Modern Pathology (2006) 19, 1324–1332

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BRAF and *NRAS* mutations in spitzoid melanocytic lesions

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BRAF **mutations are common events in a variety of melanocytic nevi and primary cutaneous melanomas. We have previously found *BRAF* mutations in 82% of nevi, consisting of congenital, common acquired and dysplastic types, and 33% of primary cutaneous melanomas other than the spitzoid type, similar to other published reports. A small number of studies have evaluated Spitz nevi and have failed to detect any lesions possessing a *BRAF* mutation. Only one study included categories of atypical Spitz nevus and borderline lesions suspected to be spitzoid melanomas, along with classic Spitz nevi and spitzoid melanomas. We examined a spectrum of spitzoid lesions that included 48 Spitz nevi, some with atypical features, seven atypical (borderline) Spitz tumors, and 13 spitzoid melanomas. *BRAF* mutations were detected in 12 of 68 spitzoid lesions, of which two were spitzoid melanomas and 10 were Spitz nevi. Five of the 10 Spitz nevi with *BRAF* mutations were altered by more than usual cytologic atypia and/or architectural atypia overlapping with dysplastic nevi, or irritation/inflammation; one desmoplastic Spitz nevus had a *BRAF* mutation. These results indicate that a small subset of Spitz nevi, some with atypical histologic features, possess *BRAF* mutations. Therefore, the *BRAF* mutational status does not separate all Spitz nevi from spitzoid melanomas and non-Spitz types of melanocytic proliferations, contrary to previous reports.**
Modern Pathology (2006) **19,** 1324–1332. doi:10.1038/modpathol.3800653; published online 23 June 2006

Keywords: BRAF; NRAS; mutation; Spitz nevi; melanoma

In 1948, Sophie Spitz described a series of 13 patients with 'melanomas in childhood', one of whom died from dissemination of metastatic melanoma. She considered these lesions to be juvenile melanomas with a generally better prognosis than conventional melanomas in adulthood.[1] Shortly thereafter, it was asserted that these lesions were, in fact, benign nevi because of their indolent behavior.[2,3] Spitz's seminal paper underscored the difficulties in distinguishing some Spitz nevi from melanomas with Spitz-like features that can metastasize and potentially eventuate in a fatal outcome. While criteria have been published over the years in an effort to distinguish Spitz nevi from Spitz-like melanomas, this diagnostic dilemma continues to plague dermatopathologists. It is readily apparent that there may be a lack of consensus in the diagnosis of spitzoid lesions even among experts in this field.[3,4]

Fortunately, most spitzoid lesions can be classified into benign Spitz nevi or Spitz-like melanomas based on published criteria.[5–15] However, a subset of spitzoid lesions remain that have histologic features that deviate from a typical Spitz nevus, yet are insufficient for a definitive diagnosis of Spitz-like melanoma. These atypical spitzoid lesions have been referred to variously in the literature as borderline and intermediate melanocytic neoplasia, minimal-deviation melanoma, nevoid melanoma, atypical Spitz nevus/tumor, malignant Spitz nevus, problematic Spitzoid melanocytic lesions, and diagnostically controversial Spitzoid melanocytic tumors.[16–22]

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Received 3 May 2006; revised 31 May 2006; accepted 1 June 2006; published online 23 June 2006

Recently, interest in the *RAS-RAF-MEK-ERK-MAP* kinase signal transduction pathway has arisen as a site for mutational analysis in a broad spectrum of melanocytic lesions, including Spitz nevi. In a landmark study by Davies *et al*,[23] 66% of malignant melanomas were shown to harbor a T1796A *BRAF* mutation in exon 15 resulting in the substitution of valine by glutamic acid at position 600 (V600E). Subsequently, Pollock *et al*[24] demonstrated a high incidence (82%) of the same *BRAF* mutation in a variety of non-Spitz nevi, similar to their primary invasive melanoma group. Since then, many studies have reported *BRAF* mutations in benign and malignant melanocytic lesions.[25–39] However, a number of studies have failed to demonstrate *BRAF* mutations in Spitz nevi.[25,40–46,57–61] Most of these studies analyzed only classic Spitz nevi. Only one study included atypical Spitz nevi and histologically borderline spitzoid lesions in their *BRAF* analysis.[40]

