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PD-L1 and Emerging Biomarkers in PD-1/PD-L1 Blockade Therapy

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Abstract

PD-(L)1 checkpoint blockade is revolutionizing cancer therapy, and biomarkers capable of predicting which patients are most likely to respond are highly desired. The detection of PD-L1 protein expression by immunohistochemistry can enrich for response to anti-PD-(L)1-blockade in a variety of tumor types, but is not absolute. Limitations of current commercial PD-L1 IHC assays and improvements anticipated in next generation PD-L1 testing are reviewed. Assessment of tumor infiltrating lymphocytes in conjunction with PD-L1 testing could improve specificity by distinguishing adaptive (interferon-gamma-driven, and cytotoxic T-lymphocyte-associated) from constitutive (non-immune-mediated) expression. The presence of a high tumor mutational burden also enriches for response to therapy and early data indicate that this may provide additive predictive value beyond PD-L1 IHC alone. As candidate biomarkers continue to emerge, the pathologist's assessment of the tumor microenvironment on H&E combined with PD-L1 IHC remains a rapid and robust way to evaluate the tumor-immune dynamic.

Introduction

Immune checkpoint pathways can be co-opted by tumors to prevent tumor-specific immune responses. The finding that therapeutic monoclonal antibodies can be used to inhibit these checkpoint interactions, unleashing pre-existing anti-tumor immunity, has revolutionized cancer therapy. One of the best studied of these immune checkpoints is the PD-1/PD-L1 receptor-ligand pair. The PD-1 receptor is expressed on activated lymphocytes, including T cells, B cells and NK cells.[1] Its ligand, PD-L1, can be expressed by an extended number of cell types, including tumor cells, lymphocytes, macrophages-lineage cells, endothelial cells, amongst others.[2] The PD-1/PD-L1 immune inhibitory pathway was originally described in

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the setting of chronic viral infections, whereby it prevents destruction of host tissues by an unrestrained immune response. In the setting of cancer, however, this homeostatic mechanism prevents tumor cell clearance by the immune system.

The power of the PD-1/PD-L1 pathway to thwart antitumor immune activity has been confirmed by the durable clinical responses achieved with therapeutic anti-PD-(L)1-blockade in patients with melanoma, non-small cell lung cancer (NSCLC), urothelial carcinoma, classic Hodgkin lymphoma, head and neck squamous cell carcinoma (HNSCC), renal cell carcinoma (RCC), Merkel cell carcinoma, hepatocellular carcinoma, and gastric carcinomas. Consequently, an unprecedented number of FDA approvals have been secured over the past 3 years for agents in this class for these different indications.[3] Among unselected patients with the eight solid tumor types listed, 10–40% show clinical response to anti-PD-(L)1 monotherapy and approximately 7–34% experience high-grade immune-related adverse events (irAEs).[4,5] Given the cost and side effect profile of these therapies, biomarkers predictive of response are highly sought after for patient selection. In the context of the first clinical trials of PD-1 blockade, it was shown that immunohistochemical (IHC) detection of tumor cell PD-L1 expression was associated with therapeutic response.[6–8] To date, PD-L1 expression in the pre-treatment tumor microenvironment (TME) represents the most well-studied potential biomarker of response to anti-PD-(L)1 therapy, though a number of other markers are currently under investigation.

PD-L1 IHC Assays

Different companion and complimentary PD-L1 IHC assays were developed for each anti-PD-(L)1 agent in association with early clinical trials, Table 1. Many parameters vary between these assays, including the primary antibody clone used. The interpretation guidelines also differ, both among assays and among indications for a given assay. The 22C3 and 28-8 assays center on tumor cell PD-L1 expression, except for the 22C3 assay in gastric cancer which also incorporates scoring immune cell PD-L1 expression. In contrast, the SP263 and SP142 assays include both tumor and immune cell PD-L1 expression for most tumor types, except for the SP142 assay in urothelial cancer, which exclusively scores immune cell PD-L1. Even for a given agent and tumor type, different assay scoring parameters have been called for in some instances, depending on the line of therapy, e.g. high PD-L1 expression (≥50%) is required for first-line pembrolizumab in NSCLC, while any PD-L1 expression (≥1%) is sufficient for use in the second-line setting.

