Fluorescence In Situ Hybridization as an Ancillary Tool in the Diagnosis of Ambiguous Melanocytic Neoplasms

A Review of 804 Cases

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Abstract: Previous studies have demonstrated the utility of fluorescence in situ hybridization (FISH) as an ancillary method in the diagnostic workup of histopathologically ambiguous melanocytic neoplasms. A combination of probes targeting 3 loci on chromosome 6 and 1 on 11q has been reported to distinguish unequivocal melanomas and nevi with a sensitivity and specificity of 87% and 96%, respectively. However, information on how FISH should be integrated into routine clinical testing is limited. We report our experience of FISH testing of 804 ambiguous melanocytic lesions performed as part of routine workup at University of California, San Francisco. The main category (47% of all cases) for which FISH testing was requested was Spitz tumors. Other categories included the distinction of possible melanoma from combined nevi (9%), acral or mucosal nevi (9%), Clark/dysplastic nevi (7%), and blue or deep penetrating nevi (6%) and to assess the possibility of nevoid melanoma (4%). Of the ambiguous tumors successfully tested, 88% received a more definitive benign or malignant final diagnosis. Of the 630 cases that tested negative by FISH, the final diagnosis was benign in 489 (78%) cases, ambiguous in 91 cases (14%), and malignant in 50 cases (8%). A positive FISH result was observed in 124 cases, with a final diagnosis of melanoma in 117 (94%). One (1%) FISH-positive case had an equivocal final diagnosis, and 6 (5%) were interpreted, despite the positive FISH result, as melanocytic nevi. We conclude that FISH testing can help reduce the number of equivocal diagnoses in ambiguous melanocytic neoplasms, in particular if FISH testing is positive, and discuss the challenges and limitations of FISH in clinical practice.

Key Words: Spitz tumor, melanoma, FISH, molecular testing, ambiguous melanocytic tumors, fluorescence in situ hybridization

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The current gold standard for melanoma diagnosis is histopathology. However, a subset of melanocytic neoplasms cannot be unequivocally separated into benign and malignant categories. Neoplasms within this category are a source of diagnostic error, as demonstrated by studies that show significant discordance rates using routine examination, ranging from 14% to 38%, even between expert dermatopathologists.[1–7] Criteria for the diagnosis of melanoma have evolved to avoid missing malignant lesions and as a consequence trade increased sensitivity for decreased specificity. In addition, the application of these criteria to thinner lesions disproportionately increases the incidence of the disease, and lowers its seeming lethality, as few thin melanomas go on to metastasize. Together, these factors have contributed to the increase in melanoma incidence observed over the last decades. This view is supported by the following: (1) despite the increasing incidence of melanoma, mortality rates have remained relatively constant[8]; and (2) melanoma incidence has increased proportionally with the number of biopsies performed.[9] Diagnostic tests that can increase the accuracy of melanoma diagnosis are therefore needed. Ideally, these tests would generate quantitative information to reduce the subjectivity inherent to the diagnostic process.

Genetic analyses of melanomas have revealed a number of recurrent aberrations that are absent in unequivocally benign lesions.[10] In an attempt to develop a fluorescence in situ hybridization (FISH) assay to detect common aberrations in melanoma, probes against 20 different genomic regions were evaluated.[11] A combination of probes targeting 6p25 (*RREB*), 6q23 (*MYB*), centromere 6, and 11q13 (*CCND1*) performed best and was able to differentiate unequivocal melanomas and melanocytic nevi with a sensitivity and specificity of 87% and 96%. A subsequent study in unequivocal melanomas showed that patients with a positive test were significantly more likely to develop metastases or die of melanoma compared with patients whose melanomas were negative by FISH.[12] This result indicates that even among melanomas, cases that are FISH negative are less likely to lead to bad outcomes than those that are positive. However, it is clear that a clinically relevant proportion of bona fide melanomas test negative by FISH. Pathologists who use FISH as part of their workup of ambiguous melanocytic

neoplasms must carefully weigh the limitation of FISH testing to integrate test results into their decision-making process. In this study, we summarize the experience using FISH in a clinical setting at a single institution and analyze how FISH testing affected diagnostic reporting on histopathologically ambiguous melanocytic neoplasms in our practice.

METHODS

We collected the following data from all melanocytic lesions in which FISH was used as an ancillary diagnostic method from 2008 through 2010 at the UCSF Dermatopathology Service: (1) the histologic classification of the melanocytic neoplasm; (2) the level of concern for melanoma before FISH testing on a 3-point scale, as determined by the wording of the preliminary report issued before testing (the lesion was considered likely benign, ambiguous, or likely malignant); (3) the FISH results including probe-level data for each enumerated nucleus; and (4) the final diagnosis rendered after FISH testing. The final diagnosis was categorized on a 5-point scale as either benign, likely benign, ambiguous, likely malignant, or malignant. FISH analysis was carried out as previously described using probes targeting 6p25 (*RREB1*), centromere 6, 6q23 (*MYB*), and 11q13 (*CCND1*).[11] Signals were scored according to previously determined criteria,[11] and the test was considered positive if 1 of the following 4 criteria was met: (1) 55% or more nuclei had higher 6p25 signal counts than CEP6 counts (relative 6p gain); (2) 40% of nuclei had lower 6q23 signal counts than CEP6 counts (6q loss); (3) 29% or more nuclei had >2 signals for 6p25 (6p gain); or (4) 38% or more nuclei had >2 signals for 11q13 (11q gain).

