OncoTargets and Therapy

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REVIEW

Potential Biomarkers for the Efficacy of PD-1-PD-L Blockade in Cancer

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Abstract: A decade ago, immune checkpoint blockade emerged as a major breakthrough in oncology, proposing a novel approach by which immune brakes could be released to enhance antitumor responses. Despite apparently modest improvement of the median duration of response, a spectacular doubling of long-term responses as compared to the available standard of care was seen, for instance, in metastatic melanoma. It soon became obvious that the percentage of patients responding to these novel approaches is relatively small, and the importance of an accurate prediction of responders became more and more clear. Strong predictive markers would allow for the administration of immune checkpoint blocker therapy to the patients most likely to benefit from it, and sparing the potential non-responders of a treatment which is far from innocuous, being associated with significant side-effects and, not least, an important price tag. A number of potential response predictors have already been investigated and partly validated, but they do not cover the major unmet need encountered in the current clinical setting. Here, we review biomarkers for immune checkpoint blockade efficacy, either clinically validated and currently in use, or which have been proposed as candidates and are currently under investigation.

Keywords: immune checkpoint blockade, predictive biomarkers, PD-L1 expression, microbiome

Introduction

Lymphocyte interaction with antigen is not enough for the initiation of an adaptive immune response. Half a century ago, the two signal models of lymphocyte activation were proposed, involving, besides relevant antigen presentation, the ligation of co-stimulatory surface molecules like CD28.[1] The notion of a third signal is used to describe a pro-activatory cytokine environment. Negative, or co-inhibitory, "second signals" were later identified, credited with a protective role for the host against the over-engagement of immune effector mechanisms. Inhibiting or blocking these "immune brakes" emerged as a paradigm shift in the immunotherapy of cancer, and earned their pioneers, Tasuku Honjo and James Allison, the 2018 Nobel Prize in Physiology or Medicine.[2]

Immune checkpoint inhibitors (ICIs) have been developed and tested in cancer over the last two decades, the first to be granted clinical approval being ipilimumab, a CTLA-4-blocking monoclonal antibody (mAb), approved by the FDA in 2011 for metastatic melanoma. Later approvals were granted to agents directed against the signaling axis represented by the programmed death (PD)-1 co-inhibitory receptor and its ligands. The advent of ICI revolutionized oncology by the introduction of a new class of agents, active in relapsed/refractory tumors for which extremely

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Received: 12 May 2021
Accepted: 24 September 2021
Published: 17 November 2021

limited therapeutic options were available. Immune checkpoint blockade also brought a reconsideration of treatment response criteria,[3,4] including the formulation of new criteria for radiologic responses such as iRECIST and imRECIST.[5]

Despite spectacular results, mostly seen in previously unmanageable tumors, it has quickly become clear that only a minority of patients respond to immune checkpoint blocker therapy.[6] Consecutively, the availability of biomarkers capable of predicting a response became of crucial importance, for a treatment approach which is far from innocuous in terms of toxicity, ease of administration, and financial burden.[7] While it was somewhat logical to focus on lymphocyte or tumor-related markers like co-inhibitory receptors or ligand expression levels, humoral, systemic markers have also been proposed for predicting outcome.

In this paper, we review established or proposed biomarkers for ICI treatment efficacy and the data that supports their use in the clinical setting.

The Immunotherapy Rationale – from History to Authorization

The immune response consists of two distinct effector arms: the innate and adaptive responses. While the innate immune cells including natural killer (NK) cells, macrophages and neutrophils react promptly, but less specifically, to external antigens, adaptive effectors such as B and T lymphocytes are part of a more accurate and precisely directed immune feedback mechanism. Nonetheless, in order to initiate such a precise action, a certain amount of time is needed. The highly discriminating adaptive immune reactions are offering excellent targets for the triggering of potent and specific immunotherapeutic responses. A model of the immune anticancer response is the cancer immunity cycle, proposed as a fundamental mechanism of immune-mediated cancer elimination.[8,9] Briefly, this multistep mechanism is initiated by the release of neoantigens by apoptotic tumor cells within the tumor microenvironment. These neoantigens are perceived as foreign, non-self, by the adaptive immune system. Cell fragments are seized by dendritic cells, which, after drifting through lymphatic vessels to the lymph nodes, present them to T-cells, triggering tumor-specific cytotoxic T-cell responses against the cancer-specific antigens. CD8+ cytotoxic T-cells migrate and infiltrate the tumor microenvironment, specifically binding to cancer antigen targets, killing tumor cells and leading to an additional discharge of tumor-associated antigens.[8] The cycle is repetitive and can lead to an optimal antitumor immune response. A disruption of this anticancer immunity course can arise, leading to tumor cells escaping immunosurveillance and consequently promoting tumor growth, progression and metastasis. One of the best known mechanisms of immune evasion to be elucidated and finally targeted is the immune regulatory checkpoints.[10,11] Immune regulatory checkpoints are represented by proteins situated on T-cells and antigen presenting cells (APCs),[12] some operating in up-regulating the immune response, some in down-regulating it. Tumor cells can escape the immunity cycle by activating immune checkpoint pathways that restrain antitumor immune responses.

A major milestone in oncology was set by the discovery of immune checkpoints such as cytotoxic T lymphocyte associated protein 4 (CTLA-4) and programmed death-1 (PD-1), molecules expressed on effector cells acting as a brake on immune feedbacks including antitumor responses.[13,14] CTLA-4 is a co-inhibitory cell surface signaling molecule that competes with CD28, co-stimulatory molecule, for the binding of CD80 and CD86.[15,16] PD-1 has two known ligands, programmed death ligands (PD-Ls) 1 and 2, and its engagement counteracts positive signaling through the T-cell receptors (TCR) and CD28.[17] By blocking these immune-silencing molecules using ICIs, a restoration of the immune response against tumor cells can be established.

