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Prognostic and predictive immunohistochemistry-based biomarkers in cancer and immunotherapy

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Introduction

Over the past decade, immunotherapy has revolutionized the treatment of cancer. Immunotherapies targeted to immune checkpoint molecules such as programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) have significantly improved the prognosis of patients with a variety of cancers.[1] The discovery of CTLA-4's function as an inhibitory molecule expressed on T cells in 1994[2,3] and the subsequent success of CTLA-4 checkpoint inhibition in clinical trials[4–8] led to the Food and Drug Administration's (FDA) approval of ipilimumab for the treatment of melanoma in 2011. More recently, inhibition of PD-1 and programmed death-ligand 1 (PD-L1) has been found to lead to durable tumor regression and prolonged disease stabilization in many types

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of solid tumors, including melanoma, non-small cell lung cancer (NSCLC), and renal-cell cancer.[9–11] Two PD-1 inhibitors (nivolumab and pembrolizumab) and three PD-L1 inhibitors (atezolizumab, durvalumab, and avelumab) have been approved by the FDA for the treatment of various cancers.[12] Finally, combined immune checkpoint blockade using nivolumab and ipilimumab has shown clinical efficacy in multiple cancer types.[13,14]

The use of immune checkpoint inhibitors must be closely monitored for severe immune-related adverse events,[15] as treatment with immune checkpoint blockade is associated with 10% to 20% grade 3 or 4 toxicities.[16] From a clinical perspective, then, the development of biomarkers to predict clinical response is critical to helping clinicians weigh the potential benefits of immunotherapy against its potential toxicities. Biomarkers may also accelerate the development of other immunotherapeutic therapies by identifying subpopulations in which these drugs would be most effective, thus allowing for clinical trial enrichment strategies.[17]

Biomarkers are biological indicators that can be subdivided into two categories: prognostic and predictive.[18] While a prognostic biomarker indicates a patient's disease outcome without treatment, a predictive biomarker indicates how a patient will respond to a given therapy and may itself be a target for therapy.[19] Thus, a prognostic biomarker may help identify patients whose disease is high risk and who would benefit from aggressive therapy; a predictive biomarker may help identify patients who will benefit from a specific therapy.

In order to be effective and practical in the clinic setting, a biomarker must be both specific and sensitive, but must also be easy to use and cost-effective.[20] Because it allows for assessment of the tumor immune microenvironment and is readily applied in the clinic, immunohistochemistry (IHC) has proven a powerful tool for the discovery and use of biomarkers.[21–23] However, many IHC-based biomarkers have struggled to reach the clinic for a variety of reasons, in particular due to challenges with validation or inaccuracy in predicting outcome.[24] A selection of key clinical and IHC-based biomarkers and the limitations of the use of IHC will be discussed further below.

Tumor-infiltrating lymphocytes

The tumor microenvironment (TME), composed of cell types including tumor-infiltrating lymphocytes (TILs),[25] is increasingly implicated in a bidirectional interplay with tumor cells capable of promoting or preventing tumor growth and invasion.[26] The recognition of such interactions has led to significant interest in TILs as a biomarker to both prognosticate disease outcomes and predict response to treatments such as immune checkpoint inhibitors. Early studies in primary melanoma identified the prognostic value of TILs using a classification of immune infiltrates as brisk, nonbrisk, or absent by conventional H&E staining.[27] A higher density of TILs has been associated with favorable clinical outcomes in various cancer types, including breast cancer and melanoma.[28,29] Immunohistochemistry has yielded further insight into the phenotypic characterization of these immune infiltrates. As melanocytic lesions progress from benign nevi to cutaneous malignant melanomas, the absolute number of TILs increases with a relative increase in the numbers of CD3+TIA-1+ resting cytotoxic T lymphocytes (CTLs) as compared to CD20+ B lymphocytes.[30]

In addition to its role as a prognostic biomarker, the density of TILs has also been described as a biomarker predictive of response to treatment. A 2014 study found that in HER2+ breast cancer patients treated with adjuvant trastuzumab, increased levels of TILs were correlated with decreased distant recurrence relative to patients receiving chemotherapy only.[31] Another study in patients with breast cancer found that density of intratumoral lymphocytes as well as protein expression of CD3, CD20, and CXCR3 are significantly associated with pathologic complete response to neoadjuvant chemotherapy.[32] Further, in melanoma, CD4+ and CD8+ lymphocyte infiltration predicts a statistically significant favorable response to anti-PD-1 immunotherapy, underscoring the role of IHC in predicting both disease outcomes and responses to therapy.[33]