The attending dermatopathologist determined the indication for FISH testing on cases received as either wet tissue or as consultations. After hybridization, all tumor areas were evaluated by a dermatopathologist (B.C.B., T.H.M., or P.E.L.) to select a minimum of 3, typically 6 to 8, high-power fields ($\times 40$ objective), which on the basis of visual inspection are most suspicious to harbor clonal chromosomal imbalances. For each of the 4 color channels from these areas, z-stacks of 9 black and white images were taken with a CCD camera at 0.2 μm intervals to capture hybridization signals across the entire focal range. One image in the center of that range was taken for the DAPI channel to identify the nuclei. The image stacks were processed using the FISH signal enumeration tool of the software package Metafer (Metasystems). A trained technician reviewed the automatic counts generated by Metafer and excluded non-neoplastic nuclei. A minimum of 30 nuclei were counted for the calculation of the parameters used to interpret the test. The selection of nuclei to be enumerated was verified by a dermatopathologist (B.C.B., T.H.M., or P.E.L.), who also performed the final interpretation of the test and subsequently sent the report to the requesting dermatopathologist within our service, who integrated the information into the final assessment of the case.

Data Analysis

For each parameter, we determined the degree of deviation from the above thresholds by dividing each parameter (relative 6p gain, relative 6q loss, 6p gain, 11q gain) by its respective threshold. Accordingly, values >1 indicate that the threshold was exceeded, whereas values <1 indicate that the threshold was not met. These weighted data were clustered using Gene Cluster 3.0 and displayed as a heatmap using Java TreeView (http://jtreeview.sourceforge.net/).

RESULTS

A total of 804 melanocytic lesions underwent FISH analysis at UCSF from 2008 to 2010. The majority (378 or 47%) were spitzoid melanocytic neoplasms. Seventy-two (9%) FISH tests were ordered for the differential diagnosis between combined nevus and melanoma arising within a nevus, 72 (9%) for the differential diagnosis of acral (n = 59) or mucosal (n = 13) melanoma versus nevus, 53 (7%) for the differential diagnosis between dysplastic nevus and melanoma (7%), 46 (6%) for the differential diagnosis between blue or deep penetrating nevus and blue nevus-like melanoma, and 31 (4%) to distinguish between nevus and nevoid melanoma (Table 1). Acral and mucosal melanomas were grouped together because of their shared non–UV-related genetic pathways that exhibit frequent genomic amplifications compared with melanomas at other anatomic sites.[13] Representative images for each histopathologic category are depicted in Figures 1–6. One hundred and fifty-two (18%) cases did not fulfill these categories and were categorized as melanoma versus nevus not otherwise specified (NOS).

FISH Results

Of the 804 cases sent for analysis, 50 cases (6%) had insufficient tissue remaining in the block for testing or failed to yield interpretable fluorescence signals. Of the 754 successfully analyzed cases, 124 (16%) were interpreted as FISH positive (at least 1 of the 4 parameters was exceeded). The category with the highest percentage of positive tests was the dysplastic nevus versus melanoma group (28% positive test results), followed by the melanoma versus nevus NOS group (24%). Cases tested to detect nevoid or acral/mucosal melanoma or to differentiate a combined nevus from melanoma in a nevus showed positive test results in 17% to 18% of cases. Thirteen percent of spitzoid tumors were positive, and 9% of the blue/deep penetrating nevus versus blue nevus-like melanoma group tested positive (Table 1). Overall, relative loss at 6q23 was the most frequent aberration detected (63% of positive cases), followed by 6p25 gain (34%). Gains involving 11q13 were found in 14% and relative 6p25 gain in 10%. There were 5 cases in which FISH signals were increased for all 3 probes on chromosomes 6 and the 11q13 probe indicative of polyploidy and 2 cases of trisomy 6. Individual results for each of the FISH test parameters in the various categories are shown in Table 1 and Figure. 7.

