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To cite this article: Christopher Ung & Mark M. Kockx (2016) Challenges & Perspectives of Immunotherapy Biomarkers & The HistoOncoImmune™ Methodology, Expert Review of Precision Medicine and Drug Development, 1:1, 9-24

To link to this article: http://dx.doi.org/10.1080/23808993.2016.1140005
Challenges & Perspectives of Immunotherapy Biomarkers & The HistoOncoImmune™ Methodology

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ABSTRACT
The confluence of the immunology and oncology tributaries has precipitated a biomarker turbulence that is novel to the cancer community. On the heels of reversing the dismal outcomes for numerous patients suffering from terminal disease, it has also left behind deep pools of biomarker questions – which ones, when to use them, how to use them and what cells express them – to name just a few. The tumor microenvironment, an arena potentially housing a complex mix of immune infiltrators in the midst of tumor cells, is a wildly heterogeneous terrain, one that severely challenges our single biomarker model. This article examines the recently FDA-approved biomarker assays for PD-1 therapeutics, considers the technical shortcomings of the single biomarker model, scans alternate biomarker technologies and proposes a model that may allow investigators a method to systematically address these complexities.

ARTICLE HISTORY
Received 18 November 2015
Accepted 6 January 2016
Published online 9 February 2016

KEYWORDS
HistoOncoImmune; immunotherapy; biomarkers; PD-L1; diagnostics; inflamed; non-inflamed; infiltrating T-cells; immuno-oncology

Introduction
For an increasing subset of metastatic non-small cell lung cancer (NSCLC) patients, checkpoint immunotherapy – a completely new mode of treatment – provides invaluable options. Our recent ability to nudge the immune system to actively mount a defense against cancer cells has created options for physicians and patients in desperate situations. In October 2015 alone, the US FDA rendered approval for two immune-oncology therapeutics that target the PD-1 receptors on T cells, doing so in accelerated cadence and providing a near photo-finish drama. On 2 October, pembrolizumab (Keytruda™) earned the coveted position of the first checkpoint immunotherapy to be approved for metastatic NSCLC patients whose disease has progressed following other treatments and whose tumors express a protein called programmed death ligand 1 (PD-L1) [1]. Exactly a week later, nivolumab (Opdivo™) also earned the right from the FDA to be used with the same patient group [2].

This October flurry of regulatory approvals has the promising likelihood of continuing in multiple tumor types as investigators and drug development companies begin to unveil the potential of immune-oncology drugs in other cancers. Previously, the FDA had already deemed pembrolizumab [3] and nivolumab [4] to be safe and beneficial for patients with advanced melanoma as single agents and even in combination with other immuno-oncology therapeutics [5]. Nonetheless, the recent approvals in NSCLC are significant since both are associated with PD-L1 biomarker assessment.

An emerging dilemma
Many studies across a diversity of tumor types identified a relationship between the pretreatment expression level of PD-L1 in the tumor microenvironment and the likelihood of response to single-agent PD-1 pathway inhibitor therapy [6]. Taube et al. conducted an enlightening literature meta-analysis of solid tumors where they observed an increased response to checkpoint therapy when PD-L1 was expressed [7]. This promising observation was rapidly tempered by numerous questions as studies emerged with conflicting results whether PD-L1 served as a predictive biomarker [8].

The simultaneous FDA approval of two similar but distinct biomarkers tests acknowledges the potential role of PD-L1 as a predictive biomarker while triggering the practical challenges of using these tools. It is instructive to trace the array of questions that lurk at the diagnostic–therapeutic interface in light of the regulatory endorsement of these novel diagnostics. The FDA approvals of pembrolizumab and nivolumab arrive with different diagnostic guidelines for use despite the similarity of the intent to treat population group. Pembrolizumab, for instance, requires the use of a
companion diagnostic test, the PD-L1 immunohistochemistry (IHC) 22C3 pharmDx test; ostensibly the first FDA-approved test to detect PD-L1 expression in non-small cell lung tumors. The approval was based on data from the phase I KEYNOTE-001 trial, in which the overall response rate (ORR) with the drug was 41% among a subgroup of 61 patients with pretreated PD-L1-positive advanced NSCLC as determined by the 22C3 pharmDx diagnostic test [9].

Nivolumab, on the other hand, is free of such a constraint. The decision to prescribe nivolumab to advanced NSCLC patients remains with the physician. Nonetheless, Richard Padzur, M.D. Director of the Office of Hematology and Oncology in the FDA’s Center for Drug Evaluation and Research, acknowledged the importance of PD-L1 as a biomarker in the approval announcement, ‘While Opdivo showed an overall survival benefit in certain non-small cell lung cancer patients, it appears that higher expression of PD-L1 in a patient’s tumor predicts those most likely to benefit’ [2]. Further, the FDA acknowledged that the level of PD-L1 IHC expression may help identify patients who are more likely to live longer due to treatment with nivolumab by approving a corresponding test. Thus, PD-L1 IHC 28-8 pharmDx test arrives with a ‘complementary diagnostic’ designation, a test capable of helping physicians determine which patients may benefit most from treatment with nivolumab, but notably is not a required test [10].

