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Utility of p16-Ki-67-HMB45 score in sorting benign from malignant Spitz tumors

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ABSTRACT

When Spitz nevi have increased vertical thickness (> 1.0 mm), show ulceration and deep seated mitoses, the differential diagnostic considerations of atypical Spitz tumor (AST) or a Spitzoid melanoma (SM) enter into consideration. While molecular genetic testing could be employed in the work up of atypical melanocytic proliferations, they are expensive and not available at all institutions. Recently, one study employed the combination of p16, Ki-67 and HMB45 (PKH) immunohistochemistry on adult melanomas and proposed a combination of the three markers with scoring of their result to support a diagnosis of melanoma. We report the utility of this antibody combination scoring in discriminating SM and AST in children. We retrospectively reviewed 30 Spitzoid lesions (7 SM, 9 AST and 14 Spitz nevi) from children. Slides from H&E staining and Immunohistochemistry for p16, Ki-67 and HMB45 were reviewed for all cases. The extent of immunohistochemical expression in the lesional cells was scored following published criteria as follows: p16 scored as 0, 1, 2, 3; Ki-67 scored as 0, 1, 2, 3, 4 and HMB45 scored as 0, 1 and 2. Thus, the total PKH score for the combination of the 3 antibodies for any case could vary from 0 to 9. The result of the immunohistochemical analysis of cases in our study revealed that the PKH score of Spitz nevus and AST was below 4 for each of the case and that of SM was ≥ 4 for each of the case. These results are significant as the previously published study found that the PKH score of equal/or > 4 correlated with melanoma and less < 4 correlated with benign nevi. Independently, the immunostains could be misleading as Ki-67 labeling index tended to be higher in young children (< 2 years of age) and HMB45 was occasionally negative in both AST and SM, and p16 could be completely lost in AST.

Our study replicates the findings of the published study of adult melanomas and nevi that showed a total PKH score of equal/or > 4 is seen in melanoma. Although, the number of SM cases in our study are few, the PKH scoring pattern of malignant and benign cases was congruent with the adult study. We suggest routine use of PKH immunohistochemistry in the work up of atypical Spitzoid lesions in children.

1. Introduction

In children, Spitz nevus is a well-known lesion that histologically has a distinctive appearance with epithelioid and spindle cells. When Spitz nevi (SN) have a diameter larger than 10 mm, a vertical thickness of > 1.0 mm, and one or more deep seated mitoses, they are usually referred to as atypical Spitz tumor [1,2]. Very rarely, melanoma with a histological appearance similar to SN or AST is encountered and could pose a diagnostic challenge [3]. An atypical Spitz tumor (AST) shares histologic features with the classic Spitz nevus, and also has some atypical features seen in melanoma. Thus, AST may show irregular shape, non-uniform color, large size, ulceration, and histologically could reveal one or more of the following features: pleomorphic cells, hypercellularity, poor cellular cohesiveness, pagetoid spread, absence of maturation, nuclear atypia, marginal or basal mitotic figures, extension into subcutis and architectural asymmetry. The biology of atypical Spitz nevus is uncertain. Some authors believe that Spitz nevi and melanoma are part of a spectrum that has classic benign Spitz nevus at one end and melanoma at the other end, with a diverse range of atypical Spitz-like lesions with features of both in between. Unfortunately, even with clinical and histologic guidelines, in many cases to distinguish classic and atypical Spitz nevi from melanoma is difficult. Many researchers have emphasized that there is no single discriminating factor for Spitz nevi and melanoma because virtually every trait of Spitz nevi has been described in melanoma. Besides using immunohistochemistry, other techniques that draw on molecular

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differentiation between these lesions, such as comparative genomic hybridization and FISH are being utilized to separate benign from malignant Spitzoid lesions [4]. Recently, one study employed the combination of p16, Ki-67 and HMB45 (PKH) immunohistochemistry on adult melanomas and proposed a scoring algorithm of the three markers to discriminate melanoma and nevi in adults [5]. In this study we employed PKH immunohistochemical panel to assess if the triple immunomarker, PKH score, could differentiate benign Spitz nevi (classic and atypical) from Spitzoid melanoma.