TABLE 1. Tumor Types for FISH Testing and Individual Probe Results

	No. Cases (%) (n = 804)	No. Positive FISH Tests (%) (n = 124)	11q Gain	Relative 6q Loss	6p Gain	Relative 6p Gain	Polyploidy/ Polysomy
Spitz nevus vs. spitzoid melanoma	378 (47)	46 (13)	7 (6)	33 (30)	9 (4)	3 (1)	0
Combined nevus vs. melanoma in nevus	72 (9)	12 (17)	4 (3)	9 (4)	4 (0)	2 (0)	1 trisomy 6
Acral/mucosal nevus vs. melanoma	72 (9)	10 (15)	5 (5)	3 (2)	1 (1)	3 (1)	1 polyploid
Dysplastic nevus vs. melanoma	53 (7)	14 (28)	1 (0)	8 (4)	10 (6)	1 (0)	1 polyploid
Blue nevus/DPN vs. blue nevus-like melanoma	46 (6)	4 (9)	1 (1)	2 (2)	1 (1)	0	0
Nevus vs. nevoid melanoma	31 (4)	5 (18)	0	2 (0)	4 (2)	0	1 trisomy 6
Nevus NOS vs. melanoma	152 (18)	33 (24)	0	21 (17)	13 (8)	3 (0)	3 polyploid

Numbers in parentheses in the individual probe columns indicate cases in which the probe was the only aberration detected.
DPN indicates deep penetrating nevus.

The Impact of FISH on Diagnosis

All cases had some degree of ambiguity that prompted FISH testing. Of the 754 tumors successfully tested, 662 (88%) were given a more definitive diagnosis of benign or malignant. Ninety-two cases were given an equivocal final diagnosis despite FISH testing, 91 of which tested negative with FISH. Of the 630 cases that tested negative by FISH, the dermatopathologist who integrated the histopathologic, clinical, and FISH test result issued a benign final diagnosis in 489 (78%) cases, an ambiguous diagnosis including both melanoma and nevus in the differential diagnosis in 91 cases (14%) and a diagnosis of melanoma in 50 cases (8%). Of the 50 cases that were diagnosed as melanomas despite a negative FISH test, 20 were spitzoid lesions. Six of these had been further analyzed with comparative genomic hybridization (CGH) because of remaining concern of melanoma despite a negative FISH test. Chromosomal aberrations not involving chromosomes 6 and 11 were detected in all 6 cases. Five of the 50 cases were of unambiguous melanoma in situ with an ambiguous dermal component for which FISH was performed to determine whether the dermal component was invasive melanoma and was found to be negative.

Of the 124 cases with a positive FISH result, 117 (94%) were reported as melanoma, 1 (1%) was interpreted as equivocal, and 6 (5%) were interpreted, despite the positive FISH result, as melanocytic nevi. In the 6 latter cases, an isolated relative 6q loss was detected in 3 cases, which only marginally exceeded the threshold in 2 cases. One case had isolated 6p gain only marginally exceeding the threshold. One case had polysomy of chromosome 6, and 1 exhibited 6p gain and 6q loss. Cases in which a benign diagnosis was initially suspected were more likely to test negative with FISH, whereas those in which a

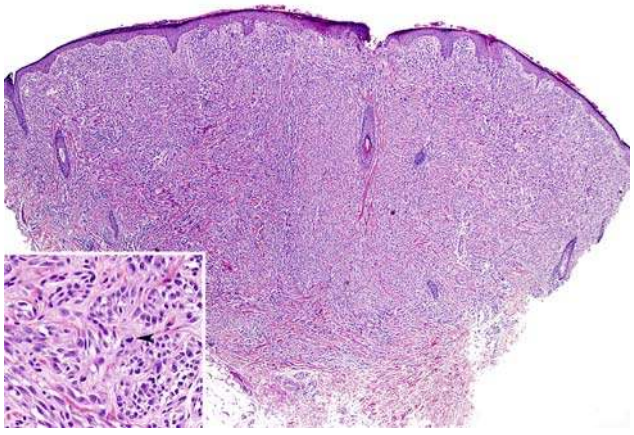

FIGURE 1. Spitzoid tumors. This neoplasm has features of a Spitz nevus (moderately large, spindled, and epithelioid cells with abundant pale cytoplasm, vesicular nuclei with small nucleoli, and clefting between cells and nests) but is large, extends to the subcutis, and has scattered deep mitotic figures (inset, arrowhead). FISH revealed no aberrations. Final diagnosis—Spitz nevus (hematoxylin and eosin).

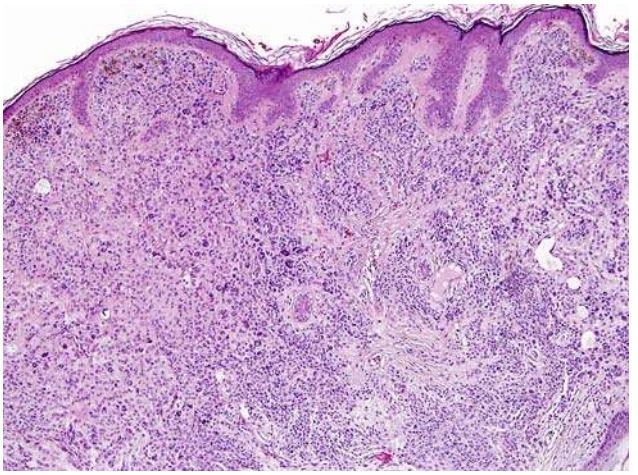

FIGURE 2. Combined melanocytic nevus or melanoma arising in a melanocytic nevus. Histopathologic image(s) of a 48-year-old man with a tumor on the postauricular scalp. Two distinct populations of melanocytes are evident. On the right, there is a population of small melanocytes typical of a congenital nevus. On the left, larger melanocytes with abundant pale cytoplasm are present. FISH showed aberrations in the larger melanocytes only. Diagnosis—melanoma arising in a melanocytic nevus (hematoxylin and eosin).

