Mechanistic insight of predictive biomarkers for antitumor PD-1/PD-L1 blockade: A paradigm shift towards immunome evaluation (Review)

WENJIE YOU[1-3], BIN SHANG[4,5], JIAN SUN[1-3], XUEQING LIU[6], LILI SU[1-3] and SHUJUAN JIANG[1-3]

[1]Department of Respiratory and Critical Care Medicine, Shandong Provincial Hospital, Cheeloo College of Medicine, Shandong University; [2]Department of Respiratory and Critical Care Medicine, Shandong Provincial Hospital Affiliated to Shandong First Medical University; [3]Shandong Key Laboratory of Infectious Respiratory Disease; [4]Department of Thoracic Surgery, Shandong Provincial Hospital, Cheeloo College of Medicine, Shandong University; [5]Department of Thoracic Surgery, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan, Shandong 250021; [6]Department of Respiratory and Critical Care Medicine, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, P.R. China

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Abstract. Checkpoint inhibitor-based immunotherapy has exhibited unprecedented success in the treatment of advanced-stage cancer in recent years. Several therapeutic antibodies targeting programmed death-1 (PD-1) or its ligand (PD-L1) have received regulatory approvals for the treatment of multiple malignancies, including melanoma, non-small cell lung cancer, kidney cancer and Hodgkin's lymphoma. However, a substantial proportion of patients still do not benefit from these agents, let alone the risk of immune-associated toxicities and financial burden. Therefore, it is imperative to identify valid predictive biomarkers which can help optimize the selection of patients. In this review, a mechanism-based interpretation of tumor PD-L1 expression and other candidate biomarkers of response to antitumor PD-1/PD-L1 blockade was provided, particularly for the tumor microenvironment-derived 'immunomes', and the challenges faced in their clinical use was addressed. Directions for future biomarker development and the potential of combined biomarker strategies were also proposed.

Correspondence to: Professor Shujuan Jiang or Professor Lili Su, Department of Respiratory and Critical Care Medicine, Shandong Provincial Hospital, Cheeloo College of Medicine, Shandong University, 324 Jingwu Road, Jinan, Shandong 250021, P.R. China
E-mail: docjiangshujuan@163.com
E-mail: sulili801@126.com

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Contents

1. Introduction

Cancer immunotherapy, which aims to foster the host immune response against cancer to obtain durable anticancer responses, has achieved marked success in the past decade (1). The programmed death-1 (PD-1)-PD ligand-1 (PD-L1) receptor-ligand pair is an important immune checkpoint pathway exploited by tumor cells to evade immune attack (2). Blockade of the PD-1/PD-L1 axis represents an effective form of cancer immunotherapy (3). To date, several anti-PD-1/PD-L1 antibodies have been designed and assessed in clinical trials for cancer treatment. Nivolumab and pembrolizumab are humanized, engineered anti-PD-1 monoantibodies that have demonstrated durable objective response and improved overall survival in patients with advanced melanoma or non-small cell lung cancer (NSCLC), supporting their approved applications in these cancer types (4-10). Nivolumab also exhibited marked therapeutic activity in patients with metastatic renal cell carcinoma (RCC) or relapsed/refractory Hodgkin's lymphoma (11,12). In addition to anti-PD-1 drugs, there are numerous agents targeting PD-L1 in clinical development at various phases. Atezolizumab, a monoclonal antibody against PD-L1, was approved for the treatment of metastatic bladder carcinoma and NSCLC, based on a prolonged overall survival compared with chemotherapy (13,14). Another recent phase Ia study revealed the potential antitumor activity of atezolizumab in metastatic RCC (15). Other anti-PD-L1 antibodies, such as

durvalumab and avelumab, have been launched for the treatment of advanced NSCLC, urothelial cancer and Merkel cell carcinoma.

Despite significant progress of PD-1/PD-L1-directed immunotherapy, the efficacy and safety profiles of these agents varies greatly across individual patients and among different tumor types. Not all patients respond to PD-1/PD-L1 blockade (5,8,9,15). Moreover, immune checkpoint inhibitors (ICIs) may have immune-associated adverse events and are usually costly (16,17). Thus, it is of utmost value to define predictive biomarkers of response, in order to optimize the application of anti-PD-1/PD-L1 ICIs. The detection of tumor-cell PD-L1 expression via immunohistochemistry (IHC) has been thus far the most widely studied biomarker for predicting response to anti-PD-1/PD-L1 immunotherapy. However, a variety of limitations have been found with the PD-L1 testing, such as different antibodies, different analysis systems and different cut-off criteria for positivity in previous clinical trials, and the heterogeneity of PD-L1 expression between serial sections of one tumor sample (18,19). While tumor PD-L1 expression is predictive of response to PD-1/PD-L1 inhibitors, a small fraction of PD-L1-negative patients can also benefit from PD-1/PD-L1 blockade (6,9,20,21). There is an urgent need to develop alternative biomarkers to identify patients who are most likely to respond. This review provides an overview of the mechanisms of action of the established PD-L1 testing and other evolving biomarkers to predict the response to anti-PD-1/PD-L1 therapies. The review also details the challenges faced by the application of predictive biomarkers, and proposes directions for future biomarker development and combined biomarker strategies. This review represents the latest evidence regarding biomarkers of response to PD-1/PD-L1 blockade for cancer treatment.

2. PD-1/PD-L1-maintained immune tolerance

Immune tolerance is considered one of the hallmarks of cancer that is exploited by tumor cells to evade immune surveillance and elimination (22) (Fig. 1). In general, antigen presenting cells (APCs) in lymphoid tissue can dispose and present mutant or non-mutant neoantigens to $CD4^+$ and $CD8^+$ T cells for priming via the interaction between major histocompatibility complex (MHC) II and T-cell receptor (TCR). The $CD4^+$ T helper cells also contribute to the priming of $CD8^+$ T cytotoxic cells via various cytokines. Both $CD4^+$ and $CD8^+$ T cells are subsequently activated by the APCs through B7.1/B7.2/CD28 co-stimulatory pathways, which trigger their proliferation, migration to tumor sites, secretion of inflammatory cytokines and cytotoxic activities, leading ultimately to tumor eradication (23). There are multiple mechanisms of immune tolerance in tumors, including the well-established B7.1/B7.2/cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and PD-L1/PD-L2/PD-1 checkpoint pathways (24). PD-1 is an inhibitory receptor found on activated T and B cells, natural killer cells and monocytes, while its main ligand, PD-L1, is expressed on tumor cells, dendritic cells, macrophages, stromal cells and activated T cells (25). Another ligand PD-L2 is mostly restricted to APCs, such as dendritic cells and monocytes (26). The mechanism of action is that PD-L1 results in the tyrosine phosphorylation of PD-1 cytoplasmic immunoreceptor tyrosine-based inhibition motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM), which recruit phosphatases, particularly Src homology region 2 domain-containing phosphatase-2 (SHP-2) (27). This leads to the dephosphorylation of TCR proximal signaling molecules, such as ZAP70, PKCθ and CD3δ, attenuating TCR and CD28 signals, which ultimately promotes T cell apoptosis, anergy and functional exhaustion (28).