The approval of multiple assays associated with unique interpretation thresholds has made it difficult to directly compare results across clinical trials. It has also created many practical concerns regarding which test to use and the interchangeability of the results. Notwithstanding, the different PD-L1 antibodies and other assay reagents, interpretation thresholds, and anti-PD-(L)1 therapeutic agents tested, the detection of PD-L1 expression in the pretreatment tumor microenvironment by IHC consistently enriches for patients harboring multiple different tumor types who are likely to respond to PD-(L)1 blockade.[9,10]

Several studies have attempted to compare the different marketed PD-L1 IHC assays. The 22C3, 28-8, and SP263 assays have been shown to detect equivalent amounts of PD-L1

tumor cell staining in NSCLC specimens in multiple studies.[11–13] In contrast, the SP142 assay has been shown to have a reduced sensitivity for the detection of tumor cell PD-L1 expression[11,13] as well as decreased sensitivity for highlighting immune cell PD-L1 expression.[11] Additionally, it was shown that there is a greater degree of variability in scoring of immune cell PD-L1 expression across all four assays, likely due to a lack of defined criteria and a lack of training, contributing to poor reproducibility amongst pathologists.[11]

It is important to note that a complete IHC assay system contains many components in addition to the antibody that is used in that assay. Studies have been performed to determine whether the 22C3, 28-8, SP263, and SP142 antibodies themselves differ in their ability to highlight PD-L1 expression on tumor cells and/or immune cells. It has been shown in both melanoma[14] and NSCLC[15] that the primary antibodies (including the SP142 clone) are no different in their capacity to stain for PD-L1 in these different cell types, when the other features of the assay are essentially held consistent. This finding indicates that it is other features of the assay system (antigen retrieval, concentration of primary antibody, amplification system, etc) that leads to the observed difference in performance of the SP142 assay and not the SP142 antibody. This has important implications for potential laboratory-developed tests that may be used by surgical pathologists for clinical care in the future.

Adaptive and Constitutive PD-L1 Expression

Current interpretation guidelines do not distinguish between adaptive and constitutive patterns of PD-L1 expression. Adaptive PD-L1 expression was first described in melanoma, and is a dynamic and geographically heterogeneous, interferon-gamma-driven mechanism of PD-L1 expression by tumor and immune cells at the host-tumor interface.[16] In contrast, “constitutive” tumor cell PD-L1 expression is defined as a population of tumor cells expressing PD-L1 on their cell surface, independent of an immune infiltrate. Constitutive expression can be driven by numerous tumor-intrinsic mechanisms associated with genetic alterations.[17,18] The frequency of constitutive PD-L1 expression appears to vary by tumor type, for example, melanoma demonstrates infrequent constitutive PD-L1 expression, i.e., ~1% of cases[16] while cutaneous squamous cell carcinoma shows a notably higher proportion of pathology specimens with some degree of constitutive expression.[19]

It has been suggested that anti-PD-1 therapy exerts its effect by inhibiting the adaptive form of the PD-L1 immune resistance mechanism.[20] It is possible that when PD-L1 expression occurs in the absence of a pre-existing anti-tumor immune response, it may not predict response to PD-1/PD-L1 blockade. This is one potential explanation for the proportion of patients that have tumors that are PD-L1+, but which do not respond to anti-PD-(L)1 therapy, Table 2. It also potentially explains the closely related finding that PD-L1 expression by immune cells is more predictive of response than tumor cell PD-L1 expression in squamous cell carcinomas of the head and neck,[21] which tend to show high levels of constitutive PD-L1 expression. Further complicating the issue, however, is the fact that the adaptive and constitutive patterns of PD-L1 expression are not mutually exclusive. For example, murine and *in vitro* studies suggest tumor cell-intrinsic PD-L1 drives expression of genes related to a tumor initiating cell phenotype and interferon gamma sensitivity;[22]

elevated PD-L1 expression associated with a JAK3 mutation was associated with a response to anti-PD-L1;[23] and histologic patterns of combined adaptive and constitutive PD-L1 expression have been observed in multiple tumor types.[3]

