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REVIEW

Dynamic Monitoring of Immunotherapy Effectiveness with Different Biomarkers in the Patients with Non-Small Cell Lung Cancer

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ABSTRACT

Non-small cell lung cancer (NSCLC) constitutes about 84% of lung cancer. Hence, increased efforts have been fueled into immunotherapy of NSCLC with immune checkpoint inhibitors (ICIs). ICIs have recently taken off as promising immune-therapeutic methods that have slowed down the progress of NSCLC and equipped patients with survival advantages. However, the long-term respondents tally is less than 20% of the population. This low response rate warrants the need for dynamic biomarkers which will provide insight into the possible response of patients to ICIs. Biomarkers are biological molecules that predict the pathologic state of patients and the potential response they will elicit to ICIs. Predictive biomarkers play a crucial role in analyzing the effects of ICI therapy on patients and potentially filter out patients who will certainly benefit from ICIs. In this way, resources in immunotherapy can be rationalised better and the healthcare system can administer an alternative mode of treatment to the non-responsive group of patients. Thus, precision therapy can be performed according to the possible responsiveness and needs of patients. PD-L1 protein is a popular biomarker that has been analyzed extensively to study the immunotherapy effectiveness of PD-L1 and PD-1 inhibitors. But as a single biomarker, PD-L1 protein levels do have their shortcomings to check for ICI therapy effectiveness. As such, this emphasizes the need for exploration of more biomarkers to be used on prediction grounds. A combination of biomarkers may also be considered for better prediction results. This review serves to highlight the importance of PD-L1 protein in dynamic monitoring of the immunotherapy effectiveness and investigates different novel biomarkers that could be plausible options to monitor immunotherapy effectiveness of NSCLC.

KEYWORDS

Immunotherapy; immune checkpoint inhibitors; dynamic monitoring; lung cancer

1 Introduction

Immunosuppression and immune evasion are hallmarks of tumour cells. Reversing immunosuppression is thus of paramount importance and it has been an increasingly plausible option with the aid of immune checkpoint inhibitors (ICI) [1]. Immune checkpoints are crucial to establish a *status quo* of self-tolerance and to regulate immune responses of peripheral tissues [2].

Currently, some FDA approved immunotherapy drugs have been regarded as part of the drug regime for certain patients under conditions deemed suitable. Programmed death ligand 1 (PD-L1) and PD-1 inhibitors

are some examples of these drugs, which have shown a certain level of overall response rate (ORR) in patients *via* the monitoring of PD-L1 levels expressed by tumour cells [3]. To date, the FDA has approved six ICIs targeting PD-1/PD-L1 for usage in the first and second line of treatment for patients with NSCLC.

The drugs comprise monoclonal antibodies such as pembrolizumab, nivolumab and cemiplimab targeting PD-1 and atezolizumab, avelumab and durvalumab targeting PD-L1 [4]. Pembrolizumab can display greatest benefit in terms of objective response rate and overall survival (OS) in patients with PD-L1 expression exceeding 50%, while atezolizumab presents with better OS in patients with PD-L1 expression of more than 1% [5].

However, the efficacy of these PD-1/PD-L1 ICIs has been reported to rarely exceed 40% in most cancer types and a large number of patients show partial responsiveness. Therefore, it is crucial to first identify and assess the credibility of the PD-L1 protein expression levels as biomarkers. If PD-L1 protein levels are not deemed to be the best option, then the healthcare system needs to seek other biomarkers to monitor the ICI therapy effectiveness. The other biomarkers may either be used together with PD-L1 or may have isolated purposes.

To monitor immunotherapy efficacy, we have used many dynamic biomarkers. This review will analyze biomarkers such as PD-L1 protein levels, tumour mutation burden (TMB), tumour neoantigen burden (TNB), exosomes, cell-free DNA (cfDNA), tumour-infiltrating lymphocytes (TILs), neutrophil-lymphocyte ratio (NLR), etc. Out of which PD-L1 inhibitors have already been administered to patients as part of the approved FDA drug regime, and TMB is still a part of clinical trials.

2 PD-L1 Protein Levels

PD-L1 protein levels are one of the first few biomarkers brought under the scrutiny of the research wing [6]. It serves as a prognostic biomarker and predicts responses to anti-PD-1/anti-PD-L1 therapy. PD-L1 is a type I transmembrane protein of 290 amino acids, and it can express in the large amounts by cells such as tumour cells, monocytes, macrophages, natural killer (NK) cells, and span across a series of immunocompetent locations like the brain, cornea and retina [7]. When the ligand, PD-L1 binds to its receptor, PD-1 expresses on T cells, it arrests the T cells in the G1 phase of the cell cycle and rapidly diminishes the immune function of T cells against tumour cells. This is duly illustrated in Fig. 1. As such, PD-L1 and PD-1 inhibitors have been constantly under the spotlight to stop the interaction between PD-L1/PD-1, invigorate the tumour-specific cytotoxic T cells against tumour cells [8] and prevent immunosuppression against tumour cells. For PD-L1 and PD-1 inhibitors, to date, PD-L1 protein levels seem to be the most widely used and dedicated biomarkers. PD-1/PD-L1 inhibitors exert a brake on the downstream line of events by preventing PD-1 and PD-L1 from binding and thus enhance antitumour immunity [9].

