

Molecular Diagnostics for Ambiguous Melanocytic Tumors

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Certain subsets of melanocytic neoplasms are difficult to classify because of conflicting histologic features and the existence of a poorly defined intermediate grade of melanocytic tumors. The integration of molecular diagnostic information with a histologic impression may contribute significantly toward improving classification. This review discusses the development of and advances in molecular techniques, including comparative genomic hybridization and fluorescence in situ hybridization (FISH) as diagnostic and prognostic tools for melanocytic neoplasms. Further, we discuss how specific molecular aberrations identified via FISH correlate with certain morphologies in melanocytic neoplasms. We also examine the prognostic value of FISH in intermediate-grade melanocytic tumors, particularly atypical Spitz tumors.

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The vast majority of conventional melanomas and melanocytic nevi may be readily classified as entirely benign or malignant using standard microscopy. However, there are at least 2 distinct factors responsible for the diagnostic discrepancy and uncertainty that dermatopathologists face when dealing with certain subsets of melanocytic tumors:¹⁻³

1. Some lesions that are completely benign or entirely malignant in their biologic potential have ambiguous histologic features that make proper classification difficult. Benign neoplasms in this category include some atypical Spitz and Reed nevi,¹ some epithelioid blue, ancient or clonal nevi, desmoplastic nevi, and nevi from special sites, such as genital,⁴ acral, or scalp. The most significant malignant neoplasm in this category is nevoid melanoma, which can have an incredibly banal-appearing silhouette and subtle

atypia with clinical behavior identical to that of similarly staged conventional melanomas.⁵

2. The presence of an intermediate grade of melanocytic tumors shows frequent involvement of sentinel lymph nodes with significantly less, but occasional, disease progression beyond the sentinel lymph node.^{6,7} The existence of intermediate-grade melanocytic neoplasms is generally gaining acceptance among the dermatopathology community.⁸

Melanocytic neoplasms, such as pigmented epithelioid melanocytoma and some atypical Spitz tumors, likely fit into this second category. The vast majority of these neoplasms have a good overall prognosis; involvement of the sentinel lymph node is frequent, whereas only a small but definitive proportion of patients end up developing distant metastasis and death. The lack of association between sentinel lymph-node biopsy (SLNB) results and prognosis as compared with conventional melanomas supports a separate classification for these neoplasms.^{6,9,10} Microscopically, these tumors may also show conflicting histologic features, some of which are more consistent with malignancy than others.

Comparative Genomic Hybridization and Fluorescence In Situ Hybridization

Integrating molecular and histologic data may allow for improved classification and risk assessment of many of these subsets of melanocytic tumors with ambiguous histopathology. Over the past 10 years, techniques, such as comparative

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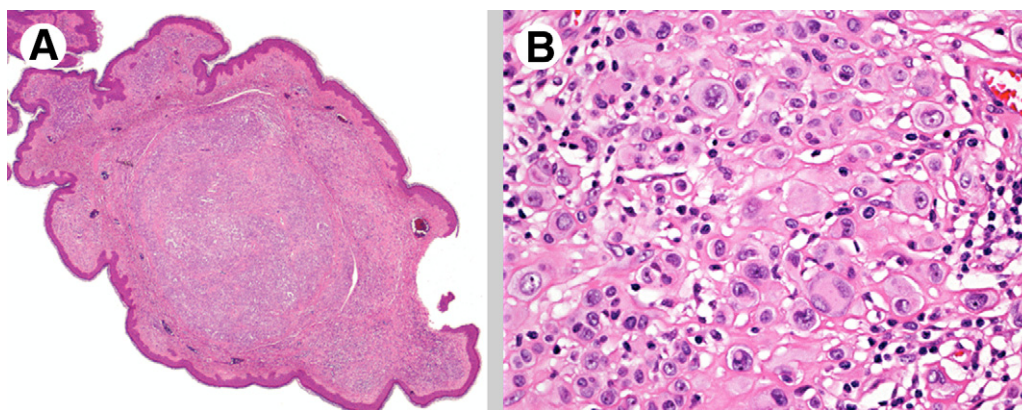


Figure 1 Typical combined melanocytic tumor with epithelioid Spitz tumor component seen in patients with BAP1 familial uveal melanoma syndrome. (A) Pedunculated melanocytic neoplasm with conventional nevocytic nests at periphery and epithelioid Spitz tumor component centrally. (B) Higher power magnification of the epithelioid Spitz tumor component. (Color version of figure is available online.)

genomic hybridization (CGH) and fluorescence in situ hybridization (FISH), have demonstrated that melanomas and benign nevi differ at the chromosomal level.

