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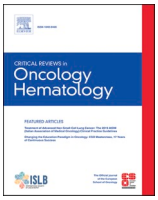

Immunotherapy-related biomarkers: Confirmations and uncertainties

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ABSTRACT

Immunotherapy profoundly changed oncology treatment, becoming one of the main therapeutical strategies. Remarkable improvement has been achieved in survival outcomes, but the percentage of patients who benefit from immunotherapy is still limited. Only one-third of patients receiving immune checkpoint inhibitors (ICIs) achieve long-term response. Several patients are not responsive to treatment or relapse after an initial response. To date, programmed death-ligand 1, microsatellite instability, and tumor mutational burden are the three biomarkers validated to predict the ICIs response, but a single variable seems still insufficient in the patient's selection. Considering the substantial and increasing use of these drugs, the identification of new predictive biomarkers of ICI response is of paramount importance. We summarize the state of the art and the clinical use of immune biomarkers in oncology, highlighting the strength and weaknesses of currently approved biomarkers, describing the emerging tissues and circulating biomarkers, and outlining future perspectives.

1. Introduction

Over the last decade, immunotherapy deeply changed the landscape of advanced malignancies treatments, becoming the fourth pillar in cancer therapy along with surgery, chemo- and radiotherapy. Immune checkpoint inhibitors including cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed death-1 (PD-1), and programmed death-ligand 1 (PD-L1) achieved success in clinical trials (Vaddepally et al., 2020; Schadendorf et al., 2015a). In 2011, ipilimumab was the first immune checkpoint inhibitor (ICI) to be approved for metastatic melanoma, subsequently followed by several other ICIs approvals in different malignancies. Immunotherapy has repeatedly shown long-term therapeutic responses, transforming the outcomes of aggressive diseases. However, response rates continue to be widely variable, with a large percentage of patients primarily refractory or just transiently responders (Topalian et al., 2019; Zaretsky et al., 2016). Immune-related adverse events have also been reported with ICIs, ranging from mild to life-threatening, and in some cases leading to tumor hyper-progression (Postow et al., 2018; Champiat et al., 2017). Therefore, identifying biomarkers with high sensitivity and specificity, able to predict the clinical benefit of ICIs, is of paramount importance. Numerous biomarkers, resulting from tumor tissue or blood circulation, have been extensively explored (Roviello et al., 2022a). PD-L1 expression, microsatellite instability (MSI), and tumor mutational burden (TMB) have received clinical validation as predictive factors in the ICI response, even though with some uncertainties. Other biomarkers, including novel circulating markers or gut microbiome and its derivates, are currently widely explored. Overall, the simultaneous utilization of more than one biomarker could be a successful strategy to overcome current limitations. Herein, we describe the current Food and Drug Administration (FDA) approved predictive biomarkers for solid malignancies treated with ICIs and reported the updated overview of the emerging biomarkers and their abilities to predict therapeutic response.

2. Essentials of cancer immunotherapy

The immune system is an interactive network of lymphoid organs, cells, humoral factors, and cytokines with the essential function of host defense against foreign pathogens. Its alteration may result in severe infections and tumors (underactive) or in allergic and autoimmune diseases (overactive). It is commonly divided into two macro systems: innate immunity, the first and the faster to intervene, and adaptive

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immunity, slower but more effective (Newman, 2018). As early as 1957 an immune surveillance of the immune system against malignant cells has been hypothesized (Burnet, 1957). However, the interaction between the immune system and cancer cells has been recently clarified and identified in a process known as immunoediting (Vesely and Schreiber, 2013). This latter is a dynamic process initiated by tumor cells in response to immunosurveillance of the immune system which can be easily summarized in three phases: elimination, equilibrium, and escape (Fig. 1).

The complex environment that surrounds solid malignancies is responsible for their growth, invasion, and metastasis. The tumor microenvironment (TME) includes tumor cells, extracellular matrix, vascular and lymphatic networks, as well as innate and adaptive immune cells. A better understanding of TME and host immune evasion mechanisms allowed the development of active ICIs in several solid neoplasms. Immune checkpoints are regulators of immune activation and play a crucial role in maintaining immune homeostasis, carefully balancing co-stimulatory and inhibitory signals, needed to maintain self-tolerance and protect the host from tissue damage (Sykes, 2007). T cells are primary mediators of immune effector functions and express several co-inhibitory receptors such as PD-1 and CTLA-4, as well as Lymphocyte activation gene-3 (LAG-3), T cell immunoglobulin and mucin-domain containing-3 (Tim 3) and others.

Both CTLA-4 and PD-1 act as negative regulators, each playing a non-redundant and complementary role in modulating immune responses. CTLA-4 binds CD80 and CD86, attenuating the early activation of naïve and memory T cells, whereas PD-1 (expressed on activated tumor-infiltrating lymphocytes [TILs]) is mainly involved in the modulation of T cells in peripheral tissues by interacting with PD-L1 and PD-L2 (commonly upregulated on the surface of tumor cells) (Ahmadzadeh et al., 2009; Sfanos et al., 2009). Treatments with anti-CTLA-4 and anti-PD1 restore antitumor immunity through the blockade of immunoregulatory checkpoints and immune recognition of cancer cells (Callahan and Wolchok, 2013; Kyi and Postow, 2014).

3. Immune-related biomarkers

Only three biomarkers have been clinically validated to date with several unanswered questions on their prognostic and predictive significance (Table 1).

The study of peripheral blood-based biomarkers continues to be an attractive field given their minimally invasive nature for detection. Circulating biomarkers such as soluble proteins, circulating peripheral lymphocytes, tumor-associated neoantigen-specific T cells, inflammatory cytokines, circulating tumor DNA and, many others, have been investigated but, their role in the ICI response needs to be confirmed yet.

3.1. Currently approved biomarkers

3.1.1. Programmed Death-Ligand 1

PD-L1 expression on tumor cells measured by immunohistochemistry (IHC) is currently the most used biomarker to select patients for ICI therapy (Herbst et al., 2014a). Different PD-L1 assays able to correlate PD-L1 expression with the objective response to ICI in some tumors have been identified: PD-L1 IHC 22C3 pharmDx, PD-L1 IHC 28–8 pharmDx and VENTANA PD-L1 (SP142), PD-L1 IHC SP263 (Tsao et al., 2018).