Immunohistochemistry (IHC) is a major method utilized in the evaluation of PD-L1 expression and improvised techniques have surfaced to better track expression levels. The results are given as the percentage of tumour cells and the area of the immune infiltrate expressing PD-L1 on its surface [10]. PD-L1 levels hold great significance as a biomarker due to the increasingly reliable methods to confirm tumour cells' expression of PD-L1 protein. A study has found that a ^{68}Ga- NOTA-Nb109 antibody is of immense use to track PD-L1 levels. The ^{68}Ga- NOTA-Nb109 has a great affinity for PD-L1 and accumulates in large amounts in tumours that are PD-L1 positive. The rapid clearance rate of the radioactive material and the high tumour-to-background ratio gives it a competitive edge as well, as such, improving the accuracy with which PD-L1 levels could be studied in patients [11]. Therefore, the PD-L1/anti-PD-L1 therapy will be more tailored and specifically administered to cancer patients with a good prognostic factor, backed up by the high expression of PD-L1 protein.

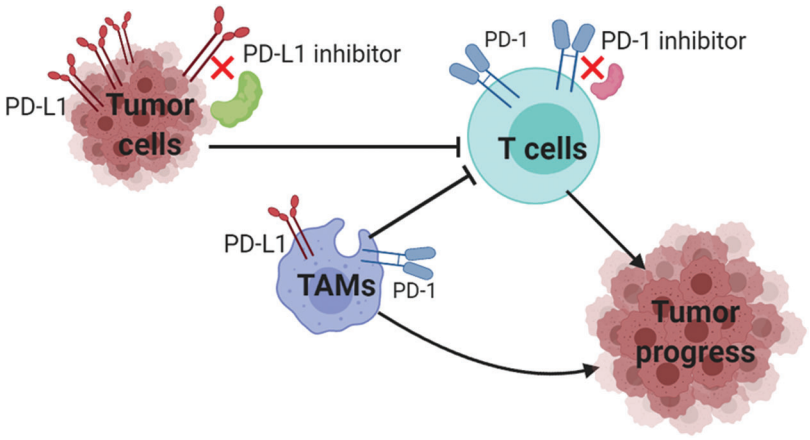

Figure 1: PD-L1 and PD-1 expression in the tumour and immune cells. The pro-tumour effect caused by PD-L1/PD-1 interaction is illustrated. T cell cytotoxic function can be inhibited by different phenotypes such as PD-L1^{+} tumour cells, PD-1^{+} T cells, PD-L1^{+} TAMs and PD-1^{+} TAMs, which can promote tumour progress

PD-L1 levels are measured in two forms as PD-L1 protein levels directly present on tumour cell surface in the tumour microenvironment (TME) and as exosomal PD-L1. Both PD-L1 forms have different sensitivity and ease of detection. Hence, they offer differing predictive spectra with respect to patient eligibility for ICI therapy.

2.1 PD-L1 Levels Measured from the Tumour Cell Surface in TME

The tumour proportion score (TPS) refers to the percentage of PD-L1 positive tumour cells detected in the TME by IHC. In the KEYNOTE-001 clinical trial, TPS has been proven to be a vital marker associated with long-term survival. Patients with ≥50% TPS showed the most 5-year OS (29.6%) in advanced NSCLC patients treated with anti-PD-1 ICI drug pembrolizumab [12,13]. The greater the expression of PD-L1 on tumour cells, the worse the prognosis turns out for the patients, according to histological settings [14]. But these patients with high levels of PD-L1 protein expression will have better response rates to ICI therapy when administered the PD-L1/PD-1 inhibitors, thus potentially challenging the expected "poor prognosis" in this group. Consequently, the survival outcomes can be improved in these patients [15].

Tumours with PD-L1 expression levels ≥ 50% qualify for the first-line single-agent pembrolizumab, while tumours with PD-L1 expression levels ≥ 1% is the criteria for pembrolizumab after progression on a platinum-based therapy according to FDA approvals and the National Comprehensive Cancer Network (NCCN) NSCLC guidelines [16]. As such, appropriate PD-L1 levels are the qualifying factors, which distinguish patients eligible for the start of PD-1/PD-L1 therapy. In this way, it is ensured that a patient's TME is understood before administration of drugs, thus paving the way for a possible positive outcome and better stratification of patient groups. Since PD-1/PD-L1 ICI therapy is based on the expression levels of PD-L1, it is vital to analyze the factors that may affect the PD-L1 expression, to better comprehend the TME in cancer patients.

Factors influencing PD-L1 expression are classified into intrinsic and extrinsic factors as shown in Fig. 2. Assessment of these factors is of paramount importance as it enables oncologists to further filter out potential responders to PD-L1 ICI therapy and leverage on the TME conditions of cancer patients. Chemotherapy and radiotherapy have a major influence on PD-L1 expression in the TME. Chemotherapeutic drugs such as oxaliplatin, cyclophosphamide, etc., carry out immunomodulatory functions by increasing antigen presentation or by inhibiting immune-suppressive mechanisms.