The purpose of this study was to evaluate *BRAF* and *NRAS* mutations in a spectrum of spitzoid lesions, which included classic and atypical Spitz nevi, atypical Spitz tumors of uncertain biologic behavior and spitzoid melanomas, to determine whether the presence or absence of these mutations can distinguish among the different groups of spitzoid lesions and to compare our results to existing data in the literature.

Materials and methods

Case Selection

A spectrum of 68 spitzoid melanocytic lesions, including 48 Spitz nevi, seven atypical Spitz tumors, and 13 spitzoid melanomas, were retrieved from the archives of the Pathology Department at the University of Michigan. The spitzoid lesions were independently reviewed by three board certified dermatopathologists (LL, LDS, and DRF) with diagnostic expertise in pigmented lesions and who are members of the Multidisciplinary Melanoma Clinic at the University of Michigan. Of the 48 Spitz nevi, 21 were classic Spitz nevi, on the basis of previously published criteria,[6,7] and two were desmoplastic Spitz nevi. The remaining 25 Spitz nevi demonstrated some atypical features, such as architectural disorder, increased cytologic atypia and/or inflammation. This subset of Spitz nevus has been referred to in the literature as atypical or dysplastic Spitz nevus.[47] Nonetheless, as these atypical lesions still retain the salient histologic criteria for Spitz nevi, they are classified within the Spitz nevus group. The seven lesions classified as atypical Spitz tumors shared some histologic criteria with conventional Spitz nevi, such as small diameter, symmetry, lateral circumscription, epidermal hyperplasia, and/or Kamino bodies. These lesions, however, showed histologic features that significantly deviated from conventional Spitz nevus, yet lacked sufficient histologic criteria for Spitz-like melanoma. These criteria included expansile or sheet-like dermal growth, incomplete to absent dermal maturation, bulbous extension into the deep dermis or subcutis, deep dermal mitoses, and/or high-grade nuclear atypia.[10,13,16,21,22,48] Although the 13 spitzoid melanomas maintained some low-power resemblance to Spitz nevi, they demonstrated at least one, and often multiple, of the following histologic features: asymmetrical growth, lack of lateral circumscription, pagetoid spread of melanocytes in the epidermis, aberrant dermal growth, dermal mitoses at all levels of the lesion, atypical mitoses, and high-grade nuclear atypia.[9,10,12,13,48] The Institutional Review Board at the University of Michigan has approved this study.

DNA Extraction

DNA was extracted from slides as previously described.[49] Briefly, lesional DNA was microdissected from unstained slides from paraffin-embedded tissue blocks. Areas of microdissection were circled on corresponding H&E stained slides by one dermatopathologist (DRF), which in turn were used as a template. Following dissection from the slides, xylene was added to remove paraffin and the DNA was precipitated with ethanol. Following centrifugation, the supernatant was discarded and the pellet was lyophilized. The pellet was resuspended in 100 μl Proteinase K buffer (50 mM tris and 200 ng/μl Proteinase K). The samples were incubated overnight at 37°C and then denatured at 95°C.

