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ABSRACT:

Background: Spitzoid melanocytic neoplasms are diagnostically challenging; criteria for malignancy continue to evolve. The ability to predict chromosomal abnormalities with IHC could help select cases requiring chromosomal evaluation.

Methods: FISH-tested spitzoid neoplasms at our institution (2013-2021) were reviewed. p16, BRAF V600E, and PRAME immunohistochemistry (IHC) results were correlated with FISH.

Results: 174 cases, 1.9F:1M, median age 28 years (range 5 months-74 years) were included, final diagnoses: Spitz nevus (11%), atypical Spitz tumor (47%), spitzoid dysplastic nevus (9%), and spitzoid melanoma (32%). 60 (34%) were FISH-positive, most commonly with absolute 6p25 gain (*RREB1* > 2). Dermal mitotic count was the only clinicopathologic predictor of FISH. Among IHC-stained cases, p16 was lost in 55/134 cases (41%); loss correlated with FISH+ ($p < 0.001$, Fisher exact test). BRAF V600E (14/88, 16%) and PRAME (15/56, 27%) expression did not correlate with FISH alone ($p=0.242$ and $p=0.359$, respectively, Fisher exact test). When examined together, however, p16-retained/BRAF V600E-negative lesions had low FISH+ rates (5/37, 14%; 4/37, 11% not counting isolated *MYB* loss); all other marker combinations had high rates (56-75% of cases; $p<0.001$).

Conclusions: p16/BRAF V600E IHC predicts FISH results. “Low-risk” lesions (p16+/BRAF V600E-) uncommonly have meaningful FISH abnormalities (11%). PRAME may have limited utility in this setting.

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INTRODUCTION:

Spitzoid tumors are diagnostically problematic, with histopathologic overlap with melanoma: enlarged atypical cells, dermal mitotic figures, and pagetoid spread[1–6]. Spitz nevi and atypical Spitz tumors (ASTs) are benign and curable by complete local excision[6–9], while spitzoid melanomas can spread systemically, hence, ancillary tests may assist with classification. Immunohistochemical (IHC) staining to evaluate p16 protein expression (a *CDKN2A* gene product) has been used for spitzoid lesions in particular[10–21]. PRAME (preferentially expressed antigen in melanoma) is reportedly helpful in a number of situations; utility is less defined in spitzoid lesions[22–27]. Comparative genomic hybridization (CGH) and array-based CGH (aCGH) demonstrate multiple partial chromosomal copy number alterations in melanoma, while nevi show no abnormalities or few (<4) isolated copy number alterations such as 11p gains and 3p losses in desmoplastic Spitz and BAP1-inactivated tumors respectively.[28,29] Findings from (a)CGH have guided selection of probes for use in fluorescence in situ hybridization (FISH), which is comparatively simple and able to select small tumor clones within a larger lesion. As such, FISH has become a more readily available and commonly used method to evaluate difficult spitzoid lesions[28–33].

Molecular understanding of melanocytic lesions has expanded rapidly in recent years. Spitz tumors are driven by *HRAS* mutations and/or 11p amplification, or kinase gene fusions[34–40]. They lack recurrent *BRAF* or *NRAS* mutations seen in conventional nevi and melanoma[41–50]. Hence, malignant Spitz tumors (Spitz melanomas) may be distinct from spitzoid melanomas with different underlying molecular drivers[51–54]. Next-generation sequencing (NGS) aids in classification, but has yet to be broadly implemented[55,56].

In this study, we hypothesized that staining patterns observed with the molecular surrogate markers - p16 and BRAF V600E, may categorize spitzoid melanocytic lesions as either highly likely or unlikely to harbor chromosomal alterations as detected by FISH. We sought to understand the true likelihood of a FISH-positive result in these risk-stratified subsets in order to guide the selection of ancillary molecular testing in clinical practice (FISH, CGH, and/or NGS); and hypothesized that PRAME may provide added benefit in this setting.

MATERIALS AND METHODS:

Cases:

FISH laboratory case records at our institution were searched for all specimens subjected to our in-house melanoma FISH panel from 2013-2021. Pathology reports were reviewed and all cases representing spitzoid lesions were included in the study. Clinical, pathologic, and immunohistochemical data were abstracted from the pathology reports and reviewed when material was available (104/174 cases in total (59%), including 33/60 FISH-positive cases (55%) and 53/114 FISH-negative cases (46%). Each diagnosis was rendered by one of six academic dermatopathologists at our institution, often with joint or group consensus. All malignant diagnoses were reviewed by at least two dermatopathologists, as were many of the benign or intermediate lesions (data not shown).

Immunohistochemistry:

Immunohistochemical staining for p16, BRAF V600E, PRAME, and ALK-1 was performed on the Ventana Medical Systems (VMS) Benchmark Automated Immunostainer (VMS, Tuscon, AZ, USA) as previously described[16]. p16 expression patterns were categorized similar to prior studies[16]. PRAME was designated as positive when greater than 75% of melanocytic nuclei stained positively compared to controls[22,23]. In cases without selected available IHC, stains were performed and blindly scored retrospectively for: p16 (5/134, 4% of cases) BRAF V600E (24/88, 27%), and PRAME (32/56, 57%). Details on processes and reagents are included in Supplementary Methods.

Fluorescence in situ hybridization:

All FISH was performed at the time of original diagnostic workup. Our laboratory uses the Abbott Vysis Cutaneous Melanoma FISH system (Abbott Laboratories, Abbott Park, IL, USA) following manufacturer instructions. Enumeration was performed by a dedicated cytogenetics laboratory technologist with subsequent

pathologist review. Lesions are deemed tetraploid when cells selected for enumeration have four signals for each probe. Details on processes and reagents are included in Supplementary Methods.

Statistics

Statistical analyses were performed using JASP (v0.14.1, JASP Team, University of Amsterdam, The Netherlands) and R (v4.1.2, R Core Team).

RESULTS:

Clinical features

Patient characteristics are listed in **Table 1**. The female-to-male ratio was 1.9:1, the median age at diagnosis was 28 years, and lesions were most common on the extremities. No clinical factors were significantly associated with FISH status. Of the 174 cases, 130 were received in consultation. Clinical follow-up information was available for 46 cases; 31 (67%) FISH-negative and 15 (33%) FISH-positive. Follow-up data are outlined in **Supplementary Table 1**. Of twelve patients who underwent sentinel lymph node biopsy, (11/12 FISH-positive), only one had positive sentinel nodes (2/2 nodes positive for melanoma). The lesion occurred in a 34-year-old woman. p16 was lost, BRAF V600E was negative, PRAME was negative (3+), and FISH showed isolated 6p25 gain. The completion lymph node dissection was negative. The patient received adjuvant pembrolizumab and was alive with no evidence of disease over a 68-month follow-up period. None of the patients with available follow-up developed distant metastases.

Histopathologic features

As noted in **Table 1**, lesions in both groups were most often compound, showed epidermal hyperplasia, and were composed of epithelioid melanocytes. The median dermal mitotic rate was higher in FISH-positive compared to FISH-negative lesions (1 versus 0 per square mm; p=0.011, Wilcoxon rank-sum test).

Final Diagnoses

As shown in **Table 1**, the final pathologic diagnoses in the 174 cases comprised benign (118/174, 68%) and malignant (56/174, 32%) entities. FISH status strongly informed final diagnosis; 87% of FISH-positive lesions were called malignant, compared with 4% of FISH-negative lesions. Eight cases (8/60, 13%) were called benign despite positive FISH results (7 AST and 1 spitzoid dysplastic nevus). Four cases (4/114, 4%) were called malignant (spitzoid melanoma) despite a negative FISH result.