This creates an advantageous inflammatory environment that is further fueled by immune checkpoint therapy (ICT) [12]. Radiotherapy increases antigen presentation, IFN-γ response and pro-inflammatory chemokine production that facilitate increased T cell trafficking to the tumours. This mounts up a stronger immune response against tumour cells [17]. The immunostimulatory effects put up by radiotherapy elevates PD-L1 expression by mounting adaptive pressures on tumour cells. ICT can be coordinated with radiotherapy to further enhance patients' systemic antitumor immunity. Other factors that produce an advantageous TME to boost PD-L1 expression levels include cytokines, growth factors, DNA damaging agents, angiogenesis, hypoxia, etc., as elaborated in Fig. 2.

Studies with PD-L1 ICI drug atezolizumab proved that higher PD-L1 expression levels are associated with better treatment effects, including higher ORR and longer survival. Similarly, the administration of pembrolizumab in high PD-L1 expression patients can promote improved treatment effects [18]. The greater TPS reflects the increased effectiveness of anti-PD-L1/anti-PD-1 drugs acting on tumour cells, thus producing gratifying improvements in terms of tumour response.

On the other extreme, there are outliers as well who present with robust responses to therapy despite the low levels of expression of PD-L1. In the early stage of NSCLC, the clonal and spatial heterogeneity of tumours may pose a challenge in determining the expression of PD-L1 protein [19]. Such controversial responses further complicate the issue of PD-L1 as an exclusionary predictive biomarker. In general perspective across all tumour types, PD-1/PD-L1 ICI therapy results in response rates of 0–17% in patients with PD-L1 negative tumours and about 36%–100% in patients with PD-L1 positive tumours [20]. Trials have satisfactorily proven that significantly longer progression-free survival and overall survival are achieved in ICI-treated patients with tumours expressing PD-L1 in at least 50% of cells. Thus, with valid results being proven over time, it is increasingly crucial to accurately filter out patients who will benefit from PD-L1/PD-1 ICI therapy, to ensure maximal benefit in terms of healthcare. PD-L1 expression in small tissue samples such as biopsy specimens might not be representative of the entire tumour specimen, due to clonal and spatial heterogeneity of PD-L1 in tumours [21]. Single biopsies or pleural effusions used for IHC assays are not characteristic of the inter-and intra-tumoural heterogeneity [13], thus hindering the comprehension of the histological settings of the tumour. Increasing the number of biopsies might be a plausible option to get a better idea of the tumour histology and to increase the reliability of the PD-L1 expression results obtained. However, being an invasive technique, it will not be for the benefit of the patient in the long run. This further reiterates the need to consider the results of PD-L1 staining across biopsies to maximize their reliability in predicting the true PD-L1 status of tumours. The usage of other biomarkers that used in combination with PD-L1 for customized treatment has to be explored. These results indicate that three and four core biopsy specimens should be taken from patients to reach an area under the curve (AUC) and sensitivity higher than 0.9 at cutoffs of 1% and 50%, respectively [22]. A microarray is a viable alternative to compare representative tissue samples from different patients by assembling them on a single histologic slide and analyzing multiple specimens at the same time. In Keynote-024 study, involving patients with tumours characterized by PD-L1≥ 50%, the ORR in the group treated with pembrolizumab is lower than expected and stands at only 45%. This further proves that outliers are possible even in a population that is scrutinized and defined by their high levels of PD-L1 expression [23].

Challenges do exist in terms of the relationship between PD-L1 expression and clinicopathological factors as well. For instance, ethnic factors may cause variations in different populations' PD-L1 expression levels, even if the stage of tumours is identical across communities. Most studies, in which high PD-L1 expression (50%–72.7%) are reported, are conducted in an Asian population whereas the western counterparts showed lower incidences [24]. In addition to that, EGFR mutations are more prevalent in Asian NSCLC patients with figures rising to 50% of patients compared to their Caucasian counterparts. EGFR-mutant NSCLC tumours are more inclined to be negative for PD-L1, and thus such

patients are poor responders for ICI therapy [25,26]. IHC staining also has its own limitations as different antibodies have different sensitivities to staining. Additionally, the cut-off value of PD-L1 staining positivity remains debatable [27].

In total, although the methods of monitoring PD-L1 expression has gained trust among researchers, the discrepancies that exist across ethnicities, outliers and other clinicopathological factors question the credibility and effectiveness of PD-L1 protein levels in TME as biomarkers. In the future, more focus has to be channelled into countering the discrepancies for establishing uniform standards for PD-L1 protein level monitoring.

