

Battling Non-Small Cell Lung Carcinoma: Applying Biomarkers Testing to Pick the Best Immune Checkpoint Inhibitors Therapy

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Abstract. Immune checkpoint inhibitors is a new treatment for Non-Small Cell Lung Carcinoma. Benefit from such ICI therapy, however, have been enjoyed by a minority of NSCLC patients, and durable clinical outcomes are scarce. Thus, identifying reliable biomarkers to predict patients' possible response, and to indicate the progression status of tumors to further refine ICIs' application in treating NSCLCs is of decisive importance. However, as ICIs are novel therapies applied for only a decade, long-term post-treatment follow-ups are scant, and the probing or detection methods for biomarkers may not be as reliable as believed. Thus, many of the biomarkers require further investigations to elucidate their exact role in varying NSCLC microenvironments. Based on previously established results and integrating updated clinical data, this review lists the 2 currently accepted ICI therapeutic regimens, presents their respective mechanisms of action and their corresponding predictive or prognostic biomarkers currently available. This systematic categorization of biomarkers to respective therapies may inform clinicians about the use of ICI therapies and raise their attention to emerging and established biomarkers in new treatment strategies.

Keywords: Immune Checkpoint Inhibitors, Non-Small Cell Lung Carcinoma (NSCLC), Biomarkers, PD-L1, CTLA-4.

1. Introduction

Non-small cell lung cancer (NSCLC), about 85% of all lung cancer cases [1]. As a heterogeneous disease, NSCLC encompasses several lung cancer subtypes. Adenocarcinoma is often seen in lifelong non-smokers, and it takes up over 50% of NSCLCs, while squamous-cell carcinoma is mostly found in male smokers [2]. The initial warning signs for NSCLCs like shortness of breath, persistent cough, chest pain, and tiredness are often mistaken as symptoms of other lung diseases. Adding that NSCLCs often progress at a slower pace than other lung cancer, the patients are thus more frequently diagnosed at advanced stages with X-ray or CT examinations. Also, this late diagnosis pattern contributes to the naming of NSCLC in that the cancer cells often appear 'non-small' by the time they were called for microscopic inspection. The bad response rate towards chemotherapy or radiation therapy further complicates the therapeutic regimen for NSCLC [3]. While patients in the early stage could still recover from a surgical resection, stage IV NSCLC patients were limited to excessively toxic, platinum-based chemotherapies (with only a 19% response rate, 7.9 months of median survival, and only <1% survived after 5 years) [4].

Thus, the advent of immunotherapy in the 2010s has given great promise as it meets the needs to improve the overall survival (OS) and quality of life for the patients [5]. Among them, ICIs are by far the most efficient treatment against NSCLC. At present, the ICIs applicable for NSCLC treatment include PD-1/ PD-L1, and CTLA-4 agents. Instead of applying the cytotoxic pathways common in chemotherapies, these engineered monoclonal antibodies bring the cancer cells back to the scope of immune surveillance by binding to and blocking the function of immune checkpoint proteins [6]. A 2018 study showed that with the administration of Nivolumab (anti-PD-1 agent), up to 16% of pretreated NSCLC patients could survive up to 5 years [7]. Nevertheless, just as how all drugs and therapies have an inherent failure rate, only a limited amount of NSCLC patients would eventually benefit from ICIs. In a meta-analysis analyzing 160 studies on anti-PD-1/PD-L1 monotherapies

across 26 cancer types, the overall objective response rate (ORR) was only 20.21% (20.06% for lung cancer) [5]. Moreover, patients who initially responded may develop resistance as time progresses and overactivated immune systems, in long term, could cause immune-related adverse events (irAEs) --- inflammation of tissues across various organs. Although often not acute and generally manageable, irAEs could become severe or even lethal in patients at more advanced stages [8]. Therefore, more precise, personalized standards for applying ICIs are acquired.