The selection of the appropriate assay is determined by the specific ICI under consideration.

For example, the PD-L1 IHC 22C3 and PD-L1 IHC 28–8 pharmDx assay are used to determine PD-L1 expression levels in patients with non-small cell lung cancer (NSCLC) who are being considered for treatment with pembrolizumab or nivolumab, respectively. VENTANA PD-L1 (SP142) assay is used to determine PD-L1 expression levels in patients with urothelial carcinoma who are being considered for

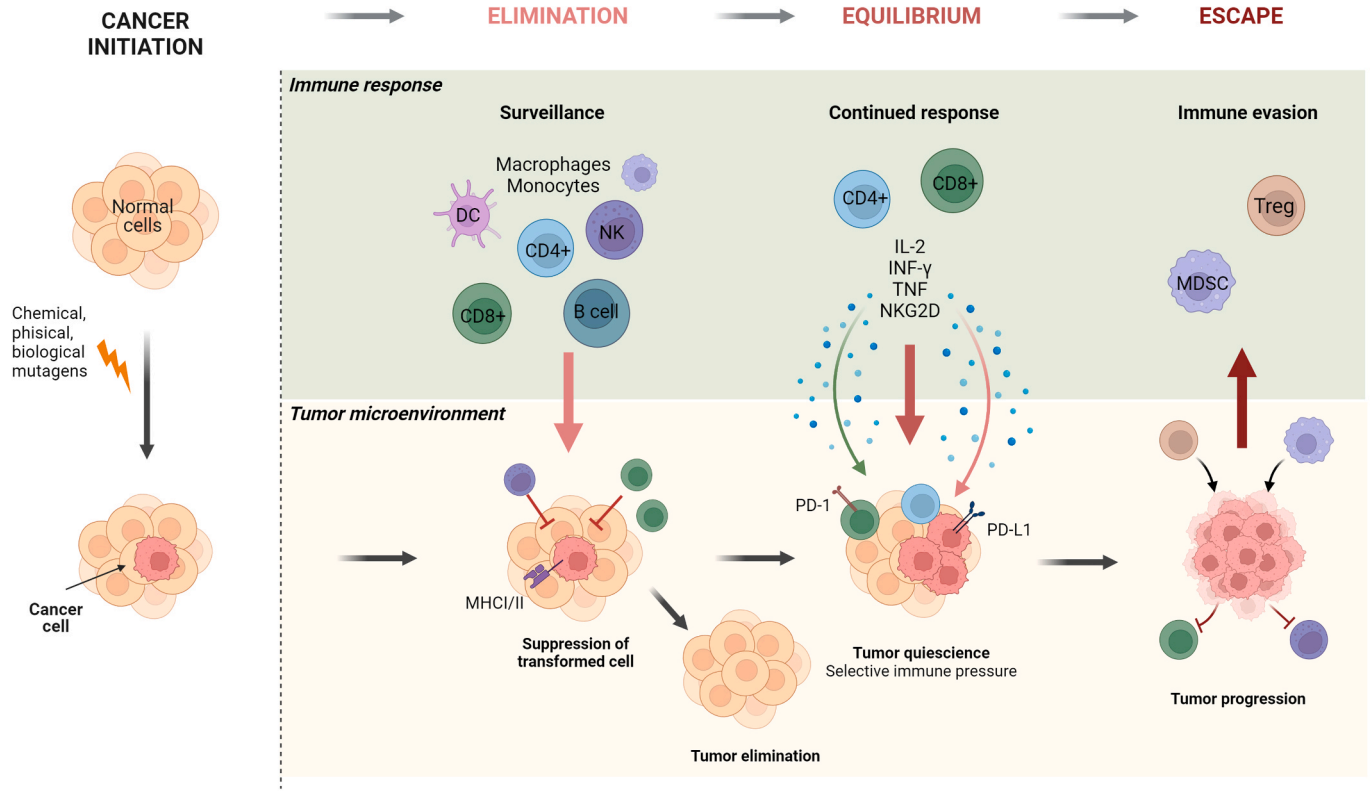

Fig. 1. Cancer immunoediting process. DC, dendritic cell; NK, natural killer; MDSC, myeloid-derived suppressor cells; Treg, regulatory T cell; IL-2, Interleukin-2; INK-γ, Interferon-γ TNF, tumor necrosis factor; PD-1/PD-L1, programmed death-1/ligand1; MSHI/II, major histocompatibility complex I/II. Created with BioRender.com.

Table 1
Current FDA approved biomarkers.

Biomarker	Role	Tissue type for assessment	FDA approval year	Clinical use	Assay for biomarker measurement	Strenghts	Limitations
PD-L1	Predictive	Tumor	2015	NSCLC, TNBC, HNSCC, Esophageal cancer, GEJ/Gastric cancer, Melanoma, Cervical cancer, Bladder cancer	Immunohistochemistry	Easily dosable and reproducible.	Variability of four PD-L1 assays using different PD-1/ PD-L1 antibodies, scoring criteria and cut-offs for PD-L1.
TMB	Predective and prognostic	Tumor and blood	2020	Advanced solid tumors that have progressed from prior treatment (TMB≥10 mut/ Mb as biomarker for administering anti-PD1 therapy)	Whole Exome Sequencing	Reproducibility and repeatability.	High cost, long turn-around time, technical complication, and availability.
					NGS targeted panels	Agreement between NGS panels-derived and WES-derived TMB data.	Difference between tumor type (different tumor types biologically have different TMB), tissue type (FFPE tissue will artificially have more mutations than fresh frozen tissue), sequencing parameters (NGS panel content, size and sequencing depth, bioinformatics pipeline), and the reporting cut-off.
MSI/ dMMR	Predective and prognostic	Tumor and blood	2017	Solid tumor progressed to following prior treatment and who have no satisfactory alternative treatment options	Immunohistochemistry	Simple, fast, cost effective widely available.	Too many variables, hard to determinate cut-off, relatively low analytic sensitivity, and accuracy.
					Polymerase Chain Reaction	Widely available, ease of use, accurate.	Capture partial MSI profiles, low prevalence in solid tumor types.
					Next Generation Sequencing	Capture full MSI profile, suitable for all tumor type. Accurate and sensitive Simultaneous detection of other potential predictors.	High cost, technical demands, lack of wide availability, need tumor-type specific cut-off.