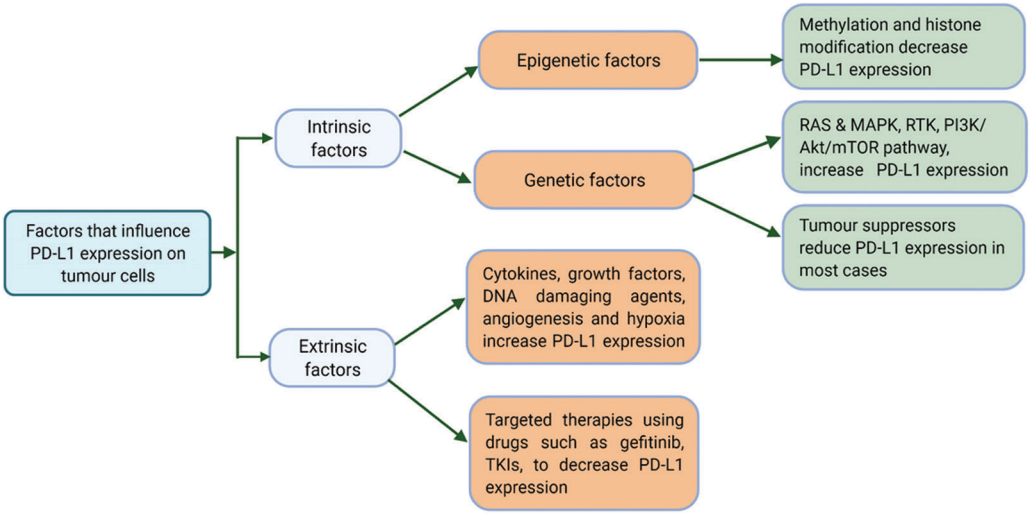

Figure 2: Extrinsic and Intrinsic factors that influence the levels of PD-L1 expression on tumour cells. The intrinsic factors can be further classified into genetic and epigenetic factors. The extrinsic factors (cytokines, growth factors, DNA damaging agents, targeted therapies, angiogenesis and hypoxia) tend to generally boost the expression of PD-L1 proteins with a few exceptions such as IL-10, etc which instead tilt the scale in the opposite direction. The genetic intrinsic factors such as RAS/MAPK, RTK, PI3K/Akt/mTOR generally cause an increase in the PD-L1 protein levels. Contrastingly, tumour suppressors seem to be fluctuating the PD-L1 levels in both directions by either increasing or decreasing expression depending on the type of tumour suppressor. The epigenetic factors are more inclined to decrease the levels of PD-L1 protein [28–31]

2.2 Exosomal PD-L1

Exosomal PD-L1 is also a prominent form used as a biomarker. Exosomes, also known as extracellular vesicles (EVs), are lipid-enclosed membranes released by tumour cells. Their contents are representative of the cells they are exocytosed from [32]. EVs have the potential to be isolated from several sources such as plasma, blood, saliva, etc. [27]. The ease of isolation and the potentially non-invasive method to monitor the effects of ICI treatment makes EVs an attractive option. Since EVs are representative of cells from which they originate further fuels the speedy detection and accuracy of diagnosis of cancer. In the early stage of NSCLC, exosomes are found to be more sensitive than cfDNA (25.7% compared to 14.2%, respectively) and more specific (96.6% and 91.7%, respectively) [33].

Recently, exosomal PD-L1 (Exo-PD-L1) has proven to be a more reliable factor than PD-L1 protein levels obtained from tumour tissue [34]. The levels of Exo-PD-L1 are notably higher than the soluble PD-L1 protein levels in the plasma of NSCLC patients. It has been proven that the change in Exo-PD-L1 values in patients before and after treatment (ΔExo-PD-L1) by more than 100 pg/mL showed an 83%

sensitivity, a 70% specificity, a 91% positive predictive value and a 54% negative predictive value for disease progression. Utilizing Exo-PD-L1 can be more potent than tumour cell-associated PD-L1 in regaining T cell function because exosomes are widely circulated and may attach to their target cells more easily than tumour cells [34,35]. The levels of circulating exosomal PD-L1 positively correlate to IFN-γ and change during anti-PD-1 therapy. The extent of increase in circulating Exo-PD-L1 serves as a distinguishing factor to clinically separate responders from non-responders by illustrating the response of tumour cells to T cell re-activation [36].

Response of exosomal PD-L1 to PD-L1/PD-1 inhibitors varies with the response from PD-L1 proteins in the TME. In a group of NSCLC patients, the use of PD-L1 inhibitors resulted in an increase in exosomal PD-L1 levels in patients with progressive disease, decrease in patients with partial response and minimal difference in patients with stable disease as compared to baseline levels of exosomal PD-L1 [37,38]. Henceforth, the basal and post-therapy exosomal PD-L1 levels may be used to determine the type of response elicited by patients with ICI therapy.

However, EVs come with their own set of challenges as well. There are multiple methods of EV isolation, which include ultracentrifugation, size exclusion chromatography, immune affinity isolation, etc. These varying methods may produce vast EV bioassay results and thus may affect the verification and reproducibility of results. Thus, standardization of methods is needed for a better combination of data [27]. Gender, physique, ethnicity, etc may also affect EVs expression. Another clinical challenge of EV-based biomarker discovery is EV heterogeneity and change in amounts of EV in different stages of NSCLC. Therefore, close monitoring of patients is mandatory if EVs are to be a viable option.

3 The other Biomarkers Different from PD-L1

3.1 Tumour Mutational Burden

TMB refers to the number of mutations present in tumour cells, which alter the amino acid sequences and thus produce TNA. TMB is measured in terms of whole exome sequencing [39]. A high TMB rate is indicative of a high mutation rate and greater genomic instability that stems from the possibility of pro-tumour changes contributed by the TNA. The use of TMB to study the effects of ICI is still very much debatable in today's context. A higher TMB corresponds to a higher response rate in patients administered with PD-L1/PD-1 inhibitors [40]. In a study [21], smokers have a higher record of TMB compared to non-smokers.

