Molecular Testing for Cutaneous Melanoma
An Update and Review

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Context.—The steady rise in the incidence of cutaneous malignant melanoma and its inherently difficult-to-interpret histopathology continues to fuel an increasing demand for diagnostically and prognostically insightful adjunctive molecular tests among both clinicians and dermatopathologists. A number of DNA, RNA, and epigenetically based assays have now been developed and are at various stages of experimental and/or clinical use.

Objective.—To examine the evidence for the utility and limitations of these leading candidates for the diagnosis and risk stratification of melanoma and related melanocytic neoplasms.

Data Sources.—The available English medical literature was reviewed in the preparation of this manuscript.

Conclusions.—Comparative genomic hybridization, fluorescence in situ hybridization, RNA-based gene expression profiling, and immunohistochemical assays for novel genetic and epigenetic markers will help bring diagnostic and prognostic accuracy to the assessment of melanocytic neoplasms.


The last 3 decades have witnessed a rise in the incidence of cutaneous malignant melanoma, with an estimated 76 380 new cases diagnosed annually in the United States alone.1 This statistic, however, is likely to underestimate by orders of magnitude the total number of cases wherein the diagnosis of malignant melanoma is additionally considered, refuted, or deferred for expert referral and consultation. The current gold standard for the diagnosis of melanoma is based primarily upon the histologic interpretation of cytomorphologic and architectural features, which remains among the most challenging areas of practice in the field of dermatopathology. This inherently and inevitably subjective practice is subject to well-documented pathologist interobserver and intraobserver variability2–4 as well as diagnostic drift.5 The latter refers to a pathologist’s tendency to render more “malignant” over “atypical” diagnoses (or vice versa) over time. Not surprisingly, the misdiagnosis of malignant melanoma is the most common reason for a medical malpractice lawsuit related to surgical pathology.6 Such practice vulnerabilities underscore the importance of adjunctive diagnostic tests to bring both increased diagnostic uniformity and prognostic accuracy to the practice of melanocytic pathology.

In addition, recent data indicate that thin melanomas, defined as 1.0 mm or less in thickness, comprise approximately 70% of new melanoma diagnoses yet account for 30% of melanoma-related deaths.7 Such findings, in part, have informed the recent additions to the 8th edition of the American Joint Committee on Cancer (AJCC) melanoma staging system.8 For instance, a 0.9-mm T1a melanoma by the 7th-edition AJCC staging criteria would be upstaged to a T1b tumor by the new 8th edition based on a thinner 0.8-mm cutoff. That efforts toward more accurate, detailed microstaging of the primary melanoma by depth and mitotic rate, the latter of which was removed from the new staging system, may not fully capture its biological potential highlights the important role for adjunctive diagnostic tests to provide important insight into malignant biological potential as it relates to risk of recurrence and/or locoregional as well as distant metastasis.

Although novel approaches at histopathologic assessment, such as the evaluation of Breslow “density,”9 require further consideration and testing, the development of molecular diagnostic tests, which probe and highlight genetic and epigenetic alterations by comparative genomic hybridization (CGH), fluorescence in situ hybridization (FISH), and RNA-based gene expression profiling, as well as certain genetically and epigenetically targeted immunohistochemical assays, has now been validated and provides important adjunctive diagnostic information for risk stratifying ambiguous melanocytic tumors of uncertain malignant potential (including so-called intermediate- or low-grade melanocytic tumors). A recent survey suggested that up to 38% of pathologists report use of FISH and/or CGH as ancillary tests for melanocytic tumors, and that these early
adopter's tend to be younger, fellowship trained, and regionally concentrated, typically at an academic medical center. 10 Although these trends may reflect access to, training in, and experience with these technologies, 11 increasing awareness about the diagnostic utility and limitations of these and other assays discussed herein is important to ensure that these important adjuncts continue to be more widely adopted. In this review, we will highlight the utility and limitations of these tests and discuss important diagnostic areas within melanocytic pathology in which further validation of these tests is needed. The major clinical indications and utilities as well as limitations of the adjunctive assays discussed below are summarized in Tables 1 and 2.