The pivotal shift of immune checkpoint blockade in the clinical setting was started by the recent approval of ICIs such as ipilimumab (anti-CTLA-4) in 2011, followed by inhibitory/blocking antibodies directed at either PD-1 like nivolumab, pembrolizumab and cemiplimab, or at its ligand PD-L1, like atezolizumab, avelumab, and durvalumab[18–20] (Table 1). The interest for immune checkpoint blockade surged, with around a thousand clinical stage immune-oncology agents (IO) under investigation in over 3000 ongoing single agent and over 1000 combination therapy trials.[21] The very promising clinical results seen with ICIs have, however, some shortcomings: only 20 to 30% of cancer patients show sustainable objective responses[6] and some might experience severe immune-related adverse events. The majority of ICIs under investigation are targeted at PD-1 and PD-L1 since the efficacy of this approach has already been proven, thus deflecting attention from other potentially interesting targets. Still, there is no consensus regarding potential biomarkers of the effectiveness of PD-1/PD-L1 blockade. Advancement of

Table 1 Immune Checkpoint Inhibitors Approved by FDA by 22nd of July 2021[20]

Drug	Mechanism	Tumor	Setting	Trial	Approval
Ipilimumab	CTLA-4 inhibitor	Melanoma	2nd line	MDX010-020	28th of March2011
			1st line (in combination with nivolumab)	CheckMate-069/CheckMate-067	1st of October 2015
			Adjuvant	CA 184-029	18th of October 2015
		RCC[a] with clear cell histology – intermediate and poor risk groups	1st line (in combination with nivolumab)	CheckMate-214	18th of April 2018
		MSI- H or dMMR CRC[b]	3rd line (in combination with nivolumab)	CheckMate-142	10th of July 2018
Nivolumab	PD-1 inhibitor	Melanoma	2nd line	CheckMate-037	22nd of December 2014
			Adjuvant	CheckMate-238	1st of December 2017
		NSCLC[c]	2nd line	CheckMate-017	4th of March 2015
			2nd line	CheckMate-057	1st of October 2015
			1st line (in combination with ipilimumab)	CheckMate-227	15th of May 2020
			1st line (in combination with ipilimumab + chemotherapy)	CheckMate-9LA	27th of May 2020
		SCLC[d]	3rd line	CheckMate-032	17th of August 2018
		Mesothelioma	1st line (in combination with ipilimumab)	CheckMate-743	2nd of October 2020
		RCC	2nd line	CheckMate-025	1st of November 2015
			1st line (in combination with cabozantinib)	CheckMate-9ER	22nd of January 2021
		Hodgkin's lymphoma	2nd line	CheckMate-205	1st of May 2016
		HNSCC[e]	2nd line	CheckMate-141	1st of November 2016
		MSI- H or dMMR CRC	2nd line	CheckMate-142	1st of August 2017
			3rd line (in combination with ipilimumab)	CheckMate-142	10th of July 2018
		HCC[f]	2nd line	CheckMate-040	1st of September 2017*
			2nd line (in combination with ipilimumab)	CheckMate-040	11th of March 2020
		Bladder cancer	2nd line	CheckMate-275	1st of February 2017
		Esophagus cancer	2nd line	ATTRACTION-3	11th of June 2020
			1st line (in combination with chemotherapy)	CheckMate-649	16th of April 2021
			Adjuvant (in combination with chemoradiotherapy)	CheckMate-577	20th of May 2021
		Gastric cancer	1st line (in combination with chemotherapy)	CheckMate-649	16th of April 2021

(*Continued*)

Table 1 (Continued).

Drug	Mechanism	Tumor	Setting	Trial	Approval
Pembrolizumab	PD-1 inhibitor	Melanoma	2nd line	KEYNOTE-001	1st of September 2014
			2nd line	KEYNOTE-002	1st of December 2015
			1st line	KEYNOTE-006	1st of December 2015
			Adjuvant	EORTC 1325/KEYNOTE-054	15th of February 2019
		NSCLC	2nd line	KEYNOTE-001; KEYNOTE-006	1st of October 2015
			1st line	KEYNOTE-024	1st of October 2016
			1st line (in combination with chemotherapy)	KEYNOTE-021	1st of May 2017
			1st line (in combination with chemotherapy)	KEYNOTE-189	20th of August 2018
			1st line (in combination with chemotherapy)	KEYNOTE-407	31st of October 2018
			1st line	KEYNOTE-042	11th of April 2019
		SCLC	3rd line	KEYNOTE-158; KEYNOTE-028	18th of June 2019**
		RCC	1st line (in combination with TKI)	KEYNOTE-426	19th of April 2019
		HNSCC	2nd line	KEYNOTE-012	1st of August 2016
			1st line (alone or in combination with chemotherapy)	KEYNOTE-048	10th of June 2019
		Hodgkin's lymphoma	2nd line	KEYNOTE-087	1st of March 2017
			2nd line	KEYNOTE-204	15th of October 2020
		PMBCL[g]	3rd line	KEYNOTE-170	13th of June 2018
		Merkel cell carcinoma	1st line	CITN-09/KEYNOTE-017	19th of December 2018
		Cutaneous squamous cell carcinoma	1st line	KEYNOTE-629	24th of June 2020
		MSI-H or dMMR tumors	2nd line	NCT01876511	1st of May 2017
		TMB-H tumors	2nd line	KEYNOTE-158	16th of June 2020
		Esophagus cancer	2nd line	KEYNOTE-180; KEYNOTE-181	31st of July 2019
			1st line (in combination with chemotherapy)	KEYNOTE-590	22nd of March 2021
		CCR	1st line	KEYNOTE-177	29th of June 2020
		Gastric cancer	3rd line	KEYNOTE-059	1st of September 2017*
			1st line (in combination with Trastuzumab and chemotherapy)	KEYNOTE-811	5th of May 2021
		Hepatocellular carcinoma	2nd line	KEYNOTE-240	9th of November 2018
		Cervical cancer	2nd line	KEYNOTE-158	12th of June 2018

(*Continued*)

Table 1 (Continued).