Melanomas carry frequent chromosomal copy-number aberrations not seen in conventional benign nevi, including copy-number gains at 1q, 6p, 7, 8q, 17q, and 20q as well as deletions at 6q, 8p, 9p, and 10q.^{11,12} Benign nevi, by contrast, typically lack chromosomal copy-number aberrations and are diploid with the exception of some subsets of Spitz nevi, which may have specific recurring copy-number aberrations.¹³ Specifically, 20% of Spitz nevi may have copy-number gains at 11p where the HRAS gene is located. These Spitz nevi are typically large bulky lesions with prominent sclerosis of the deep-dermal component. Predominantly, dermal epithelioid Spitz nevi may have deletions of all of chromosome 3, all of 3p, or only an interstitial portion around the Bap-1 locus on 3p^{12,14,15} (Fig. 1). This stark contrast allows for techniques evaluating chromosomal copy-number aberrations to be used in diagnosis. Specifically, the identification of characteristic targets has become a springboard for the development of targeted diagnostic FISH assays.

CGH was developed in the early 1990s, and for the first time, it offered the ability to assess the entire genome of a tumor for copy-number gains or deletions in 1 experiment.¹⁶ The ability to perform such a comprehensive genomic evaluation in a single experiment is among the greatest benefits of CGH. Furthermore, since the inception of CGH, newer array-CGH techniques have been developed allowing for higher-level resolution of specific copy-number gains and deletions.¹⁷ However, this technique requires a relatively pure tumor population and any low-level copy-number aberration needs to be present in approximately one-third of the tumor for it to be identified.¹⁸ Hence, the technique is ideal for large bulky melanocytic tumors. In thin lesions, small melanomas arising in a nevi or neoplasms with heavy inflammatory response are less likely to provide meaningful results.

Using data derived from CGH to identify ideal probe targets, several FISH assays were developed recently to aid in the diagnosis of melanocytic neoplasms. The very first FISH as-

say was developed through a series of 4 cohorts of melanocytic neoplasms looking at a combination of 4 chromosomal loci that together could best differentiate between conventional melanoma and nevi. The combination of probes selected included probes targeting 6p25, 6q23, Cep6, and 11q13.¹⁹ The selection of 3 probes from 1 chromosome was based on the frequent interchromosomal rearrangements of chromosome 6 in melanoma. Validation studies have documented a sensitivity of anywhere between 82% and 94% with a specificity between 90% and 98%.^{20,21} Smaller studies have further validated the utility of this probe set in several specific diagnostic scenarios, including distinguishing atypical cellular blue nevi from blue nevus-like melanomas, differentiating epithelioid blue nevi from blue nevus-like metastases, nevoid melanomas from mitotically active nevi, conjunctival nevi from conjunctival melanoma, and sclerosing nevi from desmoplastic melanomas.^{5,22-26} The probe set was subsequently CE marked for evaluation of diagnostically challenging melanocytic neoplasms in Europe.

Diagnostic Dilemmas in Melanocytic Pathology

As previously mentioned, differentiating Spitz nevi from Spitzoid melanomas and performing risk assessments of atypical Spitz tumors are among the most challenging diagnostic dilemmas in melanocytic pathology.²⁷⁻³⁰ The sensitivity of the first melanoma FISH assay for melanomas with Spitzoid morphology was determined to be approximately 70%.³¹ Through evaluation of a broad number of Spitzoid melanomas, it was noted that deletions at 9p21 were among the most frequent copy-number aberrations seen in melanoma.^{11,12} Despite this, the probe targeting 9p21 was not selected for the initial melanoma probe set because an ideal discriminatory test must identify an aberration seen in melanomas, but not in nevi. The initial probe selection studies only assessed for heterozygous/monoallelic 9p21 deletions.¹⁹ Heterozygous 9p21 deletions may be seen in some benign melanocytic neoplasms, diminishing the discriminatory value of this parameter.