Programmed death-1/ligand1, (PD-1/PD-L1); Tumor mutational burden, (TMB); Microsatellite instability, (MSI); Next generation sequencing, (NGS); Formalin fixed, paraffin embedded, (FFPE); Whole Exome Sequencing, (WES); Food and drug administration, (FDA), non-small cell lung cancer, (NSCLC);triple negative breast cancer, (TNBC); head and neck squamous cell carcinoma, (HNSCC); gastroesophageal junction, (GEJ); mutations/mega base, (mut/MB).

treatment with atezolizumab and PD-L1 IHC 73–10 pharmDx assay is used to determine PD-L1 expression levels in patients with unresectable stage III NSCLC who are being considered for treatment with durvalumab (Antonia et al., 2017; Herbst et al., 2014a; Rimm et al., 2017; Hirsch et al., 2017).

The response to immunotherapy is correlated with both the PD-L1 expression on tumor cells and immune cells. PD-L1 expression can be evaluated by measuring the tumor proportion score (TPS), which is the percentage of viable tumor cells that show partial or complete membrane staining at any intensity, and the combined positive score (CPS), which is the ratio of the number of all PD-L1-expressing cells (including tumor cells, lymphocytes, and macrophages) to the total number of tumor cells (Roach et al., 2016).

Using the TPS scoring system, mainly validated in NSCLC, the specimen is considered to have a positive PD-L1 expression if TPS $\geq$ 1% and high PD-L1 expression if TPS $\geq$ 50% (Herbst et al., 2019, 2016). Likewise, CPS$>$ 1% indicates the expression of PD-L1 and it is validated in several tumor malignancies including urothelial cancer, gastric/gastroesophageal adenocarcinoma, triple-negative breast cancer, head and neck squamous cell cancer (Kulangara et al., 2017). Alternatively, PD-L1 expression on TILs can be measured as the percentage of PD-L1 positive immune cells: IC0 ($<$1%), IC1 ($\geq$1% but $<$5%) and, IC2/3 ($\geq$5%). However, PD-L1 is a flawed biomarker that cannot single-handedly select patients for ICI treatment. Patients with a negative baseline staining for PD-L1 may respond to ICI as well as tumors with high PD-L1 expression may be resistant to treatment. In a study analyzing approximately forty-five ICI approvals, PD-L1 was predictive in only 28.9% of cases emphasizing the limitations of the PD-L1 test (Davis and Patel, 2019).

3.1.2. *Tumor mutational burden*

TMB is defined as the total number of somatic mutations per coding area of a tumor genome (Chalmers et al., 2017a). The accumulation of somatic mutations can determine the formation of tumor-specific neoantigens which can be presented by human leukocyte antigen (HLA) molecules of tumor cells. Neoantigens elicit CD8 + T cell-dependent immune responses, providing the basis for its use as an ICI's efficacy biomarker (Peggs et al., 2007; Chen and Flies, 2013). The number of somatic mutations is very variable and can range from a few to thousands of mutations and seems proportionally related to neoantigen quantity in the tumor (Devarakonda et al., 2018). The correlation of TMB with ICI response has been evaluated by whole exome genetic (WES) panel analysis or large cancer gene-targeted sequencing panels on samples from different tumor tissues and more recently, through the examination of circulating free DNA (Johnson et al., 2016; Rosenberg et al., 2016; Le et al., 2015; Gandara et al., 2018). The WES thechnique involves sequencing the entire coding regions of the genome, which consists of approximately 20,000 genes, about 1–2% of the entire genome; TMB is calculated based on the number of somatic mutations detected in the exome (Ng et al., 2009). NGS-targeted panel, are less expensive than WES and can be tailored to specific research questions (Mardis, 2017).

Several commercial-targeted NGS panels have been developed specifically for TMB testing (Mardis, 2017; Chalmers et al., 2017b). These panels typically include a set of genes that are frequently mutated in cancer and are associated with a high TMB. For example, FoundationOne CDx includes 324 genes, covering approximately 1.1 megabases (Mb) of the human genome and, is used to test TMB in various types of cancer including NSCLC, melanoma, bladder cancer, and head and neck

cancer, among others (Chalmers et al., 2017b); Oncomine Tumor Mutation Load assay includes 409 genes, covering approximately 1.4 Mb of the human genome, and it is used mainly in NSCLC, melanoma, and colorectal cancer, among others (Mardis, 2017). Other panels, such as the Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT), are designed to capture a broader set of genes and can be used for TMB testing (Cheng et al., 2015).

The cutoff value for TMB varies depending on the specific assay, tumor type, and treatment. For example, a TMB cutoff of $\geq$ 10 mutations per megabase (mut/Mb) was used to define high TMB in clinical trials evaluating pembrolizumab for first-line treatment of NSCLC. However, in the same patients treated with nivolumab plus ipilimumab, a TMB cutoff of $\geq$ 13 mut/Mb was used to define high TMB (Gandhi et al., 2018; Hellmann et al., 2018).

Other studies have employed different TMB cutoff values, ranging from 6 mut/Mb to 20 mut/Mb, to define high TMB, based on the tumor type and assay. However, further research is needed to determine the optimal cutoff value for different tumor types and treatment settings (Goodman et al., 2017; Rizvi et al., 2015a).