A physician who is considering the use of pembrolizumab or nivolumab for NSCLC patients would need to ponder several use considerations of their respectively approved PD-L1 IHC biomarker, both of which are inconveniently distinct from each other. Pembrolizumab use requires the use of a specific IHC PD-L1 test while the nivolumab use associates possible benefits of its IHC PD-L1 test. The intrigue of this decision admixture deepens since it is the same manufacturer, employing a similar technology, who makes both the pembrolizumab companion diagnostic test and the nivolumab complementary diagnostic test.

The near term NSCLC checkpoint therapy landscape promises to complicate these decisions further. Atezolizumab and durvalumab, both of which are investigational agents that target PD-L1, the ligand partner of PD-1, are also likely to be tethered to individually specified PD-L1 IHC tests that are not only different from each other, but also the ones associated with their recently approved PD-1 inhibitor counterparts. The entry of institutional cost and logistics concerns will certainly convolute the decision-making matrix for the physician and pathologist. While the physician suffers from ‘PD-L1 test confusion,’ the laboratory pathologist is susceptible to the confusion of which PD-1 or PD-L1 checkpoint therapy is being considered. The pathology laboratory will need to grapple with the cost of running multiple tests, wrestle with the ethics of using up patient material for the multiple tests, and payers may resist compensating for non-FDA-approved tests. Historical precedence does not offer much relief to the confusion, since the survival of approved similar companion diagnostics for the same population such as for BRAF mutations in melanoma is questionable [11]. If the tandem of diagnostics associated with the PD-1 inhibitors of pembrolizumab and nivolumab has potentiated companion diagnostics turbulence, the arrival of a second pair with upcoming PD-L1 inhibitors will ensure that all keenly feel the turbulence.

### Ongoing biomarker complexities in immunology drug development

Notwithstanding the FDA’s acceptance of PD-L1 diagnostics in the NSCLC indication, the debate regarding PD-L1 as a biomarker continues to proliferate in an ever-expanding matrix of issues. While physicians now possess tools to assess PD-L1 expression in the NSCLC setting, drug developers and diagnostic companies still need to overcome a long checklist of challenges, many of which still confound the medical community precipitated by HercepTest™, which began the stream of FDA-approved targeted diagnostics [12]. Table 1 summarizes these challenges. It is instructive to review these expressed challenges as we embark on the quest to refine a biomarker model for immunology drug development.

<table>
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<th>Table 1. Current impact and challenges of PD-L1 IHC testing.</th>
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<td><strong>Challenge</strong></td>
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Multiple methods and primary antibodies

The results seen in the literature associating PD-L1 expression with immunotherapeutic outcomes were obtained with multiple different IHC assays. Multiple unresolved issues confound these multiple assays. Different antibodies, different staining protocols, different target cell assessment (tumor cells, tumor-infiltrating immune cells, or both), different scoring methods (percentage of staining cells, IHC 1–3), and different thresholds for defining a positive test result, all contribute to a rather unstable PD-L1 landscape [13]. Therefore, each assay is likely to exhibit substantially different performance but without any arbitration to determine its biological relevance or clinical utility. We have recently compared several PD-L1 clones using an automated, standardized, SOP-driven protocol and demonstrated rather interesting observations [14]. For instance, the E13LN clone from Cell Signaling Technologies demonstrates a greater sensitivity on melanoma tumor cells compared to the SP142 clone from Spring Bioscience. This feature highlights potential dilemmas since tumor cell expression of PD-L1 IHC is a prominent readout for checkpoint immunotherapy patient selection.

Multiple cutoffs

The different assays employ different scoring methods and unique thresholds to define a positive result. Table 2, restricted only to NSCLC-related PD-L1 IHC cutoffs, provides a hint of the impending complexity as the immunotherapy agents for other tumor types make their way into clinical settings.

Pre-analytic conditions

The powerful and valuable morphological information from IHC is tempered by the requirement to be keenly aware of the careful and meticulous considerations of analytical and pre-analytical conditions that are necessary to ensure relevant and reproducible outputs. While diagnostic manufacturers may remove many analytical variables through their manufacturing and automated platforms, the conditions prior to analysis remain out of their reach. Lung cancer biopsies are on average very small which render them particularly vulnerable to these pre-analytical conditions. The type of fixative, length of fixation times, the amount of time it takes from surgery before the specimen is placed into fixative, cold ischemia time, and a variety of other issues affect the output of IHC [20]. Since checkpoint therapies such as nivolumab and pembrolizumab are likely to secure pillar therapy roles, and therefore we can expect PD-L1 IHC testing to increase in prominence and frequency, pathologists and physicians will need to be aware of and account for the influence of pre-analytic conditions on their workflow and subsequent patient management.