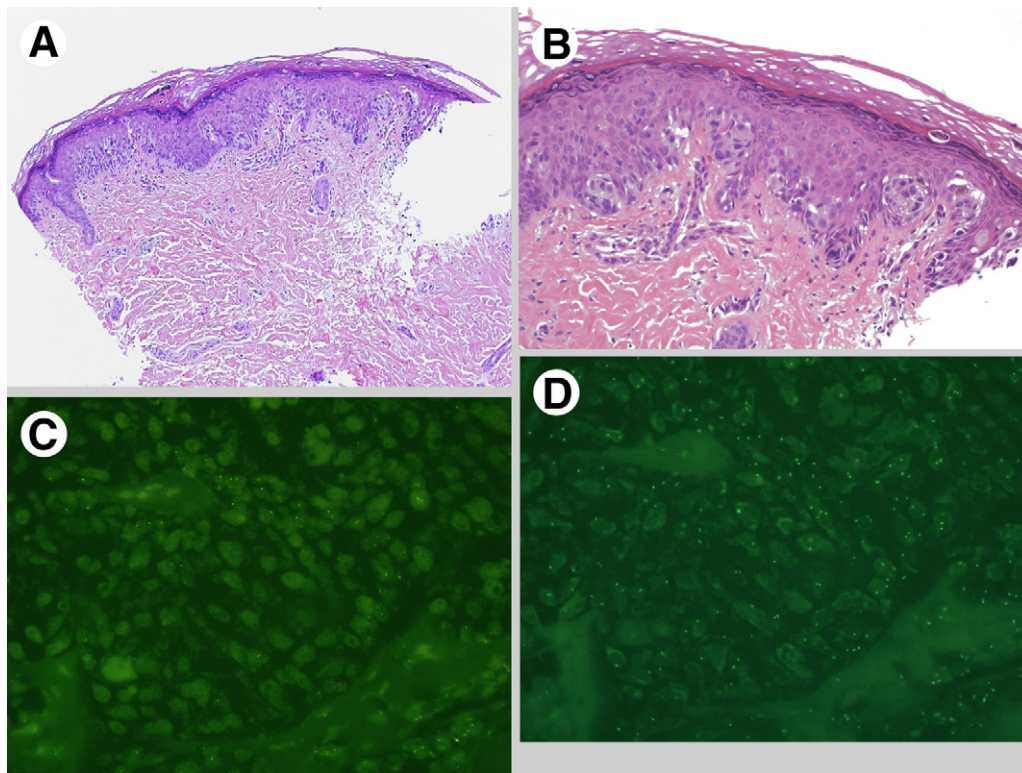


Figure 2 (A and B) Hematoxylin and eosin-stained sections at 10 \times and 20 \times of a melanoma in situ from a 5-year-old girl showing large epithelioid melanocytes with nuclear atypia, coarsely pigmented cytoplasm, and irregularly distributed pagetoid spread. The specimen was considered too superficial for reliable assessment with comparative genomic hybridization. (C) FISH in the gold channel with probe targeting 9p21 showing a large intraepidermal nest of melanocytes with homozygous deletion of 9p21. (D) FISH in the green channel of Cep9 showing the same intraepidermal melanocytic nest with most cells having 1 or 2 signals per nucleus. (Color version of figure is available online.)

In a separate study, we found that homozygous 9p21 deletions are highly specific to malignant melanocytic neoplasms and of high discriminatory value in Spitzoid melanocytic neoplasms.³¹ On this discovery, we repeated the initial process of probe selection for developing a FISH assay; however, this time, we included homozygous 9p21 deletions as one of the evaluated parameters.³² The final 4 probes selected included 6p25, 9p21 (evaluating homozygous 9p21 deletions), 11q13, and 8q24. This probe set is better tailored for evaluating Spitzoid melanocytic neoplasms because of the inclusion of the 9p21 probe. The probe set is also less vulnerable to false positivity as a result of tetraploidy because 4 loci from 4 distinct chromosomes are targeted.³² This newer probe set that includes 6p25, 11q13, 8q24, and 9p21 may be commercially available soon. In validation studies, the sensitivity was 94% and the specificity was 98%.³² Copy-number aberrations in 8q24 are uncommon in Spitzoid neoplasms, but this probe may be highly useful in diagnosing amelanotic nodular nevoid melanomas. An alternative for evaluating Spitzoid neoplasms is to run the original melanoma probe set in tandem with a second slide with probes that target 9p21 and Cep9.

