gene signatures were identified. However, there are no results on association between three immune-gram patterns with CPB efficacy (75). An increasing number of researches on machine learning emerged in different fields. Charoentong and his colleges using machine learning developed a superior predictor, termed as immunophenoscore, and verify the response to checkpoint antibodies in two independent validation cohorts. The interactive big data resource includes 28 TIL subpopulation, neoantigens, mutation load, and tumor heterogeneity. Authors illustrated a lot of associations suggesting important biological conclusions with implications for cancer immunotherapy. In this year, Zhang *et al.* published their results on the journal of *Nature Communication* which is the first report with multiple sequencing data from Chinese NSCLC patients (76). Racial differences between Chinese and Westerner could be greatly achieved by the comparing the genomic and immunological characteristics from surgical tissue and TCGA database. Although there are no data regarding the clinical efficacy of CPIs, this report provides theoretical basis for the development of future immunotherapy strategies.

Available data online provides a good opportunity for many scientists to do relevant immunological research. But the premise is that they must have a strong bioinformatic analysis team to interpret these huge and complicated data repositories and find the key determinants of favorable efficacy of CPIs in lung cancer patients.

In conclusion, PD-L1 expression by IHC testing is recommended for CPB treatment in first line and beyond line settings. With the advent of NGS, gene status could give a new sight for the guidance of immunotherapy. It should be cautious when patients with EGFR mutation, ALK rearrangement, or KRAS and STK11 or LKB1 co-mutation was treated with PD-1/PD-L1 blockade. TMB  $\geq 10$  Mut/Mb as a selected biomarker still lack sufficient evidence and further study need to confirm the predictive or prognostic role. Comprehensive analysis with multiple immune-related biomarkers is a promising research orientation on prediction of CPB (*Figure 1*). In the future, the role of liquid biopsy with CTC and exosomes, and gut microbiota on CPB prediction still need further exploration. Altogether, there is a long way for searching optimal biomarkers in anti-PD-1/PD-L1 blockade.



**Figure 1** Artificial intelligence learning is used to extract patients characteristics, such as PD-L1 expression, TMB, TILs infiltration, TCR repertoire and other immune-related factors; a final immune score will be given from machine learning to help oncologists identify the possibility that patients could benefit from immunotherapy with CPB, the yellow cartoon figures represent responders and green ones present non-responder to immunotherapy, different shades of yellow and green means heterogeneity in responders and non-responders. TMB, tumor mutation burden; TILs, tumor infiltrating lymphocytes; TCR, T-cell antigen receptor; CPB, checkpoint blockade.

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## Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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