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REVIEW

Evidence behind the use of molecular tests in melanocytic lesions and practice patterns of these tests by dermatopathologists

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Background: The gold standard for the diagnosis of melanocytic lesions is histologic examination. However, as histologic examination can have its limitations, there are many clinical scenarios in which additional testing may be appropriate in an attempt to render a definitive diagnosis.
Methods: A literature review for three ancillary tests—comparative genomic hybridization (CGH)/single-nucleotide polymorphism (SNP) array, fluorescence in situ hybridization (FISH), and gene expression profiling by quantitative reverse transcription polymerase chain reaction (qRT-PCR)—was compiled and current use patterns were tabulated. Survey of the practice patterns of these tests by dermatopathologists was also accessed in the attendees of the American Society of Dermatopathology Annual Meeting (Chicago, 2016).
Results: Here we summarize the use of these molecular tests in melanocytic lesions. We found that 54.4% of the respondents surveyed utilize (or expect consultants to utilize) molecular testing of melanocytic lesions in their practice when appropriate.
Conclusions: CGH/SNP arrays, FISH testing, and qRT-PCR applied to melanocytic lesions have allowed for more accurate classification. Just over half of those surveyed use molecular testing for melanocytic lesion with the majority sending their cases out for completion of the molecular test.

KEYWORDS

chain reaction, comparative genomic hybridization, fluorescence in situ hybridization, gene expression profiling by quantitative reverse transcription polymerase, melanocytic lesions, single-nucleotide polymorphism

1 | INTRODUCTION

To help better direct dermatopathologists in their use of ancillary tests, the American Society of Dermatopathology (ASDP) created the Appropriate Use Criteria (AUC) Task Force in 2015. The AUC Task Force was divided into four subgroups that each chose to examine 2 to 3 ancillary tests. As a part of this effort, a synopsis of the evidence behind each test was performed.[1] The reviews for each of the four subgroups are intended to be a review of the literature and highlight the data obtained during Short Course I "Best Practices" at the 51st annual meeting of the ASDP. These reviews do not have any specific recommendations. The separate manuscript that details the evidenced-based criteria to assist ordering professionals in making the most appropriate utilizations decisions for specific clinical conditions has now been published.[1]

The Melanocytic Subgroup of the AUC Task Force chose to examine the use of comparative genomic hybridization (CGH)/single-nucleotide polymorphism (SNP), fluorescence in situ hybridization (FISH), and gene expression profiling by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in the diagnosis of melanocytic lesions. As a separate assessment, the results of a survey of the practice patterns of these tests by dermatopathologists attending the ASDP Annual Meeting (Chicago, 2016) are also presented.

The gold standard for the diagnosis of melanocytic lesions is histologic examination. However, as histologic examination can have its limitations, there are many clinical scenarios in which additional testing may be appropriate in an attempt to render a definitive diagnosis. The clinical management and prognosis for the patient depends on the ability to accurately diagnose melanocytic lesions. The Melanocytic Subgroup of the ASDP AUC Task Force chose to explore the appropriate use of CGH/SNP array, FISH, and qRT-PCR assays.