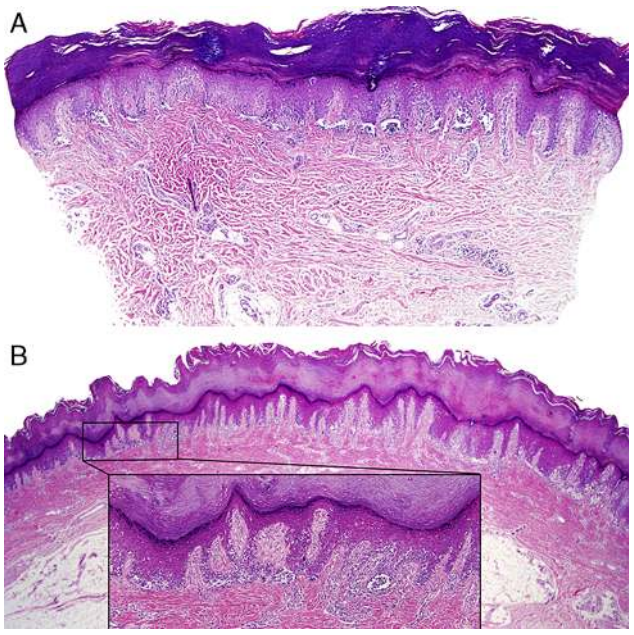

FIGURE 3. Acral neoplasms. Histopathologic image(s) of a 70-year-old woman with a foot lesion. A, Punch biopsy near a prior melanoma showing a nested proliferation of melanocytes extending to both edges of the biopsy (hematoxylin and eosin). B, Original acral melanoma showing a broad, poorly circumscribed neoplasm with many single cells in pagetoid array (inset: higher magnification) (hematoxylin and eosin). A wide excision with negative margins was performed 3 years prior. FISH showed polyploidy of chromosomes 6 and 11 in both (A) and (B). Final diagnosis—melanoma.

malignant diagnosis was initially favored were more frequently positive (Fig. 8).

DISCUSSION

The need for ancillary tests to improve diagnostic accuracy of difficult melanocytic lesions is well established. Along with the aforementioned studies demonstrating diagnostic discordance even among expert dermatopa-

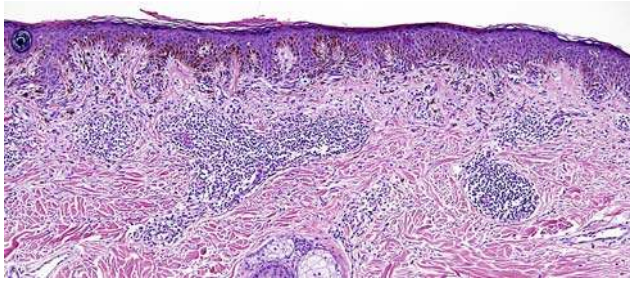

FIGURE 4. Dysplastic nevus or melanoma. Histopathologic image(s) of a 61-year-old man with a lesion on the back. This neoplasm has some features of a dysplastic (Clark) nevus with nests bridging rete and papillary dermal fibrosis with melanophages but has foci of significant pagetoid scatter (hematoxylin and eosin). FISH showed 6p25 gain and 6q23 loss prompting a diagnosis of superficially invasive melanoma.

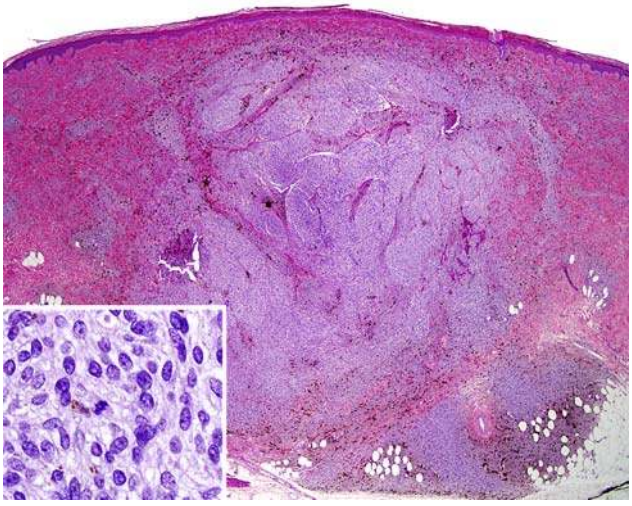

FIGURE 5. Blue nevus or blue nevus-like melanoma. Histopathologic image(s) of a 14-year-old boy with a lesion on the foot. This neoplasm has features of a cellular blue nevus with large nests and fascicles of moderately large, oval, and spindled melanocytes extending into the subcutis. However, scattered mitotic figures were present (inset), and a KI-67 immunostain showed a focus with a mildly elevated proliferation rate. FISH showed no aberrations, and a diagnosis of cellular blue nevus was rendered (hematoxylin and eosin).