2. Materials and methods

2.1. Study design

The study is retrospective, and was performed on excised skin tissue of melanocytic lesions that were received for pathological examination during the period of 2001–2018 from children aged between 1 to 17 years. The study was approved by the institutional review Board of Children's Mercy Hospital - Kansas City. Cases were searched in institutional pathology databases for the diagnosis terms "Spitz nevus", "atypical Spitz" and "melanoma." Available H&E stained and immunohistochemical stained (IHC) slides were reviewed by two pathologists independently. The tissue samples were grouped into three categories: Spitz nevus, atypical Spitz tumor and Spitzoid melanoma. All cases of atypical Spitz nevus and melanomas had been reviewed by nationally-recognized experts in melanocytic pathology as part of routine patient care around the time of their original diagnoses.

2.2. Immunohistochemistry

Immunohistochemistry for p16, HMB45 and Ki-67/MelanA was performed on each case in an automated immunostainer (Leica Bond). The antibody source and dilutions used are shown in Table 1. The tissue was fixed in neutral buffered formalin (10%) and paraffin embedded. Sections were cut at 4 μm thickness and mounted on Leica Bond plus charged slides. For the p16 and HMB45 antibodies, sections were pretreated for 20 min with Bond Epitope Retrieval Solution 2 at pH 9.0 and 25 °C and immunohistochemistry performed on autostainer with detection by Bond Refine Detection System (HRP). For the Ki67/Melan A double labeling immunohistochemistry, the sections were pretreated for 20 min with Bond Epitope Retrieval Solution 2 at pH 9.0 and 25 °C. Bond Refine Detection System (HRP) was used for 8 min at room temperature for Ki-67 and Bond Refine Polymer Red (AP) for 30 min at room temperature, respectively for the detection step. The Ki-67 stain was revealed as brown nuclear staining for positive signal. Red cytoplasmic stain was seen when Melan-A was positive.

2.3. Immunohistochemistry scoring

For each case, both H&E and IHC slides were independently analyzed by two pathologists. The protocol for assessing the immunostained slides was similar to that reported by Uguen et al [5]. Briefly, double immunostained Ki-67/MelanA slide were analyzed to determine the proliferative percentage by choosing the most proliferative area at low power scanning. In that area, the percentage of Ki-67/MelanA double-positive cells were determined by counting 100 MelanA positive (with and without associated Ki-67 positive staining) cells. The percentage of p16 positive cells was determined in all tumor cells, without discriminating between nuclear and/or cytoplasmic staining. The gradient of HMB45 gradient was considered to be positive when only the more superficial melanocytic cells were positive, and was considered to be negative when the stain was equality positive in the superficial and deeps parts of the lesion. It was considered inconclusive or doubtful when negative or only few melanocytic cells were positive. We used the same scoring which comprised of:

-p16 scored as: 0 (> 50% positive cells), 1 (11–50% positive cells), 2 (1–10% positive cells), 3 (0% of positive cells).

-Ki-67 scored as: 0 (< 2% positive cells), 1 (2–5% positive cells), 2 (6–10% positive cells), 3 (11–20% of positive cells), 4 (> 20% positive cells).

-HMB45 scored as: 0 (gradient present), 1 (doubtful/inconclusive gradient) 2 (gradient absent).

We then calculated the total PKH score by adding together individual values of P (p16), K (Ki-67) and H (HMB45) scores to yield a total score that varied from 0 to 9 for any patient. Also, we performed simple statistical analysis using *t*-test for determining significance of observed values.

Table 1
Primary antibodies showing clonality, dilution and supplier.

Antibody	Dilution	Manufacturer/Supplier	Clonality
p16	1:10	Ventana-Roche	Mouse monoclonal
HMB45	1:75	Dako (Leica)	Mouse monoclonal
Ki-67	Prediluted	Leica	Mouse monoclonal
Melan-A	Prediluted	Leica	Mouse monoclonal

3. Results

There were 14 Spitz nevus, 9 atypical Spitz tumors and 7 melanomas (1 metastatic). The staining pattern for the 3 immunohistochemical markers and scores of a typical SN are shown in Fig. 1, and the staining pattern of SM and AST cases are shown in Fig. 2. The combined scoring of Ki-67 labeling index (K), p16 (P) and HMB-4 (H) demonstrated higher scores for melanoma compared to classic Spitz nevi (Table 2). Though these individual antibody scores suggest that melanomas have a higher proliferative index, loss of expression of p16 and loss of expression gradient of HMB-45 in our study population, the groups are not distinctively separated as seen with standard deviation overlap; perhaps due to the small numbers in the study groups. Scores for a combination of two or three antibodies produces a better separation of the groups as observed when combining scores P + K or P + K + H (Table 2). Interestingly, when using a threshold PKH combined score of 4 as suggested by Uguen et al, we found that all 9 cases of AST had individual case scores of below 4, whereas all 7 SM cases had individual case scores of above 4 (score range 5–9). Thus, the lesions separated into benign (PKH score < 4) and malignant (PKH score $\geq$ 4) categories neatly. Expectedly, none of the 14 SN had a score greater than 3. Statistical analysis showed that there was a significant difference ($P < 0.0001$) between the mean PKH score of SM (7.14) versus AST (2.22).

