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Review

Emerging Biomarkers for the Selection of Advanced NSCLC-Affected Immunotherapy Patients

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Abstract: Immunotherapy in the form of ICIs has revolutionized advanced NSCLC treatment algorithms, with ICI-containing combination treatments being the latest addition to approved regimens. However, PD-L1 still represents the only routinely assessed and validated biomarker apart from genetic drivers testing, impairing our capacity to personalize and guide treatment. Therefore, this paper aims to analyze the most promising emerging predictive biomarkers that could help us in the near future to select patients more effectively.

Keywords: advanced NSCLC; immunotherapy; predictive biomarkers; PD-L1; TMB; TILS; NLR; microbiome; IL-8

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1. Immunotherapy for the Treatment of Advanced NSCLC

While for years, chemotherapy has represented the only major therapy for advanced NSCLC (non-small cell lung cancer) treatment [1–4], as of today, immunotherapy in the form of immune checkpoint inhibitors (ICIs) represents one of the cornerstones of advanced NSCLC treatment. ICIs exert their activity by binding coinhibitory receptors (e.g., PD-1, CTLA-4) or ligands (e.g., PD-L1) in the context of immune checkpoints, thus preventing T-cell deactivation and T-cell apoptosis. [5,6] Four ICIs are currently FDA- (US Food and Drug Administration) and EMA (European Medicines Agency)-approved and recommended by international guidelines for this subset of patients: nivolumab, pembrolizumab, atezolizumab and ipilimumab. These agents are employed both as single agents and in combination with chemotherapy and/or other ICIs and both in the first and in the second line setting. [7–9] (Table 1).

Nivolumab is FDA-approved in association with ipilimumab for naïve squamous and nonsquamous patients without genetic drivers with PD-L1 expression levels $\geq$1% and is FDA- and EMA-approved in combination with ipilimumab and two cycles of platinum-based doublet chemotherapy for naïve squamous and nonsquamous patients without genetic drivers; moreover, it is also FDA- and EMA-approved in a second line setting for squamous and nonsquamous patients without genetic drivers progressing after platinum-based chemotherapy [10–12].

Pembrolizumab is FDA-approved in monotherapy for naïve squamous and nonsquamous patients without genetic drivers with PD-L1 expression levels $\geq$1%, FDA- and EMA-approved for naïve squamous and nonsquamous patients without genetic drivers with PD-L1 expression levels $\geq$50%, FDA- and EMA-approved in association to cis/carboplatin + pemetrexed for naïve nonsquamous patients without genetic drivers and FDA- and

EMA-approved in combination to carboplatin + (nab)paclitaxel for naïve squamous patients without genetic drivers; furthermore, it is also FDA- and EMA-approved in a second line setting for squamous and nonsquamous patients without genetic drivers with PD-L1 expression levels ≥1% progressing after platinum-based chemotherapy [13–16].

Atezolizumab is FDA-approved in monotherapy for naïve squamous and nonsquamous patients without genetic drivers with PD-L1 expression levels ≥50% or PD-L1 stained tumor-infiltrating immune cells (IC) ≥10%, FDA- and EMA-approved in combination to carboplatin + (nab)paclitaxel + bevacizumab for naïve nonsquamous patients without genetic drivers and FDA- and EMA-approved in combination to carboplatin + nab-paclitaxel for naïve nonsquamous patients without genetic drivers; in addition, it is FDA- and EMA-approved in a second line setting for squamous and nonsquamous patients without genetic drivers progressing after platinum-based chemotherapy [17–20].

Table 1. Currently FDA-approved and international guidelines-recommended ICIs for the treatment of advanced NSCLC.