Several studies have shown a correlation between TMB and response to ICI in different tumor types, although with conflicting results. TMB does not appear to be a relevant biomarker in NSCLC patients receiving ICI plus chemotherapy, while the role of TMB in patients receiving ICI alone or in combination remains to be clarified (Gettinger et al., 2018). However, it should be noted that, unlike PD-L1, TMB has not shown a linear correlation with OS in patients with advanced NSCLC (Nie et al., 2020; Huang et al., 2018). In a pooled analysis of patients with various cancers treated with anti-PD1/PD-L1 therapies, patients with high TMB had significantly improved progression-free survival compared with patients with low TMB (Zhu et al., 2019). Pembrolizumab was recently approved for adult and pediatric patients with unresectable or metastatic TMB-high solid tumors ($\geq$ 10 mut/Mb) who have progressed on prior therapies and without other options (Marabelle et al., 2020). Recent evidence has shown that specific mutations (*e.g.*, gene fusions) or clonal mutations may correlate better with response to ICI than the total number of mutations (Yang et al., 2019; McGranahan et al., 2016). A harmonization of the different TMB testing methods and a better definition of the mutations counted in TMB are needed to standardize the use of TMB for clinical purposes.

3.1.3. *Mismatch repair deficiency/microsatellite instability*

DNA damage response (DDR) pathways aim to protect cells from exogenous or endogenous DNA damage (Chatterjee and Walker, 2017). Germline or somatic mutations in components of the mismatch repair (MMR) system (namely MLH1, *MutL Homolog 1*; MSH2, *MutS Homolog 2*; MSH6, *MutS Homolog 6*; and PMS2, *PostMeiotic Segregation increased 2*) result in the inability of cells to repair DNA damage, with consequent genomic instability (MSI).

The evaluation of mismatch repair deficient (dMMR) involves the use of multiplex immunohistochemistry techniques to assess the loss of expression of MMR proteins, which are normally expressed in the nuclei of cells. If the expression of at least one of these four MMR proteins is absent, it indicates a deficiency in MMR. The MMR system's functionality depends on the interaction of heterodimers, specifically MLH1/PMS2 and MSH2/MSH6. In their monomeric form, the MMR proteins are degraded, which often results in the loss of one MMR protein being accompanied by the loss of its partner. Nevertheless, isolated losses of PMS2 and MSH6 (which can occur in Lynch syndrome) are among less frequent/rare patterns. Therefore, the evaluation of all four MMR proteins is recommended (Willis et al., 2019).

The MSI test is conducted on DNA that is extracted from neoplastic tissue, which has sufficient tumor cellularity of over 20% (Vasen et al., 2010). This is done through molecular techniques such as polymerase chain reaction (PCR) and NGS (Luchini et al., 2019a). A tumor is classified as having MSI if the length of a marker differs from that found in the reference tissue or from the expected length in the general population. If there are no differences in marker length, the tumor is considered microsatellite stable (MSS). The Bethesda panel, which is the first reference panel for MSI analysis, includes the examination of five loci, consisting of two mononucleotide repeats (BAT-25 and BAT-26) and three dinucleotide repeats (D2S123, D5S346, and D17S250). Tumors with instability in two or more of these repeats are classified as high instability (MSI-H), those with instability in only one repeat are termed low instability (MSI-L), and tumors without any instability are considered MSS (Boland et al., 1998).

The Pentaplex panel is one of the most commonly used panels and is considered the new reference standard for diagnosing MSI (Suraweera et al., 2002; Le et al., 2017a). It analyzes five mononucleotide loci (BAT-25, BAT-26, NR-21, NR-24, and NR-27) and identifies MSI when at least three loci are unstable. In contrast, tumors with no unstable loci or only one unstable locus are considered MSS. However, when two unstable loci are observed, further comparative analysis with a non-neoplastic reference tissue is required (Suraweera et al., 2002).

Several commercial kits, such as the TrueMark MSI Assay and ThermoFisher Scientific, have been validated to allow for the simultaneous detection of more than five loci, which improves sensitivity and specificity in the identification of MSI. Using these larger panels, a tumor is considered MSS if none of the loci (0%) are unstable. A tumor is considered MSI-low if the number of unstable loci is less than 30%, and MSI-H if the number of unstable loci is greater than 30% (Morandi et al., 2012).

The most important cancer types where MSI testing should be carried out using IHC to assess MMR proteins status and MSI-PCR or NGS are endometrial, colorectal, small bowel, gastric, oesophageal, ovarian cancers and, glioblastoma. For tumor types with low frequency of MSI and limited data on the accuracy of IHC and MSI-PCR, MSI testing should be performed using NGS (Luchini et al., 2019b).

Notably, although MSI is often used as a surrogate marker of MMR deficiency, there might be discordance between MMR status and MSI status. The discordance rate can vary depending on the specific tumor type and the assay used for testing. Some studies have reported a discordance rate of 10–15%, indicating that a small proportion of tumors with MMR deficiency do not show MSI, and vice versa (DD et al., 2014; Kloor et al., 2012). This could impact patient selection and treatment decision-making in the context of immunotherapy, as MSI-high tumors are generally more likely to respond to ICI therapy, while the relationship between MMR deficiency and response to immunotherapy is less clear.

dMMR/MSI-H results in the accumulation of mutations and the production of neoantigens, which, can favor the immune response against the tumor and the sensitivity to ICI. These tumors are generally characterized by an elevated TMB with highly immunogenic neoantigens arising from frameshift mutations (Maby et al., 2015).

They are also associated with an upregulation of inhibitory checkpoints that exhaust intratumoral cytotoxic T lymphocytes and consequently protect cancer cells from their hostile immune microenvironment (Marisa et al., 2018; Llosa et al., 2015; Guinney et al., 2015).