During the work up of p16 immunohistochemical staining, we noted that compound melanocytic nevi had a "checkerboard pattern" of expression of p16 in the nevus cells wherein about 50–60% of nevus cells were positive. On the other hand, classic Spitz nevi tended to show a diffuse, strong expression in the majority (greater than 90%) of nevus cells.

4. Discussion

Earlier studies have shown that melanoma tends to have higher mitotic activity, frequent loss of p16 expression and diffuse expression of HMB45 when compared to SN ([6] [7],). Uguen et al had noted that a threshold PKH score of 4 had a high sensitivity (97.4%) and high specificity (97.3%) in discriminating benign from malignant melanocytic proliferations. Our results support the findings of their study, as utilizing the PKH threshold score of 4 could separate all benign Spitzoid lesions (Spitz nevus and AST) from SM. The value of utilizing a combination of multiple antibodies was readily apparent to us in the study because when considered individually, each of the three antibodies had variations or exceptional staining patterns across the spectrum of Spitz

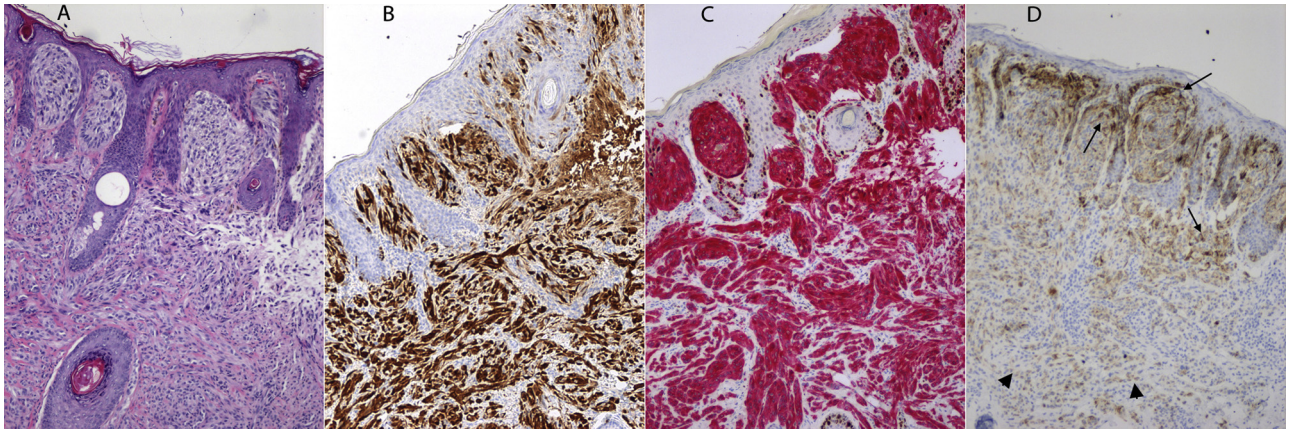

Fig. 1. Spitz nevus. A- H&E stained image (x100 magnification). B- p16 immunohistochemical stain showing strong, diffuse expression in almost all nevus cells (score 0). C- Melan-A (red)/Ki-67 (brown) dual stain showing less than 1% Ki-67 expression in nevus cells (score 0). D- HMB45 immunostain showing strong staining in superficially located nevus cells (arrows) and weakly stained in deeply located nevus cells (arrowheads) in a gradient (score 0).

tumors. But, when used in a combined manner, the unusual variation of one individual antibody tended to be neutralized by the other two antibodies in the total PKH score.