**GENOMIC CHROMOSOMAL COPY NUMBER ANALYSES**

**Fluorescence In Situ Hybridization**

Whereas CGH, discussed in greater detail below, provides a comprehensive analysis of chromosomal copy number, FISH detects chromosomal copy number alterations at predetermined, targeted genomic loci (Table 1). This technique involves the construction and hybridization of a fluorescently labeled, single-stranded DNA probe, which contains the gene of interest, directly onto formalin-fixed, paraffin-embedded tissue sections containing denatured DNA. Following additional processing steps, the results are visualized and quantified microscopically. By doing so, this technique enables the identification of chromosomal copy number alterations, such as gains and losses of entire chromosomes or targeted loci as well as of the localization of loss of heterozygosity (ie, hemizygosity) and homozygous deletions. A distinct technique known as break-apart FISH enables the identification of chromosomal translocations and fusion genes by simultaneously targeting the 2 genes of interest using 2 different colors, which when overlaid (fused) yield a third distinct signal. This latter technique is the basis upon which clear cell sarcomas (ie, melanomas of soft parts) are distinguished as an entity 12 and has also identified frequent, therapeutically targetable kinase fusions in spitzoid melanocytic tumors. 13

Genetic loci included in most commercially available FISH assays target the chromosomal loci 6p25 (REB1), 6q23 (MYB), 8q24 (MYC), 9p21 (CDKN2A), and 11q13 (CCND1). To date, FISH appears to demonstrate greatest utility in characterizing risk of metastasis in the risk assessment of atypical Spitz tumors, with those harboring the homozygous 9p21 deletion alteration in particular demonstrating the greatest risk for aggressive disease. 14,15 Fluorescence in situ hybridization has also demonstrated high sensitivity and specificity in distinguishing conventional primary cutaneous melanomas from benign nevi, 16-18 but has demonstrated considerably less than consistent results in the assessment of histologically ambiguous melanocytic tumors. 19 For instance, in the evaluation of 22 melanocytic neoplasms, including 12 ambiguous cases, Gaiser et al 20 found that a 4-probe FISH assay targeting 6p25, centromere 6, 6q23, and

| Table 1. Summary of Adjunctive Chromosome Analysis and Gene Expression Profiling Techniques for the Evaluation of Melanocytic Neoplasms |
|-----------|----------------|------------------------------------------------------------------------------------------------------------------|
| Technique                          | Optimal Clinical Use                                                                                                  | Limitations                                                                                                                                 |
| Chromosomal analysis                | Fluorescence in situ hybridization                                                                                       | Risk assessment and diagnosis of spitzoid and/or histologically ambiguous melanocytic neoplasms                                      |
|                                    |                                                                       | Distinguishing benign melanocytic nevi from malignant melanoma                                                        |
|                                    |                                                                       | Sensitivity: 43%-100%                                                                                                   |
|                                    |                                                                       | Specificity: 29%-80%                                                                                                   |
|                                    |                                                                       | Turnaround time and cost                                                                                               |
|                                    |                                                                       | Requires greater neoplastic tissue than fluorescence in situ hybridization                                            |
| Comparative genomic hybridization   | Fluorescence in situ hybridization                                                                                       | Distinguishing benign melanocytic nevi from malignant melanoma                                                        |
|                                    |                                                                       | Sensitivity: 92%-96%                                                                                                    |
|                                    |                                                                       | Specificity: 87%-100%                                                                                                   |
| Gene expression profiling          | DecisionDx-Melanoma (Castle Biosciences, Friendswood, Texas)                                                          | Assessing metastasis risk in patients with histologically confirmed diagnosis of malignant melanoma, particularly |
|                                    |                                                                       | those with negative sentinel lymph node biopsy                                                                       |
|                                    |                                                                       | Sensitivity: 85%-100%                                                                                                   |
|                                    |                                                                       | Specificity: 54%-78%                                                                                                    |
|                                    | myPath Melanoma (Myriad Genetics, Salt Lake City, Utah)                                                               | Indeterminate result may beget overtreatment                                                                        |
|                                    | Pigmented Lesion Assay (DermTech, Inc, La Jolla, California)                                                          | Cases lacking triple-concordant diagnoses upon review by experts were excluded from validation study                  |
|                                    |                                                                       | Does not provide adjunctive information in the setting of histologic ambiguity                                        |
|                                    |                                                                       | Does not assess metastasis risk                                                                                       |
|                                    |                                                                       | Patient must return for second visit for biopsy if lesion in question deemed high risk                               |
|                                    |                                                                       | Not tested or validated in the less common histologic variants of melanoma                                          |

