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Biomarkers of immunotherapy in non-small cell lung cancer (Review)

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Abstract. Immunotherapy has markedly improved the survival rate of patients with non-small cell lung cancer (NSCLC) and has introduced a new era in lung cancer treatment. However, not all patients with lung cancer benefit from checkpoint blockade, and some suffer from notable immunotoxicities. Thus, it is crucial to identify potential biomarkers suitable for screening the population that may benefit from immunotherapy. Based on the current clinical trials, the aim of the present study was to review the biomarkers for immune checkpoint inhibition, as well as other effective, invalid and hyperprogression markers that may have the potential to better predict responders to immunotherapy among patients with NSCLC. All these biomarkers may be incorporated into the predictive utility of bio-score systems and decision-making algorithms, to better guide the application of immunotherapy in the clinical setting.

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Abbreviations: APC, antigen-presenting cell; CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; IFN-γ, interferon γ; NK, natural killer; PD-1, programmed death 1; PD-L1, programmed death ligand 1; TIL, tumor-infiltrating lymphocytes; TMB, tumor mutational burden

Key words: biomarkers, immunotherapy, non-small cell lung cancer, immune checkpoint inhibition

Contents

1. Introduction

Lung cancer ranks first in morbidity and mortality rate among malignant tumors in both the United States and worldwide (1). Lung cancer is subdivided into two major categories: Non-small cell lung cancer (NSCLC), which accounts for 80-85% of all lung cancer cases, and small-cell lung cancer (2). The prognosis for patients with stage IV NSCLC is extremely poor, with reported 5-year overall survival (OS) rate of 1 to 8% in the United States in 2018 (3). Platinum-based chemotherapy has historically been the standard first-line treatment for metastatic NSCLC, although responses to these agents only range between 15-30%, with a relatively short interval until disease progression (4,5). More recently, immunotherapy has emerged as a promising treatment alternative for patients without an actionable driver mutation and has markedly altered the therapeutic approach to advanced NSCLC (6). It has been demonstrated that with immunotherapy, the 5-year survival rate of patients with advanced NSCLC increased from 4.7 to 16% in the United States in 2018 (3). A recent clinical trial also indicated that nivolumab treatment improved long-term OS rate and achieved durable responses in a proportion of patients with pretreated advanced NSCLC (3). Unlike traditional therapies for NSCLC, immune therapies exploit the host immune system to monitor and destroy cancer cells via the upregulation of key immune checkpoints; at present, NSCLC immunotherapy mainly refers to immune checkpoint inhibitors (ICIs) and anti-programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) agents (7,8).

Patients with metastatic NSCLC generally benefit from immunotherapy; however, a number of patients may not respond to therapy, exhibit a shorter lifetime with treatment

or suffer major life-threatening immunotoxicities (9,10). Immune therapies lack specific biomarkers compared with the precision of targeting genes due to the complex interactions between tumors and the immune system. Furthermore, the response to immunotherapy also varies according to the tumor characteristics (9,10). Apart from immune-associated adverse events, such as dermatitis, enteritis and hepatitis, some patients may experience clinical manifestations such as 'pseudoprogression' (PP) or 'hyperprogressive' disease (HPD) (11). PP is connected with infiltrations of active T cells and other immune cells within the lesion (12), whereas HPD is defined as a rapid increase in tumor growth rate (minimum two-fold) compared with the expected growth rate (13).

Considering both the advantages and disadvantages of immunotherapy, it is only suitable for a small number of patients (14). Moreover, indiscriminate application may significantly increase the incidence of adverse reactions. Therefore, it is crucial to establish biomarkers predictive of the response of patients with NSCLC to immunotherapy. As a result, studying host-tumor interactions and identifying predictive biomarkers for the response to immune checkpoint blockade treatment is essential for enhancing the efficacy of immunotherapy agents. In the present article, the currently approved biomarkers for immune checkpoint inhibition in NSCLC are reviewed, and the emerging effective, invalid and HPD markers are highlighted. In addition, the identification of biomarkers that predict treatment responses, and the development of rational therapeutic combinations that could enhance the efficacy of immune checkpoint blockade, are discussed.

2. Overview of immunotherapy in non-small cell lung cancer

As the understanding of lung cancer has increased, the concept of treatment has also changed. Traditional treatments for lung cancer have some insurmountable barriers. For example, molecular targeting drugs may not be effective when genomic testing reveals no targetable alteration, such as epidermal growth factor receptor (EGFR) mutations, anaplastic lymphoma kinase (ALK) or ROS1 translocation/re-arrangements (15). As traditional methods have been shown as ineffective against certain cancer types, the focus has shifted to immune cells, and immunotherapy for lung cancer is attracting increasing attention. In particular, reactivating immune cells to clear cancer cells and stopping cancer cell immune evasion are focuses of this research (16).

Immunotherapy is a process of continuous responses by activating the body's immune system to attack and kill tumor cells. First, the antitumor immune response is enhanced and prolonged by persistent recognition and memory of tumor antigens (16). Subsequently, certain cytotoxic T cells differentiate into natural memory T cells, which can provide long-term immune memory protection, even in the absence of the original antigen stimulation (16). Therefore, immunotherapy is more likely to achieve long-term survival compared with conventional treatments (17).

The immunotherapy of lung cancer is subdivided into active and passive immunotherapy. The former enhances the antitumor effect of the body by activating the patient's own immune response, and mainly includes vaccines and immunoregulatory agents. The latter provides patients with products such as anti-PD-1 and anti-PD-L1 antibodies of the immune response to enhance the body's antitumor response, mainly through adoptive cellular immunotherapy (18). When it comes to the most successful immunotherapy, ICIs have been attracting increasing attention. Immune checkpoints are important inhibitory pathways for controlling the duration and magnitude of the immune response. Tumors can use these pathways to resist immune responses. ICIs have the ability to interfere with tumor resistance and enhance the body's immune response to tumor cells, including first-generation anti-cytotoxic T lymphocyte antigen 4 (CTLA-4) antibodies, second-generation anti-PD-1 antibodies and anti-PD-L1 antibodies (7). Second-generation ICIs are more selective and safer compared with first-generation ICIs (19).