Molecular features

FISH abnormalities are listed in **Table 2**. Sixty cases showed abnormalities (60/174, 34%). Seventy cases underwent FISH testing before 8q24 (*MYC*) and 9p21 (*CDKN2A*) probes were introduced in our laboratory; 29/70 (41%) were FISH-positive and 41/70 (59%) were FISH-negative (not shown). The FISH positivity rate did not increase after 8q24 and 9p21 were introduced (41% positive before versus 32% after, p=0.201, Fisher exact test). Five cases were found to be tetraploid; these cases were included in the FISH-negative group, along with 1 case with isolated *RREB1*>2 a percentage point over the upper limit of normal as an isolated finding. These 6 cases were considered to be "negative" at the time of sign out. Absolute 6p25 (*RREB1*) gain was the most common aberration (68%). Among the 104 cases in which 9p21 was probed, 13/104 showed homozygous 9p21 loss (9p21 (-/-); 13% of total cases and 39% of FISH-positive cases). Among these 13 cases, a median of 67% of cells showed the 9p21 (-/-) (interquartile range, 37-93%). Isolated 9p21(-/-) was observed in seven cases.

Nine cases were sent for CGH testing at an outside institution (ThermoFisher/Affymetrix OncoScan platform, **Supplementary Table 2**). Seven were FISH-negative, and seven occurred in patients under ten years of age. One FISH-negative case, tested before our laboratory added 8q24 and 9p21 probes, was found to have 9p21 (-/-). This case showed striking cytologic atypia and high mitotic rate and was called a spitzoid melanoma (Supplementary Table 2, Case 1). In one other case, CGH confirmed 9p21 (-/-) seen on FISH (Supplementary Table 2, Case 8). In the other cases, CGH was negative or revealed non-specific copy number changes not associated with melanoma. In two cases, tetraploidy was suspected: one demonstrated by CGH a loss of

chromosome 17 which allowed detection of tetraploidy while the other was negative by CGH and FISH showed increased counts for multiple probes, which was considered evidence of tetraploidy[57].

Immunohistochemical features

Immunohistochemical features are listed in **Table 3;** diagnoses are provided at right for reference. Further granular detail regarding separate FISH probes with IHC findings are demonstrated in **Supplementary Table 3**.

p16

Of 134 cases stained for p16, p16 was lost in 55 cases (41%), including 21 (16%) with diffuse total loss, 19 (14%) with regional total loss, and 15 (11%) with near-diffuse loss (rare scattered positive cells only)(Table 3, top grouping). Most cases with retained p16 were FISH-negative (63/79, 80%). By contrast, similar proportions of cases with p16 loss were FISH-positive (30/55, 55%) as were FISH-negative (25/55, 45%). p16 status was significantly associated with FISH status ($p = 0.0000807$, Fisher exact test). This result was at least partially attributable to cases with 9p21 (-/-). Nevertheless, p16 was lost in several cases with retained 9p21. Sixteen of the 104 cases tested by six-probe FISH (33/104 positive overall, bottom of Table 2) showed retained 9p21 but were positive for other melanoma-associated chromosomal abnormalities (data not shown). Six of these 16 cases (6/16, 38%) showed p16 loss. Overall, these data reinforce p16 protein repression as a specific and general marker for malignancy.

Of the 25 cases that had p16 loss but were FISH-negative, most were called benign (23/25): sixteen AST (16/25) and seven Spitz nevi (7/25). Both of the remaining two cases were diagnosed as spitzoid melanomas. One case was referred to above in the CGH section (Supplementary Table 2, Case 1). The other is described with discordant cases below (Table 5, Case 9).

BRAF V600E

BRAF V600E IHC was performed on 88/174 cases. Fourteen cases (14/88, 16%) were positive, nine of which (9/14, 64%) were also FISH-positive. BRAF V600E mutation was not independently associated with FISH abnormalities (9/14 versus 5/14, p=0.242, Fisher exact test). Out of the nine BRAF-mutated, FISH-positive cases, eight were called spitzoid melanomas. One was called a dysplastic nevus with spitzoid features (Table 5, Case 8, discussed with discordant cases below). Four of the five BRAF-mutated, FISH-negative cases were called dysplastic nevi with spitzoid features due to the reassuring FISH results and retained p16 expression. One was called a spitzoid melanoma (Table 5, Case 9, discussed with discordant cases).

PRAME

Fifty-six cases were stained for PRAME. Fifteen cases (27%) demonstrated diffuse PRAME expression (staining in >75% of cells; 4+). Seven of these fifteen cases (47%) were FISH-positive. PRAME expression was not statistically associated with FISH status (p = 0.359, Fisher exact test). All seven FISH-positive lesions were called spitzoid melanomas. Of the eight cases with positive PRAME expression and negative FISH, seven were diagnosed as ASTs. The remaining case was called a spitzoid melanoma; it has been mentioned above, and is detailed below in the discordant case section (Table 5, Case 9).

ALK1

Staining for ALK1 was performed in ten cases, and was positive in three (3/10, 30%). Three (3/10) were FISH-positive; one case was both FISH-positive and ALK1-positive. All three ALK1-positive cases were called AST, regardless of FISH status; five ALK1-negative lesions were also called AST, and two were called spitzoid melanoma (both FISH-positive).

FISH Results in Low- and High-Risk Double Screen Categories

We hypothesized that combined IHC staining with p16 and either BRAF V600E or PRAME might be a superior screening tool than using markers separately. Eighty-two cases were stained for both p16 and BRAF V600E. As with the overall cohort, loss of p16 expression in these 82 cases was associated with FISH positivity (p

= 0.0000506, Fisher exact test). While BRAF V600E did not independently predict FISH status (p = 0.365, Fisher exact test), it did when examined among cases with retained p16 (p=0.00149, Fisher exact test). Fifty-three cases were stained for both p16 and PRAME expression. The majority of the lesions (30/38, 79%) were negative for PRAME expression (0-3+). p16 and PRAME double staining status was not significantly associated with FISH status (p = 0.0694, Fisher exact test, data not shown).

Cases were broken down into four groups based on combined p16 and BRAF V600E staining patterns; categories were annotated with prototypical lesion type(s) (**Table 4**). Category 1, with retained p16 and no BRAF V600E mutation, was considered "low risk," and Categories 2-4, with either p16 loss, BRAF V600E positivity, or both, were all considered "high risk," given the spitzoid morphology. The relative proportions of FISH-positive lesions differed significantly between the categories (p=0.00000991, Fisher exact test). There was no significant difference in PRAME staining between groups (p = 0.603, Fisher exact test, Table 4).

Thirty-seven cases fell into the low-risk category (37/81, 46%), predicting a negative FISH result and benign diagnosis. Five of these cases were FISH-positive (**Table 5**, Cases 1-5); thus, the risk of FISH positivity in double-screen low-risk cases was 14%. One of these five cases (**Table 5,** Case 1) showed isolated 6q23 loss, leaving four cases (4/37, 11%) in the low-risk category showing relatively worrisome positive FISH results (**Table 5,** Cases 2-5**; Figures 1-3**). Only three cases (3/37, 8%) in this low-risk category were called malignant, including two FISH-positive cases (Table 5, Cases 4 and 5, **Figures 2 and 3**) and one FISH-negative case (**Table 5**, Case 6, described with discordant cases).

Forty-four cases fell into the high-risk categories (44/81, 54%), in which FISH positivity rates ranged from 56-75%, (29/44, 66% overall). Undoubtedly in part because FISH results informed the final diagnoses, malignant diagnoses were rendered in larger proportions of cases in high-risk categories, ranging from 44-100% (30/44, 68% overall).

Unexpected or Discordant Cases

Out of interest, we examined more closely nine cases in which the final diagnosis was unexpected or discordant in light of the double-screen category and/or FISH results. Six cases derived from the low-risk category (**Table 5**, Cases 1-6) and three from the high-risk categories (**Table 5**, Cases 7-9). FISH-positive lesions were sometimes called benign in younger patients with less histopathologically concerning lesions [**Table 5**, Cases 1-3 (**Figure 1**) and **Table 5**, Cases 7-8 (**Figure 4**A-B, and C-D)]. **Table 5**, Case 8 (Figure 4B) was called a dysplastic nevus with spitzoid features, as the lesion was mitotically inactive and the degree of atypia was not severe. Some FISH-negative lesions were called malignant in relatively older patients with dermal mitoses [**Table 5**, Case 6 and Table 5, Case 9 (Figure 4C-D)]. Table 5, Case 9 occurred in a 39-year-old woman and showed p16 loss, BRAF V600E was positive, and PRAME was positive. Similarly, **Table 5**, Case 6 occurred in a 62-year-old woman and showed sun damage and extensive upward migration of individual melanocytes. These cases reinforce the fact that FISH is not entirely sensitive nor specific for malignancy, and that FISH results must be interpreted in the context of other clinical and histopathologic findings.