Mutational Analysis

Mutations in *BRAF* codon 600 were identified by direct sequencing of exon 15 of *BRAF* following PCR amplification of DNA extracted from paraffin-embedded samples. *NRAS* mutations were identified by direct sequencing of exons 1 and 2 of *NRAS* following PCR amplification. PCR reactions included 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, 100 ng both forward and reverse primer, 1.5 U AmpliTaq Gold (Applied Biosystems), and 2 μl microdissected tumor DNA in a total volume of 50 μl. Samples were denatured for 5 min at 95°C and were passed through 40 cycles of amplification, which consisted of 60 s of denaturation at 95°C, 1 min of primer annealing at 56°C, and 1 min of elongation at 72°C. The DNA sequences of the primers for exon 15 of the *BRAF* gene were forward: 5′TCATAATGCTTG CTCTGATAGGA and reverse: 5′GGCCAAAAATTT AATCAGTGGA.[23] The DNA sequences of the primers for exon 1 of the *NRAS* gene were either forward: 5′ATGACTGAGTACAAACTGGT and reverse: 5′CTCTATGGTGGGTCATATT or forward: 5′ATGACTGAGTACAAACTGGT and reverse: 5′CTCTATGGTGGGATCATATT. The sequences of

the primers for exon 2 of the *NRAS* gene were forward: 5′GGTGAAACCTGTTTGTTGGA and reverse: 5′ATACACAGAGGAAGCCTTC. All sequencing for *BRAF* and *NRAS* mutations was performed on the ABI 3700 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). *BRAF* mutations were detected by using Mutation Surveyor™ software (Softgenetics Inc., State College, PA, USA), and confirmed by visual inspection of chromatograms by two independent readers (JNP, SBG).

Statistical Methods

All statistical analyses were performed using SASv.9.1 for Windows (SAS Institute, Cary, NC, USA). Fisher's Exact test was used to test for differences in *BRAF* frequencies between groups. The Wilcoxon rank sum test was used to test the difference in median age by mutation status.

Results

The clinical features and *BRAF* status of spitzoid lesions from 68 patients are summarized in Table 1. Spitz nevi were removed from 24 males and 24 females and the patients ranged from 2 to 49 years (median = 19 years) in age. Spitz nevi were located on the head and neck (10/48, 21%), trunk (9/48, 19%), or extremities (26/48, 54%); the anatomic location was not specified for three (6%) Spitz nevi.

Atypical Spitz tumors were more common in females[5] than males,[2] and patients ranged in age from 12 to 52 years (median = 24 years). Atypical Spitz tumors were located on the head and neck (1/7, 14%), trunk (1/7, 14%), and extremities (5/7, 72%). All seven atypical Spitz tumors had sentinel lymph node biopsies that were negative for metastatic disease.

Spitzoid melanomas were diagnosed in 10 female and three male patients. The patients ranged in age from 10 to 60 years old (median = 24 years). Spitzoid melanomas were removed from the head and neck (1/13, 8%), trunk (2/13, 15%), and extremities (10/13, 77%). Five of 10 (50%) spitzoid melanomas had positive sentinel lymph nodes for metastatic melanoma. Sentinel lymph node biopsy was not performed in three patients with thin (<1 mm) spitzoid melanomas.

In 12 of 68 (18%) patients, a full set of diagnostic slides were not available for review. The initial diagnosis was rendered by one of the three dermatopathologists in this study but the slides were returned to the referring institution and were no longer available for review by the other two dermatopathologists at the time of this study. The slides from the remaining 55 of 68 patients (81%) were independently reviewed by three dermatopathologists. There was concordance among all three dermatopathologists in classifying 41 of 55 (75%) lesions into one of the three groups of spitzoid lesions. There was, however, discordance among one of the three dermatopathologists in classifying 14 of 55 (25%) spitzoid lesions. In no instance did all three dermatopathologists completely disagree in the classification of a spitzoid lesion across all three categories.

The diagnosis of record for cases where there was diagnostic discordance in this study was the diagnosis rendered by two of the three dermatopathologists. The most common cause for discordance among dermatopathologists was in classifying lesions between Spitz nevi and atypical Spitz tumors (12/14, 75%). There was discordance in classifying two lesions between atypical Spitz tumors and spitzoid melanomas, one of which had a sentinel lymph node biopsy performed that was negative for metastatic melanoma. The distinction between an atypical Spitz tumor and a spitzoid melanoma is a predictable dilemma and in our institution these lesions are treated similarly based on the maximum depth of dermal extension.