One important point to note is that it is possible to have a high TMB in the absence of MSI, whereas, except for endometrial cancer, MSI-high with TMB-low is rare. Tumors such as gliomas, colorectal and esophagogastric adenocarcinomas have shown high percentages of concordance between TMB-high and MSI-high, whereas an inverse relationship has been reported for anal cancers and esophageal squamous cell carcinomas (Weinberg et al., 2018; Vanderwalde et al., 2018; Salem et al., 2018; Hodges et al., 2017; Janjigian et al., 2018). The high sensitivity of dMMR/MS-I tumors to ICIs regardless of origin tissue, led the FDA to approve MSI as an agnostic biomarker of ICIs response (Sepulveda et al., 2017). In 2017 pembrolizumab received its first tissue agnostic approval for patients with advanced MSI-H/dMMR tumors who progressed on prior therapies with no further acceptable treatment options ($author1$ et al., 2017). Despite high response rates and sustained clinical benefit

with ICI, a high rate of tumors (*e.g.*, until 50% of MSI/dMMR mCRC tumors show primary resistance to immunotherapy, despite a significant number of these refractory tumors are misdiagnosed as MSI/dMMR (Weng et al., 2022). The quality of the neoantigens is of paramount importance in the definition of biomarkers, as demonstrated in MSI/dMMR tumors treated with ICIs (Le et al., 2017b). The presence of an elevated TMB and high neoantigen load is not synonymous with effective immune responses directed against tumor-associated neoantigens, suggesting that the immune response to ICI may be limited to only a few highly immunogenic antigens. However, these antigens mainly derive from insertion/deletion mutations, a hallmark of dMMR tumors, which are difficult to detect with currently available techniques (Mandal et al., 2019; Campbell et al., 2017).

3.2. Experimental tissue biomarkers

3.2.1. TILs and immunoscore

T cells are broadly classified based on the T cell receptor subunits (TCR αβ or γσ), which recognize peptides presented on the cell surface in the context of the major histocompatibility complex (MHC), as well as major lineage markers CD8 and CD4. In general, CD8 + and CD4 + TCRαβ T cells are the most abundant T cell subsets in tissues, including tumor tissues (Paijens et al., 2021). In addition to T cells, a successful antitumor immune response requires the presence, activation, and co-stimulation of other components of the immune system including B cells and innate lymphoid cells.

CTLA-4 or PD1 blockade also works by strengthening a preexisting antitumor immune response, therefore the density and location of TILs within the TME may predict response to ICI (Tumeh et al., 2014). Likely, ICIs are not active in tumors with an immune-excluded (T cells at the periphery of the tumor) or immune-desert phenotype (lack of pre-existing immunity) and more active in tumors with an immune inflamed phenotype (infiltration of CD4 + and CD8 + T cells in tumor parenchyma) as suggested by survival data among different tumor types (Zheng, 2022; Chen and Mellman, 2017).

In clinical practice, TILs have been suggested as potential prognostic and therapeutic biomarkers, particularly in the context of ICI therapy. In general, the presence of TILs in tumors has been associated with better clinical outcomes although all infiltrates are potentially different, due to diversity, specificity, and function (Presti et al., 2022). Therefore, the phenotype and function of TILs (e.g., cytotoxic T cell vs. regulatory T [Treg] cells), and location within the TME (e.g., marginal/stromal or intra/peritumoral) are critical aspects to be investigated. Recently, data from different cancers (melanoma, colorectal, breast, and lung cancer) have shown that immune responses active often assemble in peritumoral tertiary lymphoid structures (TLS). TLS are structures that function as mini lymph nodes capable of generating immune responses in chronic inflammatory sites (Cipponi et al., 2012; Schadendorf et al., 2015b; Germain et al., 2014; Gu-Trantien et al., 2013; Noël et al., 2021; Sautès-Fridman et al., 2019). Several studies report a correlation between cancer-associated TLS (with active germinal centers) and positive clinical outcomes (Gu-Trantien et al., 2013; Noël et al., 2021; Silina et al., 2018; Kroeger et al., 2016). Other studies on tumors treated with ICI have found a higher density of TLS in responder patients compared with non-responders and a positive association between TLS, B cells, TILs, and clinical outcomes after immunotherapy (Silina et al., 2018; Kroeger et al., 2016; Petitprez et al., 2020; Cabrita et al., 2020; Helmink et al., 2020). A recent study found that mature tertiary lymphoid structures can predict response to ICI treatment in a variety of tumor types, independent of PD-L1 expression suggesting TLS as a possible new biomarker (Vanhersecke et al., 2021). Baseline intratumoral TIL can be characterized using several technologies, including multiplex IHC and gene expression profiling (GEP). The Multiplex Immunohistochemistry/Immunofluorescence (mIHC/IF) technique can visualize multiple proteins on the same tissue section simultaneously and obtain the spatial relationships and cellular co-expression of multiple markers (Das et al., 2015). In patients with metastatic melanoma and Merkel cell carcinoma, a predictive role of response to ICI has been attributed to the spatial density of PD1/PD-L1 measured by mIHC/IF (Johnson et al., 2018; Giraldo et al., 2018). Interestingly, a systematic review reported the superiority of the mIHC/IF test over other biomarkers including PD-L1 IHC, TMB, and GEP, suggesting an additive value of the test in predicting response to PD1 block (Lu et al., 2019). This technique has overcome several limitations of IHC, allowing detailed transcript analysis (Gentles et al., 2015) and inflammation-associated gene signatures, oncogenic drivers, checkpoint protein expression, and cell subtypes. However, the lack of spatial and structural information of immune infiltrates limits their use as a complementary technique (Galon et al., 2014).

New approaches including digital immune scores and digital prognostic scores that integrate multiple immune characteristics into a single model are being developed to transform the prognostic advantage of TILs into a clinically useful diagnostic tool favoring personalized therapy.

3.2.2. DNA polymerase epsilon (POLE) and delta 1 (POLD1)

POLE/POLD1 correlate with tumorigenesis and prognosis of some tumors (Briggs and Tomlinson, 2013; Ma et al., 2022) Since the POLE/POLD1 mutation is associated with increased neoantigens and intracellular immune cell infiltration, its role as a predictive biomarker of immunotherapy efficacy has been investigated (Ma et al., 2022). Although data on the predictive value of *POLE* are very limited, cases of complete and sustained remissions have been reported in patients with *POLE* mutated and treated with ICI as well as better efficacy outcomes (Wang et al., 2018; Silberman et al., 2019). Furthermore, other studies have shown that *POLE* mutations outside the exonuclease domain are associated with improved overall survival in cancer patients receiving ICI (Dong et al., 2022). Although not correlated to a high mutational load, these mutations seem to improve immunogenicity through a mechanism independent of neoantigens (Dong et al., 2022).