Drug	Mechanism	Tumor	Setting	Trial	Approval
		Bladder cancer	2nd line	KEYNOTE-045	1st of May 2017
			1st line	KEYNOTE-052	1st of May 2017
			2nd line	KEYNOTE-057	8th of January 2020
		TNBC[h]	1st line (in combination with chemotherapy)	KEYNOTE-355	13th of November 2020
		Endometrial carcinoma	2nd line (in combination with lenvatinib)	KEYNOTE-775/Study 309	22nd of July 2021
Cemiplimab	PD-1 inhibitor	Cutaneous squamous cell carcinoma	1st line	NCT02760498	28th of September 2018
		Basal cell carcinoma	2nd line	NCT03132636	9th of February 2021
		NSCLC	1st line	EMPOWER-Lung1	22nd of February 2021
Dostarlimab	PD-1 inhibitor	Endometrial carcinoma	2nd line	GARNET	22nd of April 2021
Avelumab	PD-L1 inhibitor	Merkel cell carcinoma	2nd line	JAVELIN Merkel 200	1st of March 2017
		Bladder cancer	2nd line	JAVELIN Solid Tumor	1st of May 2017
			1st line	JAVELIN Bladder 100	30th of June 2020
		RCC	1st line (in combination with Axitinib)	JAVELIN Renal 101	15th of May 2019
Atezolizumab	PD-L1 inhibitor	Bladder cancer	2nd line	IMvigor210	1st of May 2016**
			1st line	IMvigor210	1st of April 2017**
		NSCLC	2nd line	Birch, Poplar, FIR, Oak	1st of October 2016
			1st line (in combination with bevacizumab, carboplatin and paclitaxel)	IMpower150	6th of December 2018
			1st line (in combination with carboplatin/nab-paclitaxel)	IMpower130	3rd of December 2019
			1st line	IMpower110	18th of May 2020
		SCLC	1st line (in combination with carboplatin and etoposide)	IMpower133	19th of March 2019
		Melanoma	1st line (in combination with cobimetinib and vemurafenib)	IMspire150	30th of July 2020
		HCC	1st line (in combination with bevacizumab)	IMbrave150	29th of May 2020
		TNBC	1st line (in combination with nab-paclitaxexl)	IMpassion130	8th of March 2019

(*Continued*)

Table 1 (Continued).

Drug	Mechanism	Tumor	Setting	Trial	Approval
Durvalumab	PD-L1 inhibitor	Bladder cancer	2nd line	NCT01693562	1st of May 2017**
		NSCLC	Adjuvant	PACIFIC	16th of February 2018
		SCLC	1st line (in combination with etoposide and carbo/cisplatin)	CASPIAN	30th of March 2020

Notes: [a]Renal cell carcinoma. [b]Colorectal cancer. [c]Non-small cell lung cancer. [d]Small cell lung cancer. [e]Head and neck squamous cell carcinoma. [f]Hepatocellular carcinoma. [g]Primary mediastinal B-cell lymphoma. [h]Triple-negative breast cancer. *As per April 2021, the FDA voted against maintaining accelerated approval. **Withdrawn due to failure to meet post-marketing requirements. Adapted with permission from FDA approval timeline of active immunotherapies; 2021. Available from: https://www.cancerresearch.org/en-us/scientists/immuno-oncology-landscape/fda-approval-timeline-of-activeimmunotherapies.[20]

this field is mandatory to allow for the best achievable responses with the least immune-related adverse events (irAE).

The PD-1-PD-L1/2 Axis

PD-1 (CD279) is a surface protein, a member of the CD28/CTLA4 family, found on activated T-cells, B cells and myeloid cells.[15,17,22] PD-1 is a receptor for its two ligands, PD-L1 (CD274) and PD-L2 (CD273), both B7 protein family members which share a sequence homology.[23,24] When triggered, this signaling axis behaves as an inhibitory by down-regulating T-cell signaling, effector function and killing ability.[12] Tumor cells can exploit these inhibitory signals by expressing PD-1 ligands on their surface. There are two mechanisms known to be involved in PD-1 ligand expression by tumor cells: one is constitutive, secondary to genomic alterations, whilst the other is inducible, regarded as an adaptive immune resistance:[12,25] tumor cells escape cytotoxic T-cell destruction through a natural regulatory pathway, up-regulating PD-L1 expression after the release of interferon (IFN)-γ from T-cells upon tumor recognition.[26] Consequently, a PD-L1/PD-1 mediated tumor-T-cell signaling takes place, leading to T-cell exhaustion and apoptosis, favoring tumor growth and progression.[27] Blocking this interaction has emerged as an efficient immune response-boosting action.

Biomarkers of Response to ICI – Paving the Way to Personalized Immunotherapy

One way of systematizing potential biomarkers is to follow their connection with components of the cancer immunity cycle. Meyers and Banerji analyzed biomarkers of response to ICI according to 3 fundamental elements of the cancer immunity cycle: immune stimulus, immune response and immune modulators.[28] Some biomarkers could be considered host-related (Figure 1), whilst others as tumor-associated. Taking the aforementioned elements into account, PD-L1 expression in tumors is one of the most widely investigated predictive biomarkers for the efficacy of immunotherapy. Microsatellite instability (MSI) status and deficient mismatch repair (dMMR) are two further biomarkers currently used in ICI therapeutic approaches. Tumor mutational burden (TMB) status, tumor infiltrating lymphocytes (TILs) and, more recently, tertiary lymphoid structures (TLS), POLE and POLD1 as DNA repair genes, cancer neoantigens or even RNA-signatures and the gut microbiome are emerging, potentially crucial biomarkers to be validated (Table 2). Although extensively investigated, the majority of these biomarkers are not in routine use in the clinical setting, where there is a substantial unmet need for predictive markers.

Biomarkers in Use – Status Approved

PD-L1 Expression

Being expressed on a wide range of cells from B and T lymphocytes to dendritic cells, macrophages and, not lastly, cancer cells, PD-L1 normally plays a role in preventing autoimmunity and redundant inflammation. Censoring immunogenicity, PD-L1 signaling is a target of anti-PD-1/PD-L1 mAbs, being the most investigated predictive biomarker for this therapy to date.