1.1 | CGH/SNP arrays

CGH is a molecular method that can analyze the entire genomic DNA in cells for copy number changes. Two techniques are generally employed. In one, a fluorochrome (usually green) is used to label tumor DNA. The labeled DNA is then mixed in a 1:1 ratio with a reference DNA from normal tissue that has been labeled with a different fluorochrome (usually red). The mixture is subsequently hybridized onto normal metaphase spreads (classic CGH) or hybridized onto a microarray of mapped clones of genomic DNA (array CGH; aCGH). The metaphase chromosomes or the microarrays are washed and then scanned. A green color at a certain locus indicates excess tumor DNA compared to normal and therefore a gain of that region, red color indicates a DNA loss, and yellow color indicates normal copy number compared to the normal reference. In the last decade, aCGH has largely replaced classic CGH because of its higher resolution, reproducibility, and robustness. In the second technique, only tumor DNA is labeled with a reporter fluorochrome and hybridized onto a microarray. Similar to the prior protocol, the arrays are washed and scanned. The copy number status at a certain locus is determined by comparing the signal intensity with a reference from a control series of normal tissues. More recently, SNP microarray platforms have emerged as alternatives to CGH. SNP platforms are able to provide allele frequency data as well as information regarding copy number changes. These platforms can detect copy neutral loss of heterozygosity events and can be designed to identify selected mutations. Protocols using molecular inversion probes specifically designed to work with low quantities of degraded DNA from formalin-fixed paraffin embedded tissue have also been developed in the last years.[2,3] The utility of CGH/SNP arrays is based on the principle that most melanomas to have an unstable genome with numerous chromosomal structural abnormalities while the majority of melanocytic nevi do not display chromosomal aberrations or show specific isolated abnormalities (such as 11p gains in Spitz nevi).[4–7] This non-overlapping pattern of chromosomal aberrations provided an opportunity for diagnostic strategies based on tests evaluating DNA copy number alterations such as CGH/SNP arrays. Figure 1 highlights an example of a case where SNP microarray was performed.

The advantage of CGH/SNP arrays relies in the ability to provide a global overview of the genome. However, CGH may miss changes present only in a small subpopulation of cells. Studies have shown that aCGH can reliably detect a clonal aberration when it is present in at least 30% to 40% of the lesion. For these reasons, aCGH is suboptimal in instances when there is tumoral heterogeneity or the tumor is admixed with benign elements such as inflammatory cells as dilution effect can lead to a false-negative result. Another limitation of CGH is the longer turnaround time for results (often 2-4 weeks), in comparison with other molecular technologies.[8–12]

1.2 | FISH testing

FISH is a molecular technique that uses fluorescent DNA locus specific probes to detect complementary genomic DNA sequences on metaphase and/or interphase nuclei in tissue sections, thus allowing for direct visualization of specific genomic DNA segments. Generally speaking, there are two types of probes that are used for melanocytic lesions: centromere probes that identify centromeric regions on chromosomes and locus-specific probes that hybridize onto target sequences spanning genes or regions of interest. Depending on the type of probe(s), FISH assays have the ability to detect chromosomal deletions, amplifications, and translocations. Figure 2 highlights an example of a case where FISH and SNP array testing were performed.

FISH testing has several benefits compared to CGH including the ability to detect changes in small subpopulations in a heterogeneous lesion, a faster turnaround time, and fewer tissue requirements. On the other hand, FISH also has a few disadvantages: it can only identify changes involving the probed loci resulting in false-negative results, interpretation is labor intensive and requires specialized expertise and can generate false-positive results because of tetraploidy.[13]

1.3 | Quantitative reverse transcription polymerase chain reaction

qRT-PCR is a technique commonly used in molecular biology to detect the level of expression of specific RNA transcripts. Briefly, the technique involves reverse transcription of RNA to complementary DNA followed by real-time PCR. Transcriptome data gathered from large expression array experiments can be evaluated for significant differences of RNA expression between neoplasms and used to develop gene expression signatures capable of differentiating between benign and malignant neoplasms.[14] The Myriad myPath Melanoma gene expression signature test is an example whereby a multivariate gene expression signature was developed and then validated on a training cohort of melanocytic neoplasms. The differential expression of 23 genes involved in cell differentiation and immune signaling between nevi and melanoma forms the basis of the test.[15] This includes one gene specific to melanocytic differentiation (*PRAME*), eight genes implicated in immune signaling (*CCL5*, *CD38*, *CXCL10*, *CXCL9*, *IRF1*, *LCP2*, *PTPRC*, and *SLL*), five genes with multifunctional roles (*S100A9*, *S100A7*, *S100A8*, *S100A12*, and *PI3*), and nine housekeeping genes. A proprietary weighted algorithm is applied to the expression levels to produce a melanoma diagnostic score. A negative score (−16.7 to −2.1) suggests a benign diagnosis, a positive score (0.0 to +11.1) suggests a malignant diagnosis, and a score between −2 and −0.1 refers to an indeterminante category. An advantage of qRT-PCR is that this analysis can detect gene expression changes that may not result from gains or losses.