POLE and POLD1 are genes that encode DNA polymerases, enzymes that play a key role in DNA replication and repair. Alterations in these genes have been shown to increase TMB compared to tumors without these mutations. For example, a study on endometrial cancer reported an average TMB of 150 mut/Mb in POLE-mutated tumors compared to 10 mut/Mb in tumors without POLE mutations (Church et al., 2013). Another study on advanced solid tumors reported a median TMB of 115 mut/Mb versus 5.6 mut/Mb for tumors with and without POLE mutations, respectively (Rayner et al., 2016).

While alterations in these genes can be useful biomarkers for identifying tumors with high TMB, it is important to note that not all tumors with POLE or POLD1 alterations have high TMB, and vice versa. Therefore, multiple factors, including TMB and other genomic and clinical features, must be considered when making treatment decisions (Yarchoan et al., 2017; Russo et al., 2019; Stenzinger et al., 2014; Schrock et al., 2019).

3.2.3. Tumor gene expression profiling

Gene expression-based signatures have emerged as a new generation of predictive biomarkers for ICI response (Havel et al., 2019). It is a technique used to examine the expression of thousands of genes simultaneously able to characterize tumor immune microenvironment and predict immunotherapy response, beyond the measurement of single genes. It could become a powerful tool to further stratify patients for targeted therapies and to predict potential differences in response to treatment among patients harboring the same genomic alteration (Rizvi et al., 2015a). Inflamed T-cell GEP is one of the first gene signatures reported and clinically validated to predict response to ICI in numerous solid tumors (Jamieson and Maker, 2017). Using high-throughput assays, GEP can analyze immunological transcriptomic patterns that predict the activity and toxicity of immunotherapy agents (Das et al., 2015). Interferon-γ (IFN-γ), a key cytokine of the immune system that

stimulates both innate and adaptive immune responses and produces feedback inhibition of antitumor immunity, has been evaluated with the related gene signatures (McKean et al., 2020; Ikeda et al., 2002; Liang et al., 2003; Spranger et al., 2013). The identification of a 10-gene and 28-gene IFN-γ panel was correlated with better response, progression-free survival (PFS), and OS in patients with metastatic melanoma receiving anti-PD1 (Ayers et al., 2017; Fehrenbacher et al., 2016). This suggests that GEP could become a powerful tool to further stratify patients for targeted therapies and to predict potential differences in response to treatment among patients harboring the same genomic alteration. However, it has some limitations including the inability to elucidate the cellular source of gene expression, cellular co-expression, and geographic relationships of cells within the TME (McKean et al., 2020).

3.2.4. Single-cell RNA sequencing

Single-cell RNA sequencing (scRNA-seq) analyzes complex tissue transcriptomes at the single-cell level. It can identify differential gene expression and epigenetic factors caused by mutations in single-cell genomes, as well as distinguish different cell types in tumor tissue. Furthermore, it may provide a higher resolution of cellular expression patterns and differences within the TME.

This induced a growing interest in clinicians in scRNA-seq, and its integration in several number of clinical trials with various objectives (Chisini et al., 2020). Recent evaluations of the use of RNA-seq to orient refractory patients to targeted treatments demonstrated the feasibility of such an approach in routine; however, it failed to improve patients' outcomes so far (Tuxen et al., 2019; Rodon et al., 2019).

ScRNA-seq provides a high-resolution view of the immune system and can help identify cell populations that are enriched in responders or non-responders.

In addition to predicting treatment response, scRNA-seq can also help identify new targets for immunotherapy. This information can be used to develop new therapies that target specific cell populations or molecular pathways, improving the efficacy and safety of immunotherapy.

3.2.5. HLA human leukocyte antigen status

The recognition and elimination of cancer cells by CD8 + T requires efficient presentation of tumor antigens by HLA class I (HLA-I) molecules encoded by three different genes: HLA-A, HLA-B, and HLA-C. Down-regulation of HLA-I antigen peptide complexes by tumor cells is a well-known mechanism of immune escape often associated with poor prognosis (Hicklin et al., 1999). The decrease or absence of HLA molecules and/or defects have been evaluated if they predict response to ICI. A retrospective analysis of melanoma patients treated with anti-CTLA-4 and/or anti-PD1 showed that HLA I expression was a reliable biomarker of response to ICI for anti-CTLA-4, but not for anti-PD1 (Rodig et al., 2018). Analysis of the HLA-I genotype in advanced cancer patients treated with ICI showed that heterozygosity of HLA-I loci is associated with better OS compared with patients homozygous for at least one HLA locus. Indeed, patients with heterozygous HLA-I have greater TRC clonality than homozygotes, which increases the strength of the anti-tumor T-cell response in patients receiving ICI (Chowell et al., 2018). However, the role of HLA as a predictive biomarker for ICI remains unknown and requires further prospective clinical trials (Costantini and Budillon, 2020).