Gerami et al [8] noted found that even amongst expert dermatopathologists, the interobserver agreement was low in categorizing Spitzoid lesions as malignant versus nonmalignant. In an earlier study of AST and melanoma, we found a significant overlap of histological features between the two entities, with the only exception of neural and lymphovascular invasion and sweat gland destruction occurring in melanoma [9]. The management of Spitzoid melanoma is much different from benign Spitz nevi, therefore an accurate diagnosis is very important. A study of adult patients comparing Spitzoid melanoma with conventional melanoma found no difference in outcomes when controlled for age, gender and depth of invasion [10]. On the other hand, the 5-year survival rate for Spitzoid melanoma occurring in children younger than 10 years was 88% compared to 49% for patients aged 11 to 17 years [11]. One patient with SM in our study who had distant metastases and died of the disease had a germline heterozygous mutation of BRCA2. Bahrami et al [12] reported presence of TERT promoter mutations in 2 (out of 26) children with SM who developed widespread metastases and died of disease. Thus, there appears to be a subset of SM wherein there are certain genetic aberrations such as TERT promoter mutations which portend an aggressive behavior leading to widespread distant metastases. The histological appearance of the metastatic SM in our study was not much different from AST cases, which raises the consideration if all SM should be genetically characterized by FISH, genomic microarray or next generation sequencing [13]. Redon et al in their analysis of Spitzoid lesions found that 4 cases of SM had a PKH score of > 4 each whereas 12 out 13 cases of SN had a score < 4 and all 4 cases of SM showed positive Vysis melanoma FISH panel (14). While the study found good corroboration of PKH score and Vysis melanoma FISH panel in all cases of SM, there were 3 cases of SN which

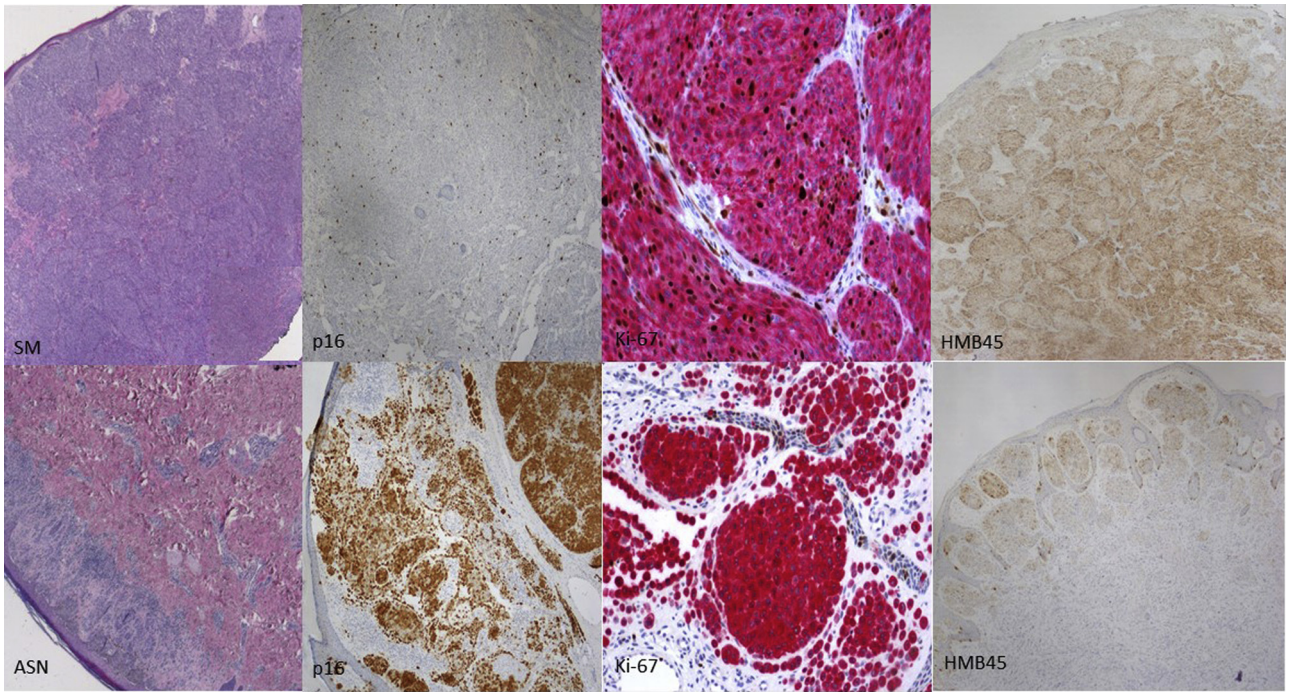

Fig. 2. Top row shows H&E stained image ($\times 20$ magnification) of Spitzoid melanoma and its immunohistochemical expression of p16, Melan-A (red)/Ki-67 (brown) and HMB45. Bottom row shows H&E stained image ($\times 20$ magnification) of atypical Spitz tumor and its immunohistochemical expression of p16, Melan-A (red)/Ki-67 (brown) and HMB45.