Potential limitations of PD-L1 IHC

In addition to the variability in PD-L1 assay performance and interpretation thresholds as well as the different physiologic implications of adaptive vs. constitutive PD-L1 expression described above, there are a number of other potential reasons why a PD-L1(+) patient may not respond to therapy and why a PD-L1(-) patient could show an objective response, Table 2. The first reason is simply the focal and heterogeneous expression of PD-L1. PD-L1 expression can also be dynamic, so it may change over time. Thus, PD-L1 expression levels in an archival, pre-treatment specimen could contribute to either a “false positive” or “false negative” value of PD-L1 expression. Similarly, there’s the possibility of limited tissue sampling, which can lead to test results that are not representative of the entire tumor.[14,24,25] Lastly, in addition to sampling issues related to PD-L1 expression, there may be other resistance mechanisms present in the tumor, including alternate PD-1/PD-L1 ligand/receptor activity (PD-L2, B7-1/CD80) or other checkpoint molecules expression, e.g. LAG-3.[26,27] Depending on the drug used (anti-PD-1 vs. anti-PD-L1), such mechanisms could be a factor underlying why some patients with PD-L1+ tumors fail to respond to anti-PD-1 therapy or some patients with PD-L1- tumors do respond.

Assessment of Tumor Infiltrating Lymphocytes (TIL)

Tumor infiltrating lymphocytes indicating a “T-cell inflamed phenotype” have been assessed for their association with response to anti-PD-(L)1 therapy. Increased CD8+ T-cell densities at the tumor’s leading edge (Figure 1A) have been associated with response to anti-PD-1 from pre-treatment tumor specimens from patients with metastatic melanoma.[20] Unfortunately, a threshold of CD8+ cell density in pre-treatment specimens is not evident that can clearly separate responders from non-responders. Similar results were seen when CD3 and CD45RO+ T-cell densities were assessed in pre-treatment melanoma specimens,[28] and when parameters such as the CD4:CD8 ratio or PD-1 or CD3 expression were assessed in a semi-quantitative fashion in a cohort that included a number of different solid tumor types.[29]

Gene expression profiling has also been used to identify an inflamed tumor phenotype by using an interferon-gamma immune-related signature score, with similar results across multiple different tumor types.[30] In fact, the receiver operating characteristic (R.O.C.) curves for the sensitivity and specificity for the IFN-gamma gene signature, CD8 density, and PD-L1 expression often overlap, indicating the connected nature of these biomarkers, Figure 2.[31]

Most of the focus to date has been on predicting response to therapy in pre-treatment tumor specimens. It is worth noting that some studies have assessed TIL from patients who have already started therapy with impressive results. A marked difference has been reported in immune cell densities in on-treatment biopsies from responders vs. non-responders to anti-

PD-1 therapy in patients with melanoma, including CD8, CD4, CD3, PD-1, PD-L1, and LAG-3 expression. When the location of CD8+ immune cells were assessed, the presence of a high density in the tumor center (Figure 1A) was associated with therapeutic response.[28] New therapeutic approaches are predicated on changing a non-inflamed TME (Figure 1B) to an inflamed one (Figure 1C) with the aim of overcoming primary resistance to anti-PD-1. Most recently, a small cohort of melanoma patients was treated with oncolytic virotherapy with talimogene laherparepvec to alter the TME to a more inflamed phenotype, contributing to an increase in anti-PD-1 efficacy.[32] Undoubtedly, many other strategies to change a non-inflamed TME to an inflamed one will be reported in the near future.

The Society for Immunotherapy of Cancer (SITC) immune biomarker taskforce has highlighted the need for "standardized protocols for the histopathologic assessment of TIL and effector cell populations."[33] Proposals have been made by the International Immuno-Oncology Biomarkers Working Group.[34,35] Such protocols are expected to become key as TIL assessments likely make their way into routine surgical pathology practice as predictive and prognostic markers.