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Since the publication of this and other landmark studies, it has become clear that chromosomal copy number variations, particularly when involving losses or gains in 11q13 had a sensitivity and specificity of 60% and 50%, respectively, for the development of metastatic disease.

Fluorescence in situ hybridization, in contrast to CGH, is widely available, requires minimal tissue, has a rapid turnaround time, and enables the testing and visualization of individual cells. However, FISH is limited by its ability to detect only increases or decreases in chromosomal copy numbers in intentionally targeted genes. Therefore, this technique may overlook pathogenetically relevant chromosomal alterations in genes not targeted by the panel of predetermined/designed FISH probes. Indeed, FISH-negative melanomas (false negatives) are not uncommon, and similarly but conversely, indolent, histologically ambiguous melanocytic tumors may also harbor FISH-positive chromosomal aberrations (false positives).21 These observations, taken together, convey the importance of furthering our understanding of the biological significance of specific FISH-detectable chromosomal aberrations within the context of a specific melanocytic histology.

### Comparative Genomic Hybridization

Unlike FISH, which is predicated upon targeting specific chromosomes of interest, CGH detects chromosomal copy number variations relative to ploidy level throughout the genome and has been used for the assessment of melanocytic neoplasms since the 1990s.22-24 This technique is based upon the extraction of lesional tissue containing neoplastic DNA from paraffin-embedded histologic sections. Tissue sections with sufficient tumor volume are stereoscopically microdissected to avoid inclusion of nonlesional tissue. DNA from this test source (eg, atypical melanocytic cells) is then fluorescently labeled (eg, red), denatured to a single strand, and hybridized to either a spread of chromosomes in metaphase (chromosomal CGH) at a 1:1 ratio or printed microarrays of genomic DNA (array CGH, or aCGH) along with DNA from a reference (normal) sample, which is similarly processed but labeled a different fluorescent color (ie, green). This is followed by what is typically a proprietary process requiring fluorescence microscopes and computer software to analyze and compare differential signals along the length of each chromosome. In so doing, aCGH enables the precise localization of small genomic changes or chromosomal breaks and also allows for screening and identification of gene fusions and fusion partners.

Although the initial application of CGH to melanoma involved metastatic tissue,25,26 the literature to date has since demonstrated that CGH can be helpful in distinguishing benign melanocytic nevi and related variants from primary cutaneous melanoma. Bastian et al23 initially demonstrated that CGH had a 94.8% sensitivity and 90.4% specificity in distinguishing unequivocally benign melanocytic variants, including Spitz, blue, and congenital nevi, from malignant melanoma, where chromosomal aberrations were detected at significantly greater frequencies in melanoma than in nevi. Of interest, all benign cases harboring chromosomal aberrations occurred in Spitz nevi, wherein the majority occurred on 11q. In addition to demonstrating diagnostic utility, aCGH shed important insight into melanomagenesis, whereby losses of chromosomes 9 and 10 were present in areas of radial growth phase in contrast to gains of chromosome 7, which occurred mostly in thicker tumors and typically were present only in conjunction with other aberrations (such as loss of the distal part of the short arm of chromosome 9, which encompasses 9p21).

Since the publication of this and other landmark studies, it has become clear that chromosomal copy number variations, particularly when involving losses or gains in
insufficient for an outright diagnosis of melanoma has helped support the concept of intermediate-grade melanocytic tumors (such as spitzoid melanocytic neoplasms and the pigmented epithelioid melanocytoma). Conversely, the absence of copy number alterations may be considered dismissive of melanoma. Importantly, an isolated genomic aberration when present may also be considered insufficient for a molecular diagnosis of melanoma, as most are biologically insignificant. Alas, as with any other adjunctive diagnostic test for melanoma, a diagnosis should not be rendered based on a single molecular test alone.

