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REVIEW

Review of diagnostic, prognostic, and predictive biomarkers in melanoma

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Abstract

Melanoma is an aggressive cutaneous malignancy with rapidly rising incidence. Diagnosis of controversial melanocytic lesions, correct prognostication of patients, selection of appropriate adjuvant and systemic therapies, and prediction of response to a given therapy remain very real challenges. Despite these challenges, multiple high throughput, nucleic-acid based biomarkers have been developed that can be assayed from histologic tissue specimens. FISH, CGH, Decision-Dx, and other multi-marker assays have been combined to improve overall predictability. This review discusses some of the most promising nucleic acid based assays that can be obtained from tissue specimens to assist with diagnosis, prognostication, and prediction of treatment response.

Keywords Diagnostic · Prognostic · Predictive · Biomarker · Melanoma

Introduction

Melanoma is an aggressive malignancy that is the fifth most common cancer among men and sixth most common among women [1]. In 2017, more than 94,000 cases of melanoma were diagnosed in the United States [1]. Despite high overall survival as well as advances in staging, classification, and treatment, there remains a critical need for biomarkers in melanoma. Controversial melanocytic lesions continue to be a diagnostic challenge. In addition, as the incidence of melanoma continues to rise and more patients are diagnosed with early-stage disease, the absolute number of "low risk" patients who ultimately relapse and die is growing [2, 3]. While conventional staging systems such as the American Joint Committee on Cancer (AJCC) can subdivide patients with clinically localized (stages I–III) melanoma into those with relative low, average, or high risk of recurrence and death, an estimated 10–20% of patients will defy the odds, and their tumors will behave contrary to predictions. Finally, in those patients that ultimately undergo systemic therapy for advanced disease, only some respond to therapy, while the remainder experience disease progression.

Consequently, many models have been proposed to fill these challenging gaps and assist with therapeutic decisions. Some tools are commercially available, including a web-based individual risk predictor [4], but we currently lack a widely accepted, rigorously validated method for prognostication or prediction of response to available therapy. In 2013, the AJCC addressed this need with the creation of the 8th edition Precision Medicine Core, tasked with performing systematic assessment of clinical prognostic tools in melanoma and four other cancers [5]. A recent systematic review of 17 available prediction tools by this group, culled from over 400 published models, concluded that there is a widespread need for methodological improvements in the development and evaluation of these tests [6]. Specifically, the authors identified frequent inclusion of cases diagnosed and treated over a decade ago, lack of external validation in over half of cases, and little consistency in data elements used to construct tools [6]. Not surprisingly, the most common data elements utilized in the tools analyzed were

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combinations of those most readily available: clinical and pathologic variables such as patient demographics and primary tumor factors.

There have been even less high-quality validations of tissue-based biomarker evaluations, such as immunohistochemistry or nuclei acid-based methods. Despite these issues, multiple biomarkers have been investigated and have shown potential to impact management in melanoma. A seminal systematic review of immunohistochemistry-based biomarkers of melanoma by Gould Rothberg and colleagues identified only 37 mostly retrospective studies from 1797 potential manuscripts that fulfilled the rigorous reporting recommendations for tumor MARKer studies (REMARK) criteria [7]. This review identified 87 assays on 62 proteins that met all inclusion criteria, and identified several emerging biomarkers [7]. A follow-up study by Schramm et al. expanded the list of potential immunohistochemical biomarkers from 62 to 81 [8]. Subsequently, there have been several reports on the use of multi-marker prognostic discriminators (MPD), which evaluate the combined effect of three or more markers in a multivariate model to enhance predictive capacity [9–11]. Teamed with the advent of high-throughput molecular techniques, there has been an increased focus on the identification of nucleic acid-based tests and signatures (i.e. Decision-Dx) for diagnosis and prognostication of melanoma from multiple sources, including recut slides from formalin-fixed paraffin embedded (FFPE) melanoma tissues [12, 13].

The following review is designed to summarize some of the most promising nucleic acid based biomarkers and biomarker panels, obtained from tissue, that may assist with diagnosis and treatment selection in melanoma. These will be discussed in categories based on how each marker or panel assists with management, namely those that assist with diagnosis (diagnostic biomarkers), those that assist with prognostication (prognostic biomarkers), and those that assist in predicting which patients will respond to a given therapy (predictive biomarkers).