Genetic biomarkers for NSCLCs may fit the role of clinical selection standards for ICIs. Biomarkers are defined as a biological signal/symptom/molecule detected that represents a medical condition of the body. For instance, elevated blood pressure could be a biomarker for hypertension. In cancer-targeting therapies, related biomarkers are either predicative, suggesting the best responders towards a specific therapy (differentiated based on: choice of drug, site of administration, the dose of administration, etc.), or prognostic, giving prudent insight on the course of the disease [9]. Several biomarkers have already been established for NSCLC treatment. For example, if a T-790-M mutation on exon 20 were to be detected in genome sequencing, the patient would be predicted as non-responder to 1st and 2nd generation of EGFR tyrosine kinase inhibitors (TKIs). Thus, a 3rd generation EGFR-TKI would be more recommended for a better prognosis [10]. Biomarkers like T-790-M provide an invaluable indication in the clinical evaluation of oncogene-targeted therapies against NSCLCs. However, such molecularly pinpointed, therapeutically tailored biomarkers are not as well-defined for ICI therapies against NSCLCs. This paucity stems from the more intricate mechanisms of immunotherapies (containing various regulators that participate in the tumor-lymphocytes interactions) compared to the clear-cut genetic markers for targeted therapies. Nevertheless, current studies in ICI-related biomarkers have seen some progress. This review summarizes the biomarkers available to be tested for currently established ICI therapies against NSCLC, discusses current issues and future directions that research in the field could focus on, and emphasizes the prognostic or predictive values and the screening methods for each biomarker.

2. Anti-PD-1 & Anti-PD-L1 ICIs in NSCLC treatments

2.1. Mechanisms of action and a brief historical background

The basis of ICI therapies stems from the mass- production of monoclonal antibodies in the 1970s, when Georges Köhler and César Milstein fused human lymphocytes with mice myeloma cells, thus creating a hybrid cell line capable of producing antibodies indefinitely in the laboratory setting [11]. With little risk of immune rejection and high specificity for the epitope targeted, these industrially manufacturable antibodies are thus exploited to target various proteins that were considered culprits of diseases. The 1st antibody-based inhibitor was developed in 2010. Named nivolumab, this PD-1 inhibitor was approved in 2014. Next, drugs like pembrolizumab (a 2nd widely applied anti-PD-1 ICI), atezolizumab (the 1st anti-PD-L1 approved in 2016), durvalumab and avelumab were developed and assessed worldly to block this checkpoint axis in various cancers. This inhibition was found to restore TCR-CD28 signaling, and activate T-cell and make it to express key transcription factors to promote the patient's rejection of tumor cells [5].

PD-1 or cluster of designation 279 (CD279) is a type I transmembrane receptor. While its ligand, PD-L1 proteins or CD 274 are limited to antigen-presenting cells (APCs), PD-1s are often constitutionally expressed on T cells. Interactions between PD-1 with PD-L1 entail a series of subsequent phosphorylation events which eventually cancel off the activation signal sent by T cell receptors (TCR) - CD 3 pathway. This T cell suppression effect makes the axis crucial in body's peripheral tolerance. However, in many cancers, this pathway axis is also exploited to escape from immune surveillance (one of the ten hallmarks of cancer). In addition, PD-1/ PD-L1 ligation may reduce the time that APCs interact with T-cells, thereby dampening T-cell activation and promoting Tregs' formation (which leads to IL-10 secretion). These various downstream pathways work synergically and thus create a tumor-promoting microenvironment around PD-L1 expressing tumor

cells [11]. Thus, interrupting PD-1/ PD-L1 interaction has always been considered a potential route to develop cancer therapies.

2.2. PD-L1: the most widely applied biomarker

Despite the widely established success story of ICIs, a meta-analysis of results from 160 trials of anti-PD-1/PD-L1 monotherapy on a range of cancers showed an overall initial efficacy of merely 10-20% [5]. Aside from that, as PD-1/PD-L1 pathway is vital in peripheral tolerance, indiscriminate administration of anti-PD-1/PD-L1 to NSCLC patients may produce off-target effects across various organs. Taken together, both facts suggest that patients must be carefully screened for the specific checkpoint expression on tumor cells to ensure the best therapeutic outcome.