Drug	PD-L1 Test	Line of Treatment	Subset of Patients	Regimen	Pivotal Trial/s
Nivolumab (Anti PD-1 ICI)	PD-L1 IHC 28-8 pharmDx *	First-line	Naïve squamous and nonsquamous patients without genetic drivers with PD-L1 expression levels ≥ 1%	Nivolumab + Ipilimumab (Anti CTLA-4 ICI)	CheckMate 227
Nivolumab (Anti PD-1 ICI)	PD-L1 IHC 28-8 pharmDx *	First-line	Naïve squamous and nonsquamous patients without genetic drivers	Nivolumab + Ipilimumab (Anti CTLA-4 ICI) + Two cycles of platinum-based doublet chemotherapy	CheckMate 9LA
Nivolumab (Anti PD-1 ICI)	PD-L1 IHC 28-8 pharmDx *	Second-line	Squamous and nonsquamous patients without genetic drivers progressing after platinum-based chemotherapy	Nivolumab monotherapy	CheckMate 017 CheckMate 057
Pembrolizumab (Anti PD-1 ICI)	PD-L1 IHC 22C3 pharmDx *	First-line	Naïve squamous and nonsquamous patients without genetic drivers with PD-L1 expression levels ≥ 1%	Pembrolizumab monotherapy	KEYNOTE-042
Pembrolizumab (Anti PD-1 ICI)	PD-L1 IHC 22C3 pharmDx *	First-line	Naïve nonsquamous patients without genetic drivers	Pembrolizumab + Cis/carboplatin + Pemetrexed	KEYNOTE-189
Pembrolizumab (Anti PD-1 ICI)	PD-L1 IHC 22C3 pharmDx *	First-line	Naïve squamous patients without genetic drivers	Pembrolizumab + Carboplatin + (Nab)paclitaxel	KEYNOTE-407
Pembrolizumab (Anti PD-1 ICI)	PD-L1 IHC 22C3 pharmDx *	Second-line	Squamous and nonsquamous patients without genetic drivers with PD-L1 expression levels ≥ 1% progressing after platinum-based chemotherapy	Pembrolizumab monotherapy	KEYNOTE-010
Atezolizumab (Anti PD-L1 ICI)	Ventana PD-L1 (sp142) *	First-line	Naïve squamous and nonsquamous patients without genetic drivers with PD-L1 expression levels ≥ 50%	Atezolizumab monotherapy	IMpower110
Atezolizumab (Anti PD-L1 ICI)	Ventana PD-L1 (sp142) *	First-line	Naïve nonsquamous patients without genetic drivers	Atezolizumab + Carboplatin + Nab-paclitaxel	IMpower130

Table 1. *Cont.*

Drug	PD-L1 Test	Line of Treatment	Subset of Patients	Regimen	Pivotal Trial/s
Atezolizumab (Anti PD-L1 ICI)	Ventana PD-L1 (sp142) *	First-line	Naïve nonsquamous patients without genetic drivers	Atezolizumab + Carboplatin + (Nab)paclitaxel + Bevacizumab	Impower150
Atezolizumab (Anti PD-L1 ICI)	Ventana PD-L1 (sp142) *	Second-line	Squamous and nonsquamous patients without genetic drivers with PD-L1 expression levels ≥ 1% progressing after platinum-based chemotherapy	Atezolizumab monotherapy	OAK POPLAR

* It is worth mentioning that, while the PD-L1 IHC 22C3 pharmDx and the PD-L1 IHC 28-8 pharmDx tests present a very high concordance, the same does not apply to the ventana PD-L1 (sp142), due to its different staining properties.

2. Currently Available Biomarkers for the Selection of Advanced NSCLC-Affected Immunotherapy Patients: PD-L1

PD-L1 (programmed death-1 ligand; also known as CD279 or B7-H1) is a ligand expressed on the surface of antigen presenting cells (APCs), that is to say macrophages, B cells and dendritic cells and on the surface of tumor cells. The binding of PD-L1 to its receptor PD-1 (programmed death 1), expressed on the surface of activated T-cells, causes T-cells deactivation and apoptosis [21,22].

As the above-mentioned data show, apart from genetic drivers testing, up to this date PD-L1 represents the only validated predictive biomarker routinely evaluated in order to stratify patients and to guide the treatment choice in relation to ICI treatment [23,24]; PD-L1, however, is far from being devoid of problems.

PD-L1 Limitations: PD-L1 as a Flawed Predictive Biomarker

In order to better explain the limitations associated with PD-L1's capacity to predict response to immunotherapy, it can be useful to take into account the data from each ICI pivotal trial (Table 1).