BRAF (V600E) mutations were detected in 12 of 68 (18%) spitzoid lesions (Figure 1). Ten of 12 (83%) spitzoid lesions with *BRAF* mutations were Spitz nevi, while the remaining two (17%) lesions were spitzoid melanomas. None of the seven atypical Spitz tumors had a *BRAF* mutation. There was no significant difference in the frequency of *BRAF*

Table 1 Clinical features and *BRAF* status in the spectrum of spitzoid lesions

	N	*Clinical features*					*BRAF status V600E/tot (%)*
		Age (years)	*Gender*	*Anatomic location*[a]			
		Range (Median)	*F/M*	*H/N*	*T*	*E*	
Spitz nevi	48	2–49 (20)	24/24	10	9	26	10/48[b] (21%)
Atypical Spitz tumors	7	12–52 (24)	5/2	1	1	5	0/7 (0%)
Spitzoid melanomas	13	10–60 (24)	10/3	1	2	10	2/13 (15%)

[a]Three Spitz nevi were from unspecified anatomic locations (H/N = head/neck, T = trunk, E = extremity).
[b]Five out of 10 were classic (typical) Spitz nevi and 5/10 were atypical Spitz nevi.

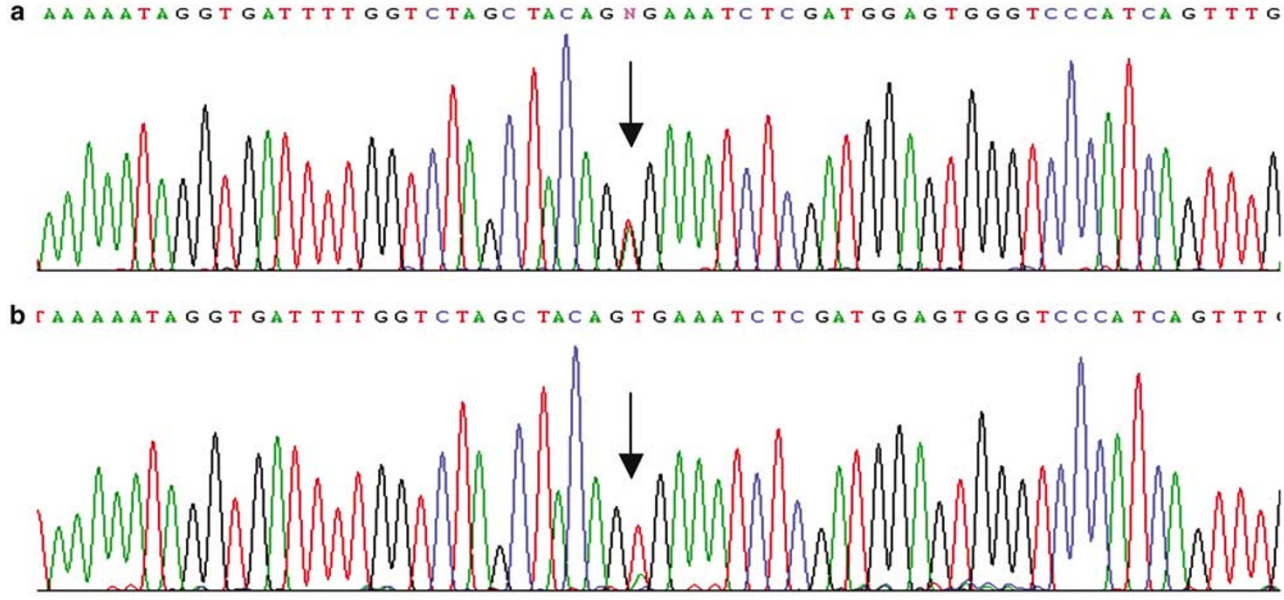

Figure 1 Chromatogram traces showing *BRAF* (V600E) mutations in (**a**) an atypical compound Spitz nevus (case 5; see Figures 2a and b for histopathology) and (**b**) a compound Spitz nevus (case 8; see Figures 2c and d for histopathology).