Several studies have concluded that patients with PD-L1 positive tumors benefit from a better response to anti-PD-1/PD-L1 antibodies as compared to those with PD-L1 negative tumors.[29] In the KEYNOTE-001 trial where pembrolizumab, an anti-PD-1 mAb was investigated as monotherapy in melanoma patients, the objective response rate

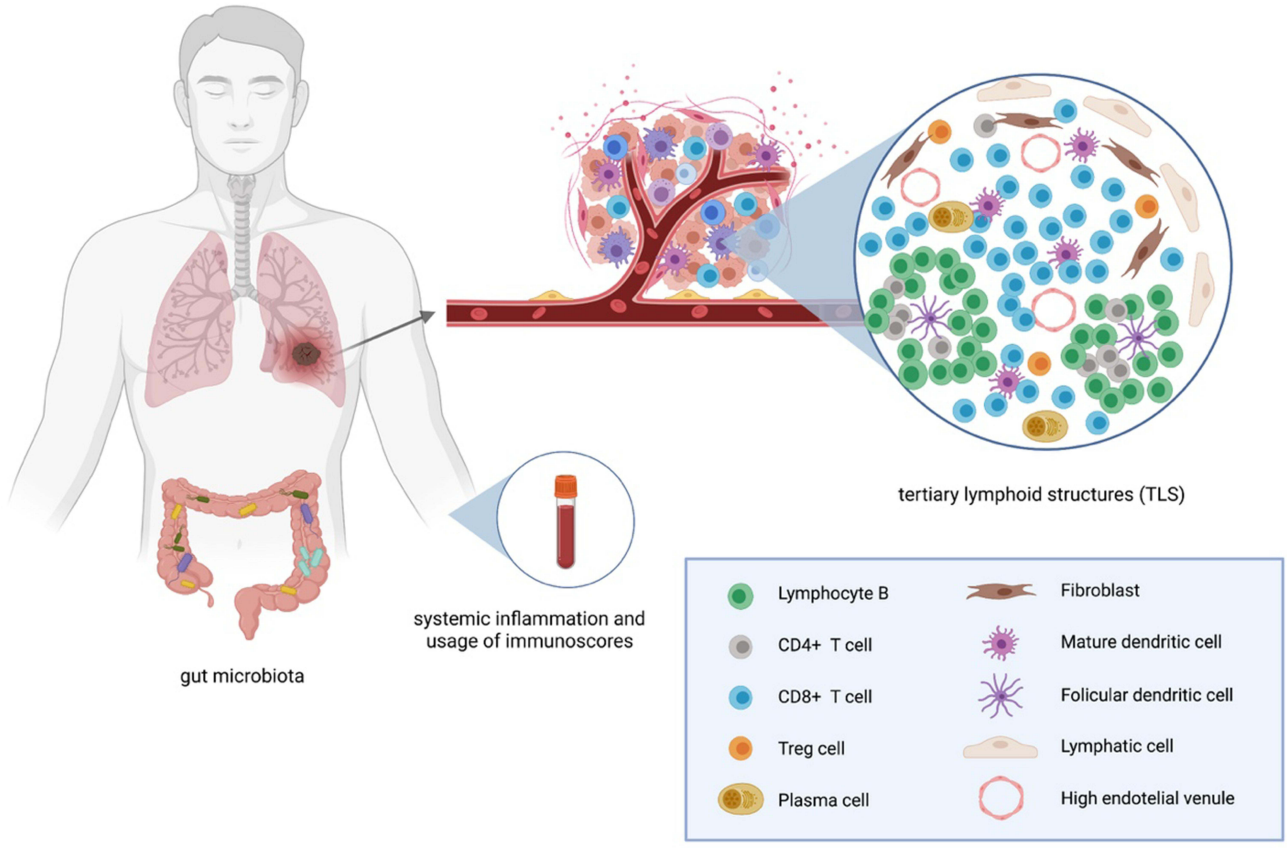

Figure 1 Host-related biomarkers. Created by https://www.Biorender.com.

(ORR) was 57% in PD-L1 positive tumors versus 8% ORR in PD-L1 negative patients.[30] However, a meta-analysis of eight prospective randomized clinical trials which included 4174 patients with advanced or metastatic tumors showed, compared to conventional therapeutic strategies, a prolonged overall survival in both patients that were PD-L1 positive (n=2254, hazard ratio 0.66, 95% confidence interval 0.59 to 0.74) and PD-L1 negative (1920, 0.80, 0.71 to 0.90).[31] Nevertheless, in this meta-analysis, the effectiveness of PD-(L)1 blockade was significantly better in PD-L1 positive patients as compared to PD-L1 negative ones, using a cut-off of 1% for defining PD-L1 positivity. In the CheckMate 067 trial where nivolumab plus ipilimumab were compared to nivolumab alone in advanced melanoma, outcomes were independent of PD-L1 status and best responses were seen in the combination arm.[32] Consequently, in melanoma, none of the presently approved ICIs requires PD-L1 status assessment.

In the non-small cell lung cancer (NSCLC) phase III trials CheckMate 017 and CheckMate 057 there was an improved overall survival (OS) for nivolumab compared to docetaxel in the second line setting, after platinum doublet chemotherapy.[33–35] A 20% ORR in the nivolumab arm was noted in the CheckMate 017 trial, seemingly independent of the PD-L1 status of the tumor, while in the CheckMate 057 trial the reported ORR was 19%, with improved OS in the nivolumab arm of 12.2 months versus 9.4 months; p=0.002, HR: 0.73. Although on subgroup analysis there is a good correlation of response to PD-L1 status as defined by ≥1% tumor membrane expression, with a better rate of response in the nivolumab arm, in less than 5% of PD-L1 positive subjects the OS did not vary between treatment arms. As a result, in the second line setting for all NSCLC subtypes, a PD-L1 status assessment is not mandatory before nivolumab administration. These results were supported by another phase III study, the OAK trial, where both PD-L1 positive and negative subgroups of patients in the atezolizumab arm had a better OS of 20.5 months (HR: 0.41) for the PD-L1 positive group and 12.6 months for the PD-L1 negative group, versus docetaxel.[36] The difference in OS according to PD-L1 expression supports its relevance as a predictive biomarker.