Multiple cell types can express PD-L1

PD-L1 can be expressed in multiple cell types, such as tumor cells, macrophages, and lymphocytes [21,22]. Figure 1 shows examples of PD-L1 IHC staining on tumor and immune cells from our laboratory. The clinical relevance of PD-L1 expression on the different cell types remains a topic for further investigation. Assessment of PD-L1 on tumor cells has dominated most of the studies, as is the case for the recently approved companion diagnostic test for pembrolizumab and the complementary diagnostic test for nivolumab. However, the assessment of PD-L1 is likely to expand to tumor-infiltrating immune cells since the atezolizumab clinical trials are studying patients based on their PD-L1 IHC expression on both tumor cells (TC) and immune cells (IC). Using the SP142 antibody, the expression of PD-L1 IHC in the atezolizumab NSCLC studies was captured using a dual scale of intensity, from low expression (TC0/IC0) to high expression (TC3/IC3). Using this combined immune-tumor cell PD-L1 expression readout, Spira et al. concluded that the efficacy of atezolizumab increased with higher PD-L1 expression in the POPLAR trial [23]. This observation appears relevant in other tumor types, as studied by Herbst and colleagues [24]. While evaluating the efficacy of atezolizumab, he found that of the 175 efficacy-evaluable patients, confirmed objective responses were observed in 32 of 175 (18%), 11 of 53 (21%), 11 of 43

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<th>Table 2. Cutoff values of PD-L1 IHC assays associated with PD-1 and PD-L1 checkpoint agents for treatment of NSCLC [15].</th>
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<td><strong>PD-L1 IHC antibody</strong></td>
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(26%), 7 of 56 (13%), and 3 of 23 (13%) of patients with all tumor types, NSCLC, melanoma, renal cell carcinoma, and other tumors (including colorectal cancer, gastric cancer, and head and neck squamous cell carcinoma).

**PD-L1 IHC is dynamically heterogeneous**

The sophisticated and dynamic biology of PD-L1 IHC expresses itself through significant variation by tumor type across disease sites making it one of the more complicated biomarkers to guide potential clinical utility. Multiple characteristics contribute to the heterogeneity of PD-L1 expression. There are inter- and intratumoral differences [25], it is inducible and dynamic [26], it expresses on membrane and in the cytoplasm and, as described above, its expressions vary by cell types.

Taube and colleagues illustrate this variability across tumor types, within the tumor itself, between the primary and metastatic sites, and between cell types [27]. The PD-L1 expression heterogeneity may silently influence downstream patient management, potentially in the form of false-negative results that result from a sample that has not included the area of the tumor that expresses PD-L1. Conceivably, because of expression heterogeneity, the biopsy of one lesion may not be predictive of an immune response ongoing at another site, offering a possible explanation for responding patients who test negative for PD-L1.

We participated in a study that investigated the PD-L1 expression heterogeneity of cell types, which revealed two distinct NSCLC subtypes, based on PD-L1 expression on immune versus tumor cells. Strikingly, the tumors with high PD-L1 expressing tumor cells

![Figure 1. PD-L1 IHC staining on non-small cell lung cancer patient specimens. (A) Low density of immune cell infiltrates expressing PD-L1. (B) Moderate density of immune cell infiltrates expressing PD-L1. (C) High density of immune cell infiltrates expressing PD-L1. (D) Moderate density of immune cell infiltrates and tumor cells expressing PD-L1. (E) Magnification of boxed area in panel C. Dense infiltrate of PD-L1 positive immune cells (arrowheads), both in the carcinoma as the stromal tissue. Other cells in the stroma (mostly B-cells) as well as the tumor cells in this patient (arrows) are PD-L1 negative. (F) Magnification of boxed area in panel D. Tumor cells are positive for membranous PD-L1 staining (arrows). In this patient, the immune cells are also PD-L1 positive (arrowheads), both the dispersed cells and immune cell aggregates (white asterisk). Scale bar: A-D = 200µm, E-F = 100µm.](image-url)
and the tumors with high PD-L1 expressing immune cells represented two distinct populations, with minimal overlap. Further examination revealed the dramatic histopathology and molecular differences for each subtype such as the positioning of infiltrating immune cells and the morphologic makeup of the tumor microenvironment [28].

**Other biomarkers may prove useful**

Numerous biomarker hypotheses focus on identifying responding populations and to deciphering the mechanisms of action of responding patients who are biomarker negative. A third investigational group introduces another round of exploratory biomarker hypotheses and analyses – the patient group who do not respond to immunotherapies. These non-responding individuals may benefit from other treatment strategies that include combinations with other immunotherapies, targeted therapies, and even chemotherapies. The search for biomarkers for all three patient groups is certainly daunting but nonetheless intriguing possibilities exist.

Le et al. helmed an interesting preliminary study, indicating microsatellite instability measurement as a biomarker for gauging the response to immunotherapy in colorectal cancer patients. In their small study, the cohort of patients with mismatch-repair deficient colorectal cancers exhibited an immune-related objective response rate of 40%. The corresponding response rate for the patient cohort with mismatch-repair proficient colorectal cancers was 0% [29]. Llosa et al. have documented similar observations showing vigorous checkpoint activity in the immune microenvironment of microsatellite instable colon cancers [30].