CD8+ TILs and the Immunoscore

The density of CD8+ T cells in the tumor has been proposed as a more precise alternative to the density of TILs.[34–36] In breast cancer, the use of IHC has demonstrated that total CD8+ lymphocytic density is an independent predictor of longer disease specific survival, and therefore that CD8+ T cell density can act as a prognostic biomarker.[35,37] In metastatic melanoma, greater numbers of CTLs with CD8 staining have been shown to correlate with longer survival,[38] a finding also demonstrated in colorectal and cervical cancer.[39,40] Further, a study by Tumeh et al. found that high density of CD8+ TILs correlates with response to anti-PD-1 therapy in metastatic melanoma, thus suggesting that density of CD8+ TILs may also have a role as a predictive biomarker.[41]

As evidence increases that CD8+ TILs confer a favorable prognosis in a variety of solid tumors including ovarian, gallbladder, and NSCLC,[34,42,43] the Immunoscore has been proposed as a method of classifying malignant tumors by quantifying the *in situ* immune cell infiltrates of two lymphocyte populations.[34,42,43] In patients with colorectal cancer, the Immunoscore has been demonstrated as a biomarker with clinical utility in predicting disease recurrence following surgical resection and therefore in identifying patients likely to benefit from adjuvant therapy.[44,45] The need to reinforce the prognostic and predictive value of the Immunoscore in other solid tumors as well as to identify follow-up parameters to modify its initial prognostic value will inevitably drive further biomarker research utilizing IHC.[46]

PD-1/PD-L1

PD-1 and PD-L1 are immune checkpoint molecules expressed on T cells, antigen-presenting cells, and tumor cells. Presence of PD1 or PDL1 have been proposed as biomarkers predictive of response to PD-1/PD-L1 blockade. These ligands inhibit T cell activity and are thus key to maintaining an immunosuppressed environment in the tumor. Both of these molecules are the target of a number of therapeutic antibodies intended to promote T cell activity within the tumor.[47] Since the majority of tumors do not respond to PD-1/PD-L1 inhibition, PD-1/PD-L1 expression has been investigated as a potential biomarker for response. A study in patients with NSCLC treated with pembrolizumab found that patients for whom at least 50% of tumor cells expressed PD-L1 had a response rate of 45.2%, whereas for all the patients combined the response rate was 19.4%, thus suggesting that PD-

L1 expression is a predictive biomarker for response to pembrolizumab and leading to FDA approval of pembrolizumab in NSCLC in the context of tumor-PD-L1 expression as a companion biomarker.[48] Further, a meta-analysis found that PD-L1 expression on tumor and tumor-infiltrating immune cells is a predictor of response across tumor types.[49] However, there remains disagreement in the field about whether PD-L1 expression alone is sufficient to accurately determine which patients will respond to checkpoint blockade. Indeed, a trial of stage III melanoma patients treated with pembrolizumab found that pembrolizumab was consistently effective both in patients with PD-L1-positive tumors and in patients with PD-L1-negative tumors, thus suggesting that PD-L1 is not a useful predictive biomarker in these patients.[50]

There are currently four IHC assays available to assess PD-L1 expression in patients who might be treated with anti-PD-L1 or anti-PD-1 in clinical trials. Three of these assays have shown consistency in direct comparisons , although the fourth assay indicates a lower PD-L1 expression in tumor and immune cells.[51] There are several challenges with these IHC assays, namely intratumoral heterogeneity, variable temporal expression of PD-L1, and prohibitive pricing.[52] As such, PD-L1 remains an unreliable predictive biomarker of response to PD-1/PD-L1 checkpoint inhibition.

Other predictive biomarkers

Although there is no definite biomarker predicting response to CTLA-4 checkpoint inhibition, several biomarkers have been proposed for this purpose. Higher protein levels of indoleamine 2,3 dioxygenase (IDO) and FoxP3 at baseline have been found to be associated with favorable clinical outcomes in patients treated with anti-CTLA-4 therapy.[53] Other studies have highlighted the importance of the ratio of effector T cells to regulatory T cells within the tumor,[54] with one study showing that the ratio of CD8+ effector T cells to FoxP3+ regulatory T cells is positively correlated with therapy-induced tumor necrosis in previously vaccinated cancer patients treated with anti-CTLA-4.[55] Further, an increase from baseline of absolute lymphocyte counts was found to positively correlate with response to anti-CTLA-4 therapy.[56] Broader changes of the immune response, such as an increase in T cell diversity, have also been noted to follow anti-CTLA-4 immunotherapy and to be associated with a higher response rate.[57,58] Other biomarkers associated with response to anti-CTLA-4 therapy play a role only during or after treatment and, as such, cannot be used to predict response prior to therapy.[59]