3. Predictive biomarkers of response to anti-PD-1/PD-L1 therapy

PD-L1 expression. PD-L1 expression in tumors has been hypothesized to be associated with response to PD-1/PD-L1 blockade. In a phase I trial, Topalian *et al* firstly demonstrated an association between PD-L1 expression in tumor cells and objective response to nivolumab in multiple cancer types (16). They determined surface PD-L1 expression of pretreated tumor samples via IHC, with a cut-off value of 5% defined to be PD-L1-positive, and found that 9 out of 25 PD-L1-positive patients had an objective response to nivolumab, while none of the 17 PD-L1-negative patients had an objective response. The KEYNOTE-024 study revealed superior progression-free survival (PFS) and overall survival (OS) in a pembrolizumab treatment group vs. a platinum-doublet chemotherapy group in patients with advanced NSCLC and PD-L1 expression in at least 50% of tumor cells (29). Thus far, several clinical trials have been performed to compare the treatment efficiency of anti-PD-1/PD-L1 antibodies between PD-L1-positive and -negative tumors (6-11,17,21,30-43), which are summarized in Table SI. Despite different pretreatments and cut-off points to define PD-L1 positivity, these studies have largely supported a role for PD-L1 expression, either on tumor cells or on tumor-infiltrating immune cells, as a predictive biomarker of response to PD-1/PD-L1 blockade in a variety of tumors. Notably, by analyzing multiple tumor types, Taube *et al* determined that membranous PD-L1 expression by tumor cells and infiltrating immune cells was most abundant in melanoma, NSCLC and RCC; tumors that exhibit objective response to anti-PD-1 immunotherapy (44).

In addition to PD-L1 expression on tumor cells or tumor-infiltrating immune cells, other forms of PD-L1 can also predict response to anti-PD-1/PD-L1 therapy. A recent study by Chen *et al* revealed the presence of PD-L1 on the surface of exosomes released by melanoma cells (45). They found that a fold change in circulating exosomal PD-L1 >2.43 at weeks 3-6 was associated with an improved objective response rate (ORR), PFS and OS to pembrolizumab. Another study of NSCLC suggested that the baseline plasma soluble PD-L1 concentration, determined using the enzyme-linked immunosorbent assay method, was significantly associated with clinical benefit in nivolumab therapy (46). However, lower response rate and shorter OS were detected in patients with NSCLC and high plasma-soluble PD-L1 levels.

In numerous tumors, PD-L1 expression can be induced either via oncogenic drivers and transcriptional factors, or via cytokines produced by tumor-infiltrating immune cells (47). Thus, PD-L1 acts as a constitutive and adaptive immune resistance against antitumor immune responses. The predictive

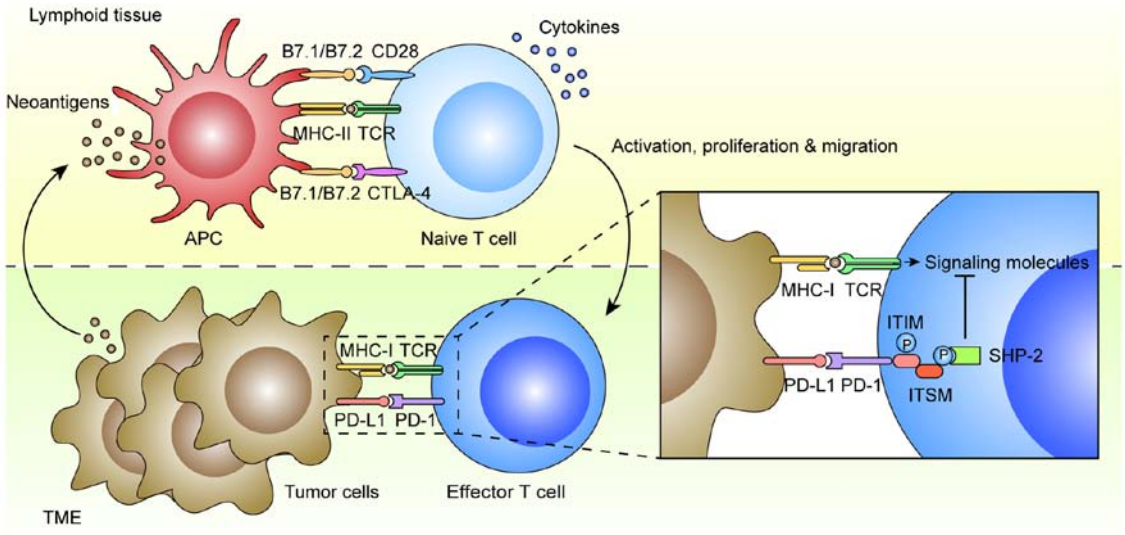

Figure 1. PD-1/PD-L1-maintains immune tolerance in tumors. In lymphoid tissue, APCs can dispose neoantigens, and then activate naive T cells through MHC-II/TCR interaction and B7.1/B7.2/CD28 co-stimulatory pathways. The $CD4^+$ T helper cells can also contribute to the priming of $CD8^+$ T cytotoxic cells via various cytokines. In early stages of T cell activation, the T-cell response can be downregulated by B7.1/B7.2/CTLA-4 checkpoint pathways. The effector T cells can proliferate and migrate to TME, leading to tumor eradication via MHC-I/TCR interaction. The PD-1/PD-L1 checkpoint pathway can maintain immune resistance of tumor cells to T-cell attack. The mechanism of action is that PD-L1 results in the tyrosine phosphorylation of PD-1 cytoplasmic ITIM and ITSM in effector T cells, which recruit phosphatases, particularly SHP-2. This leads to the dephosphorylation of TCR proximal signaling molecules, attenuating TCR and CD28 signals, which promotes T-cell apoptosis, anergy and functional exhaustion. PD-1, programmed death-1; PD-L1, PD ligand-1; APCs, antigen presenting cells; MHC, major histocompatibility complex; TCR, T cell receptor; CTLA-4, cytotoxic T lymphocyte-associated antigen-4; TME, tumor microenvironment; ITIM, immunoreceptor tyrosine-based inhibition motif; ITSM, immunoreceptor tyrosine-based switch motif; SHP-2, Src homology region 2 domain-containing phosphatase-2.