Mutational load is emerging as a crude global biomarker for checkpoint therapy benefit fueled by a growing acceptance that tumors with high mutational load respond vigorously to checkpoint therapy, irrespective of PD-L1 expression. Highly mutated tumors with 1% or even nominal PD-L1 expression would respond almost universally to the checkpoint inhibitors [31]. However, Rizvi also points out a group of patients with highly mutated lung cancers whose tumors were PD-L1 negative who failed to respond to checkpoint inhibitors. Thus, while mutational frequency serves as a ‘global’ measure across the tumors, in instances where PD-L1 IHC expression with <1% of cells in the tumor microenvironment, it may still serve as a biomarker for non-response to checkpoint blockade when it is seen in a spatial relationship with host immune cells, thus preparing both physician and patient for the potential need of additional countermeasures.

Several studies have linked the presence of tumor infiltrating immune cells to prognostic and predictive benefit [21]. Clinical trials with PD-L1 and PD-1 blockade suggested that tumors with a high number of inflammation-causing T cells were more responsive to the immunotherapy-based PD-L1 and PD-1 inhibitors. Tumors with low inflammation, or low numbers of T cells, were less responsive, highlighting the potential role of cytotoxic T-cell biomarkers such as CD8 [32].

This in turn has stimulated the identification of molecular factors that inhibit immune infiltration into tumors. Gajewski provides an intriguing description of the T-cell inflamed versus non-inflamed solid tumor, where the former is an attempt by the host to organize an antitumor response while the latter often has the hallmark of not benefiting from checkpoint therapies [33]. Thus, unpacking the molecular detail of both inflamed versus non-inflamed tumors may provide clues to checkpoint-relevant interrogative biomarkers. For example, Gajewski spotlighted beta-catenin as a factor that may distinguish immune inflamed versus non-inflamed melanomas [34]. This premise also raises the intriguing role of beta-catenin to stimulate shuttling of cytotoxic T cells into the tumor region. Interferon-gamma (IFN-γ) may provide additional definition to this inflamed phenotype. In a small but interesting study, Ribas used a gene expression signature centered on IFN-γ to show ORR and progression-free survival associations for melanoma patients treated with pembrolizumab [35]. Seiwert provided similar conclusions for head and neck cancer [36]. Both investigators relied on a combination of (1) ‘IFN-γ’, (2) ‘TCR Signaling’, and (3) ‘expanded immune’ gene expression signatures.

Epigenetic biomarkers may play an important role with the recent understanding that selective epigenetic reprogramming may increase immunotherapy responses. A team from the University of Michigan reports that the invasion of the tumor microenvironment by T cells is impeded by selective gene methylation and concludes that epigenetic reprogramming may condition tumors from poor T-cell infiltration to rich T-cell infiltration, and ultimately potentiate cancer therapy [32].

Additional biomarkers, many of them available as IHC assays, continue to emerge and are worth monitoring. The CTLA-4, TIM3, and LAG3 receptors are part of the complex set of negative T-cell regulatory switches and provide varying levels of response information depending on tumor types and therapeutic confrontation. Investigational inhibitors against these checkpoint receptors are advancing in the clinic, generating compelling biomarker data that provide further mechanistic information to predict response and resistance [26].
Programmed death ligand 2 (PD-L2), a known ligand of PD-1, has begun to attract investigational scrutiny, often in concert with its better-known counterpart, PD-L1. The recent observations from Yearley, that PD-L2 expression may be discordant to PD-L1, and that PD-L1-negative patients who respond to PD-1 inhibitors may have an active PD-L2 pathway, provide glimpses of the potential role of PD-L2 in immunotherapy [37]. Previously, Herbst and colleagues also showed that NSCLC patients who have high PD-L2 expression responded to atezolizumab [24].

The multifactorial future
The two recently regulatory-approved PD-L1 IHC tests will sculpt their roles as companion and complementary diagnostic for their respective PD-1 inhibitors, providing clinicians and NSCLC patients with a level of assurance where positive expressers have a strong probability of response. As investigational agents atezolizumab and durvalumab enter the market with their respective diagnostics, the questions will increase and only additional studies will provide guidance on how and when to use these diagnostics. The FDA convened a public workshop to surface these issues and has mandated a comparison of the different PD-L1 diagnostics [38].

Meanwhile, as the transition from chemotherapy to molecular therapeutics, and now to immune therapeutics, continues to develop, investigators will launch multifaceted studies to identify biomarkers with greater response or resistance resolution to single or multi-agent therapy regimens. Many concede that it is impossible for a single biomarker to represent the complex and dynamic nature of the human immune system, much less when confronted in tandem with the tumor microenvironment. Peters et al. express an increasingly valid perspective where ‘the complexity of immune surveillance and escape might prevent us from identifying a simple and unique predictive biomarker’ [39]. The emerging model is to review multiple immune checkpoints and other factors within the tumor that may be contributing to immune exclusion or susceptibility to immune attack. By getting a multifactorial assessment of what is happening within the tumor, investigators should be better able to derive indicators to rationally deliver individualized therapy, either monotherapy or combinatorial immunotherapy.

