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Proliferative Nodules in Congenital Nevi - A Histopathologic, Genetic and Immunohistochemical Reappraisal

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Abstract

Although histopathology is the gold standard for the classification of melanocytic proliferations, in some such as the proliferative nodule, an equivocal diagnosis can be diagnostically extremely challenging based on histopathologic features alone. The purpose of this study was to review the histologic features of proliferative nodules and to ascertain the utility of immunohistochemistry and analyses of oncogenic mutations in signaling components of the MAP kinase pathway as diagnostic adjuncts. Genomic DNA for genotyping was isolated per protocol using techniques that included laser capture microdissection to isolate nevus cells from proliferative nodules (n=3) and age-matched congenital nevi (CN, n=3). Direct DNA sequencing was performed on *BRAF* codon 600; *NRAS1* codons 12 and 13; *NRAS2* codons 60 and 61, *KRAS* codons 12 and 13 and *GNAQ*. Immunohistochemical analyses were performed using antibodies to nestin, CD133, p53, c-kit and bcl-2. While all 3 cases of proliferative nodules exhibited mitoses, features of concern were not noted in any. Of the genes analyzed, no mutations were identified in any of the PN. Immunohistochemistry revealed the following: nestin in 1 PN (3+) and in 2 CN (both 3+); CD133 negative in all PN and CN; p53 in 1 PN (2+) and 0 in CN; c-kit in 2 PN (2+/3+) and in 3 CN (2+/3+/3+) and bcl-2 in 2 PN (both 3+) and 2 CN (both 3+). Our findings, albeit limited by sample size, suggest that proliferative nodules do not appear to possess a distinctive or unifying genomic signature. In light of evidence indicating that progression to malignant melanoma involves genetic pathways instrumental to stem cell biology, that absence of a sizeable population of stem cells in 2 of 3 of proliferative nodules in the current study supports their putative benign biologic behavior.

Introduction

Congenital nevi occasionally present with areas of growth known as proliferative nodules within the dermal component [10]. The histologic appearance of these nodules consists of sheets of irregular cells with atypical pleomorphic nuclei, prominent nucleoli and scattered to even numerous mitoses [1]. Understandably enough, the "knee-jerk" reaction to lesions exhibiting such histological features is to contemplate, if not render, a diagnosis of malignant melanoma. The distinction is not merely semantics but crucial to management as, misdiagnosis of a proliferative nodule may result in unnecessary surgical intervention and perhaps even sentinel lymph node biopsy, a feature of particular concern given that these nevi typically occur in very young children.

The presence of mutations in *BRAF* in nevi implicates activation of the *RAS*/*RAF*/MEK/ERK pathway as a crucial step in the initiation of melanocytic neoplasia [14]. Studies showing *BRAF* mutations to be common in melanomas from intermittently sun-exposed sites, but rare in areas that have virtually no exposure, imply a causal relationship between UV exposure and the acquisition of oncogenic *BRAF* [4,6]. Proliferative nodules arise in association with congenital melanocytic nevi which are usually present since birth. Thus, they presumptively develop independent of UV exposure and should technically not exhibit activating *BRAF* mutations. Studies on the genomic analyses proliferative nodules are sparse, primarily because these are not common to begin with and somewhat conflicting [11,2].

In the only study detailing the immunohistochemical profile of proliferative nodules, of all the markers studied, significant differences were noted only in expression of c-kit with diffuse positive staining noted in 97% of proliferative nodules compared to 3% of congenital nevi [7]. Several lines of evidence favor the hypothesis that in select cancers, the lesional neoplastic cells may actually originate from mutated normal stem cells [16,5,15].

The purpose of verifying the frequency of mutations in the *BRAF, NRAS1, NRAS2, KRAS* and the more recently identified *GNAQ* genes in proliferative nodules was to ascertain their relative risk of progression to melanoma. Immunohistochemical reactivity for apoptotic (p53 and c-kit), anti-apoptotic (bcl-2) and stem cell (nestin and CD133) markers was also assessed to ascertain their utility as histologic adjuncts.