NSCLC patients treated with the PD-1 inhibitor, pembrolizumab, have an increased mutation burden, which shared a strong correlation with clinical efficacy. Among those with a high mutation burden (>200, above the median of overall cohort) and some degree of PD-L1 expression, the rate of durable clinical benefit (DCB) is found to be 91%. In contrast, in those with a low mutation burden and a certain level of PD-L1 expression, the rate of DCB is only about 10% [40]. As such, when TMB is coupled with PD-L1 levels, there are increased chances of better prediction of response to ICI therapy. If not as an isolated marker, TMB shares opportunities with PD-L1 to work in combination. Another study supported the fact that DCB is 64% in TMB-high patients, as opposed to 33% and 29% (together 31%) in TMB-intermediate and TMB-low patients, respectively [41]. Similarly, in the CheckMate 026 trial, a higher ORR and a longer progression-free survival (PFS) are noticed with PD-1 inhibitor, nivolumab, when compared with chemotherapy in tumours with high TMB (ORR: 47% *vs.* 28%; PFS: 9.7 months *vs.* 5.8 months). In the CheckMate 227 study, the combination of nivolumab and CTLA-4 inhibitor, ipilimumab compares to platinum-based chemotherapy. In patients with high TMB (irrespective of PD-L1 levels), a significant benefit in PFS has been reported in the combination drug trial when compared with platinum-based chemotherapy alone (7.2 months *vs.* 5.5 months, HR 0.58, 95% CI: 0.41–0.81). This is accompanied by an improvement in ORR (45.3% *vs.* 26.9%) too [19]. In NSCLC, factors such as complete response/partial response (CR/PR) rates, PFS and OS portray better outcomes as TMB increase

[42]. Patients with a high TMB had significantly higher response rates and longer PFS and OS than those with a lower TMB. Thus, the correlation between TMB and outcome is further strengthened [43].

Despite these positive findings from Checkmate 227, the role of TMB as a biomarker for immunotherapy in the advanced NSCLC remains indiscernible, as subsequent OS data has revealed a statistically insignificant benefit derived from administration of ipilimumab with nivolumab in high TMB patients (HR 0.77, 95% CI: 0.56–1.06). This statistic is comparable to the survival benefit seen in patients with lower TMB mutation/megabase (<10) (HR 0.78; 95% CI: 0.61–1.00) [44,45]. There are discrepancies in terms of TMB too. Some mutations are identified more readily by patients' immune systems and are more likely to induce a stronger antitumor immune response [27]. Translating these findings to routine clinical practice is a hassle as whole exome sequencing (WES) is expensive, time-consuming and is accompanied by technical difficulties. Profiling a smaller fraction of the genome (>1MB; approximately 3% of the exome) using next-generation sequencing (NGS) diagnostic platform may be a plausible alternative to study total mutational load and identify particular mutations related to response to anti-PD-1/PD-L1. NGS has several advantages, which includes a short processing time of about 2 weeks, standardized expertise, technical backup and its affordable nature [46]. TMB has some other drawbacks as well. Tissue biopsies are required for the analysis of TMB patterns and this can prove to be invasive. Furthermore, not all mutations may be recognized and hence the calculation of TMB may be misleading in certain cases [27]. Errors in the estimation of tumour purity are inevitable due to the practical difficulties in pathologic assessments and the limited replicability [47].

3.2 Tumour Neoantigen Burden

Tumour neoantigens (TNA) refers to the tumour-specific mutations, which produces genomic alterations and neoantigens. To date, three broad classifications of TNA are known. Namely, tumour specific antigen (TSA), tumour associated antigen (TAA) and cancer-germline/cancer testis antigens (CTAs), out of which TSA plays a predominant role. Although we have the capacity to use current models of technology to identify point mutations and indels that can form MHCI epitopes, there are many other potential TNA that pose a challenge with current standards of bioinformatics tools with existing gaps in technological advancements [46]. Neoantigens are expected to be highly immunogenic as they can be recognized by tumour-specific cytotoxic T lymphocytes. Thus, they have been explored as potential biomarkers of ICI. TNA is heterogenous from self-antigens, thus allowing the lymphocytes to spare the latter and prevent an autoimmune response. TNB is positively proportional to the number of affected DNA mutations [48].

3.3 Cell Free DNA

cfDNA has been revolving as a surrogate biomarker for tumour progression and it seems to be a promising non-invasive method for the study of tumour burden. cfDNA sets advantageous goals based on its tumour-specific mutations. cfDNA is a byproduct of tumour cells undergoing cell death and has a blood clearance half-life of about 2 h. As such, it is highly reflective of active cell death occurring in the patient's tumour environment and reiterates its stance as a reliable biomarker [49]. The distinguishing factor between cancer patients and healthy individuals is the amount of cfDNA present. The former will have a higher cfDNA count than the latter. In studies conducted with NSCLC patients administered with PD-1 antibody, nivolumab over 6 weeks, patients with an increased cfDNA count of more than 20% from baseline have a significant worse OS and time to progression (TTP) tally [50]. Similarly, another study has proven that a decrease in cfDNA by more than 50% vastly improved PFS and OS for NSCLC patients [51]. Some of these studies report observing a transient spike preceding a decline in cfDNA levels in a subset of patients, possibly due to DNA released by dying tumour cells [52].