Comparative genomic hybridization was among the first molecular diagnostic adjuncts for the assessment of melanocytic tumors. In addition to being used diagnostically, several lines of evidence suggest that CGH-detected chromosomal aberrations may distinguish metastasizing from nonmetastasizing melanocytic tumors. Moreover, this molecular technique has provided important insight into the biology of melanocytic neoplasia. For instance, it has shed light on the molecular pathogenesis of those rare melanomas arising from benign dermat melanocytes, such as the evolution of a cellular blue nevus–like melanoma arising in a nevus of Ota. Moreover, recent investigations using aCGH have supplemented and further clarified efforts at accurately classifying melanocytic neoplasms, particularly those within a difficult-to-diagnose category (ie, ALK-fusion Spitz tumors), based on shared histologic and molecular features.

Nevertheless, this important diagnostic adjunct has important limitations. Firstly, aCGH is a costly diagnostic test available only at select academic centers, thereby requiring external referral for evaluation and lengthening the comparatively long turnaround time (3–4 weeks). Furthermore, the requirement of a significant amount of lesional tissue precludes its use in select cases characterized by low tumor volume. Similarly, inaccurate or false-negative CGH results can occur because of neoplastic heterogeneity within a carefully and thoughtfully microdissected tumor cell population or the inclusion of excess normal background cells in the sample. For this reason, analysis of melanocytic tumors in situ by this technique remains a technical challenge (Table 1).

**QUANTITATIVE GENE EXPRESSION PROFILING**

Several novel quantitative gene expression profiling technologies have recently become available as adjunctive diagnostic tests for the assessment of melanocytic neoplasms. The DecisionDx-Melanoma (Castle Biosciences, Friendswood, Texas), myPath Melanoma (Myriad Genetics, Salt Lake City, Utah), and Pigmented Lesion Assay (DermTech, Inc, La Jolla, California) are 3 such tests that are all based on the algorithmic assessment of unique RNA-based gene expression profiles using biopsied or tape-stripped patient tissue samples. Whereas the DecisionDx-Melanoma assay offers a prognostic assessment of metastasis risk in those already diagnosed with malignant melanoma, the myPath Melanoma test serves as a diagnostic adjunct to distinguish benign melanocytic nevi from melanoma. In contrast, the Pigmented Lesion Assay serves as a clinical decision tool using noninvasively obtained gene expression information to aid the decision for surgical biopsy of clinically atypical melanocytic lesions (Table 1).

**DecisionDx-Melanoma: 31-Gene Expression Profiling (Castle Biosciences)**

Castle Biosciences offers the DecisionDx-Melanoma test to assess risk of metastasis in cases where the diagnosis of melanoma has already been rendered. This assay uses a messenger RNA–based gene expression profile assay using the reverse transcriptase polymerase chain reaction method and comprises 28 prognostically relevant genes and 3 control genes (Table 3). This specific panel includes a spectrum of overexpressed or underexpressed genes that were identified based upon an analysis of gene expression data from publicly available databases comparing melanoma genes that were upregulated or down-regulated in metastatic melanoma samples but not in the primary melanoma. Of interest, gene ontology analysis suggested that the
selected gene panel reflected those involved in epithelial differentiation and development, cell-cell junction, and non-membrane-bound organelle classes.

The sample set used to develop this signature included 107 stage I and II primary melanoma samples from 3 separate institutions in the United States. Twenty of these cases had evidence of metastatic disease, whereas 5 had regional recurrence. The metastasis risk prediction of the DecisionDx assay classifies tumors as either class 1 (low risk of metastasis) or class 2 (high risk of metastasis). Based on the initial study, DecisionDx-Melanoma had a 100% sensitivity and 78% specificity with a reported median time to metastasis for class 2 tumors of 2.5 years and a 38% 5-year disease-free survival compared with 100% 5-year disease-free survival for class 1 (low-risk) cases. In a subsequent multicenter cohort of 217 patients, all of whom had undergone sentinel lymph node (SLN) biopsy, Gerami et al. found that the DecisionDx-Melanoma test was a more accurate predictor of disease-free, distant metastasis-free, and overall survival than SLN biopsy status. In combination with SLN biopsy status, the RNA-based gene expression profiling assay improved prognostication. Of relevance, 16 of the 76 cases (21%) whose melanoma was deemed low risk (class 1) by the DecisionDx-Melanoma test went on to develop metastatic disease (in contrast to 91 of the 141 (65%) class II patients). Therefore, although the overall sensitivity of the DecisionDx-Melanoma test in the SLN-biopsied population may have been lower (~85%) than that of the initial development cohort, the DecisionDx-Melanoma assay may provide more prognostically accurate information than SLN biopsy itself.