CTLA-4 checkpoint inhibitors. Normally, activation of T lymphocytes requires the joint activation of two signaling pathways: The binding of the T cell receptor to the major histocompatibility complex-antigen peptide complex presented by antigen-presenting cells (APCs), and the binding of the B7 molecule (B7-1 or B7-2) to CD28 on the surface of T cells (20). CTLA-4 is expressed exclusively on the surface of T cells, where it has a higher affinity to B7 than CD28, and exerts the opposite function compared with CD28 (21). CTLA-4 competes with the costimulatory receptor CD28 for binding to the same ligands, resulting in downregulation of immune response (22). A previous study found that CTLA-4 was abnormally highly expressed on the surface of tumor-infiltrating T regulatory cells (Tregs), and its expression level in lymph nodes and tumor cells was significantly higher compared with that of peripheral Tregs and effector T cells (23). Tregs, a subgroup of $CD4^+$ T cells with notable immunosuppressive effects, can inhibit the immune response of other cells. This inhibitory function of Tregs depends heavily on CTLA-4. Thus, anti-CTLA-4 agents binding to CTLA-4 molecules can lead to Treg depletion or functional blockade, thereby contributing to T cell activation and immunological responses in cancer (24). Currently, there are two main types of antibodies targeting CTLA-4: Ipilimumab and tremelimumab (23,24). Ipilimumab has been approved by the U.S. Food and Drug Administration (FDA) as the first ICI for advanced melanoma based on several clinical trials, despite poor results in lung cancer (25). A randomized phase III trial demonstrated that ipilimumab in combination with chemotherapy did not markedly improve OS compared with chemotherapy alone in the first-line treatment for patients with advanced squamous NSCLC (26).

PD-1/PD-L1 checkpoint inhibitors. PD-1, also known as CD279, is a monomeric glycoprotein and a member of the CD28 superfamily. It is expressed on the surface of activated T cells, B cells, natural killer (NK) cells, dendritic cells and macrophages (27). PD-L1 is a ligand of PD-1 that is highly expressed in various tumor cells, such as lung cancer, malignant brain tumor and melanoma cells (28). The upregulation of PD-1 ligands in the tumor microenvironment and the connection of PD-1 to its ligands on tumor-specific T cells are the key mechanisms of escaping immune elimination (27-32). For example, PD-1 expressed on $CD4^+$ and $CD8^+$ T cells, as well as other immune cells, interacts with PD-L1. This leads to decreased activation, proliferation, survival, persistence and

effector functions of T lymphocytes, induces antigen-specific T cell apoptosis and modulates the activity of $CD4^+$ and $CD8^+$ T cells, NK cells and macrophages, thereby affecting cancer progression *in vitro* and *in vivo* (29-31). PD-L1 can be recognized by T cells, resulting in the release of cytokines, which not only attract other cytotoxic immune cells, but can also induce the expression of the checkpoints that promote immune resistance, including the metabolic reprogramming, differentiation characteristics and promotion of homeostatic proliferation of T cells (32-34).

In recent years, a growing body of evidence has shown the efficacy of PD-1/PD-L1, particularly in tumor immunotherapy. To date, the FDA has approved four immunosuppressive agents for NSCLC: Two anti-PD-1 (nivolumab and pembrolizumab) and two anti-PD-L1 (atezolizumab and durvalumab) agents. Four clinical trials (CheckMate-017, CheckMate-057, KEYNOTE-010 and OAK) have confirmed that the immunotherapy group had different benefits in terms of efficacy and survival in the second-line treatment setting of NSCLC compared with the chemotherapy group (14,35,36). Moreover, a phase III trial (KEYNOTE-024) revealed that progression-free survival (PFS) or OS with first-line pembrolizumab treatment for NSCLC expressing PD-L1 with a tumor proportion score ≥50%, were superior to those with first-line standard platinum-based chemotherapy (37). Based on results of KEYNOTE-042, in 2019 the FDA approved pembrolizumab as first-line treatment for patients with PD-L1 expression ≥1%, EGFR mutation-negative and ALK-negative advanced NSCLC (38-40). An interim analysis from the LCMC3 multicenter study at the 2019 World Conference on Lung Cancer confirmed that atezolizumab achieved over half of the pathological remissions in 49% of the patients with NSCLC on neoadjuvant therapy, suggesting the great potential of immunotherapy in the neoadjuvant setting for lung cancer (41). The clinical studies of IMpower130 and KEYNOTE-189 reported that the PFS or OS of lung cancer were significantly improved with the synergistic effect of immunotherapy combined with chemotherapy (42,43). The PACIFIC study demonstrated that durvalumab conferred OS benefits to patients with unresectable stage III NSCLC after chemoradiotherapy (44). IMpower150 indicated that immunotherapy combined with antiangiogenic agents and chemotherapy improved survival rates as a first-line treatment for advanced non-squamous NSCLC (45). The addition of atezolizumab to bevacizumab plus chemotherapy significantly improved PFS and OS among patients with metastatic non-squamous NSCLC, regardless of PD-L1 expression and EGFR or ALK genetic alteration status (45).

The mechanism of action of the CTLA-4 and PD-1 antibodies differs (46,47). A number of experiments have confirmed that the two antibody types enhance the antitumor effect through complementary mechanisms, and the combined treatment of dual immune drugs has also achieved promising results (48,49). The Checkmate-227 study first demonstrated that nivolumab combined with ipilimumab conferred a significant PFS and reduced the side effects as first-line treatment for patients with advanced NSCLC compared with chemotherapy (50).

Currently, pembrolizumab, nivolumab and atezolizumab, which target the PD-1/PD-L1 axis, are associated with a significant improvement in OS and durable antitumor responses in advanced NSCLC (37,51-53). For unresectable stage III NSCLC, the PACIFIC trial established durvalumab as a new standard for consolidation therapy, which involves continuous maintenance therapy in patients with stable disease and follow-up treatment (44). The preliminary data from several ongoing trials evaluating immunotherapy in the treatment of early and locally advanced lung cancer are promising. However, whether utilizing immune therapy in patients with early-stage NSCLC will improve survival remains uncertain.

For the aforementioned reasons, studying host-tumor interactions and establishing biomarkers to predict response to immune checkpoint inhibition are crucial steps towards using the new panel of immunotherapy agents in the most effective manner (Fig. 1). The aim of the present study was to review the biomarkers for immune checkpoint inhibition, as well as other effective, invalid and HPD markers that may have the potential to better predict responders to immunotherapy in NSCLC (Table I).