Table 2 Status of Current and Emerging Biomarkers

Biomarker	Current Status	Tumor Type	Testing Landscape	Relevance	Drawback
PD-L1	Validated	Multiple tumors (melanoma, NSCLC, HNSCC, bladder cancer, RCC, TNBC)	IHC-oriented assessment of PD-L1 expression on tumor and immune cells[26]	PD-L1 positivity associated with improved patient outcome[40,41]	Heterogeneity of PD-L1 expression and not interchangeable staining clones, defy for pathologists[49–52]
MSI and dMMR	Validated[57]	Multiple tumors, CRC	IHC for MMR proteins (MLH1, MSH2, PMS2, MSH6) PCR testing for MSI[26]	Immunogenicity due to a high amount of neoantigens secondary to MSI phenotype or deficient MMR system	Scarce prevalence of dMMR in solid tumors - 4%[61]
TMB	Under research	Melanoma, bladder cancer, NSCLC, SCLC, HNSCC	WES (whole-exome sequencing) or targeted NGS (net generation sequencing)[26]	- High mutational load correlated with T-cell enhancement[64] - Possibility of using blood TMB[75]	- Difficult access to WES - Lack of standardization of calculation and reporting TMB[26] - Access to tumor tissue
TILS and TLS	Under research	CRC, melanoma, breast cancer	Hematoxylin-eosin IHC identification of T cells in tumor stroma and in the invasive margin[26]	Elevated CD8+ TILS density and TLS assure local immune response[76]	- No standardized assessment of TILS, no cut-off value[78]
Microbiome	Under research	Melanoma, NSCLC, renal and bladder cancer	- PCR from stool samples - Shotgun-sequencing gene count - Ribosomal RNA gene amplicon sequencing[26]	Immune activating bacteria of innate and adaptive system[87–89]	Difficulty in accessing gene sequencing
Systemic inflammation	Under research	NSCLC, melanoma, clear cell renal cancer	Blood count and biochemistry	Offers insight into patient's inflammation status, predicting poor outcome[90,91]	Variety of biomarkers, need for standardization
DNA repair mutated enzymes	Under research	dMMR tumors	- Targeted NGS - Allele-specific PCR testing[26]	Predispose to an ultramutator phenotype capable of exerting a competent immune response[96,97]	- Access to testing - Less common
Genomic signatures	Under research	Melanoma, breast cancer, NSCLC, Merkel cell carcinoma	RNA gene expression assessment from tumor microenvironment[26]	IFN-γ signaling pathway major component of T-cell response[104]	Preexisting immunity of host modulates ICI effectiveness[106,107]

In first line setting, in the KEYNOTE-024 phase III trial, patients with ≥50% PD-L1 positivity treated with pembrolizumab had a longer median PFS (10.3 months) and 6 months OS (80.2%; HR: 0.60, 95% CI, 0.41 to 0.89) than those on platinum doublet chemotherapy (median PFS 6 months; OS at 6 months: 72.4%).[37] Later, the KEYNOTE-042 trial found an extended OS in the pembrolizumab arm versus platinum-based chemotherapy treated patients in locally advanced or metastatic NSCLC patients having ≥1% PD-L1 positive cell staining.[38] However, in untreated locally advanced or metastatic NSCLC patients, in the CheckMate 026 study, which used a threshold for PD-L1 expression of ≥5%, there was no significant difference in PFS or OS with nivolumab as monotherapy.[39] As a consequence, the FDA approved the anti-PD-L1 atezolizumab (based on the Impower110 trial)-[40] and, more recently, in February 2021, the antiPD-1 in cemiplimab (based on the EMPOWER-Lung 1 trial)[41] in the first line setting as monotherapy, conditional on ≥50% PD-L1 expression. In the PACIFIC trial, stage III NSCLC patients treated with durvalumab as consolidation therapy after chemoradiation experienced a significant improvement of the OS versus placebo independent of the tumor PD-L1 positivity status.[42]

In head and neck squamous cell and urothelial cancer patients, high levels of PD-L1 expression in tumor cells were associated with durable responses and improved OS after pembrolizumab, respectively avelumab.[43,44] A correspondence between PD-L1 expression in infiltrating immune cells and response to atezolizumab in urothelial cancer and atezolizumab plus chemotherapy in triple-negative breast cancer was also reported.[45,46]

All aforementioned data considered, PD-L1 is a biomarker of variable relevance, neither excluding nor warranting response to PD-1/PD-L1 blockade across different tumor types including melanoma,[30,47] squamous cell NSCLC[33–35] and renal cell cancer.[48] There are, however, situations where PD-L1 positivity can predict a better response to PD-1/PD-L1-blocking mAbs. The issue to be addressed is PD-L1 testing scores and defining the cut-off value for positivity. The vast diversity of expression of PD-L1, both intratumoral and intertumoral, is a challenge for pathologists since there are 4 FDA-approved staining assays (22C3, 28-8, SP142, SP263), one more to be validated (73-10 PD-L1 clone) and each with different staining affinity for PD-L1 on tumor and immune cells. There are several studies, like the Blueprint Project,[49,50] that showed that these clones are not interchangeable in terms of staining, consequently being discordant and with statistically significant differences at 1% and 50% cutoff values.[51,52] The most important concern is comparing the concordance of these assays in terms of primary and secondary endpoints. By doing so, several PD-L1 assays with different cut-offs for a vast majority of PD-L1 inhibitors could be validated, assuring a precise selection of patients.