The scientific evidence behind the use of CGH, FISH, and qRT-PCR for melanocytic lesions in dermatopathology was performed, itemized, and summarized. In addition, an audience response system was used at the beginning of Short Course I “Best Practices” at the

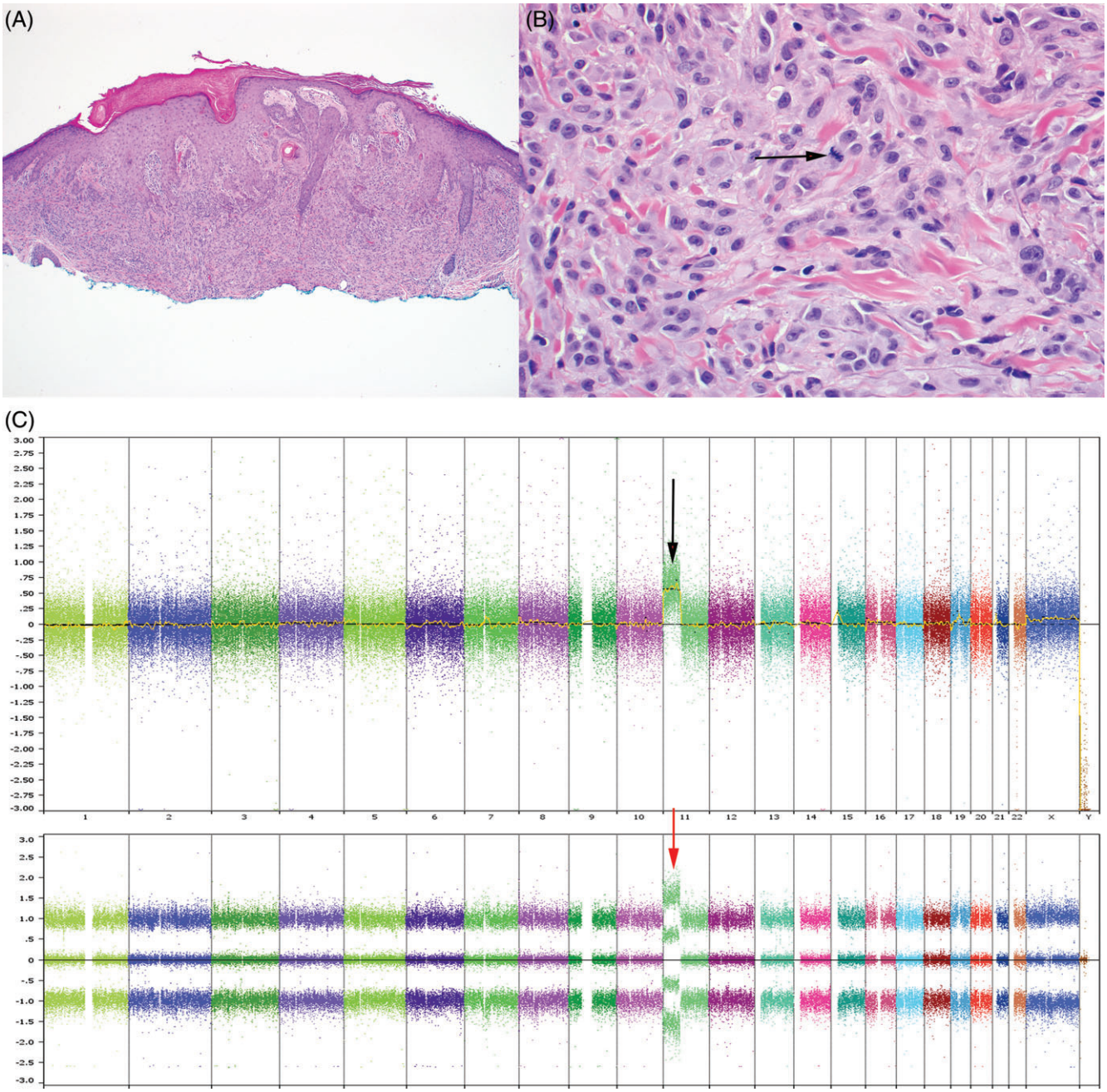

FIGURE 1 Biopsy from a 32-year old woman with a lesion on the face. A, Low-power hematoxylin and eosin (H&E): Predominantly dermal melanocytic proliferation accompanied by epidermal hyperplasia. B, High-power H&E: Melanocytes infiltrate through a sclerotic stroma and have large nuclei with vesicular chromatin, prominent nucleoli and abundant eosinophilic cytoplasm imparting a spitzoid morphology. Few mitotic figures are noted (black arrow). C, Single-nucleotide polymorphism array: Top panel represents the copy number changes and shows a gain of chromosome 11p with no additional abnormalities (black arrow). Bottom panel represents the allele ratio and shows a corresponding allele imbalance at 11p (red arrow). The findings are consistent with a desmoplastic Spitz nevus

53rd annual meeting of the ASDP in Chicago, Illinois, to explore the practice patterns of these molecular assays in the attendees of the course.

2 | MATERIALS AND METHODS

2.1 | Evidence review

Journals written in English from the years 2000 to 2016 were searched in PubMed to find and assess the use of molecular testing (FISH, CGH, and qRT-PCR) in melanocytic neoplasia. The articles were searched using "melanoma," "melanocytic neoplasm," and "melanocytic nevus/nevi" as major keywords that were then overlapped with the specific modes of molecular testing (FISH, CGH, and qRT-PCR). Only articles that mostly examined cutaneous melanomas were included. Articles dealing with non-cutaneous melanomas (e.g., uveal) were excluded, with the exception of anal and conjunctival melanoma. Both case series of fewer than three cases and individual case reports were excluded in the analysis. Research focusing on melanoma cell lines was also excluded.