Limitations of IHC for biomarker discovery and use

Despite IHC's ubiquitous presence in research and diagnostic procedures, it suffers from several limitations; most notably, the lack of strict guidelines for staining often results in conflicting results among different institutions using different protocols and different antibodies.[60,61] Indeed, McCabe et al. reported that different concentrations of HER2 antibody for staining could result in opposite prognostic implications for patients with breast cancer.[60] Beyond antibody concentration consistency, numerous other components of IHC lack quality control. For example, whether an antibody binds to its target with adequate sensitivity and specificity is not routinely tested.[62,63] The lack of staining reliability may

also stem from the absence of quality control measures beyond the antibody itself. Variations in tissue fixation times, slide thickness, and antigen retrieval all impact the sensitivity and specificity of the antibodies used.[64] As such, detailed and standardized protocols are necessary to allow systematic use IHC-based biomarkers.

Future directions

Automated IHC platforms have the potential to improve reliability and reproducibility of IHC, which so far has limited the use of IHC-based biomarkers in the clinic. Automated IHC platforms can be used in a clinical setting to create a “closed system” that prevents variations from being introduced.[61] Further, automated image analysis platforms that decrease observer variability can more reliably quantitate biomarker positivity or negativity in patient samples.[65] However, as of yet, these platforms remain only semi-automated as they require significant input from the user to aid in the machine learning process.

Although the biomarkers described in this review have been discovered and analyzed using traditional immunohistochemistry, new technologies allow for more sophisticated analyses of molecular markers. For example, technologies that allow for multiplexed immunofluorescence, such as Vectra® or AQUA, allow for analysis of multiple cell phenotypes at a time.[66,67] Importantly, the multiplexing aspect of these technologies opens the possibility of evaluating the proximity between individual cells.[68] This may allow for further specification of a biomarker. Indeed, our lab has recently used multiplexed immunofluorescence to find that a low CTL to macrophage ratio in the stroma is associated with lower overall survival, and that a closer distance of CTLs to HLA-DR^{-} macrophages is associated with poor prognosis in melanoma.[66] Such biomarker discovery has been facilitated by the use of multiplexed, quantitative IHC in many other tumor types, such as breast cancer, pancreatic cancer, and squamous cell cancer.[68–70]

Finally, while biomarkers may act as independent indicators, a single biomarker is often insufficient to clearly and safely stratify patients.[71] Combining IHC with genomic and transcriptomic techniques may help in identification of more precise and predictive biomarkers, as many biomarkers have been discovered using these techniques.[72,73] For example, Hugo et al. conducted genomic and transcriptomic analyses to define a subset of melanoma tumors with a specific transcriptomic signature (named IPRES) that are innately resistant to PD-1 checkpoint blockade.[74] Ayers et al. discovered an IFN-γ-related gene expression profile that is consistent with T cell inflammation and that is an independent predictor of response to PD-1 blockade in nine cancers.[75] Other studies have found that the tumor mutation burden is a strong predictor of response to immunotherapy in both melanoma and NSCLC.[76,77] As such, while IHC-based techniques can be powerful on their own, combining these techniques with other assays, or further developing these techniques to become multiplexed and more quantitative, may help accelerate the discovery and validation of biomarkers.

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KEY POINTS

- Immunotherapy has successfully improved the prognosis of patients with cancer, but its use must be closely monitored for severe immune-related adverse events.
- Predictive and prognostic biomarkers allow clinicians to weigh the potential benefits of immunotherapy against its potential toxicities by stratifying patients into risk groups.
- Immunohistochemistry (IHC) is a powerful tool for the discovery and use of biomarkers, but current techniques are limited due to lack of reproducibility.
- The combination of IHC with genomic and transcriptomic analyses and the use of multiplexed and automated IHC both may help accelerate the discovery and validation of biomarkers.

SYNOPSIS

Immunotherapy has drastically improved the prognosis of many patients with cancer, but it can also lead to severe immune-related adverse events. Biomarkers, which are molecular markers that indicate a patient's disease outcome or a patient's response to treatment, are therefore crucial to helping clinicians weigh the potential benefits of immunotherapy against its potential toxicities. Immunohistochemistry (IHC) has thus far been a powerful technique for discovery and use of biomarkers such as CD8+ tumor-infiltrating lymphocytes. However, IHC has limited reproducibility. Thus, if more IHC-based biomarkers are to reach the clinic, refinement of the technique using multiplexing or automation is key.