Diagnostic biomarkers

Diagnosis of melanoma via examination of histologic specimens remains a challenge as melanoma can have extremely varied cytomorphology. Interestingly, there are four molecular tests that have been validated for use in the clinical setting, and are recognized by some, if not all insurance carriers. Three of these are mainly used for diagnosis. Fluorescence in situ hybridization (FISH) and comparative genomic hybridization (CGH) are both DNA-based, while myPath (Myriad Genetics) produces an mRNA based gene expression profile. The fourth, Decision-Dx, is more useful for prognostication and will be discussed in that respective section.

FISH is a commercially available assay which was developed to aid in the diagnosis of controversial melanocytic lesions. Melanomas display numerous chromosomal aberrations such as loss of chromosomes 6q, 8p, 9p, 10q and copy number increases of chromosomes 1q, 6p, 7, 8q, 17q, and 20q, which are not found in nevi. The FISH assay is based on evaluation of individual tumor cells (≥ 30) on a recut tumor slide, and enumeration of signals from specific gene areas visualized by fluorescent probes. After extensive testing with probes for a number of chromosomal regions, a combination of four probes was demonstrated to have a sensitivity and specificity of 86.7 and 95.4%, respectively, for the diagnosis of melanoma [14]. The first version of the FISH assay employed probes targeting 6p25 (RREB1), centromere 6, 6q23, and 11q13 (CCND1) utilizing formalin fixed paraffin embedded (FFPE) tissues [15]. The exception is Spitz nevus, which shows an isolated copy number increase of chromosome 11p in one-fourth of cases, an aberration not found in melanoma [14]. The test has also been used to distinguish intra-nodal nevi from metastatic melanoma, epithelioid blue nevi from blue nevus-like melanoma, and mitotically active nevi from nevoid melanoma [16–18]. In 2011, an assay employing new probes to 9p21 (CDKN2a), 11q13 (CCND1), 8q24 (MYC) and centromere 9, and maintaining probes for 6p25 (RREB1) was promulgated to increase the sensitivity in evaluating ambiguous Spitzoid neoplasms and to address the issue of false positivity in the setting of polyploidy [19]. This test increased the sensitivity for detection of melanoma to 94% but is still hampered by lack of specificity in evaluation of atypical Spitzoid lesions, as low as 33% [20–23]. For the purposes of prognostication, the finding of homozygous deletion of 9p21 in a histologically ambiguous Spitzoid lesion is a strong predictor of metastasis and death [19].

By contrast, CGH is an assay that can be performed on FFPE tissue and detect losses or gains within portions of genomic material and map to their chromosomal regional location. It has the advantage of evaluating the whole genome of the entire tissue sample, rather than focusing on specific parts of the genome, such as with FISH, but has the disadvantage of a greater tissue requirement than for FISH (at least 20 vs. 4 sections recut at 5 μ). In CGH, the index lesion is compared to normal tissue, with the DNA from both samples allowed to compete for substrate and evaluated based upon the ratio of fluorescence intensity of tumor to normal tissue [24]. 95% of melanomas harbor whole chromosomal gains and/or losses, especially in chromosome 9, followed by chromosomes 10, 61 and 8p [25]. In contrast, benign nevi typically show normal CGH, and rarely isolated gains or losses of chromosomal regions in a pattern that does not overlap with melanoma [24]. Spitz nevi can harbor an

11p or 7q gain [15, 24, 26, 27]. One drawback of this test is that it is not widely available, further complicated by issues of difficulties in test cost reimbursement by most major insurance carriers. As such, CGH is mostly employed as part of a comprehensive, expert consultation of a diagnostic lesion at major academic centers.

A third diagnostic test became available for use in formalin-fixed biopsies of diagnostically challenging lesions in 2012. The myPath test, offered by Myriad Genetics, measures mRNA expression of 23 genes by quantitative RT-PCR. A weighting algorithm computes the expression of these genes, which are related to melanocyte differentiation, immune signaling, and others to produce a numerical score between −16 and +11. A negative score supports a benign lesion, while a positive score supports a melanoma malignancy, with a reported sensitivity of 90% and specificity of 91% in unequivocal lesions [28]. A single report testing the utility of this assay in diagnostically challenging lesions has shown that the myPath score agrees with the histologic interpretation of a panel of experts in 64% of cases. This same study showed agreement of FISH result with histologic interpretation in 70% of cases [29].

Despite these available molecular tests, rigorous validation is lacking and the National Comprehensive Cancer Network only recommends clinicians "consider the use of molecular testing for histologically equivocal lesions" [30]. With further prospective study and optimization, it is likely to see these and other tests become more main stream in the diagnosis of melanoma in the near future.