Naturally, as the drug mechanism itself suggests, PD-L1 expression could be applied as a critical positive predictive biomarker. In various studies, through both transcriptional and translational probing, an elevated PD-L1 expression, compared to normal lung tissues, was associated with over 30% of NSCLC tumors [12]. Thus, this potential predictive role of PD-L1 was confirmed. Later, it is again consolidated by that in another meta-analysis trial assessing PD-L1, recipients of pembrolizumab showed a median OS of 30 months seeing that PD-L1 was expressed on over 50% of sampled tumor cells [3]. Tailing these crucial discoveries, nivolumab and pembrolizumab or atezolizumab have received FDA approval as single agents in stage III/IV NSCLC second-line treatment (given that other therapeutic regimens were proved ineffective). Notably, pembrolizumab monotherapy may be applied first-line in selected patients. However, it is important to stress that PD-L1 overexpression in biopsied NSCLC tissues proven by immunohistochemistry (IHC) tests is a deterministic factor for consideration of anti-PD-1 or anti-PD-L1 therapies. To date, for advanced NSCLCs, PD-L1 is the only biomarker widely accepted as a standard histological evidence supporting treatment by ICI therapy [13].

Apart from the IHC results obtained from pathology samples, the enhanced expression of PD-L1 may also be detected through liquid biopsy, a less invasive test that draws evidence of metastasis from the identification of circulating/soluble pieces of malignant genetic materials. For solid tumors like NSCLCs at a deteriorating stage, the ligand PD-L1 may also be expressed in a soluble form (sPD-L1), forestalling T cells from even accessing their proximity. Although also relying on an antibody-based, enzyme-linked immunosorbent assay determination (ELISA), liquid biopsies are generally more accessible with a simple abstraction of blood (low risk) and are thus invaluable for early-stage cancer detection. This early detection benefit of sPD-L1 testing gives promising vision for NSCLC treatment as current patients often weren't diagnosed until it is too late [14].

Prognostically, and despite the varying cut-off standards for high/low sPD-L1, the level of sPD-L1 were correlated with shorter OS and a higher rate of reoccurrence/metastasis events. Similarly, recipients with higher initial and sustainingly high sPD-L1 concentrations also demonstrated a higher incidence of treatment failure in trials for second-line nivolumab administrations to NSCLC patients [15]. Taken together, these studies all demonstrated that tissue biopsy screening of sPD-L1 has great potential in NSCLC therapeutic evaluation. A universal standard on the specific methods of testing (ELISA kits) and the related cut-off values, however, should be established before its universal clinical implementation.

2.3. Tumor Mutational Burden (TMB): a controversial biomarker

Although PD-L1's primary status in evaluating response to ICIs is undeniable, other biomarkers like TMB are also under intensive investigation. TMB is characterized by the number of mutations particular cancer may express and is thus often identified through next-generation sequencing, comparing the tumor genomic profiles to that of the corresponding normal tissues (e.g., NSCLC to normal lung cells). However, as the sloppy definition may suggest, TMB's reliability as a biomarker is highly questionable. First and foremost, there is great dissent in determining the standards to quantitatively assess TMB. The total number of tumor genome [16], while other groups hold discrepancies in what type of mutations, how many such genes should be incorporated, and the timing

of genomic sequencing (some tumor may develop high TMB at later stages). Trivial differences in any of the aforementioned factors may contribute to nuances and ambiguous understanding in the TMB calculations. To add more to the complexity of the question, TMB can also be measured from both liquid and tissue biopsies, each coinciding with different facets of metastasis.

Secondly, high TMB in tumor cells, as widely hypothesized, has some conflicting ramifications. On one side, it aids in the immunogenicity of tumors as more neoantigens may thus be produced and a higher chance of immune detection may thus be achieved with more diverse populations of primed T cells. A study conducted in 2017, for example, showed that recipients' OS after anti-PD-1/PD-L1 treatment positively correlates with high TMB across 27 tumor types [17]. However, susceptibility to mutation may also drive tumor cells to adapt to the ICI-present environment and thus dampen the effect of immunotherapy. This variability and dynamic nature of TMB have rendered high TMB a poor prognosis factor for NSCLC or any type of cancer.