value of PD-L1 expression can be explained by the fact that inhibiting the PD-1/PD-L1 axis with therapeutic antibodies allows the host to overcome immune resistance and thereby activate the antitumor immunity.

Although the results suggest PD-L1 expression as a predictive biomarker, several clinical trials have repeatedly demonstrated that there is a small but definite proportion of PD-L1-negative patients who can also derive clinical benefit from PD-1/PD-L1 blockade (6,9,20,21). As summarized in Table SI, ORR to PD-1/PD-L1 antibodies in PD-L1-negative groups was revealed to be 20-40% in melanomas, 10-20% in NSCLC, and 5-20% in urothelial carcinomas. Brahmer *et al* even observed similar ORRs and survival outcomes between patients with PD-L1-positive and -negative squamous-cell NSCLC treated with second-line nivolumab, collectively revealing that there should be predictive biomarkers other than PD-L1 expression that can also determine the efficacy of PD-1/PD-L1 inhibitors (9). PD-L1 testing alone is insufficient for the selection of patients for anti-PD-1/PD-L1 immunotherapy. On the other hand, several studies indicated that anti-PD-L1 is somewhat less effective than anti-PD-1 therapy, which may be associated with slightly lower toxicity in cancer treatment (16,48). This discrepancy is potentially due to the mode of action, targeting the ligand vs. the receptor, between anti-PD-1 and anti-PD-L1 antibodies. Consistently, our data also revealed that anti-PD-1 therapy, but not anti-PD-L1, was effective against $FXR^{high}PD\text{-}L1^{low}$ mouse Lewis lung carcinoma (LLC) tumors. It speculated that the absence of targetable PD-L1 on tumor cells may be responsible for the ineffectiveness of anti-PD-L1 antibody (49). To date, no clinical trials have directly compared the treatment efficiency and toxicity between anti-PD-1 and anti-PD-L1 antibodies, particularly in PD-L1-low/negative patients.

Notably, the application of PD-L1 testing via IHC as a predictive biomarker is associated with several issues. Technically, different PD-L1 IHC antibodies with different analysis systems and different cut-off values for PD-L1 positivity were employed in early clinical trials (Table SI). The anti-PD-L1 28-2 clone and 22C3 clone were, thus far, the most prevalent antibodies for IHC. The common thresholds for PD-L1 positivity were 1, 5 and 10% in multiple cancer types (Table SI). Encouragingly, recent studies have compared three PD-L1 diagnostic assays (Dako 28-8, 22C3 and Ventana SP263), revealing preferable concordance rates at various cut-offs in resected NSCLC samples (50,51), and great efforts have been paid to develop a consensus for the use of PD-L1 IHC testing as a predictive biomarker for anti-PD-1/PD-L1 immunotherapy (52). Essentially, the expression of PD-L1 on tumor cells and infiltrating immune cells is dynamic. It has been discovered that PD-L1 expression levels can be influenced by various mechanisms, including the change in intracellular transcriptional factors or extracellular inflammatory cytokines, as well as antitumor therapies, such as radiation therapy, chemotherapy and targeted therapy (53-62). Recently, we detected an inverse correlation between FXR and PD-L1 expression in a cohort of NSCLC specimens (49). Our data demonstrated that FXR could directly bind to an FXR-responsive element in *PD-L1* promoter and repress its transcription, suggesting FXR as a novel transcriptional factor for the regulation of PD-L1. In general, contemporaneous tumor samples, obtained at the beginning of immune checkpoint blockade (ICB), should represent the PD-L1 expression status better compared with archival tumor samples. Another ineluctable variable is the heterogeneity

of PD-L1 expression, which exists both within the same tumor lesion, and between primary and metastatic lesions in the same patient. It has been reported that PD-L1 expression is widely heterogeneous within the tumor, which often accumulates at the tumor-immune interface (44). Takamori *et al* compared PD-L1 expression between lung metastases and corresponding primary tumors, including tumors in the rectum, colon, liver and bile duct. Although the proportion of PD-L1-positive tumor cells was not significantly different between lung metastases and primary tumors, PD-L1 expression on immune cells was significantly higher in lung metastases compared with the corresponding primary tumors (63). In this regard, tumor sampling aimed at a particular proportion of one tumor site may not accurately reflect on the PD-L1 status of that patient. Finally, factors enabling the prediction of response, such as tumor mutational burden (TMB) and the tumor microenvironment (TME), should be incorporated with PD-L1 IHC staining, in order to achieve a paradigm of precise anti-PD-1/PD-L1 immunotherapy.

Tumor mutational burden. Owing to advances in DNA sequencing techniques, a large amount of information on cancer genetics and genomics has been gained in the past few decades. There is increasing evidence that the TMB can predict response to ICIs, including anti-PD-1/PD-L1 drugs. The first indication was from the combined result, revealing that tumors with a high TMB (melanoma and NSCLC) often have a higher response rate to anti-PD-1 or anti-PD-L1 therapy across multiple tumor types (16,48,64-66). Moreover, within certain tumors, Rizvi *et al* revealed that patients with NSCLC and a high non-synonymous TMB exhibited durable clinical benefit to pembrolizumab, defined as a partial or stable response rate, for >6 months, compared with patients with less frequent non-synonymous mutations (67). Consistently, a pilot study of nivolumab in early-stage NSCLC revealed a significantly higher mean TMB in tumors with a major pathological response compared with tumors without a major pathological response (68). Recently, Wang *et al* reported that a higher blood TMB (bTMB), established by a 150-cancer gene panel of circulating tumor DNA, was significantly associated with superior PFS and ORR in patients with NSCLC treated with anti-PD-1 and anti-PD-L1 drugs (69), collectively suggesting the potential predictive value of TMB in antitumor PD-1/PD-L1 blockade.