mutations by type of lesion (Exact $P=0.60$). Of the 10 Spitz nevi with *BRAF* mutations, five (50%) had histologic features of Spitz nevi and five (50%) had atypical histologic features, yet lacked histologic criteria of an atypical Spitz tumor or spitzoid melanoma (Figure 2). One Spitz nevus with a *BRAF* mutation was an intradermal desmoplastic Spitz nevus. Seven of the 10 Spitz nevi demonstrated some degree of epidermal hyperplasia and four cases had Kamino bodies. Five of these Spitz nevi were inflamed, at least focally, and two were interpreted as halo Spitz nevi. One Spitz nevus was irritated or traumatized, as evidenced by epidermal ulceration. Five atypical Spitz nevi had more than usual cytologic atypia and three also had some architectural disorder similar to an atypical (dysplastic) nevus. Of the 10 Spitz nevi with *BRAF* mutations, five occurred on the extremities, three on the trunk, and two on the head and neck. However, there was no significant difference in the proportion of all spitzoid lesions with *BRAF* mutations among the extremities (17%), trunk (25%) and head and neck (16%), (Exact $P=0.89$). The age of patients with *BRAF* mutations in Spitz nevi ranged from 2 to 38 (mean = 16; median = 14) years of age; five patients were less than 10 years old. There was no significant difference in the age of patients with Spitz nevi with *BRAF* status (mean = 15.9 years) compared to those without *BRAF* mutations (mean = 20.6 years), ($P=0.32$). Both spitzoid melanomas with *BRAF* mutations arose on the extremities, and the patients were 12 and 43 years of age (Table 2).

Only one of 68 spitzoid lesions, an atypical Spitz nevus, had an *NRAS* mutation. No lesion demonstrated both *NRAS* and *BRAF* mutations.

Discussion

In a subset of spitzoid lesions, differentiating a Spitz nevus from a Spitz-like melanoma can be problematic and, subsequently, the confidence in rendering a definitive diagnosis declines. In such instances, attention has turned to ancillary techniques to aid in this distinction. Immunohistochemical studies have yielded variable results. For instance, stratification of HMB-45 has been demonstrated in Spitz nevi by some investigators but not by others.[16,18,50,51] Immunohistochemical staining with proliferation markers, such as Ki-67 and proliferating cell nuclear antigen, has yielded encouraging results in some studies.[18,51–57] The immunohistochemical pattern of S100A6 protein expression has been shown to significantly differ between Spitz nevi and other melanocytic nevi and melanomas.[58] The mean silver staining pattern of the nucleolar organizer region (AgNOR) is lower in Spitz nevi compared to melanomas but there is overlap that limits its utility.[59] Telomerase activity has been shown to be lowest in Spitz nevi by some groups,[60,61] whereas other investigators have found a similar telomerase activity in Spitz nevi when compared to ordinary nevi and melanomas.[62]

Molecular methods have been employed recently to evaluate a spectrum of melanocytic lesions, including Spitz nevi. Comparative genomic hybridization has shown chromosomal gains involving the p-arm of chromosome 11 and the q-arm of chromosome 7 in three of 17 and one of 17 Spitz nevi, respectively.[63] Mutational analysis of the *BRAF* gene has shown that *BRAF* mutations are common events in most types of melanocytic nevi