3. Predictors of immunotherapy

Predictors of effective immunotherapy. The predictive value of PD-L1 and tumor mutational burden (TMB) in lung cancer has been tested in several clinical trials (54). Facing the challenge of adaptability and dynamic changes of the immune system, combined application of biomarkers and dynamic monitoring are expected to become a popular trend of immunotherapeutic research in the future. In addition, in order to overcome the limitations of tissue sample testing, new test methods are emerging (55).

PD-L1 expression levels. The expression of PD-L1 may be a better predictive biomarker that exhibits a stronger association with the antitumor response compared with PD-1 (56). The results of KEYNOTE-024 suggested that patients with advanced NSCLC with high PD-L1 expression (≥50%) had a superior OS with pembrolizumab compared with chemotherapy (37). Data from KEYNOTE-042 indicated that the efficacy of immunotherapy was comparable to that of chemotherapy when patient expression of PD-L1 was 1-49%. Therefore, the higher the expression of PD-L1, the better the treatment effect of immunotherapy in NSCLC (38).

CheckMate017 and OAK reported that the expression levels of PD-L1 in tumor cells may not be a suitable biomarker for predicting the efficacy of immunotherapy (35,52). This may be because certain signaling pathways promote the malignant behavior of the cancer cell, such as EGFR, mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase-protein kinase (PI3K-AKT). Conversely, inflammatory response cytokines, particularly interferon (IFN)-γ, induce and stimulate PD-L1 expression in tumor cells and other types of cells in the immune microenvironment (57). In addition, different detection platforms and evaluation systems have different positive critical values, and there is no consistent standard to measure the expression of PD-L1 in tumor cells (58). Therefore, diverse biopsy sites, primary lesions, metastatic lesions and early treatment may affect the dynamic change in PD-L1 expression, and a single biopsy cannot reflect the whole picture of the tumor.

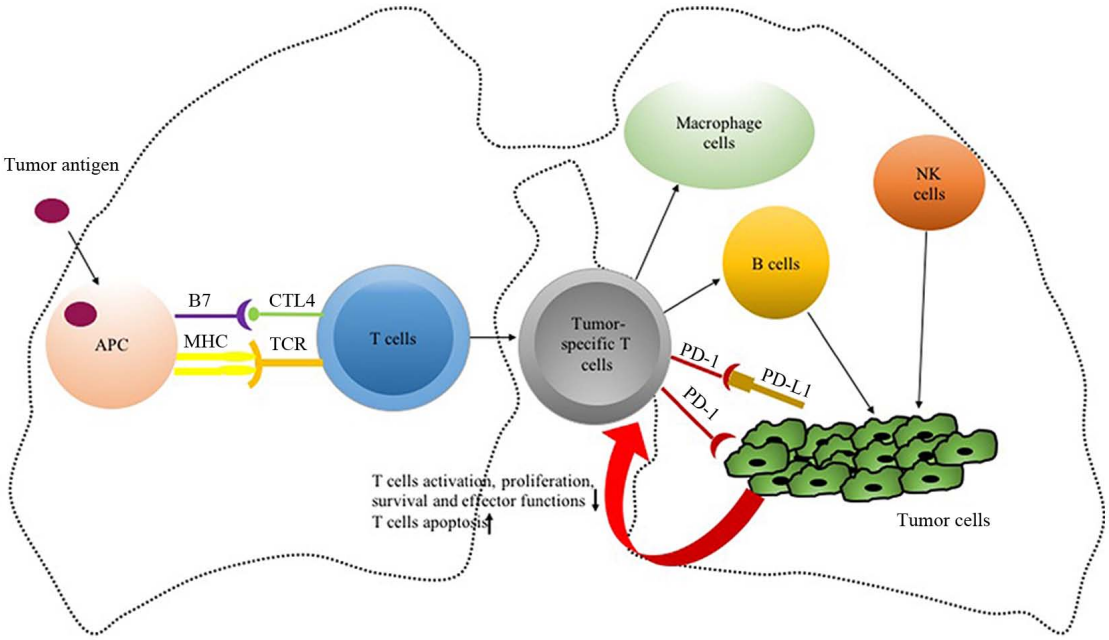

Figure 1. Host-tumor interactions for immunotherapy. APC, antigen-presenting cell; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; MHC, major histocompatibility complex; NK cells, natural killer cells; PD-1, programmed death 1; PD-L1, programmed death ligand 1; TCR, T cell receptor; TILs, tumor-infiltrating lymphocytes.

The expression of PD-L1 may be a suboptimal marker for predicting the therapeutic efficacy of NSCLC immunotherapy, but PD-L1 is currently the most established and widely used biomarker for the clinical immunotherapy of NSCLC (14,35,37, 40,42,43-45,51-53,59,60).

TMB and neoantigen burden. TMB is defined as the total number of mutations, including replacement and insertion/deletion, per Megabyte of exonic regions of the evaluated genes in the tumor specimen (mut/Mb). Mutations in somatic cells can be transcribed/expressed into RNA/protein levels, producing neoantigens, protein fragments or peptides; these new products are recognized by the immune system as non-autoantigens, activating T cells and eliciting an immune response (61). Tumors are attacked by a large number of tumor-specific T cells in patients with a high TMB (TMB-H) (61). The response to anti-PD-1/PD-L1 therapy depends on the numbers of tumor-specific T cells (61). Therefore, tumors with TMB-H are more sensitive to anti-PD-1/PD-L1 treatment, suggesting that TMB and neoantigen burden may be considered as therapeutic biomarkers of immunotherapy (62).