MSI and dMMR

Microsatellites are repetitive short sequences of DNA about 1 to 6 nucleotides long, appearing throughout the genome. These tandem sequences are located in both genes and intergenic non-coding regions, usually occurring in promoter and terminal regions, introns and in coding exons.[53,54] When the genome gains or loses ≥1 repeat during DNA replication, MSI (microsatellite instability) occurs. These faults are normally repaired by the mismatch repair (MMR) system that comprises 4 key proteins: MLH1, MSH2, PMS2 and MSH6.[53,54] A defective or deficient MMR system (dMMR) appears if there is a mutation in any of these genes or hypermethylation in the promoter of the MLH1 gene. Consequently, errors during DNA replications cannot be corrected, resulting in a MSI phenotype. According to the number of distressed microsatellites, tumors can be classified as: MSI high (MSI-H), MSI low and microsatellite stable (MSS).[55] Therefore, MMR deficiency is a mechanism that predisposes to accumulation of mutations, increasing the probability of neoantigen expression as compared to MMR-proficient tumors and consequently generating immunogenicity.

To investigate the MSI issue, pembrolizumab was administered to a heterogeneous cohort of 41 metastatic, previously treated, patients, irrespective of tumor localization, with or without dMMR.[56] There was a substantial difference in the ORR in dMMR versus proficient MMR (pMMR) patients: 40% ORR in dMMR colorectal (CRC) patients, 78% ORR in dMMR non-CRC and 0% ORR in pMMR patients, respectively. In first line setting, in the KEYNOTE-177 phase III trial, pembrolizumab was superior to chemotherapy in metastatic MSI-H-dMMR CRC patients, with a median PFS of 16.5 months vs 8.2 months (HR: 0.60, 95% CI, 0.45 to 0.80; p=0.0002), becoming a new standard of care.[57]

As a premiere, the FDA granted approval of pembrolizumab in MSI-H/dMMR previously treated metastatic or unresectable solid tumors, regardless of tumor type,

making it the first ICI to be validated based on a biomarker, based on ORR of pembrolizumab of 39.6% among 149 patients with 15 different MSI-H/dMMR tumor types and a 7% complete response rate.[58] Other ICIs to be approved in this setting were nivolumab (anti-PD-L1) and the combination of nivolumab plus ipilimumab, for MSI-H/dMMR metastatic CRC patients.[59,60]

Only a small percentage of tumors are MSI-H or harbor a dMMR system. The prevalence of dMMR is only 4% in all adult solid tumors,[61] a deficient MMR mechanism having been reported only in endometrial, gastric, small bowel, colorectal, cervical, prostate, bile duct, liver and thyroid carcinomas, neuroendocrine tumors and uterine sarcomas. A form of inherited dMMR exists, in the form of the genetic condition called Lynch syndrome. Knowing the predilection of Lynch syndrome to CRC and endometrial cancer (52–82%, respectively; 25–60% lifetime risk), it is mandatory to identify all cases of Lynch syndrome, not only for the patient himself, but also for his family members. Consequently, it is highly advised that all CRC and endometrial tumors undergo screening for defective MMR in order to benefit from ICI.

In Need of Validation – Emerging Biomarkers

TMB

TMB relates to the prevalence of somatic mutations in the genome, leading to nonsynonymous single-nucleotide variants, consequently increasing the capacity of a tumor to create neoantigens. A high TMB enables tumor cells to create unique peptides, expressing them on their surface as a major histocompatibility complex-associated neoantigen. Being interpreted as "non-self", these neoantigens are capable of generating T-cell responses. Consequently, the higher the TMB, the more immunogenic the tumor is and, as a result, the more susceptible to responding to immunotherapy.

It is well known that TMB differs across tumor types, with melanoma, lung and bladder cancer holding the highest mutation prevalence[62] with a consistent response to ICI.[63,64] In melanoma, in a CTLA-4 blockade setting by ipilimumab and tremelimumab, high mutational load was associated with a persistent clinical benefit of more than 6 months and a significant improvement of OS (p=0.04).[65] In this study, TMB cut-off was defined as more than 100 mutations per sample as identified throughout whole exome sequencing (WES).

In an NSCLC study, Rizvi et al used a cut-off of >200 mutations per sample as determined by WES, showing that high TMB is correlated with an elevated tumor objective response (63% vs 0%, p=0.03) and PFS (14.5 months vs 3.7 months, p=0.01, HR 0.19) after PD-1 blockade with pembrolizumab.[66] A distinct perspective revealed by this study is that effectiveness of antiPD-1 was associated with a molecular smoking signature, independent of smoking history, higher neoantigen burden and DNA repair pathway, raising awareness concerning other predictive biomarkers.

Besides melanoma and NSCLC, an elevated mutational load was a predictor of response to checkpoint blockade in many other tumor types such as urothelial cancer (atezolizumab),[45] small-cell lung cancer (nivolumab alone or in combination with ipilimumab)[67] and head and neck squamous cell cancer negative for human papilloma virus (antiPD-1/PD-L1).[68]

Frequently, however, rather modest responses to immunotherapy are noted in cases with a high mutational load and there are limited responses to immunotherapy, especially in proficient mismatch repair colorectal cancer.[69] Conversely, patients with a modest TMB sometimes benefit from ICI.[70,71] Many studies identified TMB and PD-L1 expression as independent predictive biomarkers, both of them associated with a better response. In this regard, in the CheckMate 026 study, patients with advanced NSCLC with nivolumab as first line treatment and having both high TMB and PD-L1 expression had a substantial response rate of 75% versus 34% for patients with only high PD-L1, respectively 32% for high TMB alone, and 16% for those with none of the two markers.[72] Also, patients with a significant mutational load and positive PD-L1 expression (>1%) treated with nivolumab and ipilimumab had an improved response and PFS in the CheckMate 227 and 012 trials.[73,74]

Limitations in validating TMB as a biomarker are difficult access to WES, lack of standardization of the assessment and reporting of TMB and, last but not least, access to tumor tissue. As an alternative to tumor tissue assessment, Gandara et al[75] used peripheral blood TMB, with a cut-off of 16 or more mutations, which was relevant for PFS, irrespective of PD-L1 status, in patients treated with atezolizumab versus docetaxel chemotherapy, making blood TMB assessment an innovative approach to be considered when tumor tissue is not available.

TILS and TLS

Tumor-associated lymphocytes are circulating lymphocytes that migrate from blood to tumor across the tumor endothelial barrier.[76] TILs depict an active, inflamed tumor microenvironment with the capacity of modulating the T-cell response in accordance to the tumor neoantigens, consequently being a good candidate for a predictive biomarker.