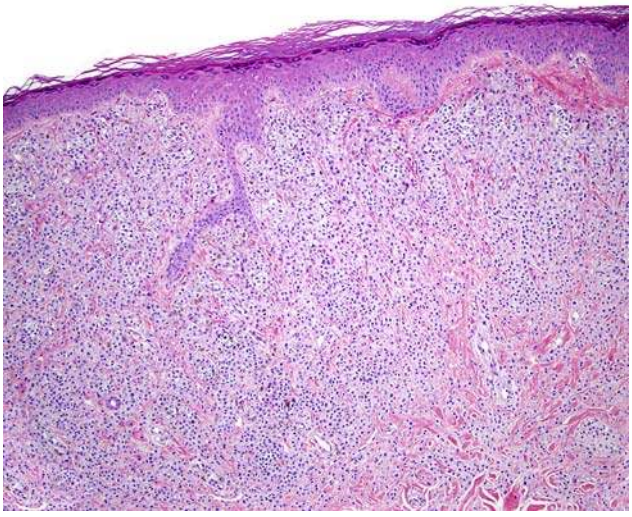

FIGURE 6. Melanocytic nevus or nevoid melanoma. Histopathologic image(s) of a 69-year-old man with a lesion on the knee. This predominantly intradermal neoplasm demonstrated maturation with descent and had a symmetrical, dome-shaped appearance. KI-67 proliferation rate was low, but an epithelial collarette was present at the periphery, suggesting an expansile lesion. FISH showed no aberrations, and a diagnosis of melanocytic nevus was initially favored. Two years later, 2 new, histopathologically similar lesions appeared at the same site exhibiting chromosomal aberrations in chromosomes 2 and 9 as detected by array CGH. The diagnosis was subsequently changed to nevoid melanoma (hematoxylin and eosin).