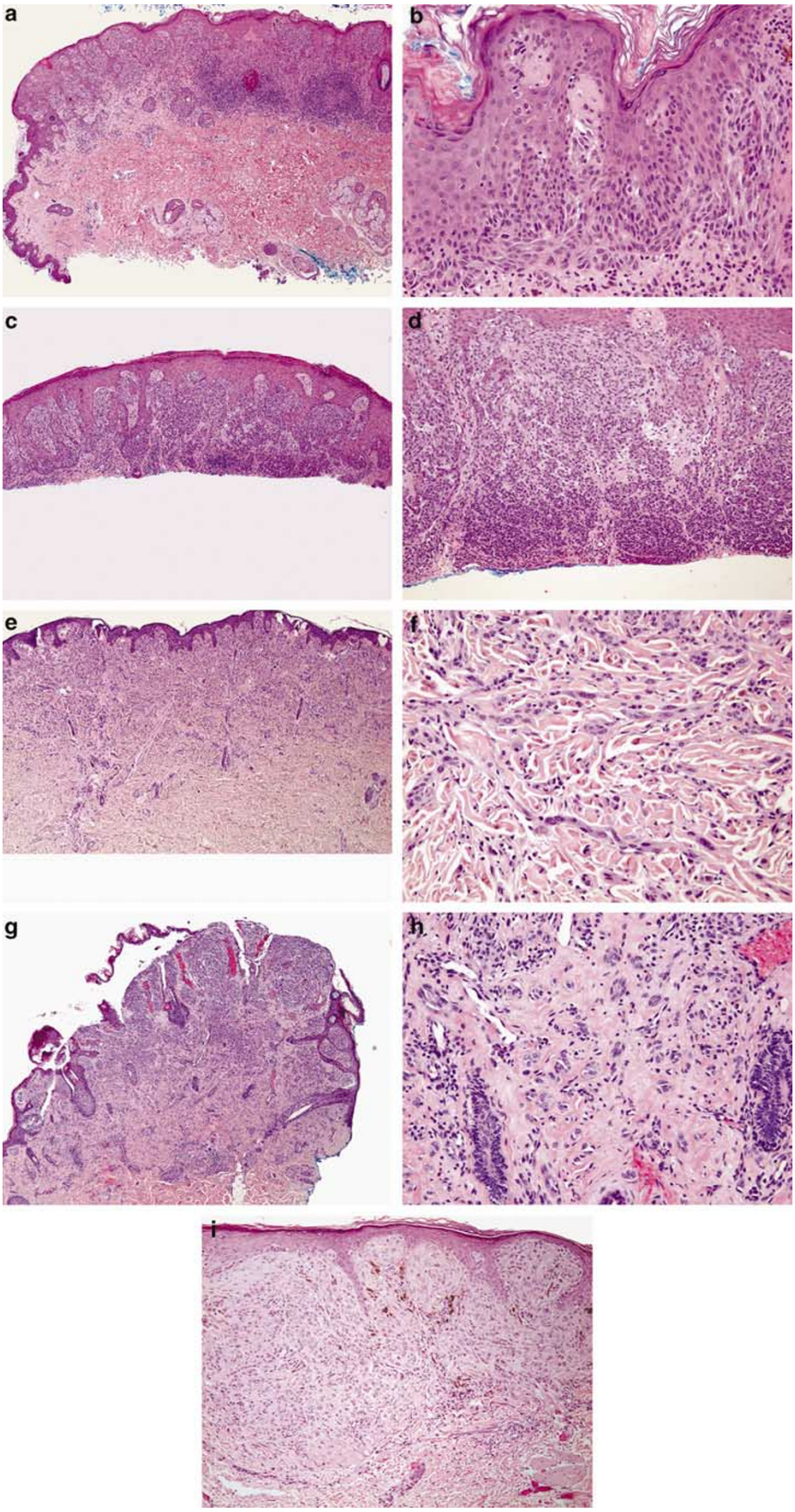
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Table 2 Clinical features and histologic diagnoses of spitzoid lesions harboring *BRAF* (V600E) mutations