A previous study summarized the association between TMB level and the effective rate of anti-PD-1 therapy in 27 different types of tumors, and demonstrated that the level of TMB was different among diverse tumors (63). Among those treated with anti-PD-1/PD-L1 inhibitors, the objective response rate (ORR) of each tumor type was positively correlated with the level of TMB. The higher the level of TMB expression, the greater the therapeutic effect of PD-1/PD-L1 inhibitors. Previous studies have also reported that patients with TMB-H have a high response rate to anti-PD-1/PD-L1 immunotherapy (61-64). CheckMate026 revealed that the ORR of nivolumab was significantly higher (47 vs. 28%) and the PFS was markedly prolonged (9.7 vs. 5.8 months) in the TMB-H arm compared with that of platinum-based chemotherapy (59). CheckMate227 reported that the PFS of patients with TMB-H (≥10 mut/Mb) treated with nivolumab and ipilimumab was superior to that of chemotherapy (43 vs. 13%, respectively) (64). Surprisingly, in the same clinical trial, PFS was improved in CheckMate 227 regardless of PD-L1 expression. CheckMate012 and CheckMate026 also observed no notable correlation between TMB and PD-L1 expression, indicating that TMB was an independent marker of immunotherapeutic response (65). Another study demonstrated that PD-L1 levels combined with TMB could better predict the efficacy of immunotherapy (66); compared with patients with both low expression of PD-L1 and TMB-L, the clinical benefit rate among those with high expression of PD-L1 and TMB-H was 50% (66). A similar conclusion was reached by CheckMate026 (59).

The POPLAR study analyzed the association between blood TMB (bTMB) and clinical benefit. For bTMB ≥10, ≥16 and ≥20, patients treated with atezolizumab had an increased PFS and OS compared with docetaxel, and the greatest benefits were obtained when bTMB ≥16 (53). The OAK study further verified that atezolizumab was associated with a PFS benefit, with a hazard ratio of 0.65 vs. 0.98 for bTMB ≥16 vs. <16, respectively (35).

Overall, TMB is considered as a good predictor in immunotherapy. As an emerging biomarker, TMB may be used to screen patients who may benefit from anti-PD-1/PD-L1 immunotherapy.

Tumor-infiltrating lymphocytes (TILs). As the function of PD-1/PD-L1 inhibitors also requires the involvement of lymphocytes near the tumor, the abundance of TILs may also be used as a biomarker to predict the efficacy of PD-1/PD-L1 inhibitors (67). In NSCLC, an abundance of TILs in primary tumor tissue has been associated with a more favorable

Table I. Efficacy outcomes of key trials of programmed cell death protein 1 and/or programmed death-ligand 1 inhibitors in non-small cell lung cancer.

Author, year	Trial	Phase	Population	Study interventions	ORR (%)	Median PFS, months (HR; 95% CI)	Median OS, months (HR; 95% CI)	Potential biomarkers	(Refs.)
West *et al*, 2019	IMpower130	III	Stage IV non-squamous NSCLC	First-line atezolizumab (1,200 mg) plus chemotherapy vs. chemotherapy	49.2 vs. 31.9	7.0 vs. 5.5 (0.64; 0.54-0.77; P<0.0001)	18.6 vs. 13.9 (0.79; 0.64-0.98; P=0.033)	PD-L1 (all benefit); EGFR/ALK (wild-type)	(43)
Socinski *et al*, 2018	IMpower150	III	Metastatic non-squamous NSCLC	First-line atezolizumab plus BCP vs. bevacizumab plus carboplatin plus paclitaxel (BCP)	63.5 vs. 48.0	8.3 vs. 6.8 (0.62; 0.52-0.74; P<0.001)	19.2 vs. 14.7 (0.78; 0.64-0.96; P=0.02)	PD-L1 (all benefit); EGFR (wild-type)	(45)
Reck *et al*, 2016	KEYNOTE-024	III	Stage IV NSCLC	First-line pembrolizumab (200 mg) vs. platinum-doublet chemotherapy, plus pemetrexed maintenance as appropriate	27.3 vs. 26.5	10.3 vs. 6.0 (0.50; 0.37-0.68; P<0.001)	NR (0.60; 0.41-0.89; P=0.005)	PD-L1 (TPS ≥50%); EGFR/ALK (wild-type)	(37)
Mok *et al*, 2019	KEYNOTE-042	III	Locally advanced, metastatic NSCLC	First-line pembrolizumab (200 mg) vs. platinum-doublet chemotherapy, plus pemetrexed maintenance as appropriate	27.3 vs. 26.5	5.4 vs. 6.5 (1.07; 0.94-1.21)	16.7 vs. 12.1 (0.81; 0.71-0.93; P=0.0018)	PD-L1 (TPS ≥1%)	(40)
Gandhi *et al*, 2018	KEYNOTE-189	III	Advanced non-squamous NSCLC	First-line pembrolizumab (200 mg) plus pemetrexed and a platinum-based drug vs. pemetrexed and a platinum-based drug	63.5 vs. 48.0	8.8 vs. 4.9 (0.52; 0.43-0.64; P<0.001)	NR vs. 11.3 (0.49; 0.38-0.64; P<0.001)	PD-L1 (TPS ≥50%)	(42)
Carbone *et al*, 2017	CheckMate026	III	Stage IV, recurrent NSCLC	First-line nivolumab (3 mg/kg) vs. platinum-doublet chemotherapy plus pemetrexed maintenance as appropriate	26 vs. 33	4.2 vs. 5.9 (1.15; 0.91-1.45; P=0.25)	14.4 vs. 13.2 (1.02; 0.80-1.30)	PD-L1 (≥5%); TMB-H	(59)

Table I. Continued.