Notably, recent evidence suggests that mismatch repair deficiency (MMRD) may be associated with an increased response to PD-1/PD-L1 blockade (70). Mismatch repair (MMR) is an intrinsic mechanism that can identify and correct errors in DNA replication and recombination, such as miss-incorporation deletions and base insertions (71). Mutations in MMR genes can produce log-fold increase of TMB, leading to the detectable DNA microsatellite instability (MSI) (23). It was estimated that tumors with a MMRD genotype possess 10 to 100-fold the mutational load of their MMR-proficient counterparts (23). Le *et al* have formally evaluated the predictive value of MMRD in patients with colorectal and non-colorectal cancer treated with pembrolizumab, and revealed that patients with MMRD colorectal cancer had a significantly improved ORR and PFS rate compared with those with MMR-proficient colorectal cancer (72). Moreover, it was demonstrated that the immune-associated ORR and PFS rate of pembrolizumab treatment were similar between MMRD colorectal cancers and MMRD non-colorectal cancers. Whole-exome sequencing revealed a mean of 1782 somatic mutations per tumor in MMRD tumors compared with 73 in MMR-proficient tumors; however, there were in total 41 cases in this phase II trial. Subsequently, the study was expanded to 86 patients of 12 tumor types with MMRD, which displayed an objective radiographic response rate of 53% and a complete response rate of 21% to anti-PD-1 therapy, although the median PFS and OS were not reached (73). Large clinical studies are required to verify the potential of MMRD or MSI in predicting the response to different PD-1/PD-L1 antibodies within different tumor types.

The association between TMB and response to anti-PD-1/PD-L1 drugs is considered to be primarily due to the generation of neoantigens, as a result of somatic mutations in tumor cells. The theory is that tumors with a high mutational load often possess more neoantigens, which can be recognized as non-self epitopes by the immune system, thereby enhancing T cell responses against tumors, as well as killing tumor cells when the PD-1/PD-L1 axis is blocked (74). It has been documented that the sensitivity to PD-1 blockade in advanced NSCLC and melanoma was increased in tumors enriched for clonal neoantigens (75). In parallel, active T cells against clonal neoantigens were detected in tumors with durable clinical benefits. Another study revealed a significant correlation between higher neoantigen burden and improved treatment efficacy in patients with NSCLC treated with pembrolizumab (67). In addition, the increased PD-L1 expression in the context of certain mutations is proposed as another determinant. A recent study demonstrated that the activating mutations in Janus kinase 3 (Jak3) promoted PD-L1 expression in lung cancer cells and the tumor immune microenvironment, thus contributing to the durable clinical benefit from anti-PD-L1 therapy (76). To date, a variety of oncogenic mutations, such as *EGFR*, *PTEN* and *ALK*, have been reported to be associated with PD-L1 upregulation in tumor cells, although further investigation is required to better define the role of PD-L1 in TMB-associated response to anti-PD-1/PD-L1 immunotherapy (77-79). In this respect, it is also reasonable to speculate that the somatic mutations in tumor cells would have a broad effect on the tumor immune microenvironment; specifically, those affecting other immune checkpoints, cytokines and chemokines that may also determine anti-PD-1/PD-L1 therapeutic response. Further studies are warranted for a comprehensive examination of host immune make-up in tumors with somatic mutations in comparison with others.

TMB is a promising predictive biomarker, albeit with its own limitations. Although a number of studies correlate TMB with tumor response to anti-PD-1/PD-L1 drugs, there has been thus far, seldom numerical cut-off value of TMB that was formally defined (16,39,48,66,68). Rizvi *et al* established a cut-off point of 178 non-synonymous mutations per sample to predict durable clinical benefit in a discovery cohort of NSCLC treated with pembrolizumab (67). In the validation set, this cut-off point yielded a clinical benefit rate of 75% in patients harboring at least 178 mutations, compared with 14% in patients with <178 mutations. Another clinical trial defined

≥10 mutations per megabase in DNA sequences of tumor cells as high TMB (34). Moreover, while somatic mutations in tumor cells may produce neoantigens that are prone to immune attack, not all neoantigens can elicit a T-cell response. A previous study has revealed that only 10% of non-synonymous point mutations of an MC38 mouse tumor model could generate peptides capable of binding to MHC-I with high affinity, and only a proportion of these peptides, rather than the overall peptide load, were necessary to elicit immunogenicity (80). Despite recent technological advances, for example, whole exome sequencing or computational algorithm, it is still challenging to identify meaningful neoantigens that are responsible for T-cell responses. In addition, intratumor mutation heterogeneity should be another obstacle for predicting response to PD-1/PD-L1 blockade. McGranahan *et al* detected considerable intratumor neoantigen heterogeneity within NSCLC tumors (75). It was demonstrated in the same study that decreased intratumor neoantigen heterogeneity was correlated with improved OS of patients with lung adenocarcinoma. Therefore, mutations that arise early and are shared by most cancer cells in an individual should elicit more potent antitumor T-cell responses compared with mutations that arise later or are limited to a fraction of cancer cells. Lastly, several studies revealed primary resistance to anti-PD-1/PD-L1 in tumors with specific mutations, for example *LKB1* loss, which may be ascribed to the impaired antitumor immune responses (81,82).

Tumor microenvironment. The TME consists of non-malignant stromal cells, such as cancer-associated fibroblasts (CAFs), bone marrow-derived cells and tumor-infiltrating immune cells, extracellular matrix, and the blood and lymphatic vascular networks (83). The tumor-infiltrating immune cells include macrophages, dendritic cells, natural killer cells, B cells, effector T helper cells, regulatory T cells (Treg) and cytotoxic T cells. The stromal cells aforementioned can release growth factors, matrix-degrading enzymes, cytokines and chemokines, responsible for either antitumor immune response or immunosuppressive response (84,85). In addition, both activating markers, such as MHC-II and CD86, and exhausting markers, such as PD-1, CTLA-4, LAG-3 and TIM-3, can be expressed in tumor-infiltrating immune cells, which collectively form a complex host of factors to either combat or promote tumor progression (86). Previous studies have revealed that the response of tumor cells to anti-PD-1/PD-L1 therapy is determined not only by intrinsic properties of tumor cells but also by cellular and molecular components of TME (87,88).