We propose a systematic application of a methodology that harnesses histopathology data of the tumor microenvironment with its corresponding molecular signatures. The activity of infiltrating immune cells into the tumor microenvironment is effectively captured through spatial and pictorial representations that viscerally inform of the immune activity. A molecular profile meanwhile provides a multiplex picture, charting out genes that are associated with such activity, often unveiling underlying networks of signaling protein activity. This ‘histoinformatics’ approach, the integration of histopathology and molecular mapping of the immune tumor microenvironment when applied in a systematic way, provides investigators a method to understand the tumor microenvironment activity and its interface with the immune system.

The HistoOncoImmune™ system
We present a methodology that attempts to encompass these elements, employing the most relevant technology to systematically capture multiple points of dynamic biomarker dynamic data across a heterogeneous terrain. There are several guiding principles underlying the design of this system (Figure 2):

- This methodology represents an organized platform that is dynamically adapted to optimize the biomarker(s) selection for the investigational immune-oncology agent.
- The selected biomarkers are rigorously validated, SOP-encapsulated and deployed, operationally scalable and can be deployed across multiple clinical trials of various sizes and geographical locations.
- The output (HistoMine™) provides a representation of histopathology and molecular data. Histopathology evaluations and scores, high-resolution whole slide images, and tissue patterns are reported in conjunction with numerical gene expression and next generation sequencing (NGS) data.

The collection of biomarkers is organized in two groups: Category 1 serves as a histopathology-centered baseline panel that can be used across multiple trials to describe the patient’s immune-tumor condition, while Category 2 provides supporting molecular data.

Category 1: description
In this histopathology driven mode, a qualified pathologist first assesses the patient’s H&E stained tumor slides, confirming tumor presence and diagnoses, reviewing for tissue and staining quality, selecting regions of interest for high definition image analyses, and identifying extraction areas that are subsequently used for the molecular analyses in Category 2 testing. This H&E assessment is a critical step and anchors all HistoOncoImmune™ biomarker activity. The data output from Category 1 consists of high-resolution images captured from microscope slides, morphological
description of the immune and tumor activity, and semi-quantitative measurements of targets of interest.

The Category 1 biomarkers provide a panoramic survey of the immune activity of the tumor microenvironment (Figure 3). These immunostains identify the presence and location of the relevant immune-oncology target (e.g. PD-L1), cytotoxic T cells (CD8), tumor-associated macrophages (CD163), immune regulatory cells (FOXP3/CD3), and B cells (CD20).

Intentionally, regulatory-approved or the most widely adopted versions of Category 1 biomarkers are used for analyses to increase the likelihood that data across multiple clinical trials can be compared and correlated. Furthermore, the validation of these biomarker assays includes an accuracy assessment, assay linearity, assay precision across users and platforms, and stability review of the target antigens and assay reagents [40,41]. Additional experiments are also conducted to understand and refine assay, tissue, or antigen parameters (e.g. heterogeneous expression, antigen stability, fixation sensitivities, etc.)

Further, we employ quantitative imaging techniques and algorithms that enable us to systematically capture immunostaining values that can be reported for regions of interest from single cells to disease compartments. The CD8 biomarker is used for illustration.

Following pathology review and CD8 immunostaining on an automated IHC stainer, imaging scientists define the pathologist-selected area into three possible compartments (Figure 4A): the tumor center (Cn), the periphery of the tumor (Pe), and the invasive margin (IM). A selection of pixels, rather than the entire pixel set, is analyzed within each component in order to enable analysis in a timely way and to work with a data file that is manageable (Figure 4B). We accomplish this by directing the software to select a random number of pixels, optimized and validated such that incremental pixels are no longer informative. The ratio of stained area versus total pixel area is captured for each pixel, essentially providing macro-immunostaining values for each component that can be used for regression and correlation across studies. This immunostaining value is available for all three compartments and can be analyzed at various levels, providing granular information to understand biological phenomena such as heterogeneity, simply by selecting the appropriate region of interest, ostensibly even at the single cell level. The relative area ratio was selected as a metric over cell counts since the deconvolution of the latter becomes exceedingly complex in certain situations such as the heavy clustering of cells.
Figure 4C illustrates the immunostaining values that are available by the relevant melanoma compartments. Thus, the immunostaining values for tumor centers, tumor periphery, and invasive margins can be compared at several levels, pre and post dose, patients within a study and tumors across multiple studies. These values are retained as metadata associated with the image and can be retrieved as needed to provide the investigator with contextual morphology.

The morphological power of Category 1 biomarkers is tempered by the requirement that each biomarker requires a single slide. (The authors are evaluating technologies that leverage the ability to align serially stained slides to create a virtual multiplex slide.) Neither does it reveal the joint complexity of immune and tumor signaling activity that lies behind the ‘sign-post’ Category 1 biomarkers. The Category 2 biomarkers assume these responsibilities.