Author, year	Trial	Phase	Population	Study interventions	ORR (%)	Median PFS, months (HR; 95% CI)	Median OS, months (HR; 95% CI)	Potential biomarkers	(Refs.)
Brahmer *et al*, 2015	CheckMate017	III	Stage IIIB, IV squamous NSCLC	Second-line nivolumab (3 mg/kg) vs. docetaxel	20 vs. 9	3.5 vs. 2.8 (0.62; 0.47-0.81; P<0.001)	9.2 vs. 6.0 (0.59; 0.44-0.79; P<0.001)	PD-L1 (all benefit)	(52)
Borghaei *et al*, 2015	CheckMate057	III	Stage IIIB, IV, recurrent non-squamous NSCLC	Second-line nivolumab (3 mg/kg) vs. docetaxel	19 vs. 12	2.3 vs. 4.2 (0.92; 0.77-1.11; P=0.39)	12.2 vs. 9.4 (0.73; 0.59-0.89; P=0.002)	PD-L1 (all benefit); EGFR (wild-type); KRAS (mutation)	(51)
Herbst *et al*, 2016	KEYNOTE-010	III	Advanced NSCLC	Second-line pembrolizumab (2 mg/kg and 10 mg/kg groups) vs. docetaxel	18 and 18 vs. 9	3.9 (0.88; 0.74-1.05, P=0.07) and 4.0 (0.79; 0.66-0.94; P=0.004) vs. 4.0	10.4 (0.71; 0.58-0.88; P=0.0008) and 12.7 (0.61; 0.49-0.75; P<0.0001) vs. 8.5	PD-L1 (TPS ≥1%); EGFR (wild-type)	(14)
Antonia *et al*, 2018	PACIFIC	II	Stage III, unresectable NSCLC	Second-line durvalumab (10 mg/kg) vs. placebo after concurrent chemoradiotherpy	30.0 vs. 17.8	17.2 vs. 5.6 (0.51; 0.41-0.63)	28.3 vs. 16.2 (0.53; 0.41-0.68)	PD-L1 (all benefit); EGFR (wild-type)	(44)
Rittmeyer *et al*, 2017	OAK	III	Locally advanced, metastatic NSCLC	Second-line or third-line Atezolizumab vs. docetaxel	14 vs. 13	2.8 vs. 4.0 (0.95; 0.82-1.10)	13.8 vs. 9.6 (0.73; 0.62-0.87; P=0.0003)	PD-L1 (all benefit); bTMB-H; EGFR (wild-type)	(35)
Fehrenbacher *et al*, 2016	POPLAR	III	Locally advanced, metastatic NSCLC	Second-line or third-line atezolizumab vs. docetaxel	15 vs. 15	2.7 vs. 3.0 (0.94; 0.72-1.23)	12.6 vs. 9.7 (0.73; 0.53-0.99; P=0.04)	PD-L1 (all benefit); bTMB-H	(53)
Garon *et al*, 2015	KEYNOTE-001	I	Locally advanced, metastatic NSCLC	Pembrolizumab 2 or 10 mg/kg (in multiple lines of treatment)	18 and 18 vs. 9	3.7 (NA)[a]	12 (NA)[a]	PD-L1 (≥1%); TMB-H; CD8+TILs	(60)

[a]Outcome reported for entire patient population, across all doses, PD-L1 expression levels and lines of treatment. HR, hazard ratio; CI, confidence interval; NA, not available; NR, not reached; NSCLC, non-small cell lung cancer; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; PD-1, programmed cell death 1; PD-L1, programmed cell death 1 ligand 1; TMB-H, high tumor mutational burden; bTMB, blood tumor mutation burden; TILs, tumor-infiltrating lymphocytes; EGFR/ALK, epidermal growth factor receptor/ALK tyrosine kinase receptor; KRAS, kirsten rat sarcoma viral oncogene homolog; TPS, tumor proportion score.

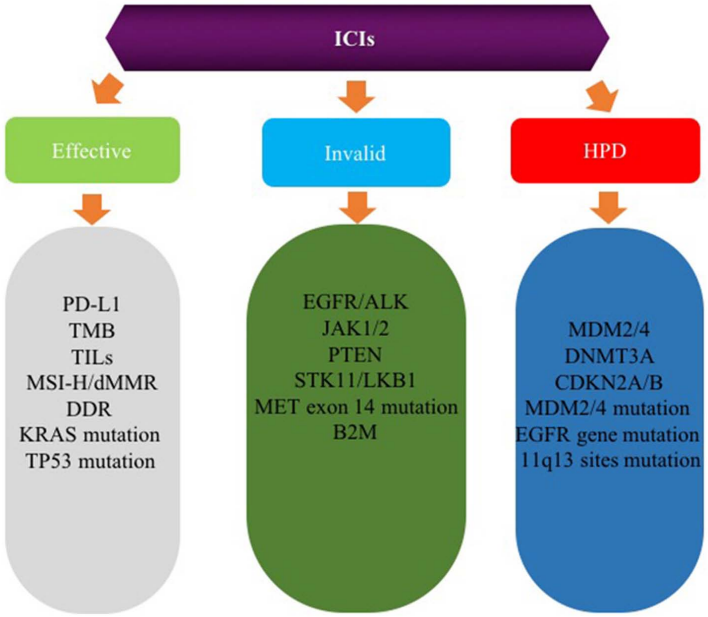

Figure 2. Overview of biomarkers for immune checkpoint inhibition in non-small cell lung cancer. ICIs, immune checkpoint inhibitors; PD-L1, programmed death ligand 1; TMB, tumor mutational burden; TILs, tumor-infiltrating lymphocytes; MSI-H, microsatellite instability, high; dMMR, DNA mismatched repair; DDR, DNA damage repair; KRAS, kirsten rat sarcoma viral oncogene homolog; TP53, tumor protein p53; EGFR/ALK, epidermal growth factor receptor/ALK tyrosine kinase receptor; JAK1/2, Janus kinase 1/2; PTEN, phosphate and tension homology deleted on chromosome ten; STK11, serine/threonine kinase 11; LKB1, Lkb1 kinase; MET, MET proto-oncogene receptor tyrosine kinase; B2M, β-2-microglobulin; MDM2/4, MDM2/4 proto-oncogene; DNMT3A, DNA methyltransferase 3 α; CDKN2A/B, cyclin dependent kinase inhibitor 2A/2B.

prognosis (67). TILs, particularly infiltration by $CD8^+$ T cells, often indicates a good response to immunotherapy and a favorable prognosis (68,69). It was previously demonstrated that patients with metastatic melanoma with high numbers of $CD8^+$ T cells in tumor tissues and tumor margins are more responsive to immunotherapy compared with conventional cytotoxic chemotherapy (12). The proliferation of $CD8^+$ T cells has been directly associated with the shrinkage of tumors on imaging after ICI treatment (70). In KEYNOTE-001, the number of $CD8^+$ T lymphocytes in the tumor parenchyma and margins of the baseline biopsy specimen of patients with effective pembrolizumab treatment were found to be higher compared with those with disease progression (60).

Recently, Sun *et al* (71) developed a radiological signature for $CD8^+$ T cells, which was validated using the gene expression signature of $CD8^+$ T cells. The imaging biomarker could estimate the $CD8^+$ T cell count and inferred the clinical outcome of patients treated with immunotherapy.