DISCUSSION:

Spitz tumors and other spitzoid melanocytic neoplasms are notoriously difficult diagnostically, with disagreement amongst expert dermatopathologists[2,7]. Expanding molecular understanding emphasizes the importance of not only risk stratifying Spitz tumors, but in distinguishing them from morphologically similar but biologically distinct spitzoid melanomas[51–54,56]. Diagnostic aids include more commonly available techniques such as IHC and FISH as well as other molecular techniques like aCGH and NGS[30,31,55,56]. In this study, we show that a combination of routine IHC markers, when applied to lesions with spitzoid morphology, helps predict which cases are more or less likely to show chromosomal abnormalities by FISH. This finding may serve as a practical tool to help dermatopathologists select cases to send for FISH/molecular testing.

In our cohort of spitzoid cases, the number of dermal mitotic figures was the only clinical or histopathologic feature that differed significantly by FISH status, aligning with several previous reports[3,4,7]. Other

groups have reported that age and sex are associated with malignancy or aggressive behavior, while mitotic rate is not[58–60]. These studies examine different outcomes, ranging from FISH status, to final diagnosis, to clinical behavior, which may partially explain different findings. Nonetheless, our study reaffirms that clinical and histopathologic features are insufficient to evaluate for malignant potential in ambiguous spitzoid cases. The difference in median mitotic rate between FISH+ and – cases, although significant, is small (0 versus 1 per square mm) and impractical for clinical application, highlighting the critical utility of ancillary testing in this setting.

We detected chromosomal abnormalities by FISH in 60 of 176 (34%) cases. Other studies report lower positivity rates, although differences in case composition (e.g. referral bias) and lesion types complicates direct comparisons[61–63]. Absolute 6p25 gain was the most common abnormality we observed (68% of FISH+ cases), followed by abnormalities at 11q13, 8q24, 9p21, and 6q23. Published rates of individual copy number changes are variable; 6q23 loss[62,63], 6p25 absolute gain[30,61], both 6q23 and 6p25[64], and 9p21 (-/-)[60,65] have all been reported as the most common finding in spitzoid lesions. Differing inclusion criteria, such as case ambiguity, BRAF mutation status, or thresholds for calling spitzoid morphology may explain variation in results[8,29–32,61–66].

Six of 114 cases that were ultimately called FISH-negative at the time of sign-out technically showed positive probes. 1 of these cases was slightly over the threshold in a single probe, and was considered negative. The other 5 cases were found to be tetraploid, either based on FISH results alone (all probes >2 being strongly positive, and loss probes or probes >CEP6 being solidly negative), or based on the recognition of borderline results with a trend that suggested tetraploidy, with confirmation from additional tests (1 outside lab FISH, and 2 outside lab aCGH). These cases highlight the expertise required in FISH testing as well as interpretation.

Several studies have indicated that isolated 6q23 loss in spitzoid lesions is not associated with adverse clinical outcomes, even in cases with nodal metastasis[8,32]. We encountered four cases of isolated 6q23 loss in our study, all of which were diagnosed as AST. It is possible that 6q23 loss is underrepresented in our cohort compared to other studies because our cohort is enriched for challenging, atypical cases. The clinical behavior of spitzoid neoplasms with isolated 9p21 (-/-) remains unresolved. While early studies demonstrated increased

aggressive behavior, later studies have not reproduced these findings[30,32,65]. We observed isolated 9p21 (-/-) in seven cases, all of which were called spitzoid melanoma, in keeping with earlier studies[32]. Our retrospective study design and limited follow-up data preclude drawing conclusions about the prognostic significance of isolated 9p21 (-/-). Regardless of its significance, knowing that a lesion demonstrates 9p21 (-/-) remains helpful. Even if isolated copy number changes are considered reassuring, tools to help dermatopathologists decide when to test for these changes are helpful.

Prior studies have shown that p16 loss correlates with malignancy in spitzoid neoplasms[17–21,67]. In our cohort, loss of p16 expression by IHC was significantly associated with FISH abnormalities, including cases with and without 9p21 (-/-)[10,11,28,68,69]. This association was largely due to cases with 9p21 (-/-), similar to other studies[17,20]. Thus, while the statistical association is not surprising, it supports sending spitzoid cases with p16 loss for evaluation. p16 loss in melanoma occurs by various mechanisms, not limited to homozygous *CDKN2A* deletion, including mutations, small deletions, promoter hypermethylation, and silencing by long non-coding RNA[69–74]. 45% of our cases with p16 loss were FISH-negative, showing p16 loss alone is not specific for predicting FISH abnormalities.

We employed BRAF V600E immunostaining to identify spitzoid nevi and melanomas that were not true Spitz tumors. A small proportion (15/89, 17%) of lesions showed mutant protein expression, suggesting our institution applies relatively strict criteria in designating spitzoid morphology. BRAF V600E immunoreactivity rates did not differ significantly by FISH status, unsurprisingly, given prevalence in both benign nevi and melanoma[47,48,69,75]. When examined in the context of retained p16, BRAF V600E positivity was significantly associated with FISH positivity, showing utility as an additional screening tool. This finding does not imply that BRAF V600E mutation itself is associated with malignancy. Rather, it excludes true Spitz lesions; and thereby a relatively lower proportion of p16-retained and BRAF V600E-negative lesions are FISH+.

In using a p16/BRAF IHC double screen for spitzoid lesions, we predicted that a Category 1 result (p16+/BRAF V600E-) would exclude many high-risk AST and spitzoid melanomas in our cohort of mostly young,

non-sun damaged patients[58]. This is a screening method commonly employed in our practice, but we wanted to quantitate the true risk in using such a method. Only 11% of cases (4 of 37) in this category showed worrisome FISH findings; all occurred in young patients (aged 1, 7, 11, and 20 years old). The fact that only two of four FISH-positive cases in this category were diagnosed as spitzoid melanoma, despite multiple FISH probes being positive (2, 2, 3 and 2 FISH probes positive, respectively), reflects the hesitancy to diagnose malignancy in this cohort. Importantly, the biology of pediatric spitzoid lesions that have chromosomal gains and losses mirroring melanoma, but that do not have BRAF V600E mutations, requires further study. These cases would merit NGS or, at a minimum, TERT promoter evaluation. The molecular criteria discriminating atypical and malignant Spitz tumors are still being established and require collaborative projects with NGS and long-term patient follow-up.

Combined with spitzoid morphology, Category 2-4 IHC results raise concern for melanoma; indeed, 66% (29/44 cases) showed melanoma-associated chromosomal alterations by FISH. Given the clinicopathologic features in our cohort, p16-negative/BRAF-negative lesions would be predicted to represent high-risk or malignant Spitz tumors. This group could also include spitzoid melanomas with other MAPK driver mutations such as MAP2K1 or NRAS. Given the atypical spitzoid morphology, the p16-positive, BRAF-positive group was predicted to be enriched along the severely dysplastic nevus-melanoma spectrum. p16 expression was heterogeneous in some of these lesions, but remained sufficient to be called retained. This finding is in agreement with published data showing that a large proportion of melanomas retain 9p21 function; indeed with improved outcome[10,11,28,68,69]. p16-negative, BRAF-positive lesions were predicted to be the most potentially worrisome. The rate of FISH-positivity in this group (4) was not higher than that in the others (2, 3), however, these lesions were rare (four total).

In the past several years, PRAME has emerged as a helpful marker for evaluating melanocytic lesions[22–26,76]. However, its role in spitzoid lesions has not been resolved. Studies have shown that only small proportions of spitzoid lesions express PRAME in general[22,24,25]. One recent study indicated higher rates of PRAME positivity in spitzoid melanoma compared with nevi and atypical Spitz tumors[27]. Concordance of PRAME staining with

chromosomal abnormalities in Spitz tumors has also been reported[23,25]. However, others have also shown benign Spitz nevi with diffuse PRAME immunohistochemical staining[22,24,25]. In our study, we found few lesions overall that diffusely expressed PRAME. There was no significant association of PRAME positivity with FISH status; in fact, diffuse PRAME expression was found in several benign lesions. Thus, our results suggest that PRAME may be less helpful in spitzoid neoplasms. Low PRAME positivity rates compared to other studies may in part be attributable to use of the Ventana Medical Systems platform in our laboratory. At least one prior study used the Leica Bond system[23]. Some authors have suggested that Leica systems may be more sensitive than Ventana[77]. Because of these incongruent results, further work in this area is warranted to evaluate the diagnostic and prognostic significance of both FISH and PRAME in spitzoid lesions, especially in young patients. Practicing dermatopathologists need to be aware of potential nuances with this stain, at a minimum. Studies with reflex NGS and CGH would be particularly insightful, but tissue size is a limiting factor.