Figure 4C. The Category 1 panel of the HistoOncoImmune™ system is a baseline panel consisting of regulatory-approved or the most widely adopted histopathology markers to increase the likelihood that data across multiple clinical trials can be compared and correlated. It starts with the H&E assessment by a trained pathologist, followed by a selected panel of markers that determine the immune activity of the tumor microenvironment. These immunostains identify the presence and location of the relevant immune-oncology target (e.g. PD-L1), cytotoxic T-cells (CD8), tumor-associated macrophages (CD163), immune regulatory cells (FOXP3/CD3) and B-cells (CD20).

Category 2: description

The biomarkers in this category correlate the signals obtained from the Category 1 grouping and also provide supplemental pathway and actionable guidance for drug development and patient selection (Figure 5). Importantly, the technologies espoused in this category use a minimum of patient material but elicit a large set of data.

We leverage the recent availability of platforms that enable the simultaneous mapping of genes in formalin-fixed, paraffin-embedded materials to assess the gene expression activity within a patient’s tumor microenvironment as indicators of tumor proliferation, susceptibility of the disease to an investigational agent, the tumor’s pathway addiction, and many other mechanistic reveals.

HistoOncoImmune™ incorporates the nCounter® PanCancer Immune Profiling Panel (Nanostring Technologies®) as its tool to document the human immune response of various tumor types [42]. This 770 gene panel combines markers for 24 different immune cell types and populations, 30 common cancer antigens and genes that represent all categories of immune response including key checkpoint blockade genes. As discussed earlier, the assessment of PD-L1 expression on tumor cells versus immune cells, or in tandem, remains an issue requiring further investigation. One limitation encountered with the nCounter platform is its inability to differentiate between the PD-L1 gene expressions on tumor versus immune...
Figure 4. Pathology evaluation of a melanoma resection and subsequent tri-compartmentalization of the image for CD8 analyses. (A) A qualified pathologist first assesses the patient’s H&E stained tumor slides, confirming tumor presence and diagnoses, reviewing for tissue and staining quality, selecting regions of interest for high definition image analyses. Following CD8 immunostaining on an automated IHC stainer, imaging scientists define pathologist-selected areas into 3 possible compartments: the tumor center (Cn), tumor periphery (Pe) and the invasive margin (IM). (B) Using imaging analysis software a selection of pixels, rather than the entire image is analyzed. The software is designed to select a random number of pixels, optimized and validated, such that incremental pixels are no longer informative. The ratio of stained area versus total pixel area is captured for each pixel. In this example 1,170 pixel tiles were analyzed and tile examples are shown to the right. (C) Example of immunostaining values that are measured in the relevant melanoma compartments. Cn, the tumor center; Pe, tumor periphery; IM, invasive margin. Scale bar = 5000µm.
As an aggregate, we observe that the PD-L1 gene expression obtained via the nCounter platform correlates exceedingly well with our validated PD-L1 IHC assays producing robust $R^2$ values (Figure 6, Schats et al., unpublished data). Furthermore, we are encouraged by an equally strong correlation with other orthogonal platforms such as real-time PCR and mRNA in situ hybridization.

Interestingly, there is an unraveling of this correlate when only the PD-L1 gene expression of immune cells was compared to the IHC analogs, a demonstration of the need for combinatorial techniques to unpack the participating mechanisms of action. Further, while it is tempting to rely on a platform such as nCounter, which offers numerical and operational simplicity compared to its IHC analog, there needs to be an in-depth understanding of...
the frequency of discrepant cases between the platforms and their significance. The relevance of high PD-L1 gene expression with concomitant low PD-L1 immune cell expression cases remains to be understood in terms of its frequency and relevance to PD-1/PD-L1 checkpoint therapy. The integrated analyses captured via the HistoOncoImmune™ methodology allows for systematic review of such cases and creating further hypotheses for clinical trials.

The targeted NGS component of HistoOncoImmune™ system acknowledges the need to identify actionable mutations while allowing for, and detecting, emerging or evolving resistance mutations. The diagnostic or clinical mode of this NGS panel has its premise on an SNP panel (26 genes, 200+ amplicons) compiled by the French Institut National du Cancer who compared and analyzed several globally well-regarded panels that guide the identification of driver and resistance mutations for multiple tumor entities [43]. This panel combines a proprietary multiplex PCR technology with subsequent NGS testing [44]. The investigational mode of our NGS panel, meanwhile, will be used to detect indels, employing a combination of split-read and paired-end approach to identify smaller and larger variants, respectively.

Combined, the nCounter platform, targeted NGS, and indel NGS provides a molecular panorama of the immune tumor environment using a standard set of panels to accommodate significant evolving and emerging resistance genes and mutations, a research mode admits the novel targets into a rigorous validation process, containing well-validated orthogonal methods. Once complete, these targets are then included into the gene expression codeset or the appropriate NGS panel.