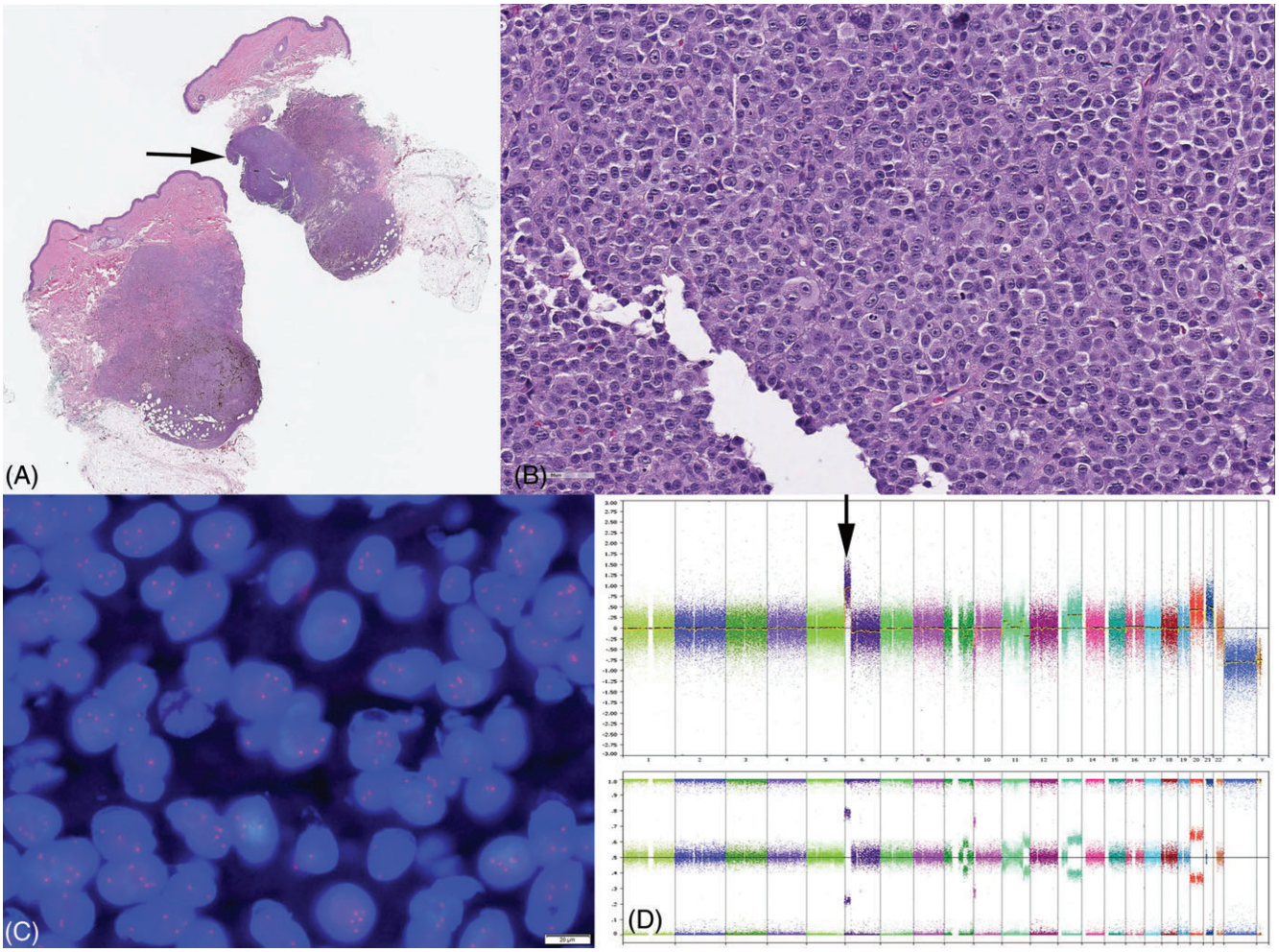

FIGURE 2 Biopsy from the right forearm of a 33-year old man. A, low-power hematoxylin and eosin (H&E): Dermal melanocytic proliferation with the appearance of a cellular blue nevus. A hypercellular nodule is noted (arrow). B, High-power H&E: Nodule composed of cytologically atypical epithelioid cells with conspicuous nucleoli and sheet-like growth pattern. C, Fluorescence in situ hybridization: A probe for 6p25 (*RREB1* gene) shows more than two copies in the majority of nuclei (80%) supporting a diagnosis of melanoma. D, Single-nucleotide polymorphism (SNP) array: Top panel illustrates the copy number changes and confirms the gain of 6p (arrow). Additional gains of 11p, 11q, 13q, 20 and 21 and losses pf 9q, 10p, 11p and 11q can be identified on the SNP array. Bottom panel represents the B-allele frequency and shows corresponding allele imbalances at affected loci. The findings are consistent with a melanoma arising in a cellular blue nevus

2.2 | Practice pattern assessment

The practice patterns of attendees of Short Course I "Best Practices" during the 53rd Annual meeting of the ASDP in Chicago, Illinois for their use of molecular testing in melanocytic lesions was evaluated using an audience response system. This was performed via a web-based platform (Poll Everywhere) that allows participation via a smartphone, tablet, or other internet-connected device by anyone in the audience. The audience was polled prior to presentation of evidence review.

3 | COMMENTS AND CONCLUSIONS

In the dawn of molecular analysis, the genetic exploration of melanocytic lesions led to the discovery that melanomas contain chromosomal gains at 1q, 4q, 6p, 7q, 11q, 17q, and 20q, as well as chromosomal deletions including 9p, 10, and 21q. Gains in 6p were found to be specifically associated with an unfavorable prognosis. Analysis of Spitz nevi highlighted that the majority of Spitz nevi do not show genomic aberrations; about 20% of Spitz nevi show gains in 11p. These crucial discoveries provided the foundation for future studies using the testing modalities described herein, and the commencement of the use of molecular diagnostic tools as an adjunct to histology. The key goals of these tests are to provide more accurate characterization of melanocytic lesions and provide some insight into prognosis.[4,6] This review summarizes the scientific evidence related to the use of CGH/SNP arrays, FISH and qRT-PCR in melanocytic lesions since these pivotal discoveries.