This study had a number of limitations. First, the retrospective nature introduces the possibility of selection bias. This study could not capture cases in which knowledge of the IHC status led the dermatopathologist not to pursue FISH testing. Second, we had follow-up data from only 46 of 174 cases (26%). Only one of these cases presented at an advanced stage, and no patients developed distant metastases over the median follow-up period of 59 months. Thus, although we can correlate IHC with FISH findings, we cannot determine how either may translate into clinical outcomes. Because long-term clinical behavior remains one of the most reliable methods for differentiating atypical Spitz tumors from true Spitz melanomas, our paucity of follow-up data means the reported final diagnoses could change with additional information[7,9].

In conclusion, we investigated a set of 174 challenging spitzoid neoplasms that were examined by FISH at our institution over an eight-year period. We found that cases with retained p16 expression, and especially the subset of those cases that were negative for mutant BRAF V600E protein, had much lower rates of melanoma FISH-associated chromosomal abnormalities than cases with p16 loss – specifically 14% (11% excluding isolated 6q23 loss). Because BRAF V600E expression helped to exclude Spitz tumors, it significantly

assisted with risk stratification among the p16 retained cases, prompting other explanations for cytologic atypia and dermal mitoses, such as melanoma, even in relatively young patients. By contrast, PRAME expression was not associated with FISH-detected chromosomal abnormalities, and in fact was positive in several benign spitzoid lesions. We conclude that knowledge of p16 and BRAF V600E may help dermatopathologists decide when to send a case for FISH and potentially other molecular tests, in the appropriate clinical and histopathologic context. PRAME should be employed cautiously in ambiguous spitzoid lesions, as results may be misleading.

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FIGURE LEGENDS

Table 1. Clinicopathologic features in FISH positive and negative cases.
†Means all cases where that feature was assessable; denominator varies between attributes.
‡Wilcoxon rank-sum test.
§Fisher exact test.
Percentages may not add to 100% due to rounding.

Table 2. Positive probes in FISH-positive cases.
†One case was called negative because the abnormality was isolated and only slightly higher than the upper limit of normal.
‡Remaining 5 cases were found to be tetraploid.
§Two of 5 tetraploid cases showed RREB1>CEP6, both with a percentage only slightly above the upper limit of normal. Repeat FISH and CGH at an outside lab were both negative and suggestive of tetraploidy.
^3 of 5 tetraploid cases analyzed with 6-probe FISH; 2 cases showed MYC>2 at near threshold for positive, both called negative and suggestive of tetraploidy at an outside lab by aCGH.
*Four cases had isolated 6q23 / MYB loss
Percentages may not add to 100% due to rounding.

Table 3. Correlation of immunohistochemical staining (IHC) and FISH results.
†Means all cases where that feature was assessable; denominator varies between attributes. Blue numbers indicate percentages obtained using the horizontal denominator to the left, as opposed to the vertical denominator above (black).
‡Fisher exact test.
Percentages may not add to 100% due to rounding

Table 4. FISH results and diagnosis in IHC-defined categories.

† Includes one case of isolated 6q23 / MYB loss
‡ Fisher exact test.
Percentages may not add to 100% due to rounding.

Table 5. Cases with unexpected or discordant FISH results and final diagnosis.
† D case: discordant case number.
‡ Isolated 6q23 loss.

Figure 1 – Table 5, Case 3 – 3-year-old boy with ear lesion, Category 1, FISH positive, called atypical Spitz tumor. A. Sections show a mostly intradermal melanocytic proliferation with ulceration and inflammatory crust (H&E, 20X); left inset shows p16 is retained in a checkboard pattern (p16, 40X); right inset shows BRAF V600E is negative (BRAF V600E, 40X). B. Cells are arranged in sheets, small nests and cords with scattered dermal mitoses (arrows) (H&E, 400X). C. Scattered multinucleated cells are noted (arrows) (H&E, 400X). D. Cells are fairly uniform, with abundant amphophilic cytoplasm, open chromatin, irregular nuclear contours (arrows) and prominent nucleoli (H&E, 400x).

Figure 2 – Table 5, Case 4 – 11-year-old boy with a forearm lesion, Category 1, FISH positive, called spitzoid melanoma. Sections show a large, mostly intradermal melanocytic proliferation with brisk inflammation (H&E, 10X); left inset shows p16 is retained diffusely (p16, 40x); middle inset shows BRAF V600E is negative (BRAF V600E, 40X); right inset shows Ki67 highlights a portion of the atypical melanocytes (Ki67, 200X) B. Cells are arranged in sheets and nests with impaired maturation, chronic inflammation, and patchy, irregular pigmentation (arrows) (H&E, 200X). C. Cells range from large to small with multinucleation, abundant amphophilic cytoplasm, open chromatin, irregular nuclear contours with multilobation, and prominent nucleoli (arrows) (H&E, 200x). D. Focal junctional activity is noted with rare pagetoid cells (arrows) (H&E, 200X).

Figure 3 – Table 5, Case 5 – 20-year-old woman with a shoulder lesion, Category 1, FISH negative, called spitzoid melanoma. A. Sections show a broad compound melanocytic proliferation with dermal fibrosis and inflammation. Cells are arranged in large and small nests and cords (H&E, 10X). B. Junctional nests show patchy pigmentation (arrow) and pagetoid spread (arrow). Cells have irregular nuclear contours with multilobation and prominent nucleoli (H&E, 200X). C. Nuclei range from large to small with open to dense chromatin and moderate amphophilic cytoplasm. (H&E, 200x). D. p16 was positive (p16, 100X); inset shows BRAF V600E was negative (BRAF V600E, 100X). E. Melan-A/Ki67 multiplex staining highlighted an elevated dermal proliferative index (Melan-A red/Ki67 brown, 100X).

Figure 4 – Table 5, Cases 7, 8 and 9. A, B. Case 7 – 13-year-old boy with a thigh lesion, Category 2, FISH positive, called atypical Spitz tumor. A. The lesion shows a large intradermal, nested melanocytic proliferation with impaired maturation (inset, H&E, 10X); cells are fusiform and uniform with amphophilic cytoplasm, open to coarse chromatin, prominent nucleoli, and scattered mitoses (arrows) (H&E, 400X). **B. Case 8 – 21-year-old woman with an abdominal lesion, Category 3, FISH positive, called dysplastic nevus with spitzoid features**. B. The lesion is a compound melanocytic proliferation with irregular nests, bridging of nests, and melanoderma (inset, H&E, 10X); cells are whorled within nests, and show a mix of cells with open chromatin and pale, dustily pigmented cytoplasm as well as more hyperchromatic nuclei (H&E, 200X). **C, D. Case 9 – 37-year-old woman with a calf lesion, Category 4, FISH negative, called spitzoid melanoma. C**. The lesion is a compound melanocytic proliferation with chronic inflammation (inset, H&E, 40X) and lateral pagetoid spread (arrows)(H&E, 200X). **D**. Cells have abundant amphophilic cytoplasm, and enlarged nuclei with inclusions, irregular nuclear membrane contours/multilobation (arrows) and prominent nucleoli (H&E, 400X).

Supplementary Table 1. Clinical Follow-up Data.
†Means all cases where that feature was assessible; denominator varies between attributes
‡Wilcoxon rank-sum test
§Fisher exact test
Percentages may not add to 100% due to rounding
SD: Standard deviation; IQR: Interquartile range

Supplementary Table 2. Cases with CGH Data.
†This case was tested by FISH prior to the introduction of 8q24 and 9p21 probes in our laboratory
‡This is the same case as Table 5, Case 1
Percentages may not add to 100% due to rounding
SD: Standard deviation; IQR: Interquartile range

Supplementary Table 3. Chromosomal abnormalities in FISH-positive cases, divided by IHC marker status.