A key step to molecular analysis especially for profiling human immune response to immunotherapeutics, such as checkpoint blockade, is the proper selection of tissue area for analysis. Critically, the selection of the region should include, where possible, the interface area between tumor and normal tissue since the infiltrating immune cells tend to congregate there. Improper selection may lead to misleading molecular data. On the one hand, a cursory extraction of the entire section without proper pathology selection would dilute active gene expression information by the inclusion of too much normal material. On the other hand, an overly enthusiastic of a tumor-only section would sacrifice the valuable information of immune entities gathered at the interface.

The final component of Category 2 testing accommodates characteristics specific to tumor types or particular immune features. As discussed earlier, microsatellite instability, other cancer testis agents, epigenetic events, certain oncogenic viruses, and other IHC biomarkers may provide data that bring focus to the molecular panorama of a complex terrain. We will also leverage the intriguing findings of Gajewski and colleagues, and incorporate markers such as beta-catenin IHC in concert with T-cell activity signatures such as IFN-γ and granzyme B to distinguish the relevance of inflamed and non-inflamed tumors [33]. Figure 7 demonstrates PD-L1 expression of an NSCLC

Figure 7. PD-L1 IHC staining of a NSCLC specimen showing distinguishing morphological features of homogenous, intense and complete membrane patterns on clustered tumor cells only and no immune cell expression. The stroma is fibrous without infiltration of immune cells. Scale bar = 100µm
specimen with distinguishing morphological features. Its PD-L1 expression has a high-intensity HER2-like homogeneity and is restricted only to tumor cells with no immune cell staining. In addition to the gene signatures mentioned above, as outcome data become available, we can begin to examine if other mechanisms such as PD-L1 amplification are associated with such staining patterns [45]. In all cases, these tests are rigorously validated for specificity, sensitivity, and reproducibility.

We have also begun validation exercises for examining relevant moieties in plasma, taking advantage of the vigorous innovations transpiring with the current interest in liquid biopsies. Nyens recently illustrated the potential utility of detecting circulating tumor DNA (ctDNA) as a monitoring tool for patients with advanced BRAFV600 mutant melanoma [46]. Significantly, their study albeit with a small patient subset highlights the superiority of the patient’s BRAFV600 mutant fraction as a predictor of proliferative tumor burden, rather than the patient’s tumor mass.

Conclusion

The range of information required to effectively select the best therapeutic combination for a patient has expanded enormously with the addition of immunology agents. Patient selection by single biomarkers in this setting is frustratingly inadequate as markedly demonstrated by IHC PD-L1 assessment which hints at patient selection strategies yet leaves many questions unanswered. Meanwhile, questions abound regarding the responding immune system and its interaction with the tumors. The additive effect of having to probe the immune system within the tumor microenvironment with multivariate biomarker analyses forces questions on several dimensions. First, what are the set of relevant biomarkers? Next, what platforms should we interrogate with? Finally, how do we integrate this information?

We designed the HistoOncoImmune™ system to systematically integrate each patient’s morphological and molecular information in global clinical trials that can subsequently be correlated to patient outcomes and be viewed according to tumor types. A mindful application of the HistoOncoImmune™ system that respectfully considers the scarcity of patient material is equally critical and we present a workflow we currently employ to mine the information with intelligent consideration to the patient’s biological material (Figure 8). Fundamentally, the HistoOncoImmune™ will enable the collection of data that lead to intelligently designed hypothesis generation with the adaptive ability to incorporate distinguishing biomarkers and extinguish non-yielding ones. Future reports will delineate the collection of such data and also review the system’s efficacy for patient selection.

Expert commentary & five-year view

Many of us in the pathology community have navigated the challenges that PD-L1 IHC has presented, acquiring lessons given to us from previous IHC companion diagnostics. The technical, standardization and interpretation issues mentioned in this review are ones we have encountered and debated extensively. We established standardization guidelines and as a result have also improved pathology laboratory systems and processes globally. One of the authors helmed the commercialization of HercepTest™ and observes the repeating themes, now being presented and debated with greater sophistication, for example, the early engagement of the FDA to create awareness of the different PD-L1 IHC tests through its town hall meetings and the ongoing comparison study. Notwithstanding these efforts, the pathology laboratory will again have to immerse themselves in numerous risk–benefit decisions that intertwine best and accurate patient care with costs and logistics decision factors, all while sifting through emerging streams of conflicting data. It is difficult to envision an average pathology laboratory committing to multiple automated staining platforms with multiple PD-L1 IHC tests on each. They will need to juggle multiple interpretation schemes, laboring from one to another depending on the tumor and intended drug of treatment in question. Many will clamor for standardization but will do so in the flavor that best suits their environment and needs, including the use of non-proprietary commercial PD-L1 IHC antibodies in a Laboratory-Developed Test format, an arguably unstandardized trend that competes vigorously with preceding companion diagnostics. Since these challenges are familiar, perhaps some of the familiar solutions might also be instructive. In the case of HercepTest™, laboratories responded positively to education and dialog. Perhaps as we sort out the similarities and differences of the regulatory-branded PD-L1 IHC diagnostics, some energy should be rationed for detailed pathology education of PD-L1 IHC expression in various tumor types. We have, as have others, begun to establish digital libraries of PD-L1 IHC in various tumor types as part of our HistoOncoImmune™ effort so that we can chart this inducible biomarker in various settings. IHC of immune cell activity has great potential value in patient selection and it too deserves
systematic cataloging and education. This acquired nuanced knowledge of PD-L1 and related expression markers, steadily accumulated by pathologists, may be the most practical and effective solution to enable therapy-guiding benefits and it will position the pathologist well for dialogs with oncologists who will also gain their own nuanced set of checkpoint therapy data.