Prognostic biomarkers

Selecting patients with melanoma that will have a more favorable or aggressive biology can also be a challenge. As a result, numerous prognostic markers have been identified in melanoma utilizing archival tumor tissues or single institutional studies [9, 11, 31–36]. Unfortunately, as with diagnostic tests, confirmative prospective validation of these assays is still lacking in most cases. While the gold standard end point for prognostic biomarker testing is survival, the validation of these molecular markers in clinical use may also provide critical information regarding the aggressiveness and metastatic potential of individual tumors. Therefore, the information provided with these prognostic molecular tests may have potential implications for both surgical and adjuvant management of melanoma.

Sentinel node biopsy is one example. It is indicated for staging of clinically node negative patients with intermediate-thickness cutaneous melanoma. Its application in thin melanoma is controversial. The recommendation for sentinel node biopsy in this subset of melanoma varies among melanoma surgeons based on patient factors and histopathological characteristics of the melanoma. Yet, sentinel node status still provides important prognostic information for patients with thin melanoma. In a cohort of 1250 thin melanoma patients, sentinel node positivity correlated significantly with worse outcome [37]. Clinical parameters, such as Breslow thickness ≥ 0.75 mm, Clark level ≥ IV, and ulceration significantly predicted sentinel node metastasis: 6.3, 7.0, and 11.6%, respectively. However, the predictive value of molecular marker assays for thin melanoma patients undergoing sentinel node biopsy has not been comprehensively evaluated.

Several molecular markers have been evaluated in correlation with sentinel node positivity [11, 35, 36, 38, 39]. However, none of these markers are commercially available assays. Osteopontin expression was examined in primary melanoma tumor tissues from 345 patients and correlated with sentinel node metastasis [38]. Among patients with low osteopontin expression, sentinel node metastasis was observed in only 8.8% compared with 32.9% in patients with high osteopontin expression. Similarly, RGS1 expression was examined in 301 primary melanomas [39] and again correlated significantly with sentinel node metastasis. Additional markers with correlation of sentinel node status include GOLPH3 expression [35] and expression of the lymphangiogenesis marker D2-40 LI [36]. D2-40 LI expression was determined in 64 melanoma patients undergoing sentinel node biopsy [36], and 12/14 patients with a positive sentinel node had D2-40 LI expression for a positive predictive value of 85.7%. Of the 50 patients without D2-40 LI expression, 44 had negative sentinel node with a negative predictive value of 88.0%. Several groups have extended these assays to include multiple markers in hopes of improving the accuracy of the tests. As an example, Kashani-Sabet et al. combined RGS1 with SPP1 and NCOA3 to create a three marker assay whereby a positive net score was not only associated with worse survival, but also with increased rates of lymph node metastasis [11]. Given the above studies, it is foreseeable that these and other molecular markers, most likely in combination as a multi-marker assay, may be promising as an adjunctive selection tool for sentinel node biopsy in patients with thin melanoma.

Another controversy in melanoma is the management of positive sentinel nodes. Since the introduction and validation of sentinel node biopsy, the management of sentinel node positive patients has entailed completion node dissection. However, recent results from the DeCOG-SLT and MSLT-II trials have demonstrated that completion node dissection does not confer survival benefit compared with nodal observation in patients with two or fewer positive sentinel nodes [40, 41]. A criticism of these studies is the inclusion of a high number of patients with relatively low burden of disease in the lymph nodes. Additionally, there was no latent subgroup analysis to determine whether early

removal of involved non-sentinel nodes improved survival compared with patients in the nodal basin observation group who subsequently developed regional nodal recurrence. As a result, there is controversy over the results of these trials. Presumably, it would be useful to predict non-sentinel node involvement in patients with a positive sentinel node. However, there is no consensus on the methodology for predicting non-sentinel node metastases. Several proposed prognostic models for non-sentinel node metastases were based on various clinical histological characteristics: (1) tumor burden/location in sentinel node; (2) serum S-100B [42]; (3) N-SCORE [43], (4) Rotterdam-Dewar combined criteria [44]; and (5) peritumoral lymphatic proliferation [45].

A relatively new, commercially available, multi-marker test was developed in 2015 and may assist with identifying these patients. Decision-Dx was developed and validated by Castle Biosciences to enhance prognostic predictions in patients diagnosed with primary melanoma. The test was originally developed based on published differences between expression in metastatic and non-metastatic melanoma samples, and assesses the expression of 28 prognostic genes and 3 control genes with a proprietary weighting algorithm to produce a binary score that divides patients into high (Class 2) and low (Class 1) risk of metastasis [12]. In a prospective cohort of 287 melanoma patients undergoing wide excision and sentinel node biopsy and GEP assay, 39 (13.6%) patients were found to have positive sentinel lymph nodes. Of these patients, 8 (20.5%) had additional positive non-sentinel nodes on completion node dissection (Personal Communication from EC Hsueh). Seven of the 8 patients (87.5%) were found to be GEP class 2, while the final patient was GEP class 1 ($p = 0.047$). Thus, molecular testing may help identify a subgroup of sentinel node positive patients with aggressive tumor biology that might benefit from completion node dissection.