Combination PD-L1 IHC and TIL assessment

The next generation of IHC assays will likely include both PD-L1 and TIL assessments. A TIL score combined with PD-L1 IHC would greatly improve our ability to categorize a tumor into one that is infiltrated, i.e., TIL+, and "immunoactive", *i.e.* PD-L1+; infiltrated, but by non-activated or exhausted TIL (TIL+/PD-L1-); or one that is non-inflamed (TIL-/PD-L1- or TIL-/PD-L1+, the latter by constitutive PD-L1 expression).[4,16,36] Such findings could have therapeutic implications. For example, patients whose tumors show PD-L1 expression in association with TIL may be more likely to benefit from anti-PD-(L)1 monotherapy, while those whose tumors are not inflamed or harboring non-activated or exhausted TIL may be more likely benefit from a combinatorial therapeutic approach. The assessment of PD-L1 and TIL could be performed using currently available PD-L1 IHC assays, simply by adding an additional parameter into the interpretation, and therefore could be a fast and inexpensive way to improve upon the currently available technology.

Other possible next generation assays combining PD-L1 with a TIL assessment require assaying for a second parameter. For example, PD-L1 protein expression and an IFN-gamma gene signature have been combined in the assessment of pre-treatment specimens from patients with NSCLC. Preliminary results suggest that this combination of markers is more predictive than either of those markers alone.[37] Another example of combining PD-L1 expression with a measure of TIL status is assaying the proximity between PD-1+ and PD-L1+ cells, *i.e.,* the dominant interaction that is ostensibly being blocked by anti-PD-1 therapy. For patients with both Merkel cell carcinoma and melanoma, the expression of these two parameters in close proximity to each other is more predictive than PD-L1 IHC expression alone.[38,39] Detailed quantitative assessments of the TME may soon be possible via multiplex immunohistochemical/immunofluorescence technologies.[40–42] This approach would have many advantages compared to traditional IHC, but streamlined assays necessary for routine use have yet to be clinically validated.

Mutational Burden

Another candidate biomarker that has been associated with a response to anti-PD-1 is the mutational load of a patient's tumor. Patients with NSCLC tumors that had a higher number of non-synonymous mutations were more likely to demonstrate an initial response to therapy as well as an increase in progression free survival.[43] Perhaps the most striking example of the correlation between mutational density and response to anti-PD-1 therapies is the differential response rate between patients with mismatch repair-deficient colorectal carcinomas and those with mismatch repair-proficient tumors (78% vs. 11%, respectively).[44] Further proof of principle is the fact that patients with microsatellite unstable tumors, regardless of the tumor's site of origin, are likely to demonstrate a response to anti-PD-1. A clinical trial of anti-PD-1 in patients with 12 different mismatch repair-deficient tumor types showed a 53% response rate, including 21% of patients with a compete response.[45] This latter finding led to an unprecedented FDA approval for a tumor-type agnostic indication, *i.e.,* pembrolizumab (anti-PD-1) for all microsatellite-unstable tumors.

The reason that mutational load is thought to be a predictor of response to anti-PD-1 is that mutations are thought to serve as the source of immunogenic neoantigens. It is recognized that some mutations are more immunogenic than others, and that increasing mutational density likely increases the chances that one or more of the antigens will elicit a notable anti-tumor immune response. Factors beyond simple immunogenicity of mutations are also important. Clonal neoantigens, *i.e.,* those that occur early in the evolution of the tumor and are present in a large population of tumor cells, are more strongly associated with response than subclonal neoantigens.[46] Neoantigen loss is also a mechanism of secondary resistance to PD-1 blockade.[47]

While mutational burden may be associated with the potential of a tumor-specific immune response, TIL/CD8 densities and PD-L1 expression are more direct measures of whether an immune response is currently present in given tumor. When principal component analysis was performed on measures of cytotoxic (*CYT*) gene expression, *PD-L1* expression, and mutational burden from melanoma tumor specimens in The Cancer Genome Atlas dataset, *PD-L1* expression and *CYT* were closely related and formed one variable, while mutational load formed a separate, independent variable.[31] These relationships can be visualized on a hive plot, Figure 2, where it can be observed that the median values for *PD-L1* and *CYT* expression profiles tend to track each other closely across multiple different solid tumor types, while mutational density does not parallel the other two parameters. Ultimately, a combinatorial biomarker representing 1) whether an immunoactive infiltrate is present within the tumor and 2) the genomic features of the tumor, is likely to have improved predictive value over either metric alone.