With cfDNA levels indicating the response capacity of patients earlier than radiographic evidence, cfDNA likely has higher efficacy for rapid assessment in NSCLC patients. Patients with decreased

cfDNA levels at week 8 experienced a better PFS than patients with persistently detectable cfDNA. OS is improved in patients with undetectable cfDNA at week 0 [53]. However, cfDNA has its own limitations as well. Some patients may not have sufficient cfDNA to allow detection in plasma. To utilize cfDNA to a certain identity, a high positive predictive value (PPV) is required. However, recent studies have shown that over the years, healthy normal individuals exhibit cancer-associated genomic alterations in tissue biopsies and cfDNA. As such, this poses a challenge to distinguishing between normal and cancer patients DNA in some cases. The metabolic activity of tumour cells and their vasculature may also be a cause for concern as these factors might lead to uneven distribution of the cfDNA.

In total, cfDNA seems to be a rather promising biomarker with its non-invasive method, relative accuracy. When patients are administered with PD-L1/PD-1 inhibitors, the changes hailed by the cfDNA do offer insight into the prognosis of patients. Thus cfDNA is a potential biomarker that may play a crucial role coupled with PD-L1 levels to determine immunotherapy effectiveness with ICI. However, the biased results due to the limiting factors is an issue that has to be addressed before considering cfDNA as an absolute biomarker.

3.4 Tumour Infiltrating T cells

TILs refer to the abundant collection of effector T cells that mount immune responses against tumour cells. Lymphocyte infiltration into tumour cells is indicative of a positive response in the OS of patients [54]. Recent progress in terms of TILs includes utilising TIL density and location of TILs subsets to create an immunoscore as a complement to the TNM system for the classification of the malignancy. TILs are observed in about 25% of resected lung neoplasms but occur rarely in neuroendocrine tumours. TILs are always detected in higher proportions in poorly differentiated tumours and tumours with microscopic vascular invasion. The presence of TILs is proven to pave way for improved survival in the early stage of squamous cell carcinomas of the lung. The survival advantage increases with the duration of follow-up.

DC, NK cells, M1 macrophages, $CD3^+$ and $CD8^+$ T cells are associated with a positive prognosis due to their anti-tumour activity, while M2 macrophages and Treg cells in the stroma are deemed to have the opposite stance due to their pro-tumour activity [55]. Recent studies in NSCLC patients has confirmed that high levels of TILs, including $CD8^+$, $CD3^+$ and $CD4^+$ correlate with improved survival [56]. A higher basal TIL count provides added survival advantages as compared to a low basal TIL count before ICI treatment with PD-1 inhibitor monotherapy. After ICI treatment administration, an increased TIL count has been observed in patients, which is indicative of a higher PFS chance. In addition to that, there is increased T cell clonality and high TMB present [57].

Recently, PD-L1 expression is markedly boosted after concurrent chemoradiotherapy (cCRT), but not after drug therapy alone. There is no significant link between baseline TPS and post-cCRT TPS. $CD8^+$ TIL density is significantly increased after cCRT. A higher post-cCRT $CD8^+$ TIL density is associated with a higher pathologic response with a favourable survival outcome.

Tumoural PD-L1 expression is elevated after cCRT (as supported by reasons as stated in Fig. 2), which provides therapeutic advantage for the administration of PD-L1 blockade following cCRT to improve the prognosis of NSCLC patients. High $CD4^+$ T cell density alone do not illustrate any significance in terms of prognosis, but a high density of $CD4^+$ T-cell combined with $CD8^+$ T-cell infiltration in cancer stroma has a desirable prognostic outcome [58]. The rationale for the addition of PD-L1 blockade following cCRT is based on preclinical evidence suggesting that chemotherapy and radiotherapy up-regulate PD-L1 expression on tumour cells. Patients with higher post-cCRT $CD8^+$ TIL density ($\geq$40) have shown significantly favourable relapse-free survival (RFS) and OS than those with lower post-cCRT $CD8^+$ TIL density [59].

Despite the promising premises of TILs, there seems to be confusion in some cases of responding patients and patients with progression due to increased $CD8^+$ T cell density in both these groups. Findings like this in certain groups of patients further hinders the process of confiding in TILs as appropriate biomarkers for ICI [60]. Besides, TILs could be rendered inactive in some ICI therapies such as PD-1/PD-L1 inhibitors and thus, their detection for post-treatment measures may prove to be difficult [61].

3.5 Tumour-Associated Macrophages

Tumour-associated macrophages (TAMs) are another kind of TME infiltrates along with TILs and generally, they have anti-inflammatory properties like M2 macrophages, although some may be anti-tumour in nature like the M1 macrophages. TAMs are believed to boost angiogenesis and lymphangiogenesis in NSCLC by increasing expression of VEGF-A and C in the TME, thus providing a lead to being potential biomarkers for NSCLC ICI therapy as well [62]. TAMs with M2 character are reflected by a $CD163^+/CD68^+$ TME and this immunostaining can be used as a means to quantify the presence of TAMs as biomarkers. In total, the presence of pre-treatment TAMs has been associated with a beneficial clinical outcome in patients, although some researchers argue that M1 is more favourable to patients over M2 due to the anti-tumour properties [63]. TAMs hold great potential in predicting patients who may respond satisfactorily to VEGF inhibitors (bevacizumab, etc.) or PD-1 inhibitors as part of ICI therapy, but they are not grounded well enough to be declared as isolated biomarkers for monitoring ICI therapy effectiveness in NSCLC patients.