3.3. Experimental circulating biomarkers

3.3.1. Immune cell subpopulation and tumor antigen-specific peripheral T cells

The immune cell subpopulations, evaluated by flow cytometry in the peripheral blood, might reflect the immune response against tumors. A high circulating memory effector (CD4 + and CD8 + T cells ratio) has been correlated with a survival benefit in NSCLC patients treated with nivolumab, as well as a delayed increase in CD4 + and CD8 + T cells or high baseline CD8 effector-memory type-1 T cells, were associated with good outcome in melanoma patients receiving ipilimumab (Manjarrez-Orduño et al., 2018; Martens et al., 2016). Conversely, increased levels of circulating myeloid-derived suppressor cells (MDSCs), or Treg, were correlated with poor outcomes in patients treated with ICIs (Martens et al., 2016; Huber et al., 2018; Arora et al., 2019). MDSCs belong to a heterogeneous population of immature myeloid cells that increase during tumor progression as a consequence of chronic inflammation. MDSCs include two major subsets named polymorphonuclear (PMN-MDSCs) and monocytic (M-MDSCs), whose prognostic impact in cancer patients has been explored in several studies (Passaro et al., 2020; Bronte et al., 2022a; Koh et al., 2020; De Cicco et al., 2020; Bronte et al., 2022b). In particular, high levels of circulating M-MDSCs in NSCLC patients correlate with a higher likelihood of recurrence/progression and mortality than lower levels in these patients (Bronte et al., 2022b). Likewise, high M-MDSC levels are strongly associated with primary resistance to immunotherapy in NSCLC patients (Bronte et al., 2022a). Conversely, PMN-MDSCs did not yield equivalent predictive outcomes, making M-MDSC a possible target in NSCLC (Bronte et al., 2022b). Monitoring of peripheral blood T-cell activation appears to predict response to PD1 blockade. It has been shown that in patients with various cancers (*e.g.*, NSCLC and thymus) treated with ICI, the early increase in Ki-67 + PD1 + CD8 + T cells correlated with the clinical benefit (Kamphorst et al., 2017; Kim et al., 2019). Immunophenotyping of peripheral T cells could enhance information regarding the peripheral systemic impact of ICI on different immune cells and the potential correlation with clinical outcomes. Recently, tumor-specific neoantigen T cells have been investigated as possible effectors of immunotherapy. A correlation between the detected number of neoantigen-specific T cells and the treatment response of melanoma patients receiving PD1 blockade has been reported (Peng et al., 2019). Furthermore, in the subset of patients with melanoma expressing NY-ESO-1, a measurable response of NY-ESO-1-specific CD4 + and CD8 + T cells correlates with greater clinical benefit to ICIs and with a significantly better survival than patients with a specific undetectable T-cell response (Yuan et al., 2011). Finally, neoantigen-specific T-cell response has been evidenced in patients with advanced melanoma or NSCLC responsive to ICI treatment (Snyder et al., 2014; Rizvi et al., 2015b).

3.3.2. Soluble PD-1/PD-L1 and CTLA-4

Previous data demonstrated that increased soluble forms of PD-1 (sPD-1) and PD-L1 (sPD-L1) in peripheral blood may correlate with ICI response (Okuma et al., 2018). Although high pretreatment levels of sPD-L1 and sPD-1 have been correlated with an increased likelihood of progressive disease and shorter survival in several malignancies, the increase in post-treatment levels has been correlated with a favorable response to ICI (Zhu and Lang, 2017; Zhou et al., 2017). The magnitude of increased circulating PD-L1 expression on the surface of exosomes, released from metastatic melanomas during the early stages of treatment, is an indicator of the adaptive response of tumor cells to T cell activation, stratifying thus clinical responders from non-responders (Chen et al., 2018). Because sPD-1 retains its ability to bind ligands and thereby disrupt the PD-1 axis, its role as a therapeutic target has been evaluated in the preclinical setting, although the lack of standardization of blood sPD1 and sPD-L1 measurement was a limitation. High baseline serum sCTLA-4 levels (>200 pg/ml) detected in anti-CTLA4-treated melanoma patients have been associated with better overall response rate (ORR) and OS compared with < 200 pg/ml serum levels, suggesting a probable predictive role of serum sCTLA-4 (Christensen et al., 2019). Large clinical cohort studies are needed to validate the role of sPD-1/PD-L1 and sCTLA-4 as therapeutic targets and predictive biomarkers (Khan et al., 2020).

3.3.3. Circulating tumor DNA and circulating tumor cell

In the last years, the diagnostic, prognostic, and predictive role of

circulating tumor DNA (ctDNA) has been extensively studied and validated for different types of tumors (Reinert et al., 2019; Maravelia et al., 2021a). Studies in patients with advanced NSCLC treated with anti-PD1/PD-L1 have shown a correlation between reduction of ctDNA greater than 50% from baseline and greater radiographic response, in PFS and OS (Goldberg et al., 2018). New NGS tests have been used to predict ICI response (Christensen et al., 2019; Reinert et al., 2019). In patients with solid tumors, an early decrease in mean ctDNA concentration and ctDNA clearance during pembrolizumab treatment was correlated with an improvement in OS (regardless of tumor type, TMB, or PD-L1 status) (Bratman et al., 2020). Even in patients with unresectable hepatocellular cancer treated with atezolizumab plus bevacizumab, on-treatment ctDNA clearance was correlated with better PFS (Maravelia et al., 2021b). Furthermore, ctDNA monitoring can detect minimal residual disease after treatment with curative intent in order to identify those who will benefit from adjuvant ICI (in patients with high-risk muscle-invasive bladder cancer) or to monitor its progression after adjuvant therapy (Survival, 2023; Azzi et al., 2022). A prospective clinical study of different tumor types has shown a correlation between ctDNA levels and the efficacy of ICI treatment (Sanmamed et al., 2017). Furthermore, early ctDNA changes in ICI-treated patients could represent a biomarker of early immune responses and survival independent of PD-L1 and TMB. However, further larger prospective cohort studies are needed to validate these findings. Regarding CTCs, their clinical utility has been reported in different cancers for early diagnosis, prognostic potential, and prediction of response/resistance to anticancer treatment. PD-L1 expression on CTC is easily detectable, with some limit on concordance with tumor tissue biopsy data, and serial sampling allows for dynamic evaluation of PD-L1 + CTC in patients who received ICI (Hofman et al., 2019). However, only one study has demonstrated a predictive value for PD-L1 + CTC for response to ICI so far (Yue et al., 2018).