The DecisionDx-Melanoma test requires a dissectible area of tumor cells to be identifiable histologically, which is then microdissected off of a 5-μm-thick histologic section. The RNA is recovered and then converted to complementary DNA through a reverse transcription assay. This complementary DNA then undergoes a 14-cycle preamplification step and is subsequently loaded onto a microfluidics gene card with primers to the 31 relevant genes. Importantly, this assay was not tested specifically on the full spectrum of histologic subtypes of melanoma, such as acral, blue nevus-like, and desmoplastic melanomas. This latter histologic subtype may, too, by virtue of the intervening desmoplasia and collagen deposition between tumor cells, be less amenable to the microdissection technique. Moreover, at present it is not clear how the results of such testing are to be integrated into the new AJCC staging criteria. Nonetheless, this tumor RNA-based gene expression profile assay may be a useful diagnostic and prognostic adjunct in the workup of intermediate-thickness to thin melanomas. Such results could be particularly clinically useful in the counseling of patients who are candidates for SLN biopsy.

**myPath Melanoma: 23-Gene Expression Profiling (Myriad Genetics)**

Myriad Genetics offers the myPath Melanoma diagnostic test to aid in the distinction of benign melanocytic nevi from malignant melanoma. This assay is also based upon reverse transcriptase polymerase chain reaction methodology, but, unlike the DecisionDx-Melanoma test, evaluates expression of 23 genes based on an algorithm that assigns various weights and thresholds of expression for each gene (Table 3). This panel was selected from a list initially composed of 79 candidate biomarker genes compiled from reports described in the literature or from internal observations regarding genes whose increased expression correlated with aggressive behavior. These includes genes of relevance to immunity, cell cycle progression, cellular differentiation, Notch signaling, cell migration, fat metabolism, and the cytoskeleton. After this set had been refined to 40 of the most promising candidates, a training cohort comprising 464 melanocytic tumors obtained from the University of Munich and Provitr (Berlin, Germany) representing a spectrum of benign (n = 210) and malignant (n = 254) melanocytic tumors underwent testing. An algorithm was applied, after which 27 genes were found to differentiate melanoma and nevi with a sensitivity of 89% and specificity of 93%. Genes with both highly correlated expression and similar biological functions were assessed as separate components. The first genetic component included PRAME (preferentially expressed antigen in melanoma), whose expression was found to be the single most effective differentiating feature to distinguish malignant melanoma from benign nevus. The remaining components reflected genes including and related to ST00A9 (n = 5) and the immune response/tumor microenvironment (n = 8), as well as housekeeper genes (n = 9) (Table 3).

This novel gene expression panel was further validated on a cohort acquired from 4 academic institutions comprising a total of 437 melanocytic tumors, including both benign (n = 211) and malignant counterparts (n = 226). The melanocytic tumors in this validation cohort as well as the training cohort described above were sequentially confirmed by 2 experts in melanocytic pathology who were blinded to the initial diagnosis. The melanocytic pathologists were encouraged to classify each case as either benign or malignant (and avoid “indeterminate” or “uncertain” designations). This exercise yielded a sensitivity of 90% and specificity of 91% in distinguishing melanoma from nevus.