In CheckMate 017, PD-L1 expression levels were found to be neither prognostic nor predictive of survival benefit, while in CheckMate 057, increasing PD-L1 expression levels were found to be associated with more favorable clinical outcomes [25,26]. In CheckMate 227, increasing PD-L1 expression levels were found to be associated with more favorable clinical outcomes; however, while the benefit was clear for PD-L1 expression levels ≥50%, patients with PD-L1 expression levels <1% presented comparable survival outcome to those with PD-L1 expression levels ≥1% and better survival outcomes than those with PD-L1 expression levels between 1–49% [27]. In CheckMate 9LA, patients with PD-L1 expression levels <1% presented comparable hazard ratios for death to those with PD-L1 expression levels ≥1% [28]. In KEYNOTE-042, increasing PD-L1 expression levels were found to be associated with more favorable clinical outcomes; this was the case for PD-L1 expression levels ≥1%, ≥20% and ≥50%; however, patients with PD-L1 expression levels between 1–49% showed worst survival data than all the other subsets of patients [29]. On the other hand, in KEYNOTE-189 and in KEYNOTE-407, increasing PD-L1 expression levels were found to be associated with more favorable clinical outcomes [30,31]; similarly, in KEYNOTE-010, increasing PD-L1 expression levels were found to be associated with more favorable clinical outcomes [32]. In the OAK, IMpower110, IMpower130 and IMpower150 trials, increasing PD-L1 or IC expression levels were found to be associated with more favorable clinical outcomes; conversely, in the POPLAR study, patients with PD-L1 expression levels ≥1% had comparable survival outcomes to those with PD-L1 expression levels ≥ 50% and better survival outcomes than those with PD-L1 expression levels ≥5% [33–37].

Globally, considering all the above-mentioned data, it clearly appears that PD-L1 represents an indeed flawed predictive biomarker: while on the one hand, greater response rates and superior survival performances are linked to higher PD-L1 expression levels (especially for PD-L1 expression levels ≥50%), on the other hand, patients with low or without PD-L1 expression can benefit greatly from ICI therapy, at times even more than patients with higher PD-L1 expression levels. In addition, while generally, patients with PD-L1 expression levels (especially if ≥50%) experience the best clinical outcomes to ICI treatment, poor responses are reported. Two main reasons could lie behind the limitations of PD-L1 as a predictive biomarker for ICI treatment: firstly, the PD-1–PD-L1 axis only represents one of the several different immune checkpoints involved in cancer immune-mediated escape mechanisms; secondly, apart from immune checkpoints, the PD-1–PD-L1 axis fits into the extremely wider (and still poorly understood) context of tumor microenvironment [38,39]

3. Emerging Biomarkers for the Selection of Advanced NSCLC-Affected Immunotherapy Patients

Therefore, several different biomarkers are currently under investigation, in order to overcome PD-L1 limitations and to better guide ICI therapy in advanced NSCLC patients.

3.1. TMB

The tumor mutational burden (TMB) represents the amount of cancer genome mutations per DNA coding region; in preclinical and early clinical trials its high expression was associated to an independent from PD-L1 expression levels increased response to ICI treatment; the reason for these results could seemingly lie in the enhanced neoantigen expression caused by high mutational loads [40,41]. In the original design of the CheckMate 227 study, TMB was evaluated as a potential predictive biomarker. In this trial, 1189 naïve squamous and nonsquamous advanced NSCLC patients without genetic drivers and with PD-L1 expression levels ≥1% were randomized (1:1:1) to receive nivolumab + ipilimumab, histology-based chemotherapy, or nivolumab monotherapy, while 550 naïve squamous and nonsquamous advanced NSCLC patients without genetic drivers and with PD-L1 expression levels <1% were randomized (1:1:1) to receive nivolumab + ipilimumab, histology-based chemotherapy, or nivolumab + histology-based chemotherapy; both subsets of patients were assessed for TMB expression. In high-TMB patients (≥10 mutations per megabase), the nivolumab + ipilimumab association performed better than histology-based chemotherapy according to every prespecified outcome measure and most importantly independently from PD-L1 expression levels: ORR (objective response rate): 45.3% vs. 26.9%, PFS (progression free survival): 7.2 months vs. 5.5 months, HR (hazard ratio) for disease progression or death: 0.58 [42]. Nonetheless, after a request by the FDA and the EMA-CHMP (EMA Committee for Medicinal Products for Human Use), OS (overall survival) data were disclosed, including both the high-TMB and the low-TMB (<10 mutations per megabase) patients. As a consequence, the nivolumab + ipilimumab experimental combination proved to be superior to histology-based chemotherapy in both high-TMB patients (OS: 23.03 months vs. 16.72 months; HR: 0.77) and low-TMB patients (16.20 months vs. 12.42 months; HR: 0.78). Due to these data, TMB effectiveness and reliability as a predictive biomarker has been significantly attenuated, seemingly re-defining it as a prognostic biomarker [43,44]. Furthermore, the TMB was also investigated in a post hoc exploratory analysis comprising the data from KEYNOTE-010 (cohorts C and G), KEYNOTE-189 and KEYNOTE-407 trials, utilizing a cut-off of 175 mutations per exome; also in this analysis, no survival benefit was noted neither in terms of ORR nor in terms of PFS or OS for patients with a high TMB (≥175 mutations per exome) [45].