Firstly, the presence of tumor-infiltrating T cells was demonstrated to be associated with clinical benefit from anti-PD-1/PD-L1 therapy. Tumeh *et al* analyzed tumor samples from 46 patients with metastatic melanoma treated with pembrolizumab (89). Higher numbers of CD8-, PD-1- and PD-L1-expressing T cells were found at the invasive tumor margin and inside tumors in responding patients compared with non-responders. Ultimately, it was validated that the presence of $CD8^+$ T cells at the invasive tumor margin serves as a potential predictive biomarker to anti-PD-1 therapy in melanoma. Chen *et al* revealed a modest association between $CD8^+$, $CD3^+$ and $CD45RO^+$ T cells in pretreated tumor samples and responsiveness to PD-1 blockade in patients with advanced melanoma (90). Notably, this association became more significant after anti-PD-1 therapy. Another study on melanoma found that an increasing proportion of PD-1^{high}CTLA-4^{high} subset within tumor-infiltrating $CD8^+$ T cells strongly correlated with objective response to anti-PD-1 therapy (91). In contrast, PD-1^+ tumor-infiltrating T cells were significantly decreased in brain metastatic lesions compared with primary lung cancer, which was associated with a lower likelihood of objective response to anti-PD-1 in brain metastases (92). Consistently, our study revealed that aside from the downregulated PD-L1 expression, enforced FXR expression generated an immunosuppressive microenvironment in mouse LLC tumors, characterized by the inactivated and exhausted $CD8^+$ T cells (shown as decreased $TNF\alpha^+CD8^+$ T cells, as well as increased LAG-3 expression on $CD8^+$ T cells), which was correlated with significant tumor growth inhibition in FXR-overexpressed LLC tumors treated with anti-PD-1 antibody (49). These findings collectively suggested that pretreated tumor-infiltrating T cells, particularly for the exhausted $CD8^+$ T cells, can act as a promising predictive biomarker for PD-1/PD-L1 blockade (Fig. 2). This phenomenon can be logically ascribed to the pre-existing T cell-mediated antitumor immunity, which is restrained by the PD-1/PD-L1-induced suppression, but can be reactivated via PD-1/PD-L1 blockade. However, it was also proposed that intratumor T cells expressing multiple exhaustion markers may be irresponsive to anti-PD-1/PD-L1 drugs (93). Kim *et al* reported that VEGF-A could induce transcription factor TOX expression in T cells to drive exhaustion-specific transcription program, accounting for the resistance to PD-1 blockade in microsatellite-stable colorectal cancer (94). Another study even defined a threshold for PD-1 downregulation on tumor-infiltrating $CD8^+$ T cells, for which the release of adaptive immune resistance could be achieved via PD-1 blockade (95). It was revealed that the functionality of PD-1^{high} T cells in resistant tumors failed to be rescued by anti-PD-1 therapy. All these lend credence to the theory that the less exhausted tumor-infiltrating $CD8^+$ T cells, rather than the over-exhausted populations, are probably the determinants of response to anti-PD-1/PD-L1 immunotherapy. A future area of research should be to evaluate the predictive value of exhausted $CD8^+$ T cells, of various phenotypes within TME, in PD-1/PD-L1-based immunotherapy.

Secondly, the immunosuppressive cell populations in TME could restrain the response to PD-1/PD-L1 blockade (Fig. 2). It has been documented that tumor-infiltrating myeloid cells and Treg cells are partially responsible for the development of anti-PD-1 resistance in mouse colorectal and mammary cancer (95). Davis *et al* revealed that the functional inhibition of myeloid-derived suppressor cells (MDSCs) could enhance the objective response to anti-PD-L1 treatment in T cell-inflamed mouse tumor models of head and neck cancers (96). In a transgenic mouse model of neuroblastoma, Mao *et al* revealed that while checkpoint inhibitors were insufficient in controlling mouse neuroblastoma growth, combining suppressive myeloid cell inhibitor with anti-PD-1/PD-L1 antibodies resulted in superior tumor control (97). In this regard, the inhibition of immunosuppressive cell subsets within TME represents a potential predictive biomarker or rational approach to enhance antitumor PD-1/PD-L1 blockade.