The myPath Melanoma algorithm produces a single melanoma diagnostic score that classifies the melanocytic lesion as benign, indeterminate, or malignant. The score is derived from a logistic regression model with a score of 0 set as the threshold to differentiate nevus from melanoma based on the quantitative reverse transcriptase polymerase chain reaction gene expression profiling. The range of scores from the validation cohort followed a bimodal distribution, where the majority of malignant lesions scored greater than 0 whereas benign lesions scored less than 0 (range, −16.7 to +11.1; area under the curve, 96%). A second validation study was performed on a cohort that ultimately comprised 736 melanocytic neoplasms that received either a benign or malignant diagnosis that was concordant among 3 experienced dermatopathologists. The myPath Melanoma gene expression signature assay in this cohort demonstrated a sensitivity of 91.5% and specificity of 92.5% in distinguishing benign nevus from malignant melanoma. Importantly, the exclusion of 407 of the initial 1400 melanocytic neoplasms initially reviewed for this study because of lack of triple diagnostic concordance, as well as the exclusion of 133 samples with triple-concordant diagnosis but indeterminate myPath melanoma diagnostic score for this study, together led to the exclusion of 540 of the 1400 initially reviewed melanocytic neoplasms. The exclusion of these diagnostically troublesome cases may limit the applicability of the assay and this study’s findings to the most histologically and/or biologically ambiguous melanocytic tumors.

A third validation study was ultimately conducted on 99 primary stage I through III cutaneous melanomas that produced distant metastases (beyond the proximal SLN[s])
and 83 benign nevi. Initially, 293 melanocytic neoplasms were submitted, but 53 were excluded because of technical limitations (ie, re-excision, <10% tumor volume) or lack of metastasis, or because the primary cutaneous melanoma was determined to actually represent a cutaneous metastasis (stage IV disease) rather than a primary. Moreover, an additional 58 cases were excluded because of insufficient mRNA quality related to the archival nature of the specimen. Notably, the myPath melanoma diagnostic score reportedly categorized 16 of 16 thin (<1.0 mm) metastasizing melanomas accurately as malignant in this cohort. This third validation study also included 8 acral melanomas, 1 spitzoid melanoma, 7 nevoid melanomas, and 3 desmoplastic melanomas. Of significance, 100% of the tested acral and nevoid melanomas as well as a single spitzoid melanoma were correctly classified by the myPath melanoma diagnostic score as malignant. In contrast, the myPath melanoma assay classified 3 of 3 desmoplastic melanomas as benign and classified 3 of 83 benign cases with benign follow-up as likely malignant. All 3 of these false-positive cases demonstrated histology consistent with atypical melanoma. In a separate study, Ferris et al evaluated dermatologist biopsy sensitivity and specificity with and without the Pigmented Lesion Assay data in a Web-based multiple-reader, multiple-case study wherein 45 board-certified dermatologists evaluated the clinical and dermatoscopic images of 8 invasive or in situ melanomas and 52 nonmelanomas (including 42 atypical nevi and 2 conventional nevi, as well as 8 lentigines or keratosis). The investigators found that this adhesive molecular skin biopsy could nearly double dermatologist biopsy specificity (from 32.1% to 56.9%), increase mean diagnostic accuracy (from 40.4% to 62.4%), and increase confidence in dermatologist decision to biopsy.

Importantly, this noninvasive molecular skin biopsy has not been tested in mucosal melanomas, acral melanomas, or nail unit melanomas, as well as the various histologic subtypes, including desmoplastic, blue nevus–like, spitzoid, and nevoid melanomas (Table 1). Moreover, its accuracy and utility have not been systematically evaluated in...
melanomas arising with preexisting nevi, which account for up to one-third of all primary cutaneous melanomas, wherein sampling error may occur.