Materials and Methods

Sample selection

This study was approved by Boston University School of Medicine institutional review board (IRB docket # H-28546). Archival materials between 2006-2009 with a diagnosis of congenital nevus were retrieved from the pathology files of Skin Pathology Laboratory, Boston University School of Medicine and Boston, MA. Histologic sections of all cases (initial sign-out on all by a dermatopathologist), were re-reviewed by the dermatopathologist (MM) to identify cases that fit the criteria for diagnosis of a proliferative nodule [12]. A total of 3 cases with a diagnosis of proliferative nodule arising in association with a congenital nevus (n=3) were identified. Three age-matched nevi with congenital features served as the control group.

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Demographics of patients with proliferative nodules are listed in Table 1.

Genomic analyses

DNA was extracted by proteinase K digestion of laser capture microdissected samples per protocol. Briefly 5-7μm thick sections of formalin-fixed paraffin-embedded archival tissue were de-paraffinized, re-hydrated and stained with hematoxylin prior to microdissection. Direct DNA sequencing was performed on the *BRAF* gene (forward gene coding strand only) spanning codon 600, *NRAS1* gene spanning codons 12 and 13, *NRAS2* gene spanning codons 60 and 61, *KRAS* gene spanning codons 12 and 13 and GNAQ gene spanning codon 209 using an ABI BigDye TerV3.1 cycle sequencing terminator ready reaction kit. Sequencing reactions were performed on an ABI 9700 thermocycler utilizing the ABI recommended protocol (Applied Biosystems, Inc., Foster City, CA) and performed on Genetic Analyzer 3100-avant (ABI). The sequencing results were analyzed with ABI DNA Sequencing Analysis Software version 3.7. A positive and/or negative control was included in each batch of sequencing analysis.

Diagnosis	Case	Age	Sex	Location	Approximate Size
Proliferative Nodule	1	3 years	Male	Scalp	1% of body surface area
	2	13 days	Female	Chest	20 cms
	3	5 years	Female	Right Temple	>3cms

Table 1: Summary of patient demographics.

Diagnosis	Case	*BRAF*	*NRAS (1&2)*	*KRAS*	*GNAQ*
Proliferative Nodule	1	WT	WT	WT	WT
	2	WT	WT	WT	WT
	3	WT	WT	WT	WT
Congenital Nevus	1	WT	WT	GG(A)T, GGC	WT
	2	T → A	WT	WT	WT
	3	WT	WT	GTT,GGC	WT

Table 2: Summary of genotypic analyses.

Diagnosis	Case	Stem cell Markers		Apoptotic markers		Anti-Apoptotic marker
		CD133	Nestin	p53	CD117	Bcl-2
Proliferative Nodule	1	0	3+	0	0	0
	2	0	0	2+	2+	3+
	3	0	1+	1+	3+	3+
Congenital Nevus	1	0	0	0	3+	3+
	2	0	3+	0	3+	3+
	3	0	3+	0	2+	0

Table 3: Summary of immunophenotypic analyses.

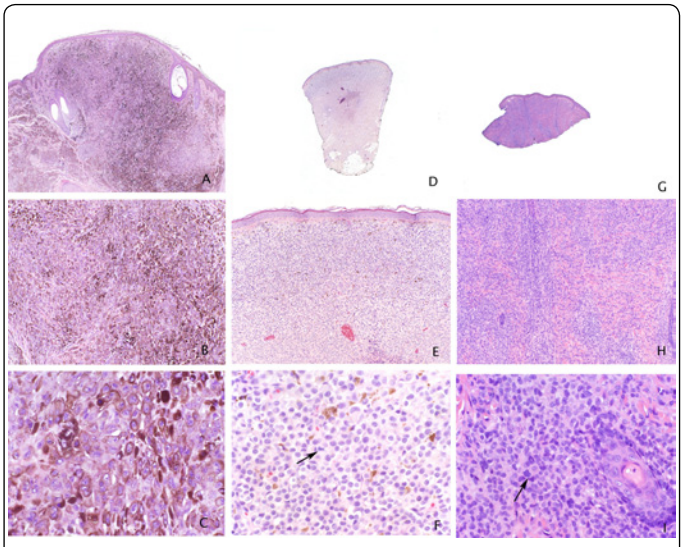

Figure 1: A-C = Proliferative nodule Case 1 H&E 4x, 10x and 40X respectively. **D-F** = Proliferative nodule Case 2 H&E 2x, 10x and 40X (arrow highlights mitosis) respectively. **G-I** = Proliferative nodule Case 3 H&E 2x,10x and 40X (arrow highlights mitosis) respectively.