Table 2
IHC staining score of P, K and H in Spitzoid lesions, shown as mean +/- standard deviation.

	P	K	H	P + K	P + K+H
Spitzoid melanoma (N=7)	2 +/- 1.15	3.43 +/- 0.97	1.5 +/- 0.54	5.43	6.93
Atypical Spitz tumor (N=9)	0.8 +/- 1.03	0.8 +/- 1.03	0.7 +/- 0.8	1.6	2.3
Spitz nevus (N=14)	0.16 +/- 0.37	0.68 +/- 0.82	0.36 +/- 0.70	0.8	1.2

had low PKH (< 4) score, but were positive for 6p25 and 11q13. Analysis of larger numbers of Spitzoid lesions may help to clarify possible gains of chromosomes 6p and 11q in benign Spitz nevi. Significantly, all cases of SM in our study had PKH score of > 4.

The entity of AST is regarded by several investigators as a Spitzoid melanocytic proliferation that has uncertain malignant potential but with a possibility for local nodal involvement. In actual practice at our institution, we consider the diagnosis of AST as provisional until a sentinel lymph node biopsy is negative for metastasis. If a draining lymph node shows metastasis, we upgrade the AST diagnosis to Spitzoid melanoma because the lesion is then treated in a manner similar to conventional melanoma. Interestingly, in one of the early reports of AST, the authors described 9 AST and 3 Spitzoid melanoma which showed no metastasis in AST and nodal metastasis in two of the 3 melanoma [15]. The accumulating evidence suggests that AST are similar to adenomatous polyps in gastrointestinal tract in the sense that they demonstrate chromosomal aberrations and gene mutations but have not developed the capacity for distant metastasis. Accordingly, subtypes of AST with ALK, BAP1, and HRAS gene mutations have been described as distinctive entities. AST that harbor ALK rearrangement or fusion were noted to have a histological appearance featuring prominent dermal proliferation of fusiform melanocytic cells in interlacing fascicles and a plexiform pattern [16]. We encountered one case of ALK rearranged AST in our experience as well. Some of the AST which have a distinctive cytological appearance of predominantly epithelioid melanocytic cells featuring amphophilic cytoplasm, nuclei with conspicuous nucleoli, and well-defined cytoplasmic borders were noted to carry loss of Bap1 and concomitant BRAF mutation [17]. Another subtype of AST that have gains of chromosome 11p, which coincided with HRAS mutations had a morphological appearance somewhat similar to Bap1-AST, but also demonstrated marked desmoplasia [18]. In one study that correlated loss of p16 expression with homozygous or heterozygous 9p21 deletion, there was retention of p16 staining in two thirds of AST cases that had heterozygous deletion and a complete loss of p16 staining in AST cases that had homozygous deletion [19]. The authors suggested that heterozygous deletion of 9p21 or partial loss of p16 immunostaining in an AST portends better prognosis and less likely to indicate malignancy. In keeping with this observation, the PKH scoring assigns a higher value to a complete loss of p16 (score of 3) and a lower value to retention of p16 immunostaining (score of 0). Significantly, all cases of AST in our study had PKH score of < 4.

There were 14 classic SN in our study and all of them had a PKH score of < 4. Interestingly, nevi from very young children (less than two years of age) showed a higher Ki-67 labeling index. If considered in isolation, the high Ki-67 index would be worrisome for the diagnosis of a melanoma but when combined with p16 immunostaining score and HMB-45 score the PKH score of such classic SN from young children would be < 4.

In summary, the histological distinction between AST and Spitzoid melanoma is difficult and genetic testing that reveals differences in chromosomal imbalances and or gene mutations could be helpful in distinguishing them. Uguen et al in a comparative study of FISH panel for chromosomes 6, 8, 8 and 11 with PKH scoring found a good concordance between FISH results and PKH scoring in melanocytic proliferations [20]. However, molecular methods are expensive and of high complexity to be routinely used. On the contrary, immunohistochemistry is a simple, and a routinely used technique. Our study, as well as that of Uguen et al, found great utility in using triple antibody panel of p16, Ki-67, and HMB45 in separating benign from malignant melanocytic proliferations. The results of our study indicate that a high PKH score could serve to mark a Spitzoid melanocytic proliferation for further molecular characterization if not aid in providing an outright diagnosis of melanoma.

Declaration of conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2019.152550.

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