Case	*Age*	*Sex*	*Location*	*Diagnosis*
1	2	M	Face	Compound Spitz nevus
2	3	F	Back	Intradermal Spitz nevus with desmoplastic stromal response
3	5	M	Arm	Atypical compound Spitz nevus
4	6	M	Leg	Halo compound Spitz nevus
5	7	F	Chest	Atypical compound Spitz nevus
6	12	F	Arm	Spitzoid melanoma
7	20	F	Leg	Atypical compound Spitz nevus
8	21	M	Leg	Compound Spitz nevus
9	23	F	Face	Atypical compound Spitz nevus
10	34	M	Arm	Atypical compound Spitz nevus
11	38	M	Chest	Compound Spitz nevus
12	43	F	Arm	Spitzoid melanoma

and melanomas. Similar to other investigators, we have previously shown that the highest incidence of *BRAF* mutations (82%) occurred in a group of congenital, common acquired, and atypical (dysplastic) nevi.[24,27,42,64] In contrast, 27% of primary invasive melanomas and 39% of metastatic melanomas had *BRAF* mutations in our series, which is similar to previously published data by other groups.[25,35,39] The high incidence of *BRAF* mutations in nevi suggests that this is an early event in melanocytic neoplasia.

Several studies have evaluated the frequency of *BRAF* mutations in Spitz nevi and, to date, there have been no reported mutations in Spitz nevi.[25,40–46] With the exception of the study by van Dijk *et al*,[40] which included an atypical Spitz nevus group, all other studies evaluated typical or classic Spitz nevi and/or spitzoid melanoma. The classic or typical Spitz nevus is usually readily distinguished from melanoma when standard histologic criteria are applied. However, when spitzoid lesions become increasingly atypical, the distinction of an atypical Spitz nevus/tumor from melanoma becomes more difficult and can be indistinguishable on histologic grounds in a small percentage of cases. Analyzing these atypical Spitz nevi and atypical Spitz tumors for *BRAF* mutations would be especially important to determine if their *BRAF* mutation status is more like that observed in classic Spitz nevi or non-Spitz nevi and melanomas.

In this study, we evaluated a spectrum of spitzoid lesions, which included typical and atypical Spitz nevi, atypical Spitz tumors of uncertain biologic potential, and spitzoid melanomas. In order to validate our classification of lesions, three dermatopathologists independently reviewed each spitzoid lesion based on previously published criteria. The concordance in the diagnosis of spitzoid lesions within our group was very good (75%), although there was discordance in 25% of the cases, which is to be expected based on previous reports in the literature.[4,5]

In contrast to other series in the literature,[25,40–46] we identified *BRAF* mutations in 10 Spitz nevi, five of which specimens had atypical histologic features such as architectural disorder, increased cytologic atypia, and/or inflammation. The somewhat underpowered sample size of our study of the group of 48 Spitz nevi does not permit firm conclusions to be drawn with respect to age and *BRAF* status; however, we did not appreciate any statistically significant differences by age. We did note that the mean age of patients with *BRAF*-positive Spitz nevi (15.9 years) was slightly lower than the mean age of patients with *BRAF*-negative Spitz nevi (20.6 years), ($P = 0.32$), which is consistent with observations in melanoma.[65] Moreover, five of 10 Spitz nevi with *BRAF* mutations occurred in children less than 10 years old. The Spitz nevi possessing *BRAF* mutations were from a similar anatomic distribution as Spitz nevi without *BRAF* mutations in our study. We noted a slightly higher proportion of all spitzoid lesions on the trunk harboring *BRAF* mutations, but this was not statistically significant. The melanoma literature suggests that *BRAF* mutations are more common in melanomas arising in intermittently sun-exposed anatomic regions,[65] but the power of the present study did not permit us to fully investigate this hypothesis for spitzoid lesions. It is not surprising that we found *BRAF* mutations in a small number of spitzoid melanomas, considering that spitzoid melanomas have been shown to have *BRAF* mutations in previous studies.[40,44]

The novel finding in our study is the presence of *BRAF* mutations in a subset of Spitz nevi, which has not been previously reported. Most previous studies have limited their analysis to Spitz nevi with classic histologic features. The exception to this is the study by van Dijk *et al*,[40] which included atypical Spitz nevi and spitzoid tumors suspected of being spitzoid melanomas along with their Spitz nevi and spitzoid melanoma groups. In contrast to our findings where we identified 10 (five atypical) Spitz nevi with *BRAF* mutations, they did not detect any *BRAF* mutations in their classic Spitz or atypical Spitz nevus groups. It is difficult to compare our findings to those of van Dijk *et al*, since they showed histologic images of a classic Spitz nevus, a spitzoid melanoma, and a