Immunohistochemical analyses

Five-micrometer-thick sections were obtained for immunohistochemical studies, which were performed on formalin-fixed, paraffin-embedded tissue using standard peroxidase immunohistochemistry techniques, heat-induced epitope retrieval buffer and primary antibodies against CD133 (clone AC133, Miltenyi Biotec, Auburn, CA), nestin (MAB5326, 1:200, Chemicon, Temecula, CA), p53 (Ab-2 1:300 and AB-6 #OP43 1:1000, Calbiochem Darmstadt, Germany), CD117 (A0452, 1:200 Dako, Carpintaria, CA) and Bcl2 (clone 124, 1:25, Dako, Carpintaria, CA). Appropriate positive and negative controls were included. Positive staining, noted by ascertaining expression of CD133, CD117, nestin and Bcl2 in the cytoplasm and, p53 in the nucleus, was scored as 0 (negative), 1+ (<10%), 2+ (10-49%) and 3+ (> 50% of the tumor cells). Cases with 1+ positivity or less were scored as negative, while those exhibiting 2 or 3+ were scored as positive.

Results

Histologic evaluation

Microscopic examination of all three cases of proliferative nodule revealed a nodular, cohesive, dermal-based cellular aggregate exhibiting a higher cellularity than the associated nevus and composed of monomorphic, pigmented (cases 1 and 2 only), oval, epithelioid cells with prominent nucleoli and scattered, albeit normal, mitotic figures (three in cases 1 and 3 and one in case 2) (Figure 1). In all three cases the proliferative nodule merged with the adjacent and/or underlying nevus. Features indicative of malignant transformation such as presence of ulceration or necrosis, a clear cut or pushing border between the nodule and adjacent/underlying nevus, abnormal mitoses, host response and/or pleomorphism were not noted in any of the three cases.

Genotyping

Summary of genotyping results are detailed in Table 2. Overall, 3/3 of control cases and 0/3 of proliferative nodule cases exhibited a mutation in one of the five genes studied.

BRAFV600E

None of the proliferative nodules (0/3) and only one of three control cases (33%) exhibited a *BRAFV600E* mutation.

KRAS

None of the proliferative nodules (0/3) and two of three control cases (67%) exhibited a *KRAS* mutation.

NRAS1/NRAS2/GNAQ

None of the proliferative nodules or control cases exhibited a mutation in *NRAS1/NRAS2/GNAQ.*

Immunohistochemical results

Summary of immunophenotyping results are detailed in Table 3.

Stem cell markers

In the proliferative nodule group, all 3 cases were negative for CD133; one of 3 cases was positive for nestin (case 1 exhibited 3+ positivity).

In the control group, all 3 cases were negative for CD133; two of 3 were positive for nestin (control cases 2 and 3, both exhibiting 3+ positivity).

Apoptotic markers

In the proliferative nodule group, one of 3 cases were positive for p53 (case 2 exhibited 2+ positivity); two of 3 cases were positive for CD117 (case 2 exhibited 2+ positivity and case 3 exhibited 3+ positivity).

In the control group, all 3 cases were negative for p53; all 3 were positive for CD117 (2 to 3+ positivity).

Anti-apoptotic marker

In the proliferative nodule group, two of 3 cases were positive for bcl2 (cases 2 and 3 both exhibited 3+ positivity).

In the control group, 2 of 3 cases were positive for bcl2 (control cases 1 and 2 both exhibited 3+ positivity).