3.1 | CGH in cutaneous melanocytic lesions since the year 2000

Within the literature review, there were 23 articles identified. The majority of these studies were retrospective case series. The number of specimens in the studies ranged from 3 to 186 with a total number

of 856 tests performed on 254 melanocytic nevi, 74 atypical melanocytic proliferations, and 528 melanomas. The melanocytic lesions studied included invasive melanoma of all histopathologic subtypes, Spitz tumors, metastatic lesions, deep penetrating nevi, blue nevi, and proliferative nodules. Eleven of these studies explored and compared the difference of CGH on nevi and melanoma (Table S1 in Appendix S1 in the Supporting Information).

Early cytogenetic studies of melanocytic lesions helped to provide the rationale behind the utility of CGH as an ancillary tool in melanocytic lesions and highlighted the differences between nevi and melanoma. Bastian et al[4] in 2003 applied CGH to 132 melanomas and 54 benign nevi and showed that 96.2% of melanomas had some form of genetic abnormality, while none of the nevi examined, except Spitz nevi (which can show gains in 11p) had any abnormalities. Since this discovery, cytogenetic analysis has also been able to differentiate between melanoma subtypes and allowed for more accurate classification of melanocytic lesions. Differences detected by CGH in acral vs non-acral, mucosal and sun exposed melanomas have highlighted the genetic heterogeneity of melanomas. It is now widely accepted that acral melanomas have copy number gains in 5p, 11q, 12q, and 4q and losses in 6q, and 15q more frequently than melanomas at non-acral sites.[5,16] Sun exposed melanoma, namely, lentigo maligna melanoma, have more frequent losses of chromosomes 17p and 13q.[4] Sinonasal melanomas have a high frequency of 1q, 6p, and 8q gains.[17] Likewise, melanomas that in the past did not fit into a traditional classification can now perhaps be better classified. For example, melanoma with large nests, a form of melanoma that lacks many other histologic features of traditional melanoma, is now regarded as a subtype of superficial spreading melanoma.[18] Although much is still left to be discovered, cytogenetic studies have also shed light on the stepwise progression of melanoma. For instance, in situ melanomas have been shown to have fewer abnormalities than invasive melanomas[19] and melanomas that do not develop metastasis have significantly less chromosomal aberrations ($P < 0.01$) compared with melanomas that develop metastasis.[20]

Moreover, cytogenetic analysis has emphasized the importance of genetics in prognosis. Hirsch et al[21] used cytogenetic analysis on a cohort of melanoma patients stratified into good and poor prognosis groups. They showed that melanomas having a good prognosis have less chromosomal imbalances than melanomas having a poor prognosis that harbor a larger number of chromosomal aberrations, including focal aberrations and chromothripsis. Similarly, it has been shown that melanomas with chromosomal gains in 6p and malignant blue nevi with loses of 3p have a poor prognosis.[22] However, some data show that not all genetic aberrations affect prognosis and one cannot rely on numbers and genetic data alone, and thus it is paramount to correlate clinical and histologic findings with molecular results when classifying melanocytic lesions.[23,24]

While studies continue to be performed with CGH to identify new aberrations associated with melanoma and their effects on prognosis, the literature suggests that CGH is an established adjunctive test in the diagnosis of melanoma in various clinical scenarios when a definitive diagnosis cannot be rendered.

3.2 | FISH in cutaneous melanocytic lesions since the year 2000

Review of the literature has shown that FISH has the potential to serve as an adjunct to morphology and immunohistochemistry in challenging cases. Within the literature, 60 articles on FISH were identified based on the search criteria. The majority of these studies were retrospective case series. Within a single study, the number of specimens ranged from 3 to 804 with a total number of 5283 tests performed on 1895 melanocytic nevi, 1398 atypical melanocytic proliferations, and 1990 melanomas. The melanocytic lesions included in the studies covered a broad range of melanocytic neoplasms, including spitzoid tumors, ambiguous melanocytic lesions, various subtypes of melanoma, metastatic melanoma, dermal melanocytosis, proliferative nodules, and conjunctival and anal melanocytic lesions. Two articles specifically included pediatric-aged cases in their series (Table S2 in Supporting Information).[25,26]