Supplementary Methods:

IHC:
Four-micrometer paraffin-embedded, formalin-fixed tissue sections were deparaffinized and treated with CC1 epitope retrieval solution (VMS, catalog number 950-124) for 32 minutes (PRAME) or 64 minutes (p16, BRAF V600E, and ALK1). Sections were incubated with antibodies in VMS antibody diluent (VMS, 251-018) at 37°C at the dilution and for the interval as follows: For p16, prediluted E6H4 (VMS, 725-4793) for 16 minutes; for BRAF V600E, VE1 (Abcam, Cambridge, MA, USA, ab228461) at 1:175 for 16 minutes; for PRAME, EPR20330 (Abcam, ab219650) at 1:500 for 32 minutes; for ALK protein, ALK1 (Agilent/Dako, M719501-2) at 1:80 for 32 minutes. Staining was revealed in brown using the OptiView DAB detection kit (VMS, 760-700) or in red using the ultraView universal alkaline phosphatase red detection kit (VMS, 760-501).

FISH:
Four-micrometer formalin-fixed paraffin-embedded tissue sections were deparaffinized and treated with target retrieval solution (Dako, Carpinteria, CA, USA, S1699) followed by proteinase K digestion (Dako S3004). Probe mixtures were added and co-denatured at 73 C for 5 minutes, then hybridized at 37 C for 12-18 hours. Slides were treated with DAPI mounting medium (Vector Laboratories, Burlingame, CA, USA, H-1200). All specimens were examined using probes for 6p25 (Abbott 04N32-020), 6q23 (04N33-020), 11q13 (01N88-030), as well as CEP6 (06J54-016). Probes for 8q24 (02N22-020) and 9p21 (02N21-020), as well as CEP 9 (06J37-019), were added in 2017.

Table 1. Clinicopathologic features in FISH positive and negative cases

*Factor**	*All cases*	*FISH+*	*FISH-*	
Sex	*n = 174*	*n = 60*	*n = 114*	
Male	59 (34%)	22 (37%)	37 (32%)	*N.S.§*
Female	115 (66%)	38 (63%)	77 (68%)	
Age at diagnosis	*n = 174*	*n = 60*	*n = 114*	
Mean (SD)	29 (17)	28 (17)	29 (17)	
Median (interquartile range)	28 (15-40)	25 (14-39)	31 (15-40)	*N.S.+*
Range	5 mo – 74 yr	1 - 74 yr	5 mo - 67	
Tumor site	*n = 174*	*n = 60*	*n = 114*	
Extremities	79 (45%)	30 (50%)	49 (43%)	
Head and neck	41 (24%)	13 (22%)	28 (25%)	
Trunk	39 (22%)	11 (18%)	27 (23%)	*N.S.§*
Acral	8 (5%)	2 (3%)	6 (5%)	
Special site (breast/genital)	9 (5%)	4 (7%)	5 (4%)	
Spitzoid cytology	*n = 169*	*n = 60*	*n = 109*	
Epithelioid	101 (60%)	43 (72%)	58 (53%)	
Epithelioid and Spindled	59 (35%)	16 (26%)	43 (39%)	*N.S.§*
Spindled	9 (5%)	1 (2%)	8 (7%)	
Dermal Mitoses	*n = 133*	*n = 58*	*n = 75*	
Mean (SD)	1.1 (1.6)	1.6 (1.9)	0.8 (1.3)	
Median (interquartile range)	1 (0-2)	1 (0-2)	0 (0-1)	*p = 0.011+*
Range	0-10	0-10	0-8	
Architecture	*n = 174*	*n = 60*	*n = 114*	
Compound	140 (80%)	45 (75%)	95 (83%)	*N.S.§*
Dermal	34 (20%)	15 (25%)	19 (17%)	
	n = 115	*n = 42*	*n = 72*	
Epidermal hyperplasia	97 (84%)	37 (88%)	60 (83%)	*N.S.§*
Final Clinical Diagnosis				
Benign/Intermediate risk				
Spitz nevus	20 (11%)	0	20 (18%)	
Atypical Spitz tumor	82 (47%)	7 (12%)	75 (66%)	
Dysplastic nevus, spitzoid	16 (9%)	1 (2%)	15 (13%)	
Malignant				
Spitzoid melanoma	56 (32%)	52 (87%)	4 (4%)	
Among malignant lesions:				
Tumor thickness (mm)	*n = 56*	*n = 52*	*n = 4*	
Mean (SD)	1.6 (1.1)	1.6 (1.1)	0.9 (0.3)	
Median (interquartile range)	1.2 (0.9 – 1.8)	1.2 (0.9 – 1.8)	0.8 (0.75 – 1.0)	*N.S.+*
Range	0.4 – 7.0	0.4 – 7.0	0.7 – 1.2	
Ulceration	*n = 56*	*n = 52*	*n = 4*	
Present	3 (5%)	3 (6%)	0	*N.S.§*
Absent	54 (95%)	50 (94%)	4 (100%)	

Table 2. Positive Probes in FISH+ Cases

	FISH+	FISH-
n = 174	*n = 60 (34%)*	*n = 114 (66%)*
6p25 / *RREB1* >2	41 (68%)	6†‡ (5%)
11q13 / *CCND1* >2	29 (48%)	5‡ (4%)
6p25 / *RREB1* >CEP6	8 (13%)	2§ (2%)
6q23 / *MYB* <CEP6	19* (32%)	0
2 probes +	12/27 (44%)	
3 probes+	7/27 (26%)	
>3 probes+	2/27 (7%)	
n = 104	*n = 33 (32%)*	*n = 71 (68%)*
8q24 / *MYC* >2	16 (48%)	1^ (1%)
9p21 / *CDKN2A* (-/-)	13 (39%)	0
2 probes +	6/33 (18%)	
3 probes+	6/33 (18%)	
>3 probes+	6/33 (18%)	

Table 3. Correlation of IHC and FISH results

Factor	All cases†	FISH+	FISH-		Spitz nevus	AST	Spitzoid dysplastic nevus	Spitzoid melanoma
p16	*n = 134*	*n = 46*	*n = 88*		*n = 16*	*n = 67*	*n = 8*	*n = 43*
Retained	79 (59%)	16 (20%, 35%)	63 (80%, 72%)	$p < 0.001$‡	9 (56%)	50 (75%)	8 (100%)	12 (28%)
Lost	55 (41%)	30 (55%, 65%)	25 (45%, 28%)		7 (44%)	17 (25%)	0	31 (72%)
p16 staining pattern								
Retained diffusely	56 (46%)	9 (15%)	53 (60%)		9 (56%)	41 (61%)	6 (75%)	6 (14%)
Retained, checkerboard pattern	17 (13%)	7 (15%)	10 (11%)		0	9 (13%)	2 (25%)	6 (14%)
Lost, rare positive cells	15 (11%)	12 (26%)	3 (3%)		1 (6%)	2 (3%)	0	12 (28%)
Lost, total regional loss	19 (14%)	8 (17%)	11 (13%)		3 (19%)	7 (10%)	0	9 (21%)
Lost, total diffuse loss	21 (16%)	10 (22%)	11 (13%)		3 (19%)	8 (12%)	0	10 (23%)
9p21 status								
Isolated 9p21 (-/-)		5/30 (17%)	*n.a.*					
9p21 (-/-) and other abn		5/30 (17%)	*n.a.*					
Other abn, 9p21 retained		6/30 (20%)	*n.a.*					
Other abn, 9p21 not probed		14/30 (47%)	*n.a.*					
BRAF V600E	*n = 88*	*n = 41*	*n = 47*		*n = 8*	*n = 36*	*n = 7*	*n = 37*
Positive	14 (16%)	9 (64%, 22%)	5 (36%, 11%)	$p = 0.242$‡	0	0	5 (71%)	9 (24%)
Negative	74 (84%)	32 (43%, 78%)	42 (57%, 89%)		8 (100%)	36 (100%)	2 (29%)	28 (76%)
PRAME	*n = 56*	*n = 33*	*n = 23*		*n = 5*	*n = 19*	*n = 3*	*n = 29*
Positive (>75%)	15 (27%)	7 (47%, 21%)	8 (53%, 35%)	$p = 0.359$‡	0	7 (37%)	0	8 (28%)
Negative	41 (73%)	26 (63%, 79%)	15 (37%, 65%)		5 (100%)	12 (63%)	3 (100%)	21 (72%)
0%	37	23	14		5	11	3	18
<25%	1	1	0		0	0	0	1
25-75%	3	2	1		0	1	0	2