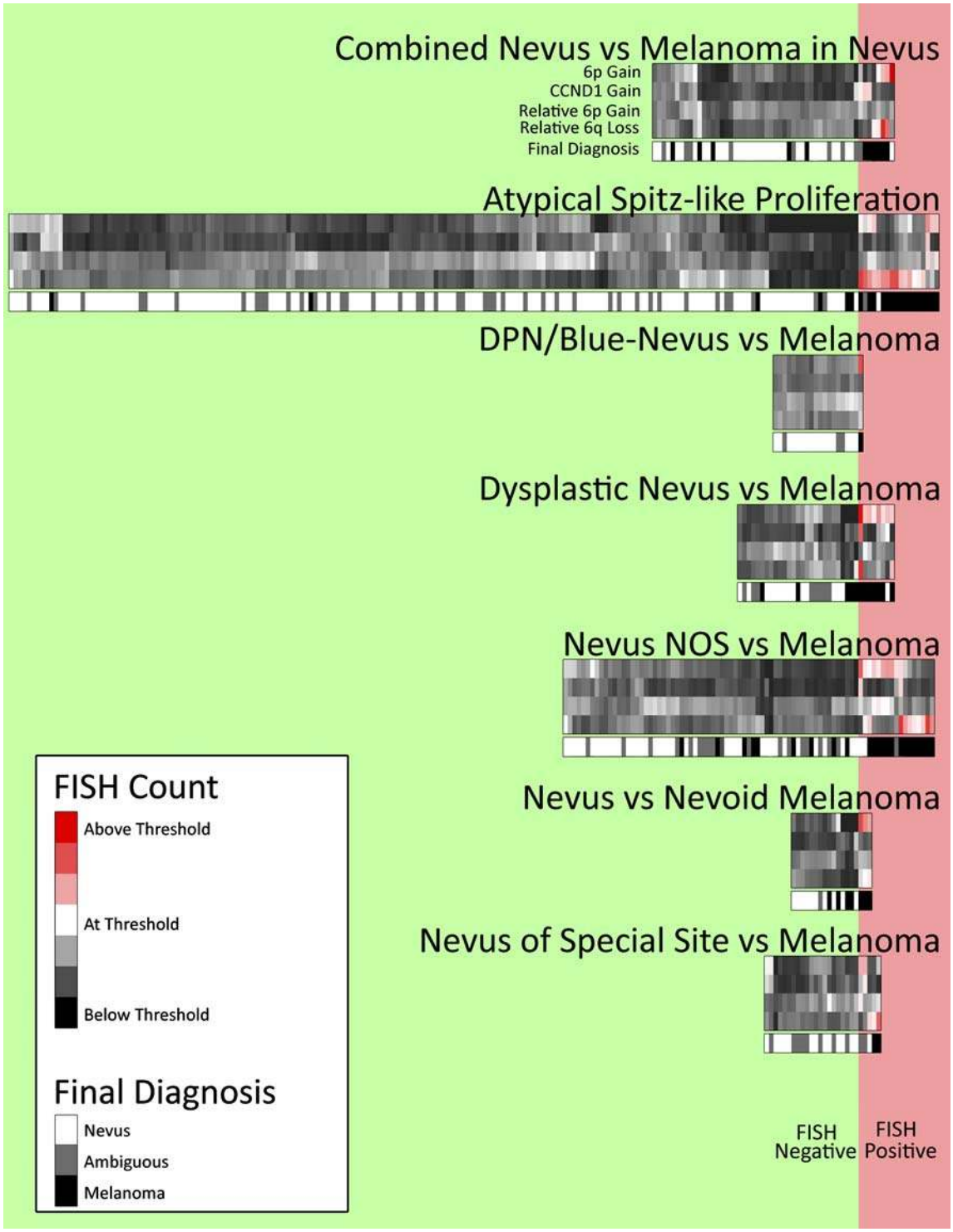

FIGURE 7. Clustering of FISH probes in different types of melanocytic neoplasms shows different patterns of FISH probe positivity among the subsets.

thologists, there is increasing concern that the threshold for diagnosing melanoma is decreasing, contributing to the steady increase in the diagnosis of melanoma.[8] Diagnostic tests that produce quantitative data have the potential to reduce the subjectivity inherent to the diagnostic process. FISH relies on the enumeration of signals within nuclei, which is objective and can be automated, provided that the nature of a signal and the conventions of

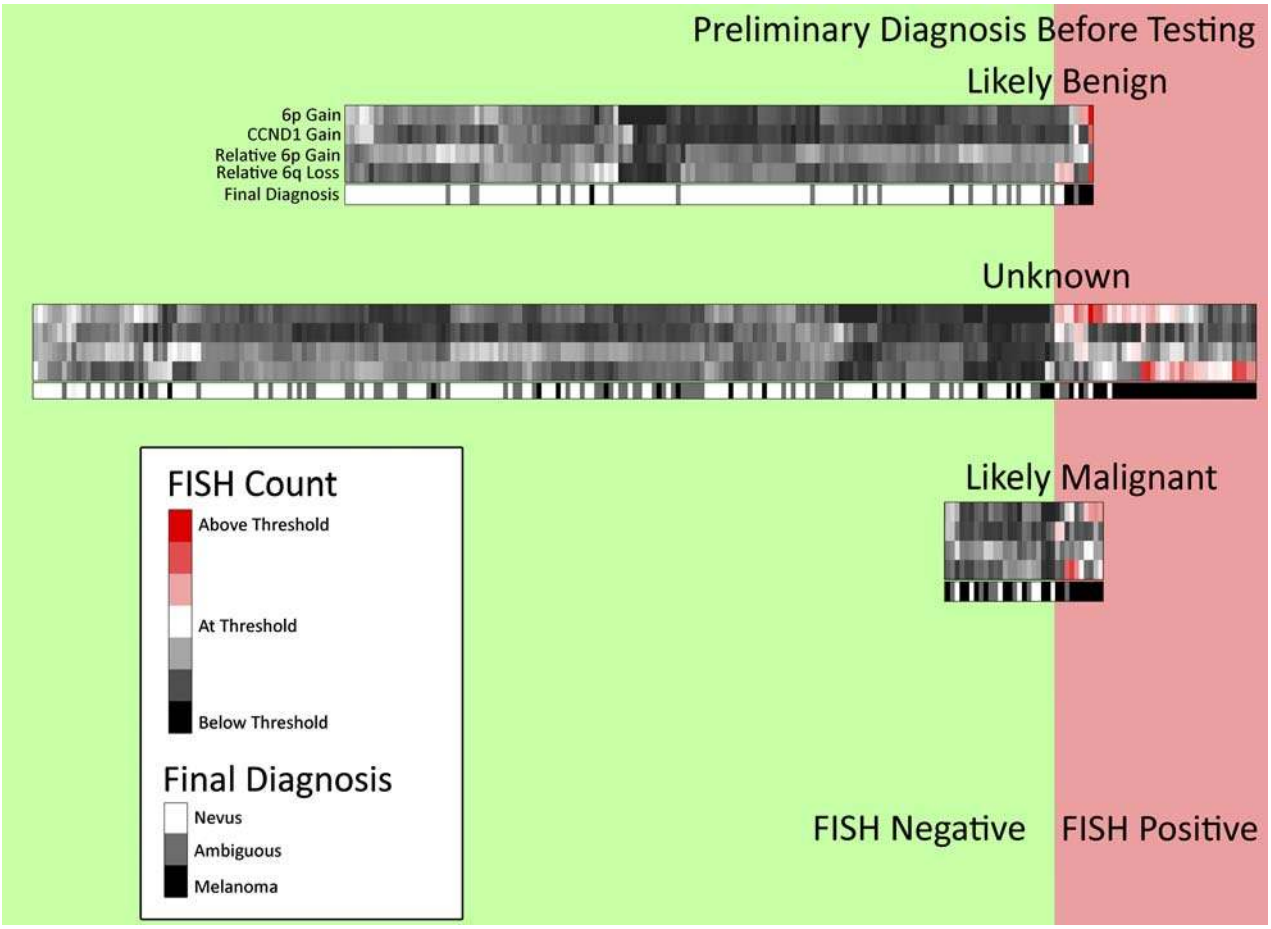

FIGURE 8. Clustering of FISH probes based on pre-FISH index of suspicion for malignancy shows good correlation between likelihood of FISH positivity and initial concern for malignancy as well as the frequency of positivity for each probe.