In general, FISH testing for melanoma is a multiplex assay including probes targeting loci that have been found to be frequently altered through CGH technology.[27] The clinical utility of FISH has been explored in a number of studies. While many studies have evaluated differences between nevi and melanoma, there are also an abundance of articles looking at ambiguous or controversial lesions.[20,21,27–37] Likewise, studies have confirmed the usefulness of FISH in the differentiation of conjunctival nevi from melanoma,[38] epithelioid blue nevi from blue nevus-like cutaneous melanoma metastasis[39] and in melanocytic lesions with a large epithelioid component.[40] FISH has also been used in the distinction of Spitz nevi from spitzoid melanoma.[41] Similarly, the utility of FISH in differentiating nodal nevi from metastatic melanoma has been reported.[42]

However, caution must still be used as limitations of this test have been identified. Certain melanoma subtypes have a higher rate of false-negative FISH results using the standard panel; therefore, more research may be needed to develop probes that yield higher sensitivities. One such example is the utility of FISH in distinguishing sclerosing nevi from desmoplastic melanoma. A positive FISH result is helpful in confirming melanoma, but a negative result does not equate with benignancy.[43]

The standard FISH panel has evolved over time. An initial study investigated the performance of 14 FISH probes targeting the loci most commonly altered in melanoma.[27] Using sets of unequivocal lesions (benign and malignant), the authors determined that a panel of probes targeting 6p25 (*RREB1*), centromere 6, 6q23 (*MYB*), and 11q13 (*CCND1*) provided the highest discrimination between the two groups with a sensitivity and specificity of 86.7% and 95.4%, respectively. The test uses a scoring algorithm that involves evaluation of at least 30 nuclei from lesional cells for the number of probes and records the percentage of nuclei with >two signals for 6p25 and 11q13 and with less signals for 6q23 compared to centromere 6. The test is considered positive if any one of these percentages exceeds the validated cutoffs.[21,28,29] Since then, several studies have examined alternative cut-off levels and used other combinations of probes.[44] Because of the relative poor performance of the initial FISH panel in the subsets of spitzoid and spindle melanomas, a second generation of the probe set was developed that included probes for 9p21 (*CDKN2A*) and 8q24

TABLE 1 Practice patterns of attendees of the American Society of Dermatopathology annual meeting (Chicago, 2016) for comparative genomic hybridization, fluorescence in situ hybridization, and quantitative reverse transcription polymerase chain reaction in melanocytic neoplasms

Audience response questions	Answer choices	Number of respondents (%)
How often do you consider insurance issues or patient-related costs before ordering molecular testing for melanocytic lesions?	Almost always	90 (53.9)
	More than half of the time	17 (10.2)
	Less than half of the time	15 (9.0)
	Rarely	45 (27.0)
Do you use molecular testing in your practice (or expect your consultants to utilize) when appropriate?	Yes	93 (54.4)
	Rarely (less than five times a year)	64 (37.4)
	Never	14 (8.2)
Do you perform molecular testing at your practice/institution?	Yes	54 (30.7)
	No, but I send out for it to be performed	103 (58.5)
	No, because I do not use the procedure/test	19 (10.8)

(*MYC*).[45] It has been proposed that adding these probes to the original probe set increases sensitivity to 94% and specificity to 98% for these lesions. In addition, it has been proposed that this probe set provides prognostic information in borderline spitzoid tumors.[46] It should be noted that these values have not yet been replicated in large subsequent studies so — as with all new ancillary tests in diagnostic pathology — these may be judged with caution at this point in time.

Overall the literature shows a relatively high sensitivity and specificity for FISH with the sensitivity ranging from 85% to 100% and the specificity ranging from 90% to 98%. The variation is because of the probe set used for the assay and the cutoff thresholds set. These ranges are significantly lower for ambiguous lesions, where the sensitivity and specificity are 43% to 100% and 33% to 89%, respectively. While it is clear when reviewing the literature that FISH is not able to give a definitive answer of benign or malignant, it does appear that in certain clinical scenarios, FISH may be informative as an ancillary study.