†Means all cases where that feature was assessible; denominator varies between attributes. Blue numbers indicate percentages obtained using the horizontal denominator to the left, as opposed to the vertical denominator above (black).
‡Fisher exact test
Percentages may not add to 100% due to rounding

Table 4. FISH results and diagnosis in IHC-defined categories

Category	1	2	3	4	
Predicted lesion type:	Spitz nevus/ AST low risk	AST/ Melanoma	Dysplastic nevus/MM high risk	Melanoma	
Factor	p16+/BRAF V600E- *n = 37*	p16-/BRAF V600E- *n = 31*	p16+/BRAF V600E+ *n = 9*	p16-/BRAF V600E+ *n = 4*	
FISH positive	5 (14%)	21 (68%)	5 (56%)	3 (75%)	*p*< 0.001‡
PRAME	*n = 15*	*n = 15*	*n = 5*	*n = 3*	
Positive	3	4	0	1	*N.S.‡*
Negative	12	11	5	2	
Diagnosis	*n = 37*	*n = 31*	*n = 9*	*n = 4*	
Benign/intermediate risk					
Spitz nevus	6	2	0	0	
Atypical Spitz tumor	26†	9	0	0	
Dysplastic nevus, spitzoid	2	0	5	0	
Malignant					
Spitzoid melanoma	3	22	4	4 (100%)	
FISH-positive, by probe	*n = 5*	*n = 21*	*n = 5*	*n = 3*	
RREB1 >2	3	15	5	3	
CCND1 >2	1	14	2	1	
RREB1 >CEP6	1	2	1	1	
MYB <CEP6	3†	7	2	0	
2 probes +	1	3	1	0	
3 probes+	0	4	1	1	
>3 probes+	0	0	1	0	
	4 / 5 tested:	*12 / 21 tested:*	*3 / 5 tested:*	*2 / 3 tested:*	
MYC >2	2	8	1	1	
CDKN2A (-/-)	0	7	0	1	
2 probes +	2	2	0	1	
3 probes+	1	3	0	0	
>3 probes+	0	4	0	0	

† Includes one case with isolated 6q23 / *MYB* loss

‡ Fisher exact test

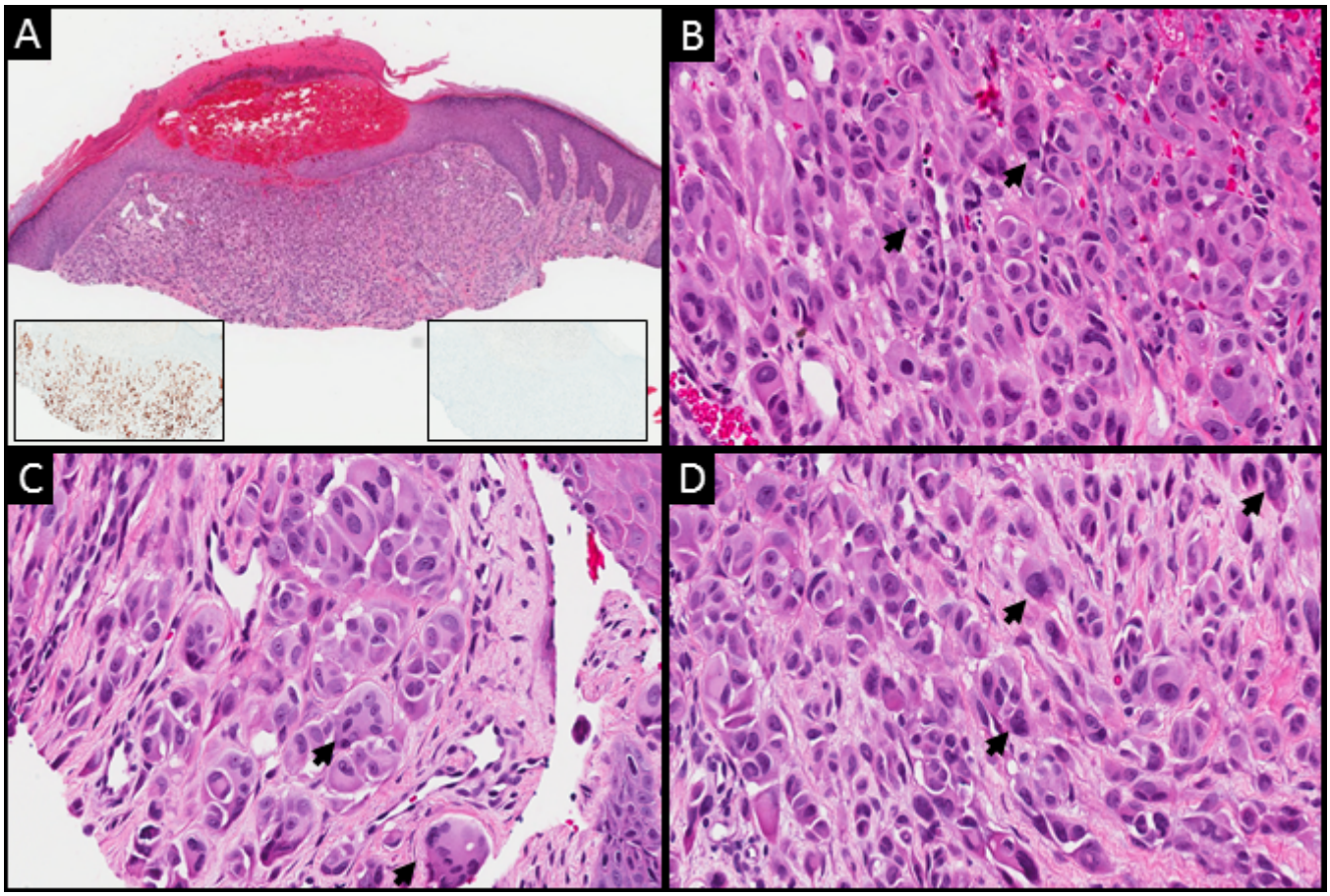
Percentages may not add to 100% due to rounding