3.6 Neutrophil to Lymphocyte Ratio

Neutrophil to lymphocyte ratio (NLR), as its name states, is a measure of the number of neutrophils in proportion to lymphocytes and it is acquired from patients' complete blood count (CBC). Tumour cells are found to recruit neutrophils to the TME as the neutrophils are role players in pro-tumour activities. Previous studies have demonstrated that NLR is used to study the responses and prognosis in many solid cancer varieties. The baseline levels and post-treatment NLR values are compared and an increase in NLR values is correlated to a significant negative impact on OS of NSCLC [64]. The NLR value increases acutely under the circumstances of tumour growth due to the release of EGF, PDGF and TGF-β [65]. As such a higher NLR corresponds to a worse prognosis as it is indicative of tumour cell proliferation. A study setting baseline NLR value as 5, showed that in the first-line treatment, the median survival times are 13.37 months and 6.77 months for NLR < 5 and NLR > 5, respectively. And similarly, patients under the second-line treatment category displayed median survival times of 13.67 and 5.63 months for NLR <5 and NLR > 5, respectively [66].

This evidence further adds weight to the fact that an increase in NLR value indicates a worse OS and PFS for the patients with NSCLC. NLR is an inexpensive and easy tool for evaluation for ICI. The higher the post-immunotherapy NLR is, the higher the risk of progression in patients with advanced NSCLC [67]. The statistical analysis data indicated that low pretreatment NLR (≤2.63) and decreased post-therapy NLR are associated with response to first-line platinum-based chemotherapy [68]. Although a high baseline NLR is linked to poor prognosis and survival in some studies, some others prioritise and show that a low NLR at the sixth week of treatment with anti-PD-1 monotherapy is correlated to better survival of the patient with NSCLC [69,70]. As such, there is a dilemma as to whether the pretreatment NLR value should take precedence over the post-treatment NLR for effective evaluation.

In total, an increase in NLR is a predictor of shorter survival in patients with advanced NSCLC and the changes in NLR during the first cycle of treatment with PD-1 inhibitors predicts survival. NLR values are easily measured and the tests are highly reproducible. However, confusions do revolve around the criteria to accept NLR as a biomarker of chemotherapy or cancer immunotherapy.

3.7 Non-Coding RNA

Non-coding RNA (ncRNA) refers to the RNA that do not translate into proteins. MicroRNAs (miRNA) and long non-coding RNA (lncRNA) make up most of ncRNAs. Many types of ncRNAs are usually present in greater quantities in NSCLC patients. They are believed to promote cell proliferation and have attracted attention in the ICI therapy [71]. For example, miR-424(322), miR-200, miR-513, miR-570, miR-217 exert the ability to suppress PD-L1 expression by binding to its 3'UTR in the NSCLC [71]. Moreover, by regulating PD-L1 expression, miR200b may be a valuable surrogate biomarker for PD-L1 expression in lung cancer patients [72].

4 Discussion

It is of utmost importance to identify reliable biomarkers in the study of ICI therapy effectiveness. Much effort and research regimes have been channelled into the study of biomarkers to establish a definite factor for the immunotherapy evaluation of NSCLC. Although cancer is an incredibly complex process with different outcomes and progressions in different patients, we strongly believe that adequate biomarkers will be utilized to overcome these differences and better aid patient treatment.

For biomarkers such as PD-L1 protein levels, which are long-time players in this field, a baseline value has to be set in order to decide when PD-L1 indicates a positive and negative prognosis for the patients. A high expression of PD-L1 in the TME positively correlates with a greater response to ICI therapy, thus resulting in better ORR and longer survival of patients. The increased TPS allows ICI drugs like PD-1/PD-L1 antibodies to have a greater impact on the tumour cells. Despite the benefit to patients, the discrepancies like heterogeneity of TME, need for invasive procedures, ethnicity and genomics have to be taken into consideration as well when configuring the baseline value as it may differ across populations. And for markers like exosomes, which prove to be more credible than soluble plasma values, a standardization of the best method of isolation should be done to ensure the best outcome for patients. Exosomes are a reflection of the tumour cells they originate from and the post-therapy levels of exosomes when compared to basal levels provide insight into the disease manifestation. An increased post-therapy exosome count is a signal of progressive disease, while a decrease indicates a partial response to therapy. Stable values signify the minimal impact of drugs on patients. TMB and TNB are still evolving sectors, which require a more in-depth study into the setting of baselines and isolation techniques. Tab. 1 serves as a summary of the basic mechanisms and significance of the various biomarkers explored in this study.