Decision-Dx has also been utilized to help understand patterns of local–regional recurrence with potential relation to margins of excision on primary tumors. Several multicenter trials have established the general guideline for the resection margins of primary melanoma based on Breslow thickness [46–50]. The majority of these trials have not shown an impact on survival with narrower margins. However, some statistically significant difference in local recurrence was observed. In addition, Doepker et al. reported increased morbidity using wider margins of resection in the form of greater need for skin graft or flap for closure when 2 cm margins area used for melanomas 1.01–2 mm in depth compared to 1 cm margins [51]. Given increased morbidity with wider resection margin and no significant impact on survival, selection of surgical margin is of importance. In a recent multicenter registry trial of 322 patients evaluating Decision-Dx, 13 patients developed local regional recurrence with median follow up of 1.5 years [52]. Ten of 13 (77%) had a high risk, class 2 signature [52]. Given that molecular testing may inform the risk for local regional recurrence, the resection margin may be tailored according to the biological behavior of the tumor based upon the results. However, the application of molecular testing in margin selection would ideally be confirmed in a multicenter study where patients with > 2 mm thickness melanoma were randomized to standard 2 cm margin versus 1 or 2 cm based on molecular testing results.

Apart from surgical management, the Decision-Dx may also help select those patients most appropriate for adjuvant systemic therapy. The test was first validated with the mindset of stage prognostication on a selected group of 268 cases from 7 independent centers, and subsequently independently validated in a selected set of 104 cases, all with either known recurrence or 5 year event-free follow-up. When combining these two groups, the test accurately classified 80% of patients who developed recurrence as "high risk" and 90% of those without a recurrence in 5 years as "low risk." When compared with other AJCC-based staging criteria, the 31-gene expression profile (GEP) was an independent predictor of metastasis [12]. Similarly, Zager et al. demonstrated Decision-Dx as an independent predictor of metastatic risk in 523 patients with cutaneous melanoma. The test ultimately identified 70% of stage I and II patients who went on to develop metastatic melanoma [53]. Several studies have also evaluated whether the Decision-Dx can be combined with other prediction tools to enhance predictive capabilities. The test was compared with the web-based AJCC Individualized Melanoma Outcome Prediction tool in a cohort of 205 patients. Specifically, the ability of the GEP to predict risk was compared to high and low-risk patient groups determined by two separate AJCC-predicted cutoffs, the 5-year survival rates for stage IIA (79%) and IIB (68%). Although both tools significantly predict risk, in multivariate analysis the GEP was more significantly associated with distant metastasis and death; it had a higher sensitivity but lower specificity when compared with AJCC at both cutoff scores [54]. Furthermore, combining both tests strengthened risk predictions [54]. The predictive value of the GEP test has also been compared with sentinel node biopsy results in a cohort of 217 patients from multiple institutions. Both tests predicted disease free, distant metastasis free, and overall survival, but in multivariate analysis the GEP test was a more significant predictor of each endpoint [13]. This does not imply that this test should replace sentinel node biopsy as a predictive tool, but that perhaps it can be used in combination with it to enhance prediction.

It should be noted that the above studies were all performed on highly selected groups, enriched in patients with recurrence events, and retrospective in nature, and to date prospective data has only been published in abstract form. Only Decision-Dx is commercially available. Furthermore,

critics of the GEP point out lack of clear guidelines for patient treatment and follow-up in the event of a high-risk result. Clearly, prospective studies are needed to further examine the role of this and other tests as this prognostic assessment may benefit patients with early stage melanoma by identifying those who might benefit from more or less aggressive screening protocols and possible need for adjuvant systemic treatment.