Other Candidate Biomarker Approaches

Numerous reports nominating additional candidate biomarkers predictive of response or resistance to PD-1/PD-L1 blockade therapy are emerging with increasing frequency. This complex and evolving landscape has been recently covered in detail elsewhere.[36,48,49] Effective T cell antigen recognition, priming, tumor infiltration, and cytotoxic effector

function are all necessary steps for tumor elimination and potential targets of therapeutic intervention. Moreover, tumor heterogeneity and evolution under the pressure of therapy add to the complexity of assessing the value of emerging biomarker candidates. In addition to tumor tissue, other sources of biospecimens are being explored in the quest to predict and monitor response to therapy in a given patient. Tumor-involved lymph nodes,[50] circulating immune cells,[50,51] circulating tumor DNA,[52] and microbiome reservoirs such as stool[53,54] have all showed early promise in predicting response, resistance, or toxicity with checkpoint blockade therapy.

Conclusions and Future Directions

The SITC immune biomarkers taskforce has proposed that for routine use, "evaluation of a novel [biomarker] should be routine, feasible, simple, rapid, robust, reproducible, objective, specific, quantitative, standardized, powerful, and preferentially IHC-based."[33] While the current value and limitations of PD-L1 IHC are widely acknowledged, the power of a pathologist's assessment of the TME on H&E combined with PD-L1 IHC has the potential to provide more information about the state of the tumor-immune dynamic than an assessment of PD-L1 expression as simply "positive" or "negative". In the future, it is anticipated that PD-L1 IHC will be incorporated into a multiplex IHC format that includes a number of potential immunotherapeutic targets or checkpoint molecules, *e.g.,* IDO[55] or LAG-3[27], likely accompanied by spatial metrics. Measures of gene expression profiles and genomic studies are also likely to provide unique information, leading to multimodality assessments of the TME. These approaches will have to be integrated and prioritized to inform testing strategies and allow for a more rational approach that consumes less time, money, and tissue. As our therapeutic arsenal and associated biomarkers continue to evolve, such an approach is essential to maximize the yield of information from a finite amount of tissue, and thus most productively guide treatment strategy for an individual patient.

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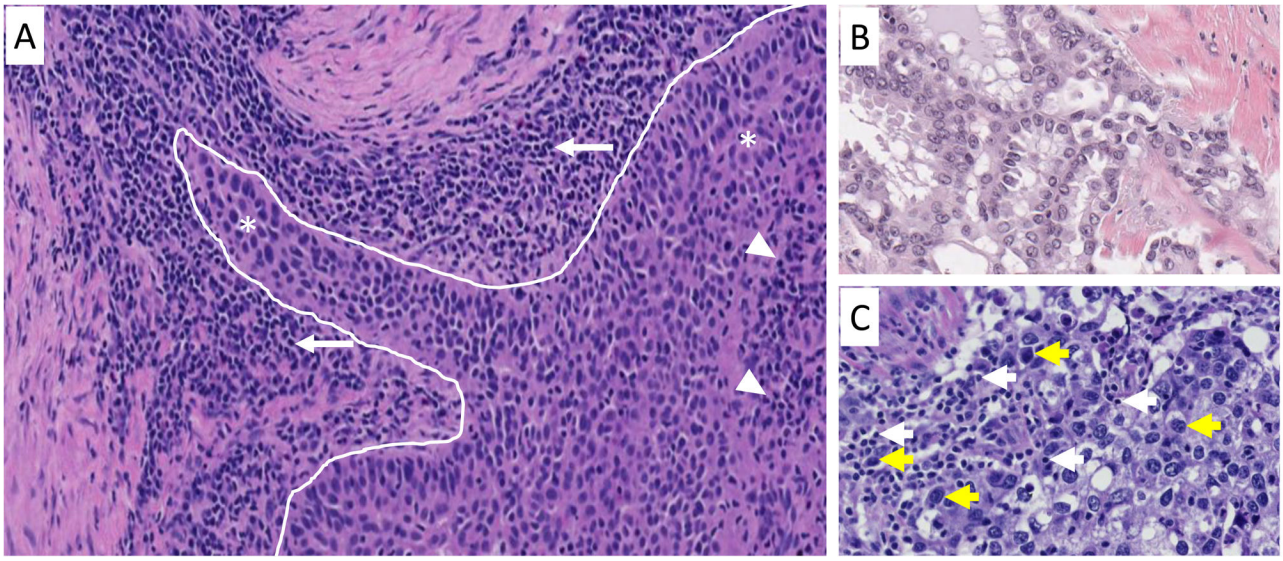