However, TMB has been assessed subsidiarily as an on-treatment (not deterministic as PD-L1) biomarker for NSCLC anti-PD-1/PD-L1 therapies. Although high TMB is often found as a concomitant factor to PD-L1 overexpression, no proven correlation has been found between the 2 biomarkers. In their 2017 study, Huang and his group found that when evaluating the non-responder cases for anti-PD-1 immunotherapy, regardless of PD-1 expression level, had a relatively high pre-treatment TMB (predictive) [18]. Adversely, in phase III studies on nivolumab or atezolizumab monotherapies, high TMB, defined with more than 150 mutations, are all responding well to the therapy with better ORR and OS data (predictive). Notwithstanding the debatable reliability of TMB as a biomarker, most researchers like Huang still consider TMB a direction worth investigating as the measurements of TMB through next-generation sequencing are much easier to interpret and conduct than IHC testing of PD-L1.

2.4. Mismatch repair Deficiency (MMRD) & microsatellite instability (MSI): one of the most successful biomarkers

MMR mechanism is an evolutionarily conserved pathway to repair errors in DNA replication. As the genes repair other genes, mutations in this genetic pathway will lead to higher and faster mutation rates in the genome, and thus a higher rate of tumorigenesis. When mutated, MMR is most likely to change the DNA tandem repeats, microsatellites, due to DNA strand slippage. Thus, MMRD may often be expressed in a subsidiary form termed MSI (microsatellites instability). This genetic marker was first identified in colorectal cancer but later found to be applicable across a wide range of solid tumors including NSCLC. MMRD, with its high susceptibility to mutations, could be counted as a subsidiary measurement of TMB. Therefore, like TMB, it is also measured through next-generation sequencing of liquid or tissue biopsy samples [19]. Nevertheless, compared to TMB, MMRD's prediction on patient response towards anti-PD-1/PD-L1 treatment against NSCLCs is much more straightforward and clear-cut. After proving that MMRD in colorectal cancers was sensitive to anti-PD-1 ICIs, a group of researchers later also identified that MMRD often leads to better prognosis and better clinical response for a solid tumor like NSCLC [18]. Also, in another study assessing patients with 12 different MMRD cancer subtypes (including NSCLC), durable response (among them, 21% showed complete response) and prolonged OS, were observed in anti-PD-1 agents recipients. Thus, MMRD or high MSI could arguably be considered an established predictive biomarker for clinical response to ICIs [20].

2.5. Gut Microbiome: a novel but informative biomarker

Aside from the markers in the tumor genome, other factors in the NSCLC patients may also contribute to providing predictive insights on ICI treatments. A typical example of them would be the intestinal or gut microbiome. As intestinal microbiota are indispensable in regulating immune homeostasis (through direct interaction with gut-associated lymphoid tissues), ICI therapies, which depend heavily on reactivating the host's T cell response, are thus inevitably affected by changes in microbiota composition. These changes may be brought by pre-treatment antibiotics consumption

and may thus manifest through symptoms like constipation. While a 2019 Japanese study has associated constipation (stool abnormality) with a lower overall response rate and shorter time to ICI treatment failure [24], a 2016 pre-clinical study demonstrated that among non-responders towards anti-PD-1 agents, antibiotic administration was common [21]. Through gene sequencing, Routy and his team found that the non-responders are lacking 2 bacteria species, namely *Enterococcus hirae* and *Barnesiella intestinihominis*. Their hypothesis that this nuance in microbiota composition leads to ICI treatment failure was later confirmed in mice experiments - after reintroducing the 2 bacteria to the intestine, mice that were exposed to the same medical conditions as the non-responders had their resistance to ICI blockade alleviated [20]. Another study conducted in 2019 also confirmed antibiotics' peril towards ICI regimen, providing that a prescription of broad-spectrum antibiotics 30 days before ICI treatment invariably worsens the patients' OS, leading to a median survival of only 2.5 months in NSCLC patients (compared to a 26 months median of the control group) [22].