Predictive biomarkers

Despite most patients presenting with early stage disease, curable by surgery [1], a significant number still present with more advanced disease or recur after initial treatment. Patients with more advanced (Stage IIIb and IIIc) resected disease have been shown to benefit from adjuvant systemic therapy [55–57]. Additionally, the outlook for patients with Stage IV disease has also meaningfully changed in recent years with multiple therapies providing substantial increases in overall survival [58–60]. This has been due, in large part, to novel immune checkpoint inhibitors such as anti-CTLA4 monoclonal antibody, ipilimumab, and anti-PD-1 monoclonal antibodies, pembrolizumab and nivolumab. These have provided significant improvements in overall survival in patients with local–regional and metastatic disease. Overall survival in patients with BRAF mutations treated with BRAF and MEK inhibitor combinations, such as dabrafenib plus trametinib or vemurafenib plus cobimetinib, have also improved overall survival [61, 62]. Despite marked efficacy of these treatments, only a subset of patients respond, and the clinical limitations and mechanisms of resistance to these treatments have been the subject of recent review [63]. As a result, predictive biomarkers and gene expression profiles associated with likelihood of response to immunotherapy have been developed.

Increased tumor PD-L1 expression assessed by PD-L1 IHC 22C3 pharmDx antibody (Agilent Pharmaceuticals) and graded by the standardized MEL scoring system has been associated with prognostic significance and is currently the only commercially available predictive biomarker in Melanoma. Biopsies that scored greater than or equal to a MEL Score of 2, indicating greater than 1% PD-L1 expression on tumor or tumor-associated immune cells achieved higher objective response rate. The higher the MEL score, the more durable the response was. However, responses were also seen in PD-L1 negative tumors [64], thus making use of PD-L1 expression via IHC pre-immunotherapy controversial and not standard of care in melanoma.

As a result, research groups are actively investigating promising nucleic acid-based methods to explore other high throughput biomarkers and improve predictive capacity. Whole exome sequencing (WES) and next generation sequencing (NGS) are two such examples. Snyder et al. first noted that a pilot group of 25 patients (11 responders, 14 non-responders) could be stratified into high likelihood of response and low likelihood of response based on WES mutational burden and other clinical factors [65]. High mutational burden was associated with response, but not sufficient to independently predict response in this study published in the New England Journal of Medicine. Similarly, Johnson et al. demonstrated that high mutational load determined by targeted NGS of 315 genes in responders versus non-responders could correctly stratify the two groups of patients by response and also predict progression free and overall survival [66].

In addition, recent characterization of the immunobiology of melanoma metastases has identified characteristic genetic signatures associated with improved response to immunotherapy [67, 68]. These include interferon-γ inducible genes, Th-1 markers, and other chemokines such as CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10. These inflammatory signatures have been shown to support the T-cell inflamed tumor microenvironment: a phenotype characterized by infiltrating CD8 effector T-cells and tumor reliance on immunosuppressive pathways for immune escape [67, 69]. Ayers et al. have demonstrated that both a preliminary 10-gene and expanded 28-gene interferon-γ related profile predict best overall response, progression free survival, and overall survival in 81 patients with melanoma treated with Pembrolizumab under the KEYNOTE-001 clinical trial [70]. Together, these predictive assessments have shown promise in selecting patients who are more likely to respond to immunotherapy and are currently being further investigated in clinical trials using anti-PD1 agents.

Conclusions

The aforementioned advancements in biomarkers and treatment stand to benefit patients with both early and metastatic melanoma. Diagnostic assays such as FISH, CGH, and myPath may help improve the accuracy in diagnosis of challenging melanocytic lesions and are available commercially. Single and multi-marker assays such as the Kashani-Sabet score and Decision-Dx may help to better identify aggressive biology and better select patients for either more aggressive surgical treatment and/or systemic adjuvant therapy. Only Decision-Dx is widely available. Finally, advancements in sequencing and the understanding of the tumor microenvironment in melanoma have led to the use of genome sequencing and development of multi-marker T-cell inflammatory assays to better predict response to immunotherapy. Unfortunately, these assays currently operate in isolation and likely need to be studied in combination and across different disease risk populations to identify improved multi-marker

assays. Biomarker platforms developed in early stage melanoma tend to define high-risk tumors by their risk of tumor recurrence while biomarker platforms in late stage tumors predict likelihood of response to systemic therapy. Given recent appreciation for benefit of immunotherapy and targeted therapy in the adjuvant setting, early stage melanoma with unclear risk of recurrence may benefit from a synthesis of these two types of biomarker platforms by classifying high or low risk of recurrence and the likelihood of response to treatment after resection. Given frequency and severity of immune-related adverse events associated with immunotherapy, patients stand to benefit from a reliable prediction of treatment necessity and efficacy. Integration of these molecular tests in this way may provide more comprehensive insight into an individual tumor's behavior and ultimately guide these difficult management decisions in melanoma patients. While the National Comprehensive Cancer Network falls short of recommending these tests currently, the great number of ongoing studies makes it more and more likely they will hopefully enter the mainstream clinical arena in the coming years.

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