Figure 1. Photomicrograph showing the geographic assessment of lymphocyte infiltrates in the tumor microenvironment

(A) A dense population of small lymphocytes (white arrows) is present in the peritumoral stroma along the leading edge of the tumor (white line). Although fewer in number, tumor infiltrating lymphocytes (white arrowheads) are also intermixed among larger tumor cells (asterisks) more centrally within the tumor. **(B)** A non-inflamed tumor composed almost exclusively of large tumor cells without admixed lymphocytes. **(C)** An inflamed tumor with numerous small lymphocytes (white arrows) among the larger tumor cells (yellow arrows). Hematoxylin and eosin (H&E) stain, Original magnification 200x.

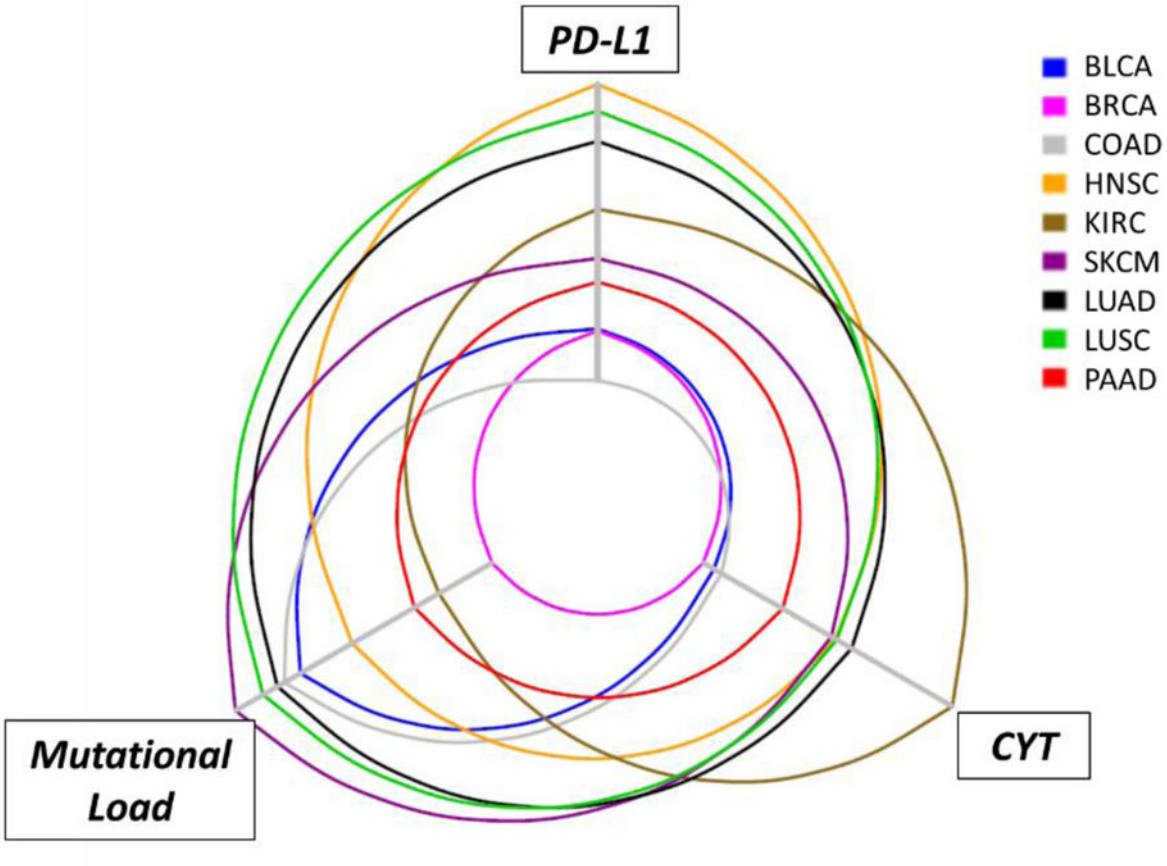

Figure 2. Hive plot for demonstrating the relationship between *PD-L1 expression*, *CYT* (cytotoxic gene signature expression), and mutational load in multiple common solid tumor types

Comparisons of *PD-L1* and *CYT* expression levels amongst different tumor types have been challenging due to the diverse assays used across studies. TCGA public datasets were used to determine the relative expression levels of PD-L1 and *CYT* (combined *GZMA_PRF1* transcript levels, indicating cytolytic effector activity[56]). for nine solid tumor types (SKCM, melanoma; KIRC, renal cell carcinoma; LUSC, squamous cell carcinoma of the lung; LUAD, lung adenocarcinoma; COAD, colonic adenocarcinoma; HNSC, head and neck squamous cell carcinoma; PAAD, pancreatic adenocarcinoma; BRCA, breast carcinoma; BLCA, bladder carcinoma). Each axis increases in value as it extends from the center of the plot. The highest levels of median *PD-L1* expression are seen in the tumor types with squamous differentiation, consistent with the known constitutive expression in these tumor types. Notably, *PD-L1* expression levels and *CYT* track each other closely, while mutational density tends to be less aligned.