3.2. TILS

$CD3^+$, $CD4^+$ and $CD8^+$ tumor infiltrating lymphocytes (TILS) are the main effectors of the ICI-mediated anticancer activity and higher levels of TILS are associated to a better prognosis in NSCLC patients [46]. However, enhanced TILS levels (in particular $CD8^+$

TILS) also seem to be linked superior responses to ICI therapy. In a recent trial, Uryvaev et coll. analyzed a subset of 26 advanced NSCLC patients, assessing $CD4^+$ and $CD8^+$ TILS levels in tumors tissues: while $CD8^+$ TILS levels <886/mm^2 were associated with lower response rates to anti PD-1 ICI therapy (16.7% of treated patients), $CD8^+$ TILS levels between 886–1899/mm^2 were associated with superior response rates (60% of treated patients); furthermore, $CD8^+/CD4^+$ TILS ratios <2 were linked to lower response rates (13.3% of treated patients), while $CD8^+/CD4^+$ TILS ratios >2 were linked to superior response rates (43–50% of treated patients) [47].

3.3. NLR

The neutrophil-to-lymphocyte ratio (NLR) is simply calculated by dividing the number of neutrophils by the number of lymphocytes after a blood sample; a neutrophil-rich TME is believed to promote tumor progression via the production of cytokines that enhance angiogenesis and inhibit cell death. In this vein, baseline increased NLR has proved to be a negative prognostic biomarker (in terms of inferior OS and PFS results) in advanced NSCLC patients treated with chemotherapy, immunotherapy or targeted therapy [48]; for example, in a very recent series of retrospective trials analyzing 784 and 132 advanced NSCLC patients with PD-L1 expression levels ≥50%, Banna et coll. showed that an NLR >4 or >5, respectively, represents an independent negative prognostic factor also in this subset of patients [49,50]. Moreover, the NLR could also serve as a predictive biomarker for advanced NSCLC undergoing ICI treatment, according to several recent studies. In a recent retrospective trial, Bagley et coll. reviewed the medical records of 175 pretreated advanced NSCLC patients receiving subsequent-line nivolumab and evaluated their NLR adopting a ≥5/<5 cut-off; as a result, a baseline NLR ≥5 proved to be associated with inferior survival outcomes when compared to a baseline NLR <5: PFS: 1.9 months vs. 2.8 months (HR for death or progression: 1.43), OS: 5.5 months vs. 8.4 months (HR for death: 2.07) [51]. Similarly, in another trial, Nakaya et coll. retrospectively evaluated 101 pretreated advanced NSCLC patients receiving subsequent-line nivolumab analyzing their NLR, employing a ≥3/<3 cut-off not only before ICI therapy, but also after 2 and 4 weeks after the first administration; as a result, not only the PFS data favored patients with low baseline NLR (3.4 months vs. 2.9 months), but they also favored patients with a low NLR at two weeks after treatment (5.3 months vs. 2.1 months) and at 4 weeks after treatment (5.3 months vs. 2.0 months) [52].

3.4. IL-8

Interleukine-8 (IL-8) is a pro-inflammatory chemokine overexpressed by tumor cells (often alongside its receptor, also known as IL-8R); the activation of the IL-8–IL-8R pathway promotes angiogenesis, proliferation, tumor cells survival, the formation of metastases as well as the creation of an immunosuppressive TME [53]. IL-8's possible predictive role with respect to ICI treatment is currently the subject of debate. In an interesting recent trial, Sanmamed et coll. analyzed 19 advanced NSCLC-affected pretreated patients receiving subsequent-line nivolumab or pembrolizumab, assessing serum IL-8 levels before ICI treatment and 2 and 4 weeks after the first administration. Interestingly, taking into account baseline levels, among the 12 responders, the median serum IL-8 levels declined when the patients presented the best response; at the same time, among the seven non-responders, median serum IL-8 levels rose when they experienced a progression of disease, suggesting that variations of serum IL-8 levels could represent a predictive biomarker with reference to ICI treatment; on a side note, it is worth mentioning that when two patients showed radiological signs of progression that was confirmed to be a pseudoprogression at a later follow-up, a decrease in serum IL-8 levels was recorded [54].