counting closely juxtaposed signals are standardized. The original study on which the thresholds for positivity were based found the test to distinguish unambiguous melanoma from unambiguous melanocytic nevi with approximately 85% sensitivity and 95% specificity. Other studies reached comparable results using unambiguous melanomas and melanocytic nevi.[14,15] However, the selection of the nuclei for analysis can be subjective and decrease reproducibility of the procedure. In 1 study, there was considerable interobserver disagreement (19% to 25%) in the interpretation of the FISH results.[16] The source of this disagreement was not further detailed, but selection bias of nuclei enumerated is a likely cause. This difference in selecting cells for enumeration could have led to the discordant results between FISH and CGH testing in some cases of that study. Although it is conceivable that CGH misses aberrations detected by FISH when they are present in a minority population of tumor cells, finding aberrations by CGH that are not detected by FISH indicates a technical problem with either method.

In our experience with FISH, it is paramount for reproducible results that the nuclei selected for enumeration are selected randomly from a predefined region of the neoplasm. The goal of the assay is the detection of any clonal chromosomal imbalances, that is, imbalances that are shared among cells with a recent common progenitor cell. Clonal chromosomal imbalances indicate failure of critical tumor-suppressor checkpoints that normally prevent further cell division after a mitotic error occurs. Cells that are closely spaced together within a neoplasm are expected to be more closely related genetically than cells separated by greater distances. To increase the sensitivity to detect such a clone, representative areas of the entire tumor should be visually inspected for abnormal FISH signals for each probe. Areas with the most abnormal FISH signals should then be counted. As there is variation of hybridization signals due to nuclear truncation during tissue sectioning, interspersed polyploid nuclei, and random (ie, nonclonal) chromosomal aberrations present in tumors, it is important to randomly choose the nuclei to be counted from the preselected areas.

Whereas numerous studies of FISH in melanocytic tumors have been reported, only a few studies have evaluated the FISH technique in ambiguous melanocytic tumors with long-term clinical follow-up. One such study by Vergier et al[17] reported a dramatically lower sensitivity

and specificity (43% and 80%) for detection of malignancy. Although the use of lymph node positivity as a criterion for malignancy in this study is questionable, the results do raise a warning that the chromosome 6 and 11 FISH assay may not be as sensitive in detecting melanoma in ambiguous neoplasms as in unambiguous cases. Most likely, the sensitivity of FISH varies depending on the type of melanocytic neoplasm being tested.

The development of a single assay to detect all types of melanoma is an admirable goal but presents a daunting challenge because of genomic heterogeneity in melanoma. Using CGH, Curtin et al[13] demonstrated that significant differences exist among different types of melanomas in the number of chromosomal gains and losses, as well as the locations of such aberrations. For example, 85% to 89% of acral and mucosal melanomas possess chromosomal amplifications, whereas melanomas arising in chronically sun-damaged or intermittently sun-exposed skin have smaller copy number gains with relatively few amplifications. With this genetic heterogeneity, one would expect variable sensitivity and specificity among types of melanocytic tumors subjected to FISH testing.

In a review of FISH performance in melanoma subtypes, Gerami et al[15] found gain of 6p25 to be the most frequent aberration in all subtypes of melanoma tested (superficial spreading, lentigo maligna, acral lentiginous, nodular, melanomas in chronically sun-damaged skin, and melanomas in non–chronically sun-damaged skin). This is consistent with a large-scale study of CGH in unambiguous melanoma and nevi in which 6p was the region most frequently gained in melanomas (37%), whereas 6q loss was seen to a lesser degree (26%).[10] Gain of 11q13 was seen in only 9% of nonacral melanomas but in 36% of acral melanomas.[10] Our review shows variable importance of FISH probes depending on the tumor type (Table 1, Fig. 7). 6p25 gain was the most frequent aberration (79% of positive cases) in the dysplastic nevus versus melanoma group. This group contains melanomas of the superficial spreading or lentigo maligna type, the most common forms of melanoma. In contrast, 6q23 loss was the most frequent aberration in spitzoid cases (72% of positive cases), with 6p25 gain in only 26%. The lower fraction of 6p25 gain could stem from different genetic alterations that result in a spitzoid phenotype. Recent study of CGH in spitzoid neoplasms showed no aberrations in either chromosome 6 or 11 in l6 "atypical Spitz tumors," 6 of which had aberrations involving other chromosomes.[18]

The distribution of 11q13 gain is also noteworthy. Chromosomal gains of 11q13 are most often seen in acral melanomas and melanomas on chronically sun-damaged skin.[13,15] In our review, 11q13 gain occurred primarily in the acral, spitzoid, and combined nevus versus melanoma arising in nevus groups (Table 1). In the 45 positive cases in the dysplastic nevus versus melanoma, nevus versus nevoid melanoma, and melanoma versus nevus NOS groups, only one 11q13 gain was detected. Because of the limited number of probes that can be incorporated into a cost-effective FISH assay, selection of the highest-yield probes is critical, and perhaps a probe targeting a different locus could be substituted for 11q13 in such cases.