The prognostic significance associated with FISH in melanocytic lesions has also been studied. In looking at paired primary and metastatic melanomas, primaries that were FISH positive had a higher rate of metastasis and melanoma-associated deaths compared with FISH-negative cases, although in this study only the primary melanoma was studied with FISH.[47] Research has shown that gains in 11q13 and 8q24 are thought to be predictive of metastasis.[48] Likewise, in a study examining atypical spitzoid tumors with borderline histology, cases with homozygous losses of 9p21, gains in 6p25 or 11q13 were at a higher risk for aggressive clinical behavior compared to atypical spitzoid tumors that were FISH negative or had isolated 6q23 deletions that did not show clinical progression.[49]

3.3 | qRT-PCR in cutaneous melanocytic lesions

Within the literature, there were four articles on qRT-PCR for review. The studies included a case series, a validation, a validation with a prospective cohort, and a prospective cohort. Within a single study, the number of specimens ranged from 117 to 1695 with a total number of 3649 tests performed on 2001 melanocytic nevi, 481 atypical melanocytic proliferations, and 1167 melanomas (Table S3).

The two validation studies suggest qRT-PCR has a sensitivity of 90% to 91.5% and a specificity of 91% to 92.5% for melanoma.[25,50] In one study, there was 97% and 83% concordance with histology for FISH and qRT-PCR in a group of unequivocal melanocytic lesions resulting in a sensitivity and specificity of 93% and 100% for FISH and 62% and 95% for qRT-PCR. The intertest agreement was found to be 80%.[51] In another study, for diagnostically challenging cases initially diagnosed as indeterminate by histomorphology and immunohistochemistry, definitive diagnoses increased by 56.6% following qRT-PCR testing.[52] As the literature for qRT-PCR is in its infancy, future studies will probably dictate the utility and clinical scenarios where this ancillary test may be helpful.

3.4 | Practice patterns of FISH, CGH, and qRT-PCR in cutaneous melanocytic lesions

To identify the practice patterns of CGH, FISH, and qRT-PCR, an audience response system was used to survey attendees of Short Course I "Best Practices" during the 53rd annual meeting of the ASDP (Chicago, Illinois, 2016). The number of respondents ranged from 167 to 176 for each question related to molecular testing of melanocytic lesions. It is difficult to provide a precise overview of the expertise level of these participants and "expertise" certainly has an element of subjectivity. This could be considered a limiting factor in the interpretation of the results. But given the nature of this subspecialty conference, it can be assumed that the vast majority were practicing dermatopathologists or dermatopathologists in training. Fifty-four percent of respondents reported routine use (or expecting consultants to use) of molecular testing for ambiguous melanocytic lesions with an additional 37% reporting rare use (less than five time each year), while only 8% of respondents never used molecular testing for this scenario. Not surprisingly, only 31% of respondents reported performing molecular testing at their practice or institution, with an additional 59% report using outside labs to perform these tests. This probably reflects the technical difficulty in performing these assays and the fact that qRT-PCR is generally only available as a send out test (Table 1).

While cost is not a factor when creating AUC, more than half (54%) of respondents report "almost always" considering insurance or patient-related costs prior to ordering molecular testing for melanocytic lesions and only 27% rarely consider cost. Because cost is a significant factor in ordering these ancillary studies, criteria that identify clinical scenarios where testing improves diagnosis and outcomes with

a high degree of specificity and sensitivity will be helpful to practicing dermatopathologists.

To reiterate, this article is intended to be a review of the literature and highlight the data obtained during Short Course I "Best Practices" at the 51st annual meeting of the ASDP. This review does not have any specific recommendations. As the work of the AUC task force of the ASDP continues, appropriateness ratings to better help guide dermatopathologists in the selection of these tests in commonly encountered scenarios in clinical practice will be reported.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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