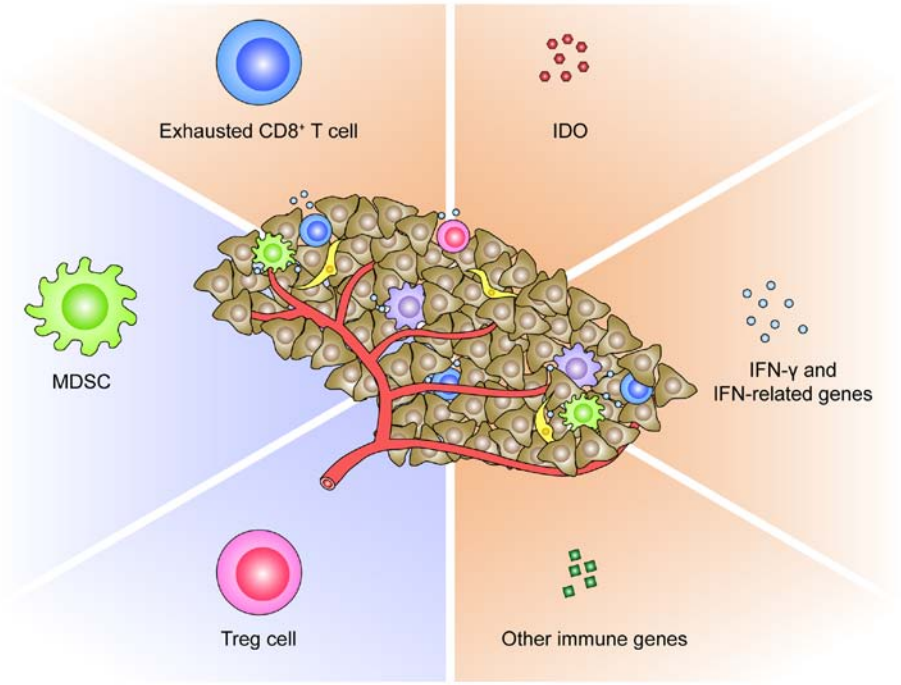

Figure 2. TME-derived predictive biomarkers for PD-1/PD-L1 blockade. The TME consists of non-malignant stromal cells (cancer-associated fibroblasts, MDSCs, effector T helper cells, cytotoxic T cells, Treg cells and macrophages), extracellular matrix, and the blood and lymphatic vascular networks. Those stromal cells can release growth factors, matrix-degrading enzymes, cytokines and chemokines. The components of TME, including exhausted CD8+ T cells, MDSCs, Treg cells, IDO, IFN-γ and IFN-related genes (CXCL9, CXCL11, and IFN receptor-associated Jak1 and Jak2), and other immune genes (BACH2 and CCL3), proposed as biomarkers of response to anti-PD-1/PD-L1 therapy were categorized. TME, tumor microenvironment; PD-1, programmed death-1; PD-L1, PD ligand-1; MDSCs, myeloid-derived suppressor cells; Treg cells, regulatory T cells; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; CXCL, C-X-C motif chemokine; Jak, Janus kinase; BACH2, BTB domain and CNC homolog 2; CCL, C-C motif chemokine.

Thirdly, the molecular profiles of TME pre- or post-anti-PD-1/PD-L1 immunotherapy represent alternative biomarkers of response (Fig. 2). Indoleamine 2,3-dioxygenase (IDO), a rate-limiting enzyme in the degradation of tryptophan via the kynurenine pathway, plays a critical role in suppressing T-cell immunity within tumors (98). A recent study revealed that MSI colorectal cancer overexpressing IDO were more responsive to anti-PD-1 treatment compared with microsatellite-stable cancer (99). The IFN-related genes are also relevant in patient selection for anti-PD-1/PD-L1 therapies. In a phase I/II study of durvalumab, an ORR of 33% was revealed in NSCLC patients positive for IFN-γ mRNA and 8% in those negative for IFN-γ mRNA (100). Consistently, in another study on atezolizumab, pretreatment melanoma samples from responding patients had increased expression of IFN-γ and IFN-related genes compared with non-responding samples (66). In contrast, Zaretsky *et al* revealed that defects in IFN receptor signaling pathways resulted in acquired resistance to anti-PD-1 therapy in melanoma (81). In addition, in a study characterizing the gene expression profiles of RCC treated with anti-PD-1 antibodies, immune genes such as *BACH2*, a regulator of $CD4^+$ T cell differentiation, and *CCL3* involved in leukocyte migration were revealed to be overexpressed in responding patients as compared with non-responders (101). Chen *et al* analyzed immune signatures in longitudinal tumor samples obtained at multiple time-points during anti-PD-1 therapies, and identified numerous genes to be differentially expressed between responders and non-responders (90). However, there is thus far no conclusive data on the predictive power of either IDO, or IFN-related genes, or other immune genes in TME for anti-PD-1/PD-L1 immunotherapy.

The close association between the aforementioned TME components and treatment efficacy in PD-1/PD-L1 blockade can be explained by the following theory. Immune recognition of tumors results in a host-immune response, which promotes tumor eradication through immune mechanisms, including antigen presentation, T cell priming and trafficking, cytokine production, cytotoxic activity and the expression of other immune genes. However, the antitumor Th1 and $CD8^+$ T cell responses are negatively regulated by PD-1/PD-L1-mediated adaptive immune resistance (89). Other immunosuppressive constituents also contribute to the adaptive immune resistance. This is supported by the combined result, revealing that the upregulation of PD-L1 and IDO in response to IFN-γ promotes the development of tumor-infiltrating myeloid cells and Treg cells, thereby facilitating tumor immune evasion (102-104). Response to anti-PD-1/PD-L1 immunotherapy occurs primarily in cancer patients with a T cell-inflamed but adaptive immune-resistant TME (105). In addition, the potential interactions between TME components and PD-L1 expression have partially been disclosed before. PD-L1 expression in tumors represents a negative feedback to IFN-γ released by tumor-infiltrating immune cells, including macrophages, dendritic cells, natural killer cells, B cells and effector T helper cells (56,106). Other immune factors within TME, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-12, IL-4, IL-10 and transforming growth

factor-β (TGF-β), have also been revealed to induce the expression of PD-L1 (57-59,107-109). In return, PD-L1 was documented to compromise the effector T-cell responses, promote the differentiation of induced Treg cells, as well as mediate the immunosuppressive activity of myeloid cells in tumors (89,102,103).

Traditionally, the immune profiles of tumors can be classified into three main phenotypes: The immune-inflamed phenotype, the immune-excluded phenotype and the immune-desert phenotype; based on whether tumors harbor an inflammatory TME or not (93). In this scenario, the immune-inflamed phenotype is postulated to correlate with a higher response rate to anti-PD-1/PD-L1 therapy. Recently, a new theory arose that according to both tumor-infiltrating lymphocytes (TILs) and PD-L1 expression in tumors, the TME can be stratified into four groups: $TILs^{-}PD\text{-}L1^{-}$, $TILs^{-}PD\text{-}L1^{+}$, $TILs^{+}PD\text{-}L1^{-}$ and $TILs^{+}PD\text{-}L1^{+}$ (88). While the $TILs^{-}PD\text{-}L1^{-}$ group may exhibit lack of response to anti-PD-1/PD-L1 antibodies, and the $TILs^{+}PD\text{-}L1^{-}$ group is irresponsive to anti-PD-L1, the $TILs^{+}PD\text{-}L1^{+}$ group would expect the best response to anti-PD-1/PD-L1 treatments. An alternative classification of the TME immune types provided by Kondou *et al* was determined by the expression level of the PD-L1 and CD8b genes (110). The aforementioned stratifications should enable the selection of optimal treatment strategies for patients with cancer. More recently, the effects of TME on PD-L1 expression have attracted much attention, particularly for those with a pre-existing T cell-inflamed phenotype. The adaptive $CD8^{+}$ T cells, CAFs, M2-like macrophages, and corresponding cytokines were revealed to engage in the dynamic change of PD-L1 within local tumors (106,108,111). These findings support the predictive value of TME-derived cellular and molecular elements as a whole population, rather than in individuals, for anti-PD-1/PD-L1 immunotherapy. With the discovery of more determinants for ICB in the past decade, more attention is expected to be devoted to evaluating a spectrum of 'immunome' before anti-PD-1/PD-L1 therapies.