The immune microenvironment of PD-L1 and TILs is divided into four states as follows (72): I (TIL^+/PD-$L1^+$), II (TIL^-/PD-$L1^-$), III (TIL^+/PD-$L1^-$) And IV (TIL^-/PD-$L1^+$). Based on this method, the association between PD-L1 and $CD8^+$ TIL density in patients with stage III NSCLC receiving concurrent chemoradiotherapy was evaluated (73). The PFS in patients with type I, II, III and IV was 17.6, 13.1, not reached (NR) and 8.6 months (P=0.02), respectively, and the OS was 35.3, 36.9, NR and 13.9 months (P=0.11), respectively. The results demonstrate that the PFS and OS are longer in patients with high numbers of $CD8^+$ TILs, and suggests that the abundance of TILs may be used as a biomarker for immunotherapy.

Microsatellite instability (MSI)-H/mismatched repair-deficient (MMR). DNA MMR (dMMR) is an important replication error avoidance mechanism that prevents mutation and is essential for maintenance of genetic information, since it repairs polymerase errors during replication and prevents recombination between closely related sequences (74). MSI is a form of genomic instability due to reduced fidelity during the replication of repetitive DNA (74). The highly unstable state of microsatellites is referred to as MSI-H or dMMR, which is easily recognized by the immune system (74).

It was demonstrated that patients with MSI-H/dMMR are more likely to benefit from immunotherapy (75,76). Pembrolizumab has been approved by the FDA for use in MSI-H/dMMR-positive solid tumors that are unresectable or metastatic in patients who receive no other treatments (77). In addition, nivolumab and pembrolizumab were considered as alternative second- or third-line treatments for dMMR/MSI-H metastatic colorectal cancer (mCRC) in the 2017 NCCN guidelines (78). The CheckMate142 study confirmed that mCRC with MSI-H/dMMR treated with nivolumab and pembrolizumab had an increased immune response compared with nivolumab monotherapy (79). Moreover, nivolumab has been approved for mCRC with MSI-H/dMMR following unsuccessful standard chemotherapy (80). Thus, MSI-H/dMMR has emerged as another immunotherapy-related biomarker for screening the subpopulation of patients that are likely to benefit from immunotherapy.

Others. Genes and signaling pathways associated with DNA damage repair (DDR) in tumor cells may lead to genomic instability. A previous study suggested that the mutation status of DDR was correlated with the level of TMB, and that patients with co-mutation may benefit more from immunotherapy (81). A retrospective analysis also found that the PFS and OS of patients with metastatic urothelial carcinoma with DDR

mutations were significantly improved with anti-PD-1/PD-L1 antibody treatment (82).

KRAS mutations are found in 15-20% of patients with NSCLC, particularly in smokers with lung adenocarcinoma (83). KRAS mutations are implicated in tumor formation, proliferation, migration, diffusion and angiogenesis (84). A retrospective study demonstrated that patients with KRAS mutations exhibited low expression of PD-L1 and a high somatic mutation load (85), whereas others also suggested that KRAS was positively correlated with PD-L1 expression, while it did not regulate PD-L1 expression (86). Notably, CheckMate-057 confirmed that patients with KRAS mutations benefited more from nivolumab compared with those without KRAS mutations (51). The BIRCH study also reported that patients with advanced NSCLC with KRAS mutations receiving atezolizumab had better outcomes compared with those with wild-type KRAS (87).

It has been described that the mutation rate of TP53 was 39-46% in adenocarcinomas, 81% in squamous cell carcinomas and 68% in large-cell carcinomas (88). Dong *et al* (89) reported that mutation of TP53 or KRAS increased the expression of PD-L1 and infiltration by $CD8^+$ T cells. DNA polymerase deltacatalytic subunit gene 1 (POLD1), DNA polymerase epsilon catalytic subunit (POLE), Breast cancer susceptibility gene 1 (BRCA1), Breast cancer susceptibility gene 2 (BRCA2), the catalytic subunit of the DNA-activated protein kinase (PRK-DC), DNA ligase 3 (LIG3), RAD17 checkpoint clamp loader component (RAD17), RAD51 paralog C (RAD51C), FA complementation group F (FANCF), endonuclease non-catalytic subunit of ERCC excision repair 1 (ERCC1) and other rare genetic changes associated with equilibrium and repair of functional proteins in the process of DNA replication also affect the efficacy of immunotherapy. These mutations lead to an increase in the load of non-synonymous mutations and the number of TILs, making patients more sensitive to immunotherapy (90,91). Anti-PD-1 antibodies have been reported to be highly effective in endometrial, bowel and lung cancer patients harboring the POLE mutation (92-94).

Predictors of invalid immunotherapy. Molecular targeted therapy has been found to prolong the OS and PFS of patients with advanced NSCLC; however, it is difficult to effectively treat this type of cancer due to the instability of the driver genes (95). Interactions between the tumor driver gene pathway and the PD-1/PD-L1 pathway have been demonstrated previously (14,35,37,43-45,51). In addition to driver gene mutations, factors in immunotherapy-resistant pathways appear to be involved (96,97). For example, it has been demonstrated that IFN-γ is able to recognize the corresponding receptors on tumor cells or APCs (98), and that mutations and deletions of the IFN-γ receptor chains, such as Janus kinase (JAK)1 and JAK2, STATs and INF regulatory factor 1, lead to resistance to ICIs. Moreover, multiple mechanisms may stimulate high expression of PD-L1, including phosphatase and tensin homolog (PTEN) deletion or PI3K/AKT mutation, EGFR mutation, MYC overexpression, CDK5 gene fragmentation and 3'-untranslated region truncation of PD-L1 (99,100). It remains unknown whether the high expression of PD-L1 affects the response to ICIs, but it may indeed lead to a lack of therapeutic response in antitumor immunotherapies by inhibiting the activation of antitumor T cells (99,100).