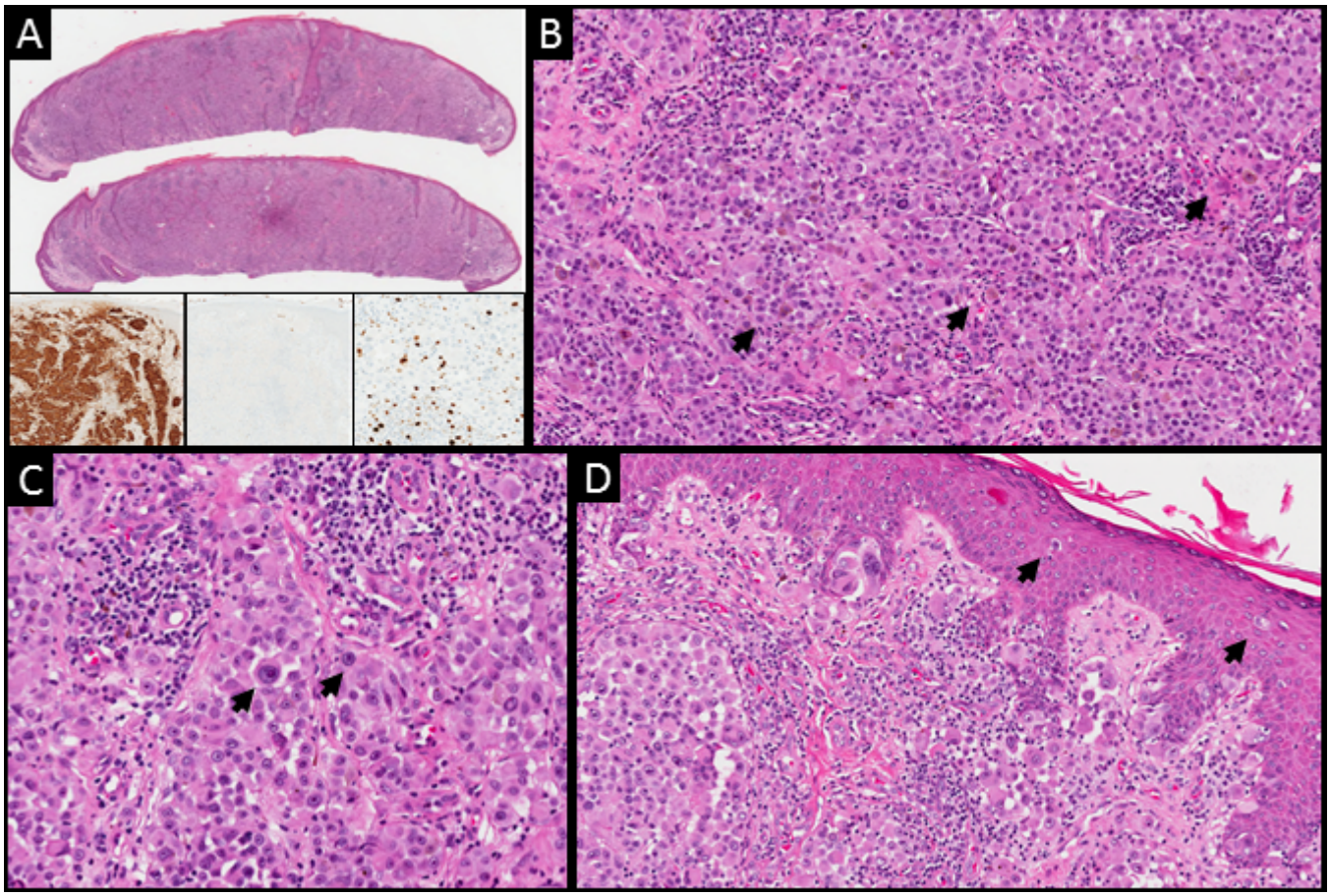
Table 5. Cases with unexpected or discordant FISH results and final diagnosis

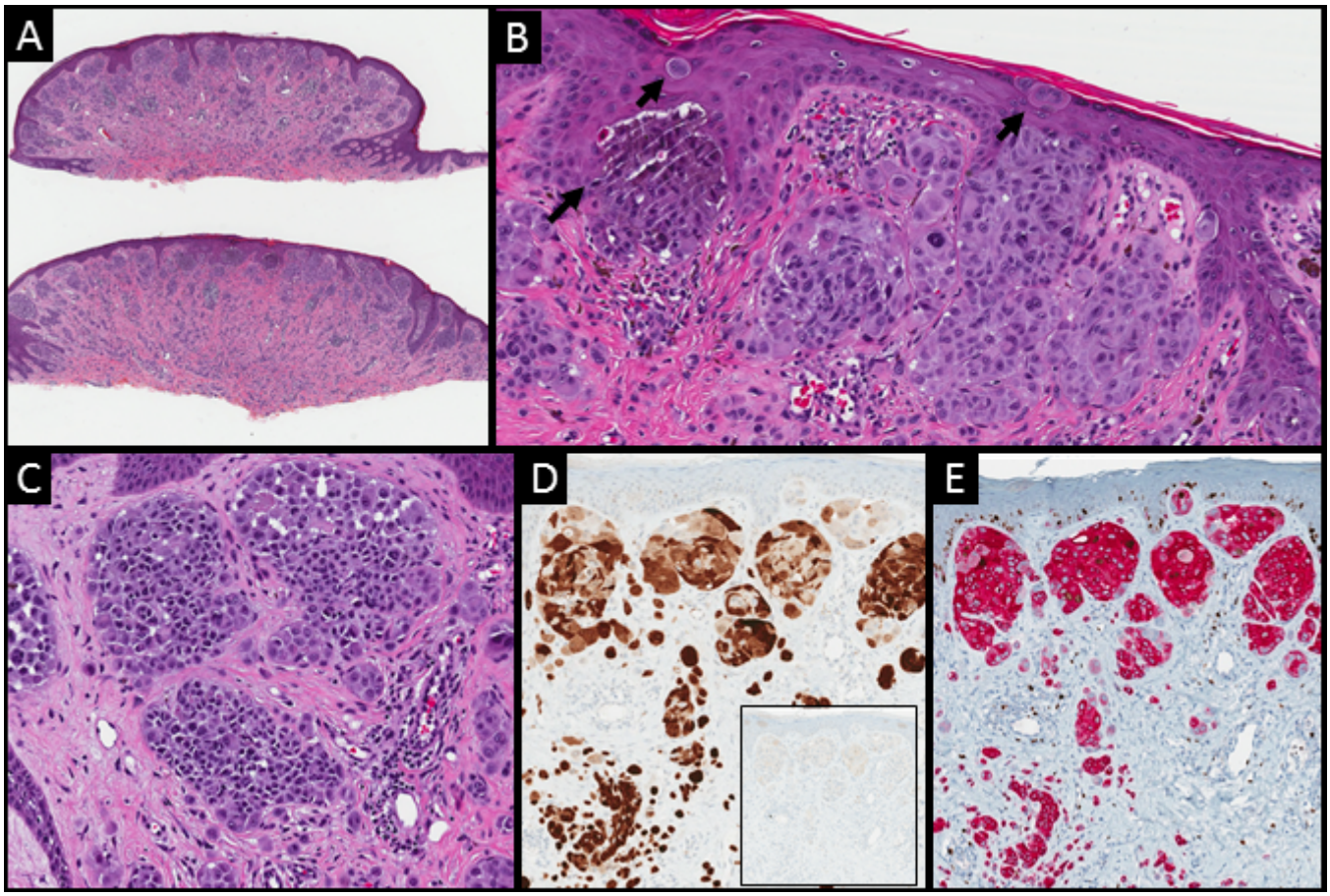
D Case†	Age	Sex	Site	*RREB1* > 2	*CCND1* > 2	*RREB1* > CEP6	*MYB* < CEP6	*MYC* > 2	*CDKN2A* (-/-)	PRAME	Diagnosis
Category 1 - p16 retained, BRAF V600E negative; FISH positive											
1‡	*3*	*F*	*Cheek*	*-*	*-*	*-*	*+*	*-*	*-*	*N*	*AST*
2	1	F	Leg	+	+	-	-	nd	nd	N	AST
3	7	M	Ear	+	-	-	-	+	-	N	AST
4	11	M	Forearm	+	-	+	+	-	-	N	Spitzoid melanoma
5	20	F	Shoulder	-	-	-	+	+	-	N	Spitzoid melanoma
Category 1 - p16 retained, BRAF V600E negative; FISH negative but called malignant											
6	62	F	Forearm	-	-	-	-	-	-	nd	Spitzoid melanoma
Category 2 - p16 lost, BRAF V600E negative; FISH positive but called benign											
7	13	M	Thigh	-	+	-	+	nd	nd	N	AST
Category 3 - p16 retained, BRAF V600E positive; FISH positive but called benign											
8	21	F	Abdomen	+	-	-	-	-	-	N	Dysplastic nevus with spitzoid features
Category 4 - p16 lost, BRAF V600E positive; FISH negative but called malignant											
9	37	F	Calf	-	-	-	-	-	-	+ in area of p16 loss	Spitzoid melanoma