3.5. IDO1

Indoleamine 2, 3-dioxygenases (IDO1) is an enzyme normally responsible for the conversion of tryptophan into kynurenine, whose metabolites exert an immunosuppressive

role and contribute to immune tolerance; however, IDO1 levels can also be enhanced in several tumors, NSCLC included, leading to an immunosuppressive TME. In this vein, in an intriguing study by Botticelli et coll, the kynurenine/tryptophan ratio was assessed as a predictive biomarker with reference to ICI treatment. Considering 26 advanced NSCLC-affected patients, a statistically significant correlation (p: 0.017) was noted between a short time to the progression of disease (<3 months) and higher kynurenine/tryptophan levels, while lower kynurenine/tryptophan levels were significantly linked to improved PFS and OS results (p: 0.018) [55]

3.6. Microbiome and Antibiotic Treatment

Preclinical and clinical trials have established that bacterial diversity in the context of gut microbiome plays an important role with respect to immune response against several different cancers, NSCLC included. In the same vein, pre-ICI therapy antibiotic treatment has been linked to decreased immunotherapy effectiveness in terms of PFS and OS, seemingly due to decreased gut bacteria diversity [56,57]. Therefore, gut microbiome is currently being assessed as a potential predictive biomarker. In a recent study by Hakozaki et al., baseline stool samples were collected from 70 advanced NSCLC patients before administering first or subsequent-line ICI monotherapy, then the median clinical outcome measures were reported: mORR: 34%, mPFS: 5.2 months, mOS: 15.2 months. On the one hand, patients who received pre-immunotherapy antibiotic treatment presented a reduced gut bacteria diversity with a particularly diminished representation of Ruminococcaceae UCG 13 and Agathobacter, resulting in worse OS results when compared to patients who did not receive pre-immunotherapy antibiotic treatment: 12.1 months vs. 16.1 months; on the other hand, patients who did not receive pre-immunotherapy antibiotic treatment presented a superior gut bacteria diversity with an overrepresentation of Ruminococcaceae UCG 13 and Agathobacter, resulting in better ORR (>34%), PFS (>6 months) and OS (>16 months) [58]

3.7. Immune Gene Signatures

An immune gene signature can be defined as the combination of all the alterations of the immune-related gene expression [59,60]. In this vein, several different immune gene expressions are presently being investigated in order to assess if they can predict response to immunotherapy. In a very recent and interesting study, Hwang et al. collected tumor samples from 21 advanced NSCLC-affected ICI-treated patients, proving that patients harboring an M1 signature (including the CBLB, CCR7, CD27, CD48, FOXO1, FYB, HLA-B, HLA-G, IFIH1, IKZF4, LAMP3, NFKBIA, SAMHD1 genes) or a peripheral T signature (including the HLA-DOA, GPR18, STAT1 genes), as well as those expressing CD137 and PSMB9 mRNAs showed superior survival performances when compared to those who did not and that this immune gene signature proved to be far more predictive of ICI response than PD-L1, TMB or TILS [61].

3.8. Established and Emerging Mutations

According to the more recent and larger literature data, classic driver mutations (EGFR, ALK, BRAF, ROS1, MET, KRAS, HER 2, RET) are considered negative predictive biomarkers, due to their association with very poor response to ICI treatment and thus, immunotherapy should only be considered after the failure of all the other available therapies [62,63]. Furthermore, this association also seems to apply to more uncommon mutations, e.g., STK11. Taking into account both the available literature data and the data coming from a very large and recent trial by Ricciuti et coll., it appears that STK11 mutations represent a negative predictive biomarker in ICI-treated advanced NSCLC-affected patients in terms of poor ORR, PFS and OS when associated to a KRAS co-mutation, while they seem to be uninfluential when associated to a wild type KRAS gene [64,65].

4. Conclusions and Future Perspectives

Immunotherapy in the form of ICIs has revolutionized advanced NSCLC treatment algorithms, with ICI-containing combination treatments being the latest addition to approved regimens [66,67]. However, if on the one hand treatment strategies have consistently evolved since the first FDA-approved ICI in this subset of patients (nivolumab, 2015) [68], on the other hand, our biomarker availability still lags behind, with PD-L1 still representing the only routinely assessed and validated apart from genetic drivers testing; moreover, taking into account the different PD-L1 tests used, it is worth mentioning that while the PD-L1 IHC 22C3 pharmDx and the PD-L1 IHC 28-8 pharmDx tests present a very high concordance, the same does not apply to the ventana PD-L1 (sp142), due to its different staining properties [69]. Nevertheless, as the above-mentioned data extensively show, there is a growing and thriving interest towards emerging predictive biomarkers, that in a near future will undoubtedly help us to further tailor treatments, optimizing current treatment options and maximizing survival results. In this vein, further and larger studies will be needed in order to validate, standardize and shift these experimental biomarkers into clinical practice.

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