Nevertheless, 2 other studies conducted in 2019 on Japanese or Chinese patients have suggested the role gut microbiota may play in strengthening anti-PD-1 agents. Through 16S ribosomal RNA gene sequencing or Illumina sequencing platform, the researchers have found that abundance in species like *Alistipes putredinis*, *Bifidobacterium longum*, and *Prevotella copri*, or in bacteria that fall in the phyla of *Lactobacillus* or *Clostridium* may help identify the responders to anti-PD-1 drugs among NSCLC patients [23, 24]. Generally speaking, having greater intestinal microbiome diversity is predictive of better response to PD-1 inhibition. But the last point worth mentioning is that all studies listed in this section have a small sample size of no more than 200 patients (since NSCLC patients with long-term ICI treatments are hard to find in a regional clinical study), a further study of larger scale should be conducted to evaluate the current findings.

3. Anti CTLA-4 ICIs in NSCLC treatment

3.1. Mechanisms of action and a brief historical background

CTLA-4 participates in managing immune responses under homeostasis. It is similar to CD28 in structure and has a much higher affinity for APC B7 ligand. As B7s on APCs engage with receptor CD28 on naïve-T cell, an indispensable 2nd signal for T cells to proliferate and differentiate would be sent. Therefore, overexpression of CTLA-4 on tissues effectively increases the motility of, and tunes down the survival and proliferation signal for T-cells and modulate optimal T cell (Treg) functions [25]. All these effects lead to a suppression of T cell response. Thus, like PD-L1, CTLA-4 is also utilized as a 'learned' disguise from immune surveillance. The antibody can block the series of immunosuppressive mechanisms mentioned above. Through allowing the B7 molecules to interact with CD28, the uninhibited co-stimulation can activate NF- κ B and NFAT, which in return stabilize the IL-2 mRNA for better transcription, increasing the T-cell survival and preventing anergy or apoptosis [26]. The neoplastic cells can then be effectively eliminated by the effector T-cells.

Despite the wider application of those antibodies in treating lung cancers, ipilimumab (anti-CTLA-4) monotherapy against melanoma, a type of skin cancer, was the first established ICI approved by FDA in 2011. Like PD-L1, CTLA 4's overexpression on NSCLC tumor tissues and cell lines has been confirmed since 2012 [27]. However, anti-CTLA-4 inhibitors against NSCLCs, currently, are still undergoing clinical tests due to its limited success as a monotherapy. Nevertheless, lower doses of ipilimumab have been widely adopted by clinicians to subscribe along with anti-PD-1/PD-L1 agents (particularly nivolumab, as this combination is an established and most promising therapy in advanced melanoma) to patients that acquired resistance towards anti-PD-1 monotherapy regimen [28]. Together, the combined ICI agents alternates according to cellular environment, thus amplifying and prolonging T cell activation against NSCLCs.

3.2. CTLA-4 and PD-L1: the most apparent biomarkers

Due to its clinical insignificance, unlike PD-L1, CTLA-4, as the checkpoint a monotherapy may directly target, is currently a not-so-well-defined biomarker for anti-CTLA-4 therapies. Since CTLA-

4 is a crucial in hampering the action of tumor-infiltrating T cells, preserving the immune system's tolerance towards the tumor, the presence of CTLA-4 in NSCLC samples is thus considered of noticeable prognostic and predictive values. A 2017 study set out to explore CTLA-4's prognostic value in 536 surgically removed tumor samples from stage I-IIIa NSCLC patients. Applying IHC-based analyses, the researchers were able to show that high CTLA-4 expression at primary NSCLC tumor sites is positively correlated with better prognosis. However, they have also indicated that high CTLA-4 expression at tumor-related lymph nodes may lead to reversed prognostic indications [29]. However, in a 2020 study applying similar IHC tests on resected primary NSCLC tumors, high CTLA-4 expression, positively associated with poliovirus receptor expression on tumor cells, were proved to be great prognostic factors indicating poor prognosis results (poor OS in 108 Chinese patients) [30]. This contrast in CTLA-4's prognostic value on primary tumor suggests that CTLA-4's expression, put in a different context, should be interpreted differently to obtain a reliable estimate on an NSCLC patient's progression stage or tumor microenvironment. Furthermore, few published studies have set out to study the predictive indication CTLA-4 expression may have in anti-CTLA-4 therapies due to its limited success in treating NSCLC patients.