EGFR and ALK mutation. Based on the findings of KEYNOTE-024, the FDA approved pembrolizumab for initial treatment of NSCLC with high PD-L1 expression (≥50%) and EGFR/ALK mutation-negative, which accounts for approximately one-third of these cancer types. In the phase II trial of KEYNOTE-001, 64% (seven out of 11) of patients were positive for EGFR mutations (exons 19 and 21), and 73% (eight out of 11) of patients exhibited high PD-L1 expression (60). Among patients with NSCLC, the rate of effectiveness of anti-PD-1 treatment was almost zero. In the IMpower150 study, patients with advanced NSCLC with EGFR mutations did not benefit from the combination of PD-L1 and chemotherapy (45). Similar results were reported by phase III of the KEYNOTE-010, CheckMate057 and OAK trials (14,35,51). Multiple clinical trials and retrospective studies have demonstrated that the ORR of patients with ALK mutations treated with anti-PD1/PD-L1 inhibitors is lower compared with that of patients with the wild-type (14,35,45,51,69). The reasons for the poor therapeutic effect of anti-PD-1/PD-L1 agents in patients with EGFR/ALK mutation is that these patients may have a lower proportion of $PD\text{-}L1^+/CD8^+$ cells, as well as having a non-inflammatory phenotype and weak immunogenicity (101).

Phase III clinical trials have confirmed that NSCLC with EGFR mutations exhibited a lower response to ICIs; however, ICIs were found to be effective against some patients with NSCLC with EGFR mutations (98-101). Hastings *et al* (102) found that EGFR mutations in different alleles may affect the response to immunosuppressive agents. In addition, smoking may be associated with the opposite result. Another study demonstrated that the effectiveness of anti-PD-1 therapy, regardless of EGFR mutations, was >20% in patients with NSCLC who were smokers (103). The latest ATLANTIC trial confirmed that patients with EGFR mutations and PD-L1 expression ≥25% may benefit from durvalumab (104). The efficacy of durvalumab was the lowest in patients with NSCLC with low expression of PD-L1 and $EGFR^+/ALK^+$ [only one of 128 patients (4%) reached OR].

Recently, Su *et al* (105) published a retrospective study on the association between the expression of PD-L1 in patients with NSCLC with EGFR mutations and the therapeutic effects of EGFR-TKI. Compared with the low or no expression of PD-L1, high expression of PD-L1 was associated with a worse ORR (35.7 vs. 63.2 vs. 67.3%, respectively; P=0.002) and PFS (3.8 vs. 6.0 vs. 9.5 months, respectively; P<0.001). In addition, PD-L1 expression and the proportion of $PD\text{-}L1^+$ and $CD8^+$ T cells in patients with primary resistance to EGFR-TKIs were higher compared with those with acquired EGFR-TKI resistance. In conclusion, the higher the expression of PD-L1 in patients with EGFR mutations, the poorer the efficacy of EGFR-TKIs. Therefore, patients with primary resistance to EGFR-TKIs might benefit from PD-1 immunotherapy.

JAK1/2 mutations. Upon tumor antigen recognition, T cells produce IFN-γ, which leads to the expression of IFN-stimulated genes through the IFN-γ receptors, including JAK1 and JAK2, and also activates STAT signaling (106). JAK1/2 mutations have

been found to be associated with loss of PD-L1 expression upon IFN-γ exposure mediated by disabling the receptor signaling pathway (106,107). Shin *et al* (107) reported two cases with JAK1/2 loss-of-function mutations and lack of reactive PD-L1 expression. Therefore, patients with JAK1/2 mutations may not be not suitable candidates for PD-1 blockade therapy (108).

PTEN deletion. As a tumor suppressor gene, *PTEN* serves a regulatory role in some of the key cell processes in tumor proliferation. The deletion of *PTEN* increases the activity of the PI3K-AKT signaling pathway in various types of tumors (109,110). In a study on melanoma, *PTEN* deletion decreased the effectiveness of anti-PD-1 inhibitors through upregulation of the expression of tumor immunosuppressive factors, suggesting that the absence of *PTEN* is associated with resistance to ICIs (96,111). The melanoma database of The Cancer Genome Atlas (http://cancergenome.nih.gov) shows that the deletion of *PTEN* is significantly correlated with the downregulation of IFN-γ and the infiltration of granzyme B and $CD8^+$ T lymphocytes. In addition, it shows that the deletion and mutation of the *PTEN* gene occurs more frequently in tumors without T cell inflammatory infiltration (112). However, the role of the *PTEN* gene in immunotherapy of lung cancer remains unclear and requires further investigation.

Serine/threonine kinase (STK)11 deletion and LKB1 kinase mutation. Immunotherapy may not be effective in patients with a *STK11* gene deletion due to the lower PD-L1 expression in this population (113). Dong *et al* (89) demonstrated that patients with co-mutation of KRAS/TP53 had the greatest clinical benefit (ORR 30%) compared with those with co-mutation of KRAS/STK11, suggesting that *STK11* deletion may be one of the main reasons for primary resistance to ICIs in patients with KRAS mutations in lung adenocarcinoma. Skoulidis *et al* (114) reported that patients with KRAS-mutated lung adenocarcinoma (7.4 vs. 35.7 vs. 28.6%; P<0.001) and those treated with nivolumab (0 vs. 57.1 vs. 18.2%; P=0.047) differed significantly in ORR. Therefore, STK11/LKB1 mutation may be the main driver of primary resistance to PD-1 inhibitors in KRAS-mutated lung adenocarcinoma.

Others. The incidence of MET proto-oncogene receptor tyrosine kinase (*MET)* exon 14 mutations in patients with NSCLC is 2-4%. Patients usually have a smoking history and high expression of PD-L1, inducing more prominent tumor immune cell infiltration. In contrast, low levels of TMB have been found to be associated with worse response to immunotherapy (115).

β-2-microglobulin (*B2M*) was found to be inactivated in ~5% of all primary NSCLCs and SCLCs, and the presence of mutations was strongly correlated with the loss of the human leukocyte antigen-I complex, which strengthens the specificity of cytotoxic T lymphocyte activation against tumor cells (116). *B2M* loss-of-function mutations are also involved in the acquisition of resistance to ICI treatment, as was first described in patients with melanoma (117) and later in a patient with NSCLC who progressed to anti-PD-1 (118).