3.3.4. Inflammatory cytokines

Cytokines play a critical role in the activation of host immunomodulation. They regulate the trafficking of immune cells into tumors, are implicated in tumor development, progression, and development of drug resistance (Lan et al., 2021; Catalano et al., 2023). Measurement of several inflammatory blood cytokines including IFN-γ, IL-6, IL-8, IL-11, and IL-2 has been evaluated across tumor types in patients receiving ICI (Sanmamed et al., 2017). Various cytokines such as INF-γ and TNFα have been shown to induce PD-L1 expression in cancer cells, as well as increase sPD-L1 secretion through the splicing activities of PD-L1 in cancer cells (Zhou et al., 2017). Some studies have reported that circulating cytokines in plasma can also predict responsiveness to ICI (Herbst et al., 2014b). Tumor vascular dysfunction and high VEGF levels have been correlated with decreased T-cell infiltration into tumors. Notably, elevated baseline VEGF levels and high pre-treatment plasma levels of angiopoietin-2 in melanoma patients receiving ipilimumab have been correlated with decreased OS (Hofman et al., 2019). Furthermore, a significant increase in the baseline plasma level of IL-8 was detected during the progression of patients with melanoma and NSCLC treated with anti-PD-1 (Hofman et al., 2019). However, several factors related to the tumor (e.g., tumor burden, the presence of brain metastases) and host (e.g., stress or infection) may influence inflammatory cytokine levels in peripheral blood limiting their sensitivity and specificity as predictive biomarkers (Lim and Yoon, 2021).

3.3.5. Neutrophil-to-lymphocyte ratio

The neutrophil-to-lymphocyte ratio (NLR) is considered a balance between pro-tumor inflammatory status and antitumor response, and its alteration can symbolize the systemic inflammatory response in patients. Several studies have demonstrated that the NLR is a potent prognostic biomarker related to a worse OS in several tumor types in the pre-immunotherapy era (Guthrie et al., 2013; Leitch et al., 2007; Nunno et al., 2019; Su et al., 2019; Chen et al., 2021). Currently, the NLR ratio was found to have predictive and prognostic value in patients receiving ICI (Wu et al., 2021). In patients with different cancer types, a low NLR/high TMB correlated with a significantly higher ICI benefit than a high NLR/low TMB (Valero et al., 2021). Recent evidence in patients with stage III NSCLC treated with or without consolidation immunotherapy has confirmed the correlation between a higher NLR before treatment and lower PFS, more evident in the group of patients treated with ICI. These results suggest a potential role of NLR pre-treatment as a predictive biomarker of ICI benefit (Bryant et al., 2022).

Additionally, a derived neutrophil-to-lymphocyte ratio (dNLR) has been integrated into a prognostic assessment known as the Lung Immune Prognostic Index (LIPI), along with lactate dehydrogenase (LDH) measurements. The pre-treatment LIPI, which combines a dNLR greater than 3 and LDH greater than the upper limit of normal, has shown a correlation with poorer outcomes in ICI therapy, but not in chemotherapy (Mezquita et al., 2018). This suggests that this index could potentially serve as a valuable tool for guiding the selection of ICI treatments. However, subsequent studies have demonstrated the prognostic significance of both LDH levels and dNLR, regardless of the treatment approach, for patients with metastatic NSCLC (Kazandjian et al., 2019). Nonetheless, there remains insufficient evidence that LIPI can effectively identify patients who are unlikely to benefit from specific treatments (Hopkins et al., 2021).

3.4. Gut microbiota

Finally, the gastrointestinal microbiota, acting on adaptive and innate immunity, is recognized as a relevant factor for antitumor immunity. Indeed, the immunoregulatory role of the microbiota has emerged as a predictor of response and toxicity in patients treated with immunotherapy (Roviello et al., 2022b). Differences in the bacterial species determining the microbiota appear to have been correlated with response to ICI (Shoji et al., 2022). Overall, a *Bacteroides* enriched and an *Akkermansia* enriched gut microbiota were correlated with a lower and higher response to ICI, respectively (Routy et al., 2018; Brandt et al., 2012; Chaput et al., 2017). However, most studies have yielded conflicting data regarding the predictive role of microbiota-specific composition. Dietary control (e.g., the amount of dietary fiber) and probiotic and prebiotic intake may be one approach to manipulate microbiota composition and improve response to immunotherapy (Young, 2016). In a study on melanoma patients, a high-quality diet rich in whole grains positively correlate with ICI response (Huang et al., 2021). In addition, fecal microbiota transplantation (FMT) from healthy subjects or responders to a specific treatment seems to be promising to overcome resistance or toxicities ICI correlated. Initial results showed that some patients with metastatic melanoma who previously failed ICI treatment had partial or complete responses to anti-PD-1 upon receiving FMT from melanoma patients who achieved a durable complete response (Baruch et al., 2021). Further research is needed to define the predictive role of microbiota composition and validate microbiota remodulation methods.

4. Conclusion

The currently FDA-approved predictive biomarkers, PD-L1, MSI, and TMB, play a key role in patients' selection to be treated with ICI (Wang et al., 2021). However, some limitations exist, such as the interpretation of positivity cut-offs, the low prevalence in metastatic tumors, the high costs, and technical complications. Furthermore, it should be considered that high response rates to ICIs were recorded in patients whose tumors did not express PD-L1, with low TMB, or in the absence of MSI and vice versa. Simultaneously, many other tissue or circulating biomarkers have been analyzed, obtaining interesting but not yet adequate results. Likely, the current biomarkers tend to capture a unique contributing factor of ICI response, while data suggest that patient response to ICIs is a complex quantitative characteristic determined by multiple factors (Lee

et al., 2022). Recently, gene expression-based signatures have emerged as a new generation of predictive biomarkers for ICI response. To date, GEP is the only clinically validated gene signature for predicting response to pembrolizumab across multiple solid tumors (Ayers et al., 2017). T cell dysfunction and exclusion gene signature (TIDE), melanocytic plasticity signature (MPS), and B cell-focused gene signature are being validated. Next-generation expression signatures derived from RNA sequencing data and based on molecular pathway activation profiles appear to better guide ICI treatment customization (Buzdin et al., 2018; Borisov et al., 2020; Moisseev et al., 2020). An integrated nucleic acid biomarker signature assembling information from different DNA and RNA biomarkers in one single assay seems to be a promising means of overcoming potentially conflicting results from different biomarkers (Teder et al., 2018). Only the combination (such as PD-L1 and TMB, PD-L1 + CD8 +TILs, GEP+TMB, and MPS+TIDE) of different biomarkers through complementary or additive effects will lead to better predictive performance.

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Declaration of Competing Interest

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