There are 3 distinct types of CD-8+ T cells infiltrating tumors, as described by Naito et al[77] after examining 131 patients with resected colorectal cancer: T-cells infiltrated within cancer nests, in the cancer stroma and at the junction between tumor and host (invasive margin). In this study, only the TILS within the cancer nest had a significant independent correlation with patient survival (p=0.016; HR: 0.52) due to the fact that earlier stages were associated with a greater infiltration.

TILs' density, both at the invasive margin and within the cancer nest, was considered a predictive biomarker in the baseline biopsies of melanoma patients responding to pembrolizumab versus the progressing group.[25] Also, an elevated density of CD8+, CD3+ and CD45RO+ T-cells in pretreatment melanoma patients was associated with a better response to CTLA-4 blockade.[78] However, in both of these studies, there was no clear cut-off value for TILs to distinguish between responders and non-responders to ICI therapy. Moreover, Chen et al[78] demonstrated that there is also an enrichment of T-cells in the tumor center versus the margin in responders compared to non-responders, indicating an ICI-induced tumor infiltration.

Also, in breast cancer trials the presence of TILs was associated with an immune checkpoint inhibitor response. In the phase IB/II PANACEA trial, the use of pembrolizumab-trastuzumab in HER2 positive metastatic patients was associated with a better ORR and longer period of disease control in the presence of TILs at baseline.[79]

Furthermore, there are studies that evaluated residual disease (RD) TILs after neoadjuvant chemotherapy in triple-negative breast cancer, revealing there is a positive correlation between RD TILs and CD8+ T-cell density, with an enhanced OS of these patients.[80,81] Consequently, RD TILs could be a independent biomarker, serving as a predictive one in the adjuvant setting.

All these studies demonstrate the existence of TILs as a noteworthy cancer immunotherapy biomarker candidate; however, like with other predictive aforementioned biomarkers, there is an unmet need for a standardized assessment, with a quantification of TILs using a homogenous scoring, not a manual IHC interpretation. Nowadays, in the artificial intelligence era, machine learning-based algorithms could be the key in identifying the best subset of immunoresponsive patients.

Lately, besides TILs, tertiary lymphoid structures (TLS) are of extreme interest as potential biomarkers. TLS are defined as de novo ectopic lymphoid structures that develop in non-lymphoid tissues because of chronic exposure to inflammatory signals mediated by chemokines and cytokines[82] such as IL-7 and CXCL13.[83] TLS are usually found in the peritumoral stroma, invasive margin, and/or core of different tumor types, forming a unique lymphoid structure similar to secondary lymphoid organoids, resembling a B-cell follicle, with a germinal center and an enriched T-cell area with mature dendritic cells.[84] TLS assure a local immune response by the induction of effector functions, antibody generation, and clonal expansion.[76] Therefore, TLS are associated with favorable prognosis and sustained response to ICI, especially in melanoma patients where they were correlated with increased survival after CTLA-4 blockade.[85] Seeing the strong link between TLS and the antitumor immune response, there is ongoing research on the therapeutic induction of TLS, combined with immune checkpoint blockade.[82]

Microbiome

Microorganisms found in the gastrointestinal tract, on the skin and on mucosal surfaces represent the human microbiome. Their immunomodulatory role has been recognized since the 2013 description by Viaud et al of gut microbiota modulating the antitumor immune response and influencing the efficacy of cyclophosphamide.[86] In the PD-1/PD-L1 setting, Routy et al[87] described an interaction between gut microbiota and ICIs, showing that patients with NSCLC, renal and urothelial cancer treated with broad spectrum antibiotics prior to antiPD-1/PD-L1 therapy had a significantly shorter OS and PFS. Furthermore, shotgun sequencing showed that fecal samples from PD-1 blockade responders were more abundant in a particular commensal bacteria, *Akkermansia muciniphila*. They concluded that bacterial diversity and specific bacteria such as *A. muciniphila* restore barrier integrity, reduce systemic inflammation and enhance immunosurveillance.[87]

In the same regard, Matson et al[88] found 8 bacterial species to be more abundant in metastatic melanoma patients responding to PD-L1 blockade, and 2 species to

be more abundant in non-responders. The authors even proposed a ratio between beneficial immune-activating bacteria and deleterious bacteria that negatively regulate innate and adaptive immunity as a predictive biomarker for immunotherapy. Another study reported an association between CD8+ tumor T-cell infiltrates and the richness of Fecalibacterium genus, Ruminococcaceae family and Clostridiales order in the gastrointestinal tract in responder metastatic melanoma patients, proposing the hypothesis of an improved antitumor systemic response due to increased antigen presentation and refined T-cell function.[89]

All of the above considered, the microbiome, through its diversity and composition, is a strong candidate for an immune predictive biomarker, though it requires clinical validation.

Systemic Inflammation and Immunoscores

Blood biomarkers are preferred over tumor tissue biomarkers because of their better availability and their objective reflection of the patient's systemic inflammation status. High levels of serum lactate dehydrogenase (LDH), associated with an elevated tumor load and cellular turnover, were predictive of poor outcome in NSCLC and melanoma patients.[90,91] In this setting, one experimental study investigated in vitro the antitumor effects of an LDH A inhibitor that suppresses tumor growth through apoptotic cell death,[92] proposing combination approaches including PD-1/PD-L1 ICIs. Another study investigated soluble forms of immune checkpoint molecules in metastatic clear cell renal carcinoma patients treated with nivolumab, showing that high plasma levels of sPD-1, sPD-L1 and BTN3A1 were correlated with a longer PFS.[93]

Additionally, there are some immunoscores that could help clinicians in identifying subsets of patients with a worse outcome. For example, lung immune prognostic index (LIPI), based on a derived neutrophils/ leukocytes minus neutrophils ratio (dNLR) greater than 3 and LDH greater than the upper limit of normal permitted to distinguish 3 prognostic groups of patients: good (0 factors), intermediate (1 factor) and poor (2 factors).[91] To date, PILE is another immunoscore based on pan-immune inflammation value (PIV), a recently developed peripheral blood count biomarker, lactate dehydrogenase level, and Eastern European Oncology Group performance status.[94] The higher the immunoscore, the poorer OS noted in ICI-treated patients.