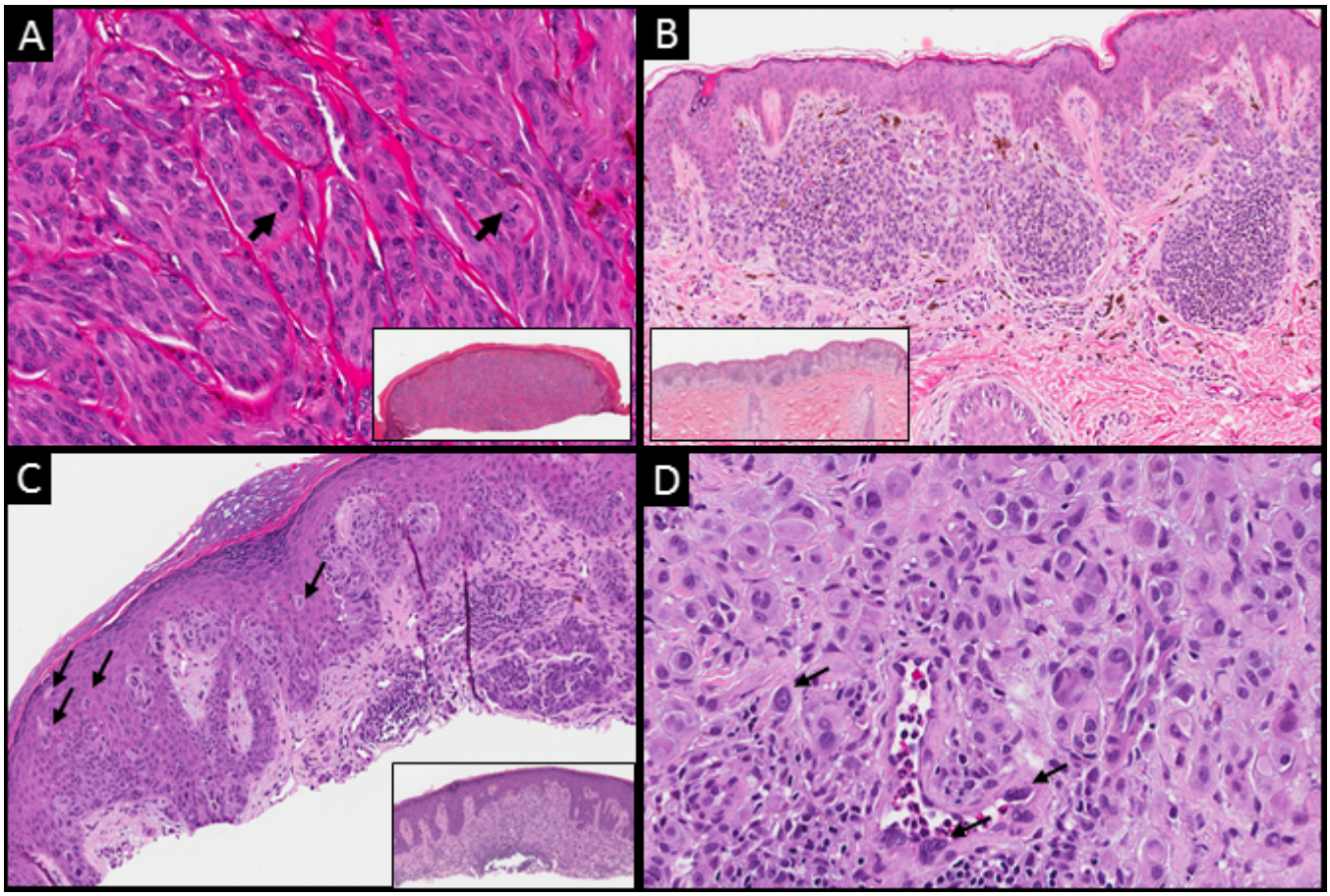
†D case: discordant case number

‡ Isolated 6q23 loss









Supplementary Table 1. Clinical follow up data

*Factor**	*All cases*	*FISH+*	*FISH-*	
	n = 46	*n = 15*	*n = 21*	
Length of follow up (months)				
Mean (SD)	57 (30)	63 (29)	54 (30)	
Median (IQR)	59 (26 - 82)	63 (46 - 87)	51 (33 - 79)	*N.S.#*
Range	2 - 99	2 - 96	5 - 99	
Age at diagnosis				
Mean (SD)	28 (18)	28 (15)	29 (19)	
Median (IQR)	28 (15 - 40)	22 (16 - 34)	20 (12 - 42)	*N.S.#*
Diagnosis				
Spitz nevus	7 (15%)	0	7 (33%)	
AST	16 (35%)	2 (13%)	16 (51%)	
Dysplastic nevus, spitzoid	8 (17%)	1 (7%)	7 (33%)	
Spitzoid melanoma	13 (28%)	12 (80%)	1 (5%)	
Lymph node status				
SLNBx performed	12 (26%)	11 (73%)	1 (5%)	
SLNBx positive	1 (8%)	1 (9%)	0	
Tumor site				
Extremities	20 (43%)	8 (53%)	12 (59%)	
Head and neck	9 (20%)	2 (13%)	7 (33%)	
Trunk	11 (24%)	4 (27%)	7 (33%)	
Acral	2 (4%)	0	2 (6%)	
Special site (breast/genital)	4 (9%)	1 (7%)	3 (10%)	
Dermal Mitotic Rate				
Mean (SD)	1 (1.2)	1.5 (1.5)	0.7 (1.1)	
Median (IQR)	0 (0 - 2)	1 (0 - 2)	0 (0 - 1.5)	*N.S.#*
BRAF V600E	*n = 21*	*n = 12*	*n = 9*	
Positive	5	4 (33%)	1 (11%)	*N.S.§*
Negative	16	8 (67%)	8 (89%)	
p16	*n = 22*	*n = 10*	*n = 22*	
Retained	22 (70%)	3 (30%)	20 (87%)	*p = 0.003§*
Lost	10 (30%)	7 (70%)	3 (13%)	
HRAS	*n = 12*	*n = 10*	*n = 3*	
Positive (>75%)	2 (15%)	1 (10%)	1 (33%)	*N.S.§*
Negative (<75%)	11 (85%)	9 (90%)	2 (67%)	

Among malignant lesions:

Tumor thickness (mm)	*n = 13*	*n = 12*	*n = 1*
Mean (SD)	1.2 (0.8)	1.2 (0.8)	0.7 (n.a.)
Median (IQR)	0.95 (0.7 - 1.2)	1.0 (0.7 - 1.4)	0.7 (n.a)
Range	0.4 - 3.2	0.4 - 3.2	n.a.
Ulceration	*n = 13*	*n = 12*	*n = 1*
Present	0	0	0
Absent	13	12	1

Supplementary Table 2. Cases with CGH data

Case	Age	Sex	Site	p16	BRAF V600E	PRAME	FISH abnormality	CGH abnormality	Diagnosis
1	2	M	Thigh	L	nd	nd	-†	9p21 homozygous loss	Spitzoid melanoma
2	7	M	Finger	nd	nd	nd	-	-	AST
3	7	F	Arm	nd	nd	nd	-	Heterozygous loss of chr 9	AST
4	4	F	Calf	R	N	nd	-	-	AST
5	9	F	Thigh	L	N	nd	-	Tetraploidy; one copy of chr 17 lost	AST
6	31	F	Groin	R	N	N	-	Nonspecific copy number changes on chr 3	AST
7	28	M	Arm	R	N	P	-	-	AST
8	4	M	Arm	L	nd	nd	9p21 (-/-)	9p21 homozygous loss	Spitzoid melanoma
9‡	3	F	Cheek	R	N	nd	6q23 (*MYB*) loss	Possible ROS1 break	AST

†This case was tested by FISH prior to the introduction of 8q24 and 9p21 probes in our laboratory
‡This is the same case as Table 5, Case 1

Supplementary Table 3. Chromosomal abnormalities in FISH-positive cases, by IHC marker status

	p16 lost	p16 lost, 9p21 retained	BRAF V600E +	PRAME +
	n = 30	*n = 6*	*n = 9*	*n = 3*
6p25 / *RREB1* >2	26 (87%)	6 (100%)	8 (89%)	1 (33%)
11q13 / *CCND1* >2	21 (70%)	5 (83%)	3 (33%)	1 (33%)
6p25 / *RREB1* >CEP6	6 (20%)	0	2 (22%)	0
6q23 / *MYB* <CEP6	8 (27%)	2 (33%)	2 (22%)	0
	n = 16	*n = 6*	*n = 6*	*n = 3*
8q24 / *MYC* >2	10 (63%)	5 (83%)	2 (33%)	1 (33%)
9p21 / *CDKN2A* (-/-)	10 (63%)	(n/a)	1 (17%)	2 (67%)