Table 1

Companion/complementary PD-L1 IHC diagnostics by tumor type

Many of the scoring parameters have the potential to change as number of patients examined and disease indications expand.

	Ab	Melanoma	NSCLC	Urothelial	HNSCC	Gastric
Anti-PD-1						
Nivolumab (BMS)	28-8 Dako	1% (TC)	1%, 5%, 10% (TC)	1% (TC)	1% (TC)	
Pembrolizumab[a] (Merck)	22C3 Dako		1%[b], 50%[c] (TC)			1% (TC/IC)
Anti-PD-L1						
Durvalumab (AstraZeneca)	SP263 Ventana			25% (TC/IC)		
Atezolizumab (Roche/Genentech)	SP142 Ventana		50% (TC)/10% (IC)	5% (IC)		

[a] FDA approved use requires PD-L1 positivity using the indicated assay and thresholds

[b] Second-line

[c] First-line

Abbreviations: (Ab) antibody; (NSCLC) non-small cell lung cancer; (HNSCC) head and neck squamous cell carcinoma; (TC) tumor cell PD-L1; (IC) immune cell PD-L1.

Table 2

Patients whose tumors are PD-L1+ and who do not respond to anti-PD-(L)1 as well as patients whose tumors are PD-L1- and who do respond may be attributable to numerous factors potentially limiting the sensitivity and specify of the available IHC assays.

PD-L1(+) non-responders	**PD-L1(−) responders**
Assay performance and threshold for positivity	Assay performance and threshold for positivity
Exclusive constitutive PD-L1 expression	Exclusive immune cell PD-L1 expression
Spatial and temporal expression heterogeneity	Spatial and temporal expression heterogeneity
Limited sampling	Limited sampling
Alternate PD-1/PD-L1 ligand/receptor activity	Alternate PD-1/PD-L1 ligand/receptor activity
Other inhibitory checkpoints/mechanisms	