4. Future biomarker considerations

Despite the known predictive biomarkers, such as PD-L1 expression, TMB and TME profiles, additional research is still necessary to explore other reliable candidate biomarkers for predicting the response of patients to anti-PD-1/PD-L1 therapies. In fact, emerging data have indicated future directions for biomarker development.

Immunogenic cell death. Immunogenic cell death (ICD) in tumors has been implicated in the response to PD-1/PD-L1 blockade. Recently, Zhao *et al* reported that irreversible electroporation could induce ICD in pancreatic ductal adenocarcinomas, which activated dendritic cells and alleviated stroma-induced immunosuppression (112). The combination of irreversible electroporation and anti-PD-1 resulted in a significantly longer median survival in mouse orthotopic pancreatic ductal adenocarcinoma models compared with mice administered either treatment alone. Another study revealed that the combination of cisplatin and high-dose crizotinib induced ICD in NSCLC cells, which synergized with anti-PD-1 to induce a superior cure rate and long-term survival in mouse orthotopic NSCLC models (113). It is considered that the dying tumor cells may function as tumor vaccines to stimulate antitumor immune responses during ICD.

TCR clonality. The diverse T-cell repertoire, corresponding to different antigenic peptides bounding to class I or II MHC, is generated by random recombination of discrete TCR-αβ gene segments (114). Tumeh *et al* analyzed tumor samples from 46 patients with metastatic melanoma obtained before and during anti-PD-1 therapy (89). It was revealed that patients who met the criteria for radiographic response had more than 10 times as many TCR clones expansion after pembrolizumab treatment, suggesting that the TCR clonality may represent a promising on-treatment predictive biomarker for anti-PD-1 therapies. The predictive value of TCR clonality during anti-PD-1 can be interpreted as an enrichment of a diverse T-cell repertoire, which will eventually be tailored as antigenic peptides on MHC interaction with TCR, reflecting an activated immune response against tumor cells (115).

Gut microbiome. Recently, intensive studies have been conducted to dissect the impact of the gut microbiome on response to anti-PD-1/PD-L1 immunotherapy in human malignancies, including melanoma, hepatocellular carcinoma, gastric cancer and NSCLC (116-119). It has been acknowledged that patients responding to PD-1/PD-L1 blockade often harbor higher diversity of gut microbiome compared with non-responders (116,118,119). This effect can be partially attributed to the ability of the gut microbiome to activate the host innate immune responses (120). In addition, subsequent studies revealed a clearer association between an intact commensal bacterial community and robust antitumor T-cell responses (121,122). Other studies revealed that interventions to modulate gut bacterial profiles exhibited great promise for improving the therapeutic responses (121,123). Dong *et al* reported that diosgenin, a natural steroidal saponin, could modulate the composition of the intestinal microbiome in melanoma-bearing mice, thereby enhancing the growth-inhibitory effect of anti-PD-1 antibody (123). Another study revealed that oral administration of *Bifidobacterium* collaborated with anti-PD-L1 therapy to control tumor growth in mouse melanoma models (121). Although promising, the favorable bacterial species that improve antitumor PD-1/PD-L1 blockade remain to be determined.

Peripheral blood biomarkers. The evaluation of peripheral blood could be another interesting approach. It has been reported by Weide *et al* that relative eosinophil count (≥1.5%), relative lymphocyte count (≥17.5%), LDH level (≤2.5-fold elevation), and the absence of metastases other than soft-tissue/lung were confirmed as independent baseline characteristics associated with favorable OS in patients with melanoma treated with pembrolizumab (124). Another study reported that low neutrophil to lymphocyte ratio and absolute neutrophil count during treatment was correlated with superior objective response and treatment duration in patients with advanced NSCLC cured by PD-1/PD-L1 inhibitors (125). Similarly, data from a study on patients with advanced/metastatic melanoma treated with nivolumab or pembrolizumab revealed that patients with

Table I. Predictive biomarkers for antitumor PD-1/PD-L1 blockade.

Biomarkers	Details of clinical use	Tumors	Clinical outcomes	(Refs.)
PD-L1	IHC analysis of tumor cells, tumor-infiltrating immune cells, or both	NSCLC, melanoma, RCC, squamous cell carcinoma of the head and neck, and urothelial carcinoma	ORR, PFS, OS, DoR and DCR	(6-11,16,17,21,29-44)
	ELISA examination of circulating exosomal PD-L1	Melanoma	ORR, PFS and OS	(45)
	ELISA examination of plasma soluble PD-L1	NSCLC	ORR and OS.	(46)
TMB	Whole-exome sequencing to identify somatic non-synonymous mutations in tumor or plasma samples	Melanoma and NSCLC	ORR, PFS and pathologic response	(16,29,64,65,67-69)
MMRD	PCR analysis of microsatellite sequences or IHC analysis of MMR proteins	Colorectal, ampulla of water, chol-angiocarcinoma, endometrial, pancreas, gastroesophegeal, neuroendocrine, osteosarcoma, prostate, small intestine and thyroid cancer	ORR, PFS and radiographic response	(72,73)
TME				
Tumor-infiltrating T cells	Multiplex IHC, immunofluorescence or flow cytometric analysis of tumor samples	Melanoma, lung cancer, colorect-al cancer and mammary cancer	ORR, radiographic response and PFS	(49,89-92,94,95)
Tumor-infiltrating myeloid cells and Treg cells	Flow cytometric analysis of tumor samples	Colorectal cancer and mammary cancer	ORR	(95)
IDO	RT-PCR analysis of tumor-infiltrating T cells	Colorectal cancer	ORR	(99)
IFN-γ and IFN-related genes	Fluidigm BioMark HD RT-PCR platform to detect IFN-γ and IFN-related genes in tumor samples	NSCLC and melanoma	ORR and PFS	(66,100)
Other immune genes	Whole genome microarray, multiplex quantitative RT-PCR or NanoString platform to analyze gene expression in tumor samples	RCC and melanoma	ORR and radiographic response	(90,101)
ICD	Flow cytometry, ELISA, ATPLite bioluminescence and Fluorescence microscopy to detect ICD markers	Pancreatic cancer, melanoma and NSCLC	OS, radiographic response and cure rate	(112,113)
TCR clonality	Next generation sequencing of TCRβ CDR3 region	Melanoma	ORR and radiographic response	(89)
Gut microbiome	16S ribosome RNA gene sequencing, metagenomic sequencing or exon sequencing to evaluate gut bacterial in fecal samples	Hepatocellular carcinoma, gastric cancer, melanoma and NSCLC	ORR, radiographic response and PFS	(116-119,121,123)

Table I. Continued.