In a recent study of spitzoid neoplasms, the addition of 9p21 and Cep9 probes to detect homozygous loss of 9p21 increased sensitivity for detection of spitzoid melanoma from 70% with the standard 4-probe FISH panel to 85% with the addition of the chromosome 9 probes.[19] A study of a new 4-probe set targeting 9p21, 11q13, 6p25, and 8q24 was reported to have a sensitivity of 94% and specificity of 98% in a validation set of a mixed group of melanomas and melanocytic nevi.[20] These results are promising and need to be confirmed in additional studies from other groups.

Another area of uncertainty in FISH involves polyploidy (Fig. 9). Polyploidy has been detected in a small percentage of Spitz nevi. In a study of 41 Spitz nevi, polyploidy was detected in 4 cases.[21] However, 3 of the 4 were from the same patient with agminated Spitz nevi. All 4 polyploid cases had 3 or 4 copy numbers of each probe, indicative of tetraploidy. Polyploidy can also be seen in melanoma. One acral melanoma in our cohort was polyploid (Fig. 3). This patient developed a new pigmented lesion near a skin graft from a past melanoma. FISH demonstrated polyploidy in both the original unambiguous melanoma and the new lesion.

Two studies have analyzed the DNA content of enlarged nuclei in Spitz nevi and melanoma using DNA cytometry. The first showed that the mean DNA content in superficial melanocytes was 1.157 times greater than reference keratinocytes in Spitz nevi and 1.238 times greater in melanomas.[22] The second study reported that a small percentage of Spitz cells are polyploid with a copy number of 3 to 4, whereas the copy number in melanoma ranged from 2 to 9.[23] As uncertainty exists in the meaning of polyploidy, any FISH result showing polyploidy

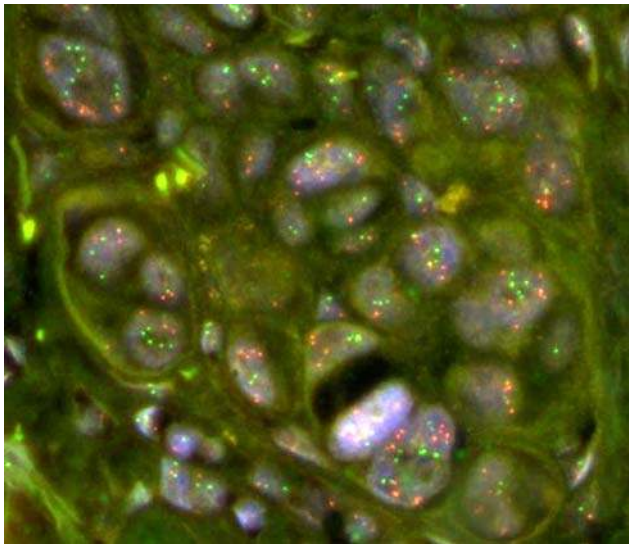

FIGURE 9. Polyploidy in FISH. All 4 FISH probes showing >2 signals. Red—6p25, Green—11q13, Yellow—6q23, Aqua—centromere 6.

should be interpreted with caution. However, analysis of the available data indicates that polyploidy exceeding the tetraploid state (ie, >4 copy numbers) is more consistent with melanoma than a nevus.

It should also be noted that there is considerable skill involved in performing FISH to characterize melanocytic tumors. Selecting which nuclei to count in DAPI-stained sections requires expertise both on the part of the pathologist choosing fields for measurement and on the part of the technician who looks at these fields and discards truncated nuclei and those thought to be the nuclei of stromal cells, lymphocytes, or keratinocytes. Inexperience among those newly performing FISH could lead to spurious probe counts and could be a contributing factor to the discordance in sensitivity of FISH found in some studies.

In conclusion, this review of FISH at UCSF describes the types of tumors most frequently sent for FISH analysis and the role of FISH in decreasing equivocal diagnosis in difficult melanocytic tumors. Results from the different FISH probes in these tumor types suggest that the current test would benefit from further studies matching tumor type with more specific FISH probes. FISH is a useful ancillary test in melanoma diagnostics, and studies have shown high positivity rates in cases with documented adverse outcomes.[12] Unfortunately, there is still uncertainty regarding the sensitivity of FISH in the various types of neoplasms for which the test is most frequently obtained. FISH results should be interpreted in conjunction with all other available data before rendering a final diagnosis.

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