Both FISH and CGH can be powerful tools assisting dermatopathologists in the diagnosis of histologically challenging melanocytic tumors, with each offering distinct advantages. Advantages of FISH include:

1. The minimal tissue requirement, the procedure can be performed on a single slide with tissue cut at 5 μ m.
2. The procedure is rapid and can be completed in a 48-hour window.
3. There is an easy correlation with histology because one can visualize the copy-number aberrations within the specific cells on immunofluorescent sections. Hence, smaller superficial lesions, including melanoma in situ (Fig. 2) and melanomas arising in nevi, can be evaluated.
4. Furthermore, because the copy-number aberrations within specific cells can be seen, it is easy to determine if there are heterozygous or homozygous deletions, which as noted earlier are particularly important for 9p21. The distinction between homozygous 9p21 deletions and heterozygous 9p21 deletions may not be evident by CGH.

Advantages of CGH include a more comprehensive genomic evaluation identifying copy-number gains and losses throughout the entire genome in a single experiment. This includes identification of 3p losses common in epithelioid Spitz tumors, 11p gains also seen in a subset of Spitz nevi, and other copy-number gains, such as 7q34 typical of conventional melanomas as well as others.

Both CGH and FISH have clearly shown that there are differences at the chromosomal level between conventional

melanomas and benign nevi. However, the ultimate question is how well these tools can predict prognosis in intermediate-grade melanocytic tumors, such as atypical Spitz tumors. There is accumulating evidence of a strong correlation of prognosis with some specific chromosomal copy-number aberrations; however, there has been some variability in the results of different investigators. There are several reasons underlying this variability. The first and foremost of these is our current incomplete and continuously evolving understanding of atypical Spitz tumors.

Recently, Ludgate et al⁶ provided data to support conventional wisdom among expert dermatopathologists regarding sentinel lymph-node involvement of atypical Spitz tumors. In a series of 67 patients with atypical Spitz tumors, of whom 57 underwent SLNB, 27 (47%) had involvement of the sentinel lymph nodes with an atypical Spitz' tumor. All 27 patients with positive SLNB were alive with no evidence of disease at past follow-up.⁶ The discordance between sentinel lymph-node involvement and distant metastasis has also been supported by several other recent studies.^{9,10} Distant metastasis or death of disease from a Spitz tumor is an infrequent event, and even collaborative studies involving multiple major tertiary care centers often struggle to identify a handful of such cases. However, such cases do exist, and identifying those cases at the most risk remains a highly enigmatic process.

FISH and CGH studies offer a potential avenue to perform a more objective risk assessment of atypical Spitz tumors. However, in our opinion, it is insufficient to assume that because a specific chromosomal copy-number aberration is more typical of conventional melanomas than benign nevi, that the presence of such an aberration is diagnostic of malignancy or the aggressive clinical course in intermediate-grade melanocytic tumors, such as atypical Spitz tumors. The data need to be validated within the appropriate patient population.

Among 27 patients with atypical Spitz tumors, we found that 6 cases exhibited bulky lymph-node metastasis, distant metastasis, or death.¹⁹ In 2 of the 6 cases, the disease only progressed to lymph-node metastasis. As a result, distant metastasis or death was seen in only 4 of our patients. A positive FISH result was obtained for all 6 cases using the conventional CE marked probe set for melanoma targeting 6p25, 6q23, Cep6, and 11q13. Using the same probe set, 6 of the 21 nonmetastasizing atypical Spitz tumors were also FISH positive. A Fisher's exact test comparing the frequency of metastasis in the FISH-positive versus the FISH-negative cases produced a highly significant *P* value of .003.¹⁹ When Vergier et al²¹ studied 113 ambiguous melanocytic lesions, distant metastasis or death of disease was seen in 13 patients. This study also led them to conclude that using FISH in conjunction with histologic evaluation can improve diagnostic accuracy.²¹ In another study, Massi et al³³ found that among 38 atypical Spitz tumors, only 1 patient had distant metastasis and death. FISH results revealed this patient also had multiple chromosomal copy-number aberrations.³³