Predictors of 'toxic' immunotherapy. HPD, also known as the 'toxic' response, may occur in targeted therapy and chemotherapy; however, the incidence of HPD after immunotherapy is significantly increased to >29%, including 10-16% in patients with NSCLC compared with those that did not receive immunotherapy (119). Patients with HPD have a poor overall prognosis, with an OS of only 3-4 months (120). In early 2017, HPD occurred in ~9% of patients treated with ICIs and 19% of the patients were aged >65 years (121). In addition, immunotherapy-induced HPD was not correlated with tumor load, tumor type, number of treatment lines or PD-L1 expression level, but was associated with advanced age (>65 years) and poor OS (121). HPD is principally observed with PD-1/PD-L1 inhibitors; however, there is no significant difference between PD-1 inhibitors and PD-L1 inhibitors in the occurrence of HPD (121). In the clinical setting, patients with lung cancer with driver gene mutations have higher rates of HPD. A retrospective study found that >500 patients with eight common lung cancer gene mutations had a high incidence of HPD in all the mutations after using PD-1/PD-L1 alone. Among these mutations, the incidence of *EGFR*, *ALK* and *RET* mutations were 44.8, 45.5 and 43.8%, respectively (122). In regard to the factors and mechanisms of HPD, studies have demonstrated that certain clinical characteristics are associated with HPD, such as age >65 years, number of baseline metastatic sites >2 or local recurrence, although these characteristics have inconsistent results in different studies and are not sufficient as predictors (13,119,123,124). A review summarized the five biological mechanisms by which PD-1/PD-L1 inhibitors may cause HPD; four of those formed an immunosuppressive microenvironment to facilitate the immune escape of tumor cells and indirectly accelerate tumor growth, while one directly promoted tumor cell proliferation through the activation of oncogenes (125). Gene variation has been associated with HPD; however, basic experiments and further investigation, including studies about mouse double minute (*MDM*)2/4 amplification, DNA methyltransferase 3 α (*DNMT3A*) mutation and cyclin dependent kinase inhibitor 2A/2B (*CDKN2A/B*) deletion, are required.

MDM2/4 amplification. MDM2 is a critical negative regulator of p53, and plays a key role in controlling its transcriptional activity, protein stability and nuclear localization (126). MDM2 expression is upregulated in numerous cancer types, resulting in a loss of p53-dependent functions, apoptosis and cell cycle arrest (126). Previous studies have demonstrated that the MDM2 protein has low expression levels in normal tissues, and that amplification of the *MDM2* gene may lead to tumorigenesis (126). Kato *et al* (121) reported that 67% (four out of six) patients with *MDM2/4* gene amplification-induced HPD and that the clinical symptoms of the other two patients rapidly deteriorated, suggesting that *MDM2/4* gene amplification may be associated with HPD. In another article published in 2018, Kato *et al* (127) further explored the amplification status of *MDM2* after immunotherapy in various cancer types. *MDM2* amplification accounted for 3.5% (3,650 cases) of the tumors, among which 99.0% (3,613 cases) had genomic co-mutations, and the most common co-mutated genes were *CDK4* (43.6%), fibroblast growth factor receptor substrate 2 (40.8%), *TP53* (20.1%) and *CDKN2A* (18.2%). Various pathways, including those associated with tyrosine kinase (37.9%; 1,385/3,650), PI3K signaling (25.4%; 926/3,650), TP53 (24.9%; 910/3,650) and MAPK signaling (23.6%; 863/3,650) were involved. In

addition, *MDM2* amplifications were less frequently associated with TMB-H compared with the *MDM2* wild-type population (2.9 vs. 6.5%, respectively; P<0.001).

DNMT3A mutation and CDKN2A/B deletion. Kato *et al* (121) demonstrated that *DNMT3A* gene mutation was closely associated with immunotherapy-related HPD. It was further confirmed that *CDKN2A/B* deletion and *MDM2* mutation were strongly correlated with HPD after immunotherapy at the 2019 American Society of Clinical Oncology Meeting (abstract no. e20628) (128). Tumor growth was >50% after treatment with pembrolizumab in advanced NSCLC. *MDM2* gene amplification was observed in all patients, and deletion of *CDKN2A/B* was reported in four patients. Moreover, none of the patients with non-HPD had amplification of the MDM2 gene or protein.

Others. Kato *et al* (121) revealed that *MDM2/4* (66%), *EGFR* (50%) and 11q13 mutations (43%) were associated with HPD. However, their role as expected biomarkers for HPD must be further validated in a larger cohort.

4. Conclusions and future perspectives

Immunotherapy for NSCLC has recently evolved into a new standard treatment modality primarily through PD-1 and PD-L1 inhibitors. However, patient selection is currently at the discretion of the treating physician. The predictors mentioned in the present review are based on the latest research results, and are innovatively classified into three categories: Effective, invalid and 'toxic'. All the biomarkers aforementioned may be incorporated into the prognostic bio-score systems and decision-making algorithms to better guide the application of clinical immunotherapy.

Despite efforts focusing on immunotherapy in NSCLC, a number of issues remain to be addressed in future studies. Currently available evidence indicates that PD-L1, TMB and MSI-H/dMMR have been acknowledged for screening the population in whom immunotherapy is effective of immune drugs, thus it is crucial to improve drug efficiency and reduce the incidence of adverse reactions. However, these markers allPD-L1, TMB and MSI-H/dMMR have certain drawbacks, for example some of the predictors have not been identified due to the limited clinical validation sample size and contradictory research results, which requires further confirmation by prospective studies with larger sample size. In addition, biomarkers and their mechanisms of action remain under investigation, therefore the role of gene mutations in immunotherapy of lung cancer requires further clinical research and experiments to verify in the context of precision medicine. Scoring tools based on blood indicators or characteristic expression of tumor gene profiles, including LIPI, TIDE and IMPRES scores, have not been widely used due to their respective drawbacks (129-131), and an immune prognosis assessment scale must be developed by combining various predicted molecules. Positron emission tomography combined with CT, dynamic contrast-enhanced CT and diffusion-weighted magnetic resonance imaging have demonstrated promising results for diagnosing and staging patients with lung cancer (132), and improvement of the evaluation criteria for immunotherapy and the risk of HPD are expected to make these predictions more precise (133,134).

Immunotherapy has long-lasting therapeutic activity and appears to hold promise for patients with NSCLC (135). Efforts must be focused on identifying patients who may benefit from this type of treatment through biomarkers, and on effectively controlling adverse reactions.

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Authors' contributions

YH and XCW conceived and designed the study. LLW, YH and SCW wrote the manuscript. XCW and JLS revised the article for important intellectual content. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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