Biomarkers	Details of clinical use	Tumors	Clinical outcomes	(Refs.)
Peripheral blood biomarkers	Peripheral blood routine and biochemical examination, and flow cytometric examination	Melanoma and NSCLC	ORR, DCR, DoR, PFS and OS	(21,124-127)
Imaging biomarkers	Positron-emission tomography imaging with radio-labeled antibodies or radiomic feature analysis of targeted molecule	Lung, urothelial, kidney, gynaeco-logical, liver, breast, colorectal, head and neck, gastric, oesopha-geal, thyroid, prostate cancer, mel-anoma, sarcoma and lymphoma	ORR, PFS and OS	(129,130)

PD-L1, PD ligand-1; IHC, immunohistochemistry; NSCLC, non-small cell lung cancer; RCC, renal cell carcinoma; ELISA, enzyme-linked immunosorbent assay; TMB, tumor mutational burden; MMR, mismatch repair; MMRD, mismatch repair deficiency; PCR, polymerase chain reaction; RT-PCR, real-time polymerase chain reaction; TME, tumor microenvironment; Treg cells, regulatory T cells; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; ICD, immunogenic cell death; TCR, T cell receptor; ORR, objective response rate; PFS, progression-free survival; OS, overall survival; DoR, duration of response; DCR, disease control rate including CR, PR, SD ≥6 weeks; CR, complete response; PR, partial response; SD, stable disease.

an increased baseline LDH had a significantly shorter OS compared with those with normal LDH. Furthermore, patients with a relative increase of >10% from elevated baseline LDH during treatment had a significantly shorter OS compared with those with ≤10% change (126). Other parameters, such as peripheral Treg cells, antigen-specific $CD8^+$ T cells and MDSCs, were also revealed to be associated with response to nivolumab in patients with melanoma (21,127). These factors may be associated with adaptive immune resistance, which can be overcome when the PD-1/PD-L1 axis is blocked, although the detailed mechanisms need to be elucidated in future. In clinical practice, these peripheral blood biomarkers have the advantage of being readily assessable, and are suitable for serial sampling during treatment.

Imaging biomarkers. Owing to advances in technology, medical imaging can not only assess macroscopic features, but also characterize the cellular and molecular properties of malignant lesions, which may serve as a novel approach to select patients for PD-1/PD-L1 blockade. It has recently been revealed that PD-L1 and PD-1 expression in NSCLC could be quantified non-invasively with PET-CT imaging using the radiotracers ^{18}F-BMS-986192 and ^{89}Zr-nivolumab, respectively (128). Bensch *et al* conducted another first-in-human study characterizing the biodistribution of zirconium-89-labeled atezolizumab via PET within 22 patients across three tumor types (129). Notably, they found that zirconium-89-labeled atezolizumab tumor uptake was positively correlated with ORR, PFS and OS of enrolled patients treated with atezolizumab. TILs can also be traced by medical imaging. In a retrospective study by Sun *et al* a radiomic signature that included eight variables was established for assessing tumor-infiltrating $CD8^+$ T cells in solid cancer types. Their data revealed that a high baseline radiomic score was associated with a higher proportion of patients who had an objective response at 3 or 6 months, as well as associated with improved OS either in univariate or in multivariate analysis (130). These imaging biomarkers were designed to detect and monitor antitumor immune activities within tumors, thereby predicting response to PD-1/PD-L1 blockade. Although just the beginning, image-driven biomarkers have the unique advantage of being non-invasive, which should make them promising candidates to aid future anti-PD-1/PD-L1 immunotherapy.

5. Conclusions

The clinical use, applicable tumors, as well as predicted clinical outcomes of each biomarker discussed in this review are summarized in Table I. ICIs, particularly PD-1/PD-L1-targeted antibodies, are proven to be effective in a variety of cancer types. The establishment of reliable predictive biomarkers to ensure the rational use of these agents is crucial, given the reality that only a subset of patients can benefit from PD-1/PD-L1 blockade, and that treatment with these agents carries a risk of immune-associated toxicities and substantial financial burden (5,8,9,15). Conversely, based on the result of multiple validated biomarker assays, even patients who are stratified as non-responders for anti-PD-1/PD-L1 monotherapy may be treated with other

antitumor regimens or combined treatment strategies to maximize clinical outcomes (131).

Combined biomarker strategies may enrich responders to anti-PD-1/PD-L1 immunotherapy in future. Factors enabling response prediction, such as PD-L1 IHC testing, TMB and TME profiles, should be incorporated together, since it has been indicated that high tumor PD-L1 expression does not equate to a T cell-inflamed content, and that high TMB does not always indicate pre-existing antitumor immune activities (132,133). In fact, evidence has been increasingly showing that combining data from tumor immune profiling, including CD3, CD8, FoxP3, CD163, PD-L1, PD-1 and CTLA-4, could be more ideal approaches to predicting response to PD-1/PD-L1 blockade, although the optimal model has yet to be determined (91,134).

Overall, anti-PD-1/PD-L1 immunotherapy is one of the predominant methods for cancer treatment. The improvement in the understanding of the interactions among multiple factors, as well as the dynamic changes of certain variables within different tumor types will certainly help identify more reliable and effective predictors for PD-1/PD-L1 blockade, thereby paving the way for a framework that allows treatment decisions to be made on a personalized basis.

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Authors' contributions

LS and SJ devised the conceptual idea. WY, BS and JS performed the literature search. WY prepared the figures. WY, XL, LS and SJ wrote, reviewed and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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