Discussion

The presence of nodules, similar to proliferative nodules, is not unique to large congenital nevi and has been documented in non-cutaneous neoplasms as well [13,3,18]. Histologic features noted in these, similar to those in proliferative nodules include varying degrees of increased cellularity, cellular pleomorphism, increased proliferative activity and a host response. While all three cases in the current study exhibited mitotic figures ranging from 1-3/10 HPFs, no atypical mitotic forms were noted and cytologic atypia and a host response were not appreciated in any of the cases. Furthermore, in the two cases in which adjacent or underlying normal nevus was apparent (cases 1 and 2), the proliferative nodule merged imperceptibly with the adjacent and/or underlying nevus, features arguing against malignant transformation.

The biologic course of proliferative nodules arising in congenital nevi, like that of nodules arising in non-cutaneous neoplasm, is believed to be banal as most proliferative nodules become static after reaching a certain size and regress or involute with age [7]. However, the limited number of reports on follow-up data on patients with proliferative nodules in nevi and scattered reports indicating that patients with a proliferative nodule may have an increased risk of developing melanoma confound the issue [17]. Briefly, the lifetime risk of melanoma for patients with giant congenital nevi believed to vary anywhere from 4-50% while the cumulative risk of melanoma incidence in small congenital nevi is lower and ranges form 2.6 – 4.9%. Thus, despite the reassuring histologic features in all three cases in the current study, given the clinical features "changing nevus" (case 1), "dark papule" (case 2) and "dysplastic nevus" (case 2), a recommendation for clinical follow-up of the area was made; as the large size of the congenital nevus precluded complete excision. Only one of the three cases (case 1) underwent further excision of residual "atypical" nevus which showed histologic features identical to those observed in the initial biopsy and was completely excised. Given the large size, a recommendation for clinical follow-up was made for cases 2 and 3 and both have remained unchanged in the 52 and 22 month follow-up period respectively.

We found no somatic mutations in any of the proliferative nodules in any of the genes analyzed including the more recently identified *GNAQ* [19]. Thus, our findings limit the utility of ascertaining the mutational status of the MAP kinase pathway as a histologic adjunct. While our results are in keeping with a previous report in which no aberrations were detected by conventional cytogenetic analysis on two nodular proliferations, they conflict findings by Bastian *et al* who found frequent chromosomal aberrations and *NRAS* mutations in atypical nodular proliferations arising in congenital nevi [11,2]. This higher proportion may be attributable to different methodologies used in mutation analysis (comparative genomic hybridization versus direct sequencing in the current study) and the cohort studied (proliferative nodules with atypia versus those without in the current study). Of interest, all three of our control cases exhibited mutations in *KRAS* (control cases 1 and 3) or *BRAFV600E* (control case 2). Although few studies have detected *KRAS* mutations in either melanoma or nevi, there is conflicting evidence regarding the significance of the same [20,21]. For example, Shukla et al. [21] believe it to be an early event, while Ball et al. [20] have shown it to be a feature of tumor progression in malignant melanoma.

Like Herron et al. [7] we observed diffuse positive expression of c-kit in 2 of 3 of PN [7]. However, in the two cases in which adjacent or underlying normal nevus were no difference in staining intensity between PN and adjacent normal nevus was observed. Furthermore, all 3 nevi in our control group exhibited diffuse and strong c-kit expression. These findings argue against the potential utility of c-kit in distinguishing PN from surrounding normal nevus. A universally accepted paradigm is that to attain the complete transformed phenotype, a cell must accumulate multiple "hits" in the form of progressive alterations within its chromosomes and irreversible changes in a number of genes [8]. A stem cell with its lifespan comparable to the organism and inherent characteristic of "slow cycling" is the perfect candidate for accumulations of such hits [9]. Evidence for the role of stem cells in cancers is continually mounting with cells with stem cell-like features being identified in several malignancies [16,5,15]. The paucity of stem cells in 2 of the 3 cases of PN argues against their potential for malignant transformation.

While the obvious limitation of the current study is the number of cases, for now it appears that despite their alarming clinical and histopathological appearance, proliferative nodules are no different from congenital nevi. Although the solution is to study more cases, these are relatively rare lesions and more importantly, given the age of patients difficult to biopsy. Future studies need to include studying more cases of proliferative nodules and, in light of findings from the control group, to obtain data from the non-proliferative component to serve as internal control in cases where such is available.

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