In contrast, Gaiser et al classified³⁴ 12 histologically ambiguous lesions as benign or malignant based only on clinical endpoints. They came to the conclusion that FISH did not reach a level of sensitivity or specificity that was clinically useful. Inves-

tigators did have some discordant CGH and FISH data. Certain copy-number changes were identified only by CGH, even though the locus was also targeted by the FISH assay.³⁴ Because at least 30%-50% of cells collected must have a specific chromosomal aberration for it to be detectable by CGH, the aberration should also be detected by FISH because FISH can identify smaller aberrant tumor populations.¹⁸ This result raises concern of possible methodological difficulties. It should also be noted that investigators did not use the criteria reported in the multi-site FISH validation study. Among the 16 patients with AST identified by Raskin et al,³⁵ 15 were alive with NED at the most recent follow-up and only 1 died of metastatic disease. None of the AST cases had copy-number aberrations at chromosomes 6 or 11. In this study, an outside laboratory performed the FISH assays.³⁵

Our lab has been using FISH as a diagnostic tool for approximately 4 years, and we have had extensive personal experience regarding the benefit and utility of identifying chromosomal copy-number aberrations as a discriminatory tool for melanocytic neoplasms. What has become readily apparent to us is that not all chromosomal copy-number aberrations are of equal value, and therefore, a "positive FISH" result may not have nearly the same significance as another "positive FISH" result. Hence, what is needed is a larger study of atypical Spitz tumors with complete follow-up assessing how each individual chromosomal aberration relates to the risk for aggressive behavior that could provide significant objective measures for risk assessment of atypical Spitz tumors. Such a study is underway and thus far, our results suggest that atypical Spitz tumors with homozygous 9p21 deletion present the greatest risk of more aggressive clinical behavior (personal data).

Multiple studies have shown that specific chromosomal copy-number aberrations identified by FISH may provide significant prognostic information in conventional melanomas. In a series of 144 melanomas of Breslow depth > 2 mm, FISH-positive lesions had a significantly increased risk of metastasis or melanoma-specific mortality when compared with the FISH-negative lesions.³⁶ A separate study showed that among a series of potential chromosomal copy-number aberrations seen in cutaneous melanoma, copy-number gains of CCND1 and MYC at loci 11q13 and 8q24 were most prognostic. In a case-control study of 55 metastasizing and 42 nonmetastasizing melanomas of similar Breslow depths, gains at 8q24 and 11q13 were second only to ulceration in their prognostic potential.³⁷ Amplification of CCND1 may be seen in the form of homogeneous staining regions, which when present seem to consistently correlate with an adverse prognosis.³⁷ We previously reported the case of a relatively thin melanoma with cyclin D1 homogeneous staining regions that followed an aggressive course.³⁸ We subsequently reported similar results in a follow-up series of 7 additional patients.³⁹

Conclusion

In conclusion, there are strong data showing that conventional melanomas and benign nevi differ in patterns of possible chromosomal aberrations and that among conventional melanomas, certain specific aberrations correlate with more aggressive be-

havior. Less data are available for prognostication and risk assessment of intermediate-grade tumors. To definitively validate the utility of FISH and CGH for the identification of chromosomal copy-number aberrations in intermediate-grade tumors, a large sample of atypical Spitz tumors with prolonged follow-up, including some cases with adverse events, need to be evaluated. Furthermore, it would be important to assess these cases for a broader spectrum of FISH probes than just the original 4-probe melanoma FISH assay because not all copy-number aberrations have the same significance and that certain aberrations, such as homozygous 9p21 deletion, are especially frequent in Spitzoid melanomas. We are in the process of finalizing a multicenter study, which should provide clinically meaningful and objective data for risk assessment of atypical Spitz tumors.

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