Financial & competing interests disclosure

This work was supported by the Agency for Innovation by Science and Technology (IWT O&O grant 130894). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Key issues

- Cancer cells can escape the immune system by overexpressing immunosuppressive factors. The PD-1/PD-L1 checkpoint axis plays a key role herein. Expression of PD-L1 on the tumor cells protects the tumor cells from cytotoxic CD8+ T cells.
- The development of checkpoint blocking antibodies, such as those directed against programmed death-ligand 1 (PD-L1) or its receptor PD-1, has demonstrated significant recent promise in the treatment of an expanding list of malignancies.
- Anti-PD-1 immunotherapeutics nivolumab (Opdivo™) and pembrolizumab (Keytruda™) received FDA approval for the treatment of advanced melanoma and metastatic NSCLC patients whose disease has progressed after other treatments and whose tumors express PD-L1. Anti-PD-L1 agents atezolizumab and durvalumab are under intense investigation.
- The FDA approval of two similar but distinct PD-L1 IHC tests (22C3 pharmDx and 28-8 pharmDx) acknowledges the potential of PD-L1 as a predictive biomarker and helps physicians decide which checkpoint therapy to prescribe. Unfortunately, not all patients respond to these therapies, and evaluation of biomarkers associated with clinical outcomes is crucial and ongoing.
- We propose a systematic application of a methodology that harnesses histopathology data of the tumor with its corresponding molecular signatures. The selected biomarkers are rigorously validated and can be implemented across multiple clinical trials of various sizes and geographical locations.
- The HistoOncoImmune™ system is the integration of histopathology and molecular technology and provides investigators and physicians a method to understand the tumor microenvironment activity and its interface with the immune system. It offers a methodology to attain a biomarker profile that predicts for response or resistance to checkpoint therapy.
References

Papers of special note have been highlighted as:
• of interest
○ of considerable interest


• Meta-analysis of 20 trials (1475 patients) reporting PD-L1 expression and overall response rate to checkpoint inhibitor therapy, showing benefit even in the absence of PD-L1 expression.


• KEYNOTE-001 clinical trial in which the efficacy and safety of pembrolizumab in patients with advanced NSCLC was assessed.


• Review of the early clinical experience and development of PD-L1 immunohistochemistry (IHC) as a predictive biomarker for anti-PD-1 directed therapy in human cancer.

14. Schats K, Van Vreë E, De Schepper S, et al. PD-L1 IHC validation and comparison of E1L3N & SP142 antibodies in melanoma. Poster session at the meeting of the European Society for Medical Oncology; 2015; Lausanne, Switzerland.


• Pre-existing CD8 T cells distinctly located at the invasive tumor margin (melanoma) are associated with expression of the PD-1/PD-L1 immune inhibitory axis and may predict response to anti-PD1 therapy.


• These data suggest that azetolizumab is most effective in patients with high PD-L1 expression PD-L1 on tumor-infiltrating immune cells.


• Demonstrates that the mismatch-repair efficiency is predictive for clinical benefit of immune checkpoint blockade with pembrolizumab in colorectal cancer patients.


• Excellent paper in which the authors performed whole-exome sequencing of non-small cell lung cancers treated with pembrolizumab, and showed that higher nonsynonymous mutation burden in tumors was associated with improved objective response, durable clinical benefit, and progression-free survival.


• These data show that hypermethylation of distinct genes represses the tumor production of TH1-type chemokines and T-cell trafficking to the tumor microenvironment. Treatment with epigenetic modulators removes this repression and increases effector T-cell tumor infiltration, slows down tumor progression, and improves the therapeutic efficacy of PD-L1 inhibitors.


• Describes one of the next significant hurdles to develop new therapeutic interventions to be effective in patients with the non-T-cell-inflamed phenotype. Proposed is a detailed molecular understanding of the mechanisms that explain the presence or absence of the T-cell-inflamed tumor microenvironment, which in turn will benefit from focused interrogation of patient samples. It is envisioned that the end result of these investigations will be an expanded array of interventions that will broaden the fraction of patients benefiting from immunotherapies in the clinic.


• Initiation of activities to increase the understanding of the analytic performance of companion diagnostic assays to facilitate testing and decision-making in the clinic.


46. Schreuer M, Meersseman G, Jansen Y, et al. Quantitative assessment of BRAF V600 mutant cell-free tumor DNA from plasma as a diagnostic and therapeutic biomarker in pts with BRAF V600 mutant melanoma. Poster session at the meeting of the Association of Molecular Pathology; 2015; Austin, TX.