NOVEL GENETIC AND EPIGENETIC TARGETS FOR IMMUNOHISTOCHEMISTRY

BRCA-Associated Protein 1

Novel immunohistochemical probes against genetic and epigenetic targets may serve as important diagnostic adjuncts in the evaluation of melanocytic tumors and shed light on melanomagenesis (Table 2). For instance, the loss of immunohistochemical expression of BRCA-associated protein 1 (BAP-1), which functions as a histone deubiquitinase, was shown to be important in the pathogenesis of sporadic and familial metastasizing uveal melanomas and also to mark the so-called sporadic BAPoma (also known as Weisner nevus or sporadic BAP-1-inactivated spitzoid nevus).39–41 The latter is a histologically distinct, biphasic, combined nevomelanocytic tumor composed of sheets of small, plasmacytoid nevomelanocytes with admixed larger, plump nevoid melanocytes that can arise sporadically but may also be seen in BAP-1 tumor predisposition syndrome (Figure 1, A through C).40 Although loss of BAP-1 in the setting of this more or less benign histology is suggestive of the benign BAPoma, a subset of conventional malignant melanomas as well as melanomas arising in BAPoma may also demonstrate BAP-1 loss.21 Therefore, its immunoreactivity or lack thereof must be interpreted within the appropriate histologic context, and thus may be of less utility as a routine adjunct for the risk stratification of most melanocytic tumors.

Bivalent Histone Modifications and EZH2 Expression

Immunohistochemical expression patterns of bivalent histone modifications and the enhancer of zeste homolog (EZH2), the catalytic subunit of the histone 3 lysine 27 (H3K27) methyltransferase polycomb repressive complex 2 (PRC-2), may provide important diagnostic information during the assessment of a melanocytic tumor in addition to providing novel insight into the tumor biology of melanoma stem cells. Kampilafkos et al42 demonstrated that histone 3, lysine 27, trimethylated (H3K27me3) and histone 3, lysine 4, dimethylated (H3K4me2) expression as detected immunohistochemically were increased at the invasive front of vertical growth phase melanomas, a critical region of the tumor-stroma interface where cancer stem cells are thought to concentrate. The investigators hypothesize that these changes may reflect epigenetic modifications relevant to the epithelial to mesenchymal transition. Of interest, they also observed that metastatic melanomas demonstrated significantly greater loss of H3K27me3 and H3K4me2 expression than did primary melanomas. The immunohistochemical expression of EZH2, which is known to be essential to stem cell renewal,43 was also noted to be significantly greater in dense nests are visible along its periphery. B, Aggregates of small melanocytes adjacent to larger, epithelioid melanocytes with round to oval nuclei and abundant pink-gray cytoplasm. C, Immunohistochemistry for BAP-1 demonstrates positive nuclear labeling of small melanocyte aggregates along the periphery whereas the larger, epithelioid melanocyte nuclei remain negative or demonstrate loss of BAP-1 (brown, nuclear). Case contributed by Arivarasan Karunamurthy, MD (hematoxylin-eosin, original magnifications ×10 [A] and ×40 [B]; original magnification ×20 [C]).
melanoma cells than in benign nevomelanocytes. Taken together, the investigators hypothesized that such testing could help distinguish benign from malignant melanocytic proliferations and, in addition, help better characterize melanoma stem cells and, potentially, their therapeutic targeting. Of note, immunohistochemistry for H3K27me3 has also been used to distinguish malignant peripheral nerve sheath tumor, wherein H3K27me3 is lost, from spindle cell melanomas, wherein H3K27me3 is strongly positive.44

5-Hydroxymethylcytosine

Finally, loss of the epigenetically modified DNA base 5-hydroxymethylcytosine (5-hmC) as detected by immunohistochemistry (and direct immunofluorescence) has also been shown to be a potentially useful diagnostic and prognostic adjunct in the assessment of melanocytic proliferations.45 5-hmC is the key intermediate of ten-eleven translocase 2 (TET2)–mediated DNA demethylation, a pathway that has been demonstrated to be critical to marking sites of DNA damage as well as the maintenance of telomeres and genome stability.46,47 Loss of 5-hmC has been shown to be a diagnostic hallmark of malignant melanoma and to distinguish among a spectrum of histologic and genetically distinct subtypes of melanoma and benign nevi with high sensitivity and specificity.46–53 In general, benign melanocytic proliferations demonstrate strong nuclear reactivity for 5-hmC, whereas those with increasing malignant potential demonstrate increasing loss of 5-hmC, a finding that has been reproducibly observed in a number of independent studies. In the most recent and largest independent validation study to date, Saldanha et al54 demonstrated that 5-hmC loss was, after controlling for existing staging and prognostic pathologic parameters, independently associated with worse prognosis and that the assay was easily and reproducibly performed. Their experience with 5-hmC led them to propose that this mark be included as a prognostic adjunct to be integrated with AJCC staging criteria.54 A recent study also demonstrated its ability to consistently distinguish malignant melanomas from benign proliferative nodules arising within precursor giant congenital melanocytic nevi.51