Table 1: Summary of biomarker mechanisms and their potential/significance in terms of immunotherapy in NSCLC patients

Biomarkers	Mechanism of action	Significance
PD-L1 protein levels	Transmembrane protein expressed on tumour cells, monocytes, etc. PD-L1 binds to PD-1 on T cells and diminishes immune function of T cells against tumour cells.	Patients with significantly greater PD-L1 levels had greater OS and better prognosis as the anti-PD-L1 drugs put forth better response in patients.
Extracellular vesicles/ exosomes	Lipid-enclosed vesicles that are characteristic of tumour cells that release them. Exosomes are more easily detected in patients as compared to soluble biomarkers.	Increased exosome levels are used to filter out responders from non-responders. It reflects the response of tumour cells to T-cell reactivation.
TMB	Refers to the amount of mutations present in tumour cells which produce TNA. Higher mutation rate is reflective of greater genomic instability due to pro-tumour changes.	TMB-high patients displayed a higher DCB rate, ORR and longer PFS than TMB-low patients when treated with PD-L1 inhibitors. High-TMB patients coupled with significant PD-L1 expression had an elevated DCB rate as high as 91%.

(Continued)

Table 1 (continued).

Biomarkers	Mechanism of action	Significance
TNB	Refers to measure of TNA produced by tumour-specific mutations. It is positively proportional to number of mutations.	Current standards of technological advancements do not seem to be on par with identification and study of TNAs. Therefore, more gaps have to be bridged to work out the full use of TNAs as predictive biomarkers.
cfDNA	Serves as byproducts of tumour cells undergoing apoptosis and has a rapid clearance rate from blood.	cfDNA levels plunge initially and radiography shows diminished tumour size gradually in responders. cfDNA has the potential to act as an earlier signal than radiography in responders.
TILs	Refer to the amount of effector T cells that initiate immune responses against tumour cells. TIL density and location is used to compute an immunoscore to aid classification of malignancy.	Higher pre-treatment TIL levels possibly confer a survival advantage to patients. In some patients, chemoradiotherapy increases TIL levels and it is associated with a higher pathologic response and favourable survival outcome.
TAMs	PD-1/PD-L1 can express in the TAMs, which modulates anti-tumor immune response.	TAMs hold great potential in predicting patients who may respond satisfactorily to PD-1 inhibitors as part of ICI therapy.
NLR	The baseline NLR value and post-treatment NLR values are noted in patients. It is indicative of tumour cell proliferation and increases significantly during tumour growth due to influence of EGF, PDGF, etc.	An increase in NLR post-therapy is indicative of a poor survival outcome, worse OS and PFS for patients.
Non-coding RNA	Some miRNA can directly regulate PD-1/ PD-L1 expression by binding to its 3'UTR in the NSCLC.	Some miRNA can pose the ability to suppress PD-L1 expression and be a valuable surrogate biomarker for PD-L1 expression in lung cancer patients.

When it comes to dynamic monitoring of immunotherapy effectiveness, some biomarkers may seem more feasible and attractive as compared to others. The entire purpose of dynamic monitoring is to simplify tracking disease progression with biomarkers and save as much time as possible in the testing period. Biomarkers such as PD-L1 protein levels, TMB and TNB are more time-consuming in the usage of tools such as WES and require the usage of biopsy specimens, which is invasive on the patients. While on the other hand, biomarkers such as exosomes and cfDNA provide easy isolation with just blood and plasma samples of patients. As such, with the ease of extraction and convenient testing standards, the latter group of biomarkers may seem to be plausible and more attractive options to monitor the immunotherapy effectiveness. However, the advantages and disadvantages of each biomarker have been highlighted in this review too and if one is to take a closer look at the comparison between biomarkers, different credibility will be attributed to the different biomarkers. Some biomarkers are more popular and widely accepted by global standards like the PD-L1 protein levels for instance. While some others are still emerging factors that may have a huge treasure of potential hidden. But overall, singling out a

biomarker is challenging and can be biased because patients have different genetic makeups that will affect the way the biomarkers monitor immunotherapy effectiveness. Different biomarkers can be utilized at different period intervals to enhance the credibility of analyzing immunotherapy effectiveness. For instance, biomarkers involving invasive or time-consuming techniques are used less frequently with a wider time interval whereas those biomarkers that are easily extracted from patient plasma/blood could be utilized more frequently in shorter time intervals. Hence, a combination of biomarkers for monitoring still proves to be a more ideal option.

Contradicting findings in both the progressive disease group and the improving group has proven to be a challenge to date. A composite score is one of the advantages of blood-based biomarkers as it produces a combined evaluation of multiple biomarkers. As such, more than one biomarker has to be employed in a single patient to increase the credibility of the prognosis prediction and to better cater to the patients' neoplastic needs. With more technological tools such as the multiplex IHC, high-throughput sequencing technology and microarray technology, an increased number of biomarkers can be screened on the genomic scale and quantified with respect to better outcomes for the patients with NSCLC. With a multiple biomarker approach, the reliability of the predictive response in patients is brought to greater heights. As of now, many biomarkers are still under thorough conceptualization and are yet to be deemed as definite predictive biomarkers. But surely, in the near future, these biomarkers will bring about a significant breakthrough in predictive immunotherapy response of NSCLC and other types of cancers.

5 Future Perspective

This review has analyzed the roles of PD-L1 protein levels mainly, along with a few other popular biomarkers. However, the list of possible biomarkers is not exhaustive. Some biomarkers may improve the precision of prognosis prediction of ICI therapy in patients when combined with other biomarkers. A rational system can be looked into to achieve the plausible combined review of various biomarkers to cater to patient needs and requirements. Since not all the biomarkers can be utilized at once, in the future more studies can be invested into settling on feasible biomarker combinations for patients, thus catering to the different genetic makeup and needs of the patients.

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