However, since anti-CTLA-4 agents in current trials are often co-applied with anti-PD-1/PD-L1 agents, PD-L1 expression could still be counted as a valuable prognostic and predictive factor in these therapeutic regimens. To start, in a 2021 review assessing the success of the two antibodies combined therapy against chemotherapy for NSCLC IIB/IV patients, it was found that recipients of combined therapy with a lower than 1% PD-L1 expression level saw a statistically significant increase in OS. However, a higher than 50% expression level showed similar bettering of therapeutic outcomes, while a medium value in between was associated with poor ramifications. Based on this unexpected outcome, the researchers thus speculated that different mechanisms of action were at play at extreme PD-L1 levels: at low PD-L1 levels, the main effector would be the CTLA-4 inhibitors, acting to block the complementary immune checkpoints; at high PD-L1 level, the anti-PD-1/PD-L1 agents would instead play the major role to inhibit tumor evasion. This flexible shift in major therapeutic agents may thus well explain the clinical success of combined therapy over monotherapies [29].

3.3. TMB: not so controversial in clinical evidence

TMB leads to less uncertainty in predicting outcomes for ipilimumab plus nivolumab combined therapies than for anti-PD-1/PD-L1 therapies. As a subsidiary on-treatment biomarker, high TMB expression in the aforementioned 2021 meta-analysis was assessed with PD-L1 expression and was found to suggest weakening effects on combined ICI therapies [31]. However, the 2018 CheckMate 227 study confirmed that a TMB level larger than 10 mutations per megabase showed a much superior median OS and prolonged PFS in combined therapy recipients [32]. Furthermore, in the 2019 CheckMate 586 study, the same expression level of TMB in CheckMate 227 emerged as an independent biomarker. But it was found to also indicate better ORR and progression-free survival in 288 CT naïve advanced NSCLC patients treated with combined therapy [33]. A last piece of clinical evidence regarding TMB's predictive role in combined therapeutic regimens comes from a phase III trial. In this 2019 study, the same larger than 10 mutations per megabase TMB was again identified as a significant predictor of improved OS and reduced hazard ratio [34]. Despite the unifying results indicating TMB as a strong predictive biomarker for better outcomes in combined ICI therapies, it should be noted that the issues with inconsistent measuring approaches (the 3 clinical studies used similar calculation methods for TMB) and the intricate complication of TMB still call for further probing in its predictive value.

4. Conclusions

NSCLC patients expressing high PD-L1 or MMRD have seen the promised success of anti-PD-1/PD-L1 monotherapies despite the controversies around the predictive value of high TMB. In contrast, although the blockage against both CTLA-4 and PD-1/PD-L1 has been efficacious, most of

its related biomarkers have given contrary results in different clinical assessments because a predictive or prognostic biomarker for immunotherapy often subjects to multifarious and dynamic factors, and the same measurement could have completely different therapeutic indications when placed in different contexts. Thus, the future direction for exploring new predictive or prognostic biomarkers for ICIs could be precise but ‘broad’ tests on tumors, encompassing a matrix of multiple variables, just as how high TMB may have different predictive implications when tested as a subline factor to PD-L1. Identifying and verifying the distinctive predictive and prognostic values of biomarkers in NSCLC treatments fits the spirit of this era of precision medicine, as it contributes to refining the established therapeutic regimen while giving insights on potential immune treatments for cancer in the future. However, due to limitations in detection methods and the gaps in understanding of immuno-oncology, ambiguity still exists for biomarkers in ICI treatments against NSCLC. Hopefully, with the ever-expanding understanding of the tumor microenvironment, the search and establishment of biomarkers may address more vital problems in fine-tuning ICI therapies for NSCLC patients.

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