Unlike the assays discussed above that require macro/microdissection of tumoral melanocytes, which are left vulnerable to inclusion of nuclei from benign precursor nevus cells, epidermal keratinocytes, or stromal/connective tissue cells, this immunohistochemistry-based assay for 5-hydroxymethylcytosine enables evaluation at a molecular level of each individual cell of interest within the context of an entire histologic section (Figure 2, A through C). To this end, we have also demonstrated that this epigenetic biomarker may facilitate (1) microstaging and Breslow depth assessment of vertical growth phase melanomas associated with either small cell change, pseudomaturation, or preexisting nevus at the deepest aspect of the tumor55; (2) risk assessment of heavily pigmented, histologically ambiguous, and malignant melanocytic tumors, including atypical deep penetrating nevi, pigmented epithelioid melanocyto-

Figure 2. Spitzoid melanoma harboring homozygous 9p21 deletion from thigh of 17-year-old girl. A, Dual-label immunohistochemistry for MART-1 (blue, cytoplasmic) and 5-hydroxymethylcytosine (5-hmC) (brown, nuclear) showing distinct zones of 5-hmC immunoreactivity. B, A predominant pattern of 5-hmC loss is seen within MART-1–positive cells having plump, epithelioid morphology at deeper aspects of the tumor. C, Strong 5-hmC immunoreactivity is seen within spindled MART-1–positive cells within the superficial dermis. This pattern of staining is most consistent with a spitzoid melanoma harboring homozygous 9p21 deletion in association with a preexisting nevus. This patient had a negative sentinel lymph node biopsy without recurrence or further disease progression at 22 months (original magnifications ×20 [A] and ×60 [B and C]).
mas, and blue nevus–like melanomas; and (3) the distinction of nodal nevi from micrometastatic melanoma in the evaluation of SLN biopsies. The latter diagnostic scenario, to date, has the strongest indication for use of 5-hmC by either immunohistochemistry or direct immunofluorescence, which may be because of a greater degree of epigenomic differences inherent to nevomelanocytic rests within the sentinel node and that of micrometastatic melanoma. Although this antibody is commercially available and its immunohistochemical assay easily implemented in most surgical pathology laboratories, in our experience, its interpretation, particularly when faced with the most histologically challenging melanocytic tumors, requires significant experience and may necessitate computer-assisted digital image analysis for the most reliable, objective, and reproducible quantification.

**CLOSING REMARKS**

The assessment of melanocytic tumors by traditional light microscopy is challenging and a subject matter within dermatopathology where experience, expertise, and consensus still provide the highest standard for accurate diagnoses. Thus, for the evaluation of challenging melanocytic tumors, referral to local, national, and international experts in consultation remains a best practice. Alongside the novel genetic and epigenetic molecular tests, which are attractive approaches for diagnostic and prognostic improvements, refining our techniques at interpreting routine histopathology, such as the evaluation of a Breslow density in place of depth and detailed characterization of tumor infiltrating lymphocytes, should not be overlooked. Furthermore, the application of cutting-edge image analysis techniques to whole slide images may also open new doors for technology-assisted histologic assessments. Nonetheless, the development and refinement of diagnostic adjuncts such as FISH, CGH, gene expression profiling, and epigenetically targeted immunohistochemical techniques will significantly aid this practice. Continued, ongoing, and widespread independent, non–industry-led validation studies that apply these techniques to the scenarios of most practical clinical interest and diagnostic relevance, in addition to those that illustrate the ways in which such testing could inform AJCC staging, are needed. Importantly, studies that attempt to align and identify the areas of diagnostic overlap between one methodology and another (ie, 5-hmC immunoreactivity and FISH) are critical, as no single adjunct may be entirely diagnostic and the use of more than one adjunct when considered in light of the histology may provide greater accuracy. In light of cost and economic considerations however, increased consideration of low-cost, high-impact adjunctive diagnostic assays may be preferable.

**References**


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