Arbour et al[95] showed that immunosuppressive agents such as steroids, used at the beginning of PD-1/PD-L1 therapy, were significantly associated with a decreased PFS (p=0.03; HR: 1.3) and OS (p=0.001; HR: 1.7). Even though this study was conducted on a small subset of patients, systemic corticoid use is generally discouraged in the antiPD-1/PD-L1 setting, especially in the absence of irAE.

POLE and POLD1 Along with Other DNA Repair Mutated Enzymes

Polymerase ε (POLE gene) and δ (POLD1 gene) are DNA polymerase enzymes participating in DNA replication in the S phase of the cell cycle. Through their exonuclease domain, the enzymes allow excision and replacement of incorrect bases, ensuring a correct DNA replication. Mutations of the exonuclease domain lead to an accumulation of mutations in the genome, promoting an ultramutator phenotype.[96]

POLD1 mutations are less common than POLE mutations and occur mainly in dMMR tumors, but nevertheless can lead to an ultramutator phenotype.[97] POLE mutations are mainly found in MSS/pMMR tumors but some cases were described in MSI patients with unexplained Lynch syndrome.[98] POLE mutations associated with an ultramutator phenotype have a plethora of mutations across the genome, an inflamed tumor microenvironment and up-regulated PD-L1; consequently, they were linked to a better prognosis, similar to dMMR tumors.[26] In this regard, there is a strong scientific rationale to assess these mutations with targeted NGS or allele-specific PCR testing not only for POLE/POLD1, but also for other DNA repair enzymes and mechanisms such as MGMT, homologous recombination, base excision repair and nucleotide excision repair[71] since they can significantly influence the response to immunotherapy. Further clinical studies are awaited for validation.

Genomic Signatures Responsible for Immune Feedback

Several studies used RNA-based gene expression profiling to identify underlying mechanisms of tumor response to immunotherapy. Taube et al, by a whole genome sequencing approach, showed that factors like IL-10 and IL-32 gamma induce PD-L1 expression on monocytes but not tumor cells, in melanoma patients, as an adaptive immune regulatory mechanism.[99] Adaptive PD-L1 expression was

also observed in other tumors such as NSCLC,[100] breast cancer,[101] and Merkel cell carcinoma.[102] The IFN-γ signaling pathway is considered a sine qua non component of a pertinent T-cell response. This is in accordance with the fact that Janus kinase (JAK) 1 and JAK 2 immunotherapy-resistant mutations prevent up-regulation of IFN-γ target genes.[103]

Gene signatures such as an IFN-γ 10-gene and expanded-immune 28-gene signatures described by Ribas et al[104] were correlated with an improved ORR and PFS in melanoma patients treated with pembrolizumab. In the POPLAR study, NSCLC patients with IFN-γ gene signature and 8 gene T-effector had a better OS when treated with atezolizumab versus docetaxel.[105]

As stated, these specific mutational signatures shape the response to PD-1/PD-L1 blockade and point out interactions between the host and the tumor. For example, NSCLC patients with tumors having molecular smoking signatures had higher response rates compared to those without such mutational patterns.[66,106] Another gene signature associated with durable clinical benefit in NSCLC patients treated with anti-PD-1 therapy, was the apolipoprotein B mRNA editing enzyme catalytic polypeptide-like (APOBEC) mutation.[107] Patients with head and neck squamous cell carcinoma and bladder cancer with APOBEC-related mutational signature responded to immune checkpoint blockade.[106] In melanoma patients, those with mutational signatures related to ultraviolet exposure or prior treatment with alkylating agents had a consistent clinical benefit versus other dominant mutational signatures.[106]

These studies suggest that preexisting host-related immune status plays a role in predicting the effectiveness and benefit of immune checkpoint blockade.

Lately, there has been a focus of attention on the role of long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) in regulating PD-L1/PD-1 axis signaling.[108] Both lncRNAs and oncogene miRNAs are upstream modulators of the axis with the ability of hampering anti-tumor immunity, including molecular pathways such as STAT3,[109] PI3K/Akt[110] and MAPK.[111] Consequently, these molecules became potential targets for a genetic therapeutic approach.

Conclusion

As with other anticancer treatments, the availability of reliable biomarkers predictive for the efficacy of ICI remains a cobsiderable challenge. One crucial concern regards the significant proportion of patients with concomitant driver alterations and PD-L1 expression, raising questions regarding patients' optimally tailored treatment.[112] To date, only two biomarkers have been validated for clinical use: PD-L1 expression in selected tumor types and MSI-H/dMMR for all types of solid tumor.

There are many other promising biomarkers such as TMB, TILS and TLS, and the microbiome, which have to be validated. In this regard, the development of robust, appropriate assays for their investigation is mandatory. We believe that the impressive efficacy seen with ICIs in selected patients warrants the effort to identify biomarkers for predicting treatment efficacy. Besides this, predictors of non-response may also prove of huge clinical relevance, allowing for the avoidance of submitting patients to an ineffective, but not innocuous, treatment course. Finally, predictors for the risks of side effects are also of potentially great clinical benefit. Therefore, a systematic recording of patient and tumor-related characteristics in both clinical trials and real life is crucial, allowing for the retrospective identification of correlations with treatment outcome.

In the years to come, this setting will be a thriving territory of research and validation.

Disclosure

Prof. Dr. Mihnea Zdrenghea received clinical study funding from GlaxoSmithKline, personal fees from Amgen, non-financial support from Astra Zeneca, personal fees from Bristol Myers Squibb, personal fees from Novartis, personal fees from Janssen, personal fees from Pfizer, grants and personal fees from Roche, personal fees from Sanofi, personal fees from Servier and personal fees from Takeda during the conduct of the study.

The authors report no conflicts of interest in this work.

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