Figure 2 Histopathologic features of representative Spitz nevi with detectable *BRAF* mutations. Case 5 shows an atypical compound Spitz nevus with focal inflammation (**a**) and evidence of Kamino bodies within the epidermis (**b**). Case 8 demonstrates a compound Spitz nevus (**c**) with maturation of Spitz nevus cells with descent in the dermis (**d**). Case 2 shows a predominantly intradermal Spitz nevus with desmoplastic stromal response (**e, f**). Case 1 shows an irritated compound Spitz nevus with small nests of spitzoid melanocytes in the deepest portion of its dermal component (**g, h**). A spitzoid melanoma corresponding to case 12 has an aberrant dermal growth pattern of epithelioid spitzoid melanocytes with striking cytologic atypia (**i**).

lesion suspected of being a spitzoid melanoma but they did not show any histologic images of an atypical Spitz nevus. It is possible that different criteria were applied to these lesions between our and their groups of observers. Interestingly, one of our cases that possessed a *BRAF* mutation was an intradermal desmoplastic Spitz nevus. We had one other desmoplastic Spitz nevus in our series that lacked a *BRAF* mutation. Prior studies did not describe any lesions that were desmoplastic Spitz nevi, so there is no data available for comparison in the literature. Our low number of desmoplastic Spitz nevi precludes drawing any conclusion regarding *BRAF* status in this variant and requires accrual of additional cases for further evaluation.

Atypical Spitz tumors are problematic lesions because they possess overlapping histologic features between Spitz nevus and spitzoid melanoma. In our study, none of our atypical Spitz tumors had *BRAF* mutations, which is consistent with the results reported by van Dijk *et al*.[40] This interpretation is limited, however, by the small sample size in this group. More cases of atypical Spitz tumors are required for analysis to add strength to this data.

NRAS mutations involving codon 61 and, to a lesser extent, codons 12 and 13, have been previously reported in melanomas and nevi occurring at sites of ultraviolet light exposure.[26,30,32,34,36–38,66–71] Only one atypical Spitz nevus from the arm of a 22-year-old female had an *NRAS* mutation in our study. As expected, this lesion lacked a *BRAF* mutation, since *NRAS* and *BRAF* mutations are mutually exclusive events. An increased copy number of chromosome 11p by fluorescence *in situ* hybridization, corresponding to the *HRAS* gene, has been previously reported by Bastian *et al*.[72] Subsequently, another group of investigators confirmed *HRAS* mutations in a low percentage of Spitz nevi and atypical Spitz nevi/tumors, but not in spitzoid melanomas, and did not identify any *NRAS* mutations.[40] Other investigators, however, have failed to identify *HRAS* mutations in Spitz nevi or spitzoid melanomas.[41] We did not perform *HRAS* mutational analysis in our spitzoid study group.

In conclusion, we report the presence of *BRAF* (V600E) mutations in a small subset of Spitz nevi, some demonstrating atypical histologic features, at a major melanoma referral center. This finding is contrary to previously published studies that have not detected *BRAF* mutations in any Spitz nevi to date. Thus, *BRAF* mutation status does not reliably distinguish all Spitz nevi from non-Spitz nevi and melanomas, as previously touted, and cannot be relied upon as a specific ancillary diagnostic tool in the evaluation of melanocytic lesions.

Acknowledgements

This work was supported in part by NCI U01 CA83180 (SBG) and NIH T32 HG00040 (JNP), as well as a generous gift from Lewis and Lillian Becker. JTE and RPN were supported by the Babcock Memorial Trust. JTE is supported by the Ann Arbor Veterans Affairs Hospital.

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