Received: 6 September 2018 | Revised: 26 February 2019 | Accepted: 11 March 2019

DOI: 10.1111/cup.13457

JOURNAL OF CUTANEOUS PATHOLOGY WILEY

ORIGINAL ARTICLE

Immunohistochemical characterization of benign activation of junctional melanocytes and melanoma in situ of the nail unit

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Background: Immunohistochemical (IHC) stains that distinguish benign, pigmented nail lesions from malignancy are needed. Candidate markers of malignant transformation include p16, HMB45, and Ki-67, with p16 being of particular interest. There is limited knowledge about the spectrum of p16 expression in pigmented lesions, especially junctional melanocytic proliferations of the nail. The objective of this study was to determine if any of these markers demonstrate diagnostic utility in distinguishing between benign activation of junctional melanocytes (BAM) and melanoma in situ (MIS) of the nail unit.

Methods: In this retrospective study, ten cases of BAM and eight cases of MIS were identified. Archival slides available for review included H&E (hematoxylin and eosin), Fontana-Masson, and MelanA (Mart1) IHC slides. IHC studies for p16, HMB45, and dual-color Ki-67/MelanA (Mart1) were then performed.

Results: None of the tested IHC stains distinguished BAM from MIS. p16 IHC expression was uniformly negative with the exception of two cases of MIS. HMB45 was positive in all BAM and MIS cases. Ki-67/MelanA showed positive Ki-67 staining of MelanA-positive melanocytes in two cases of MIS, and all other cases of MIS and BAM were negative for Ki-67. The two positive p16 and two positive Ki-67/MelanA cases were non-overlapping.

Conclusion: p16, HMB45, and Ki-67/MelanA IHC studies show no apparent utility in distinguishing BAM from MIS in the nail unit.

KEYWORDS

benign activation of junctional melanocytes, HMB45, Ki-67, lentigo, melanoma in situ, nail, p16

1 | INTRODUCTION

Evaluation of pigmented lesions in the nail unit remains a diagnostic challenge, both clinically and histopathologically.[1] Distinguishing benign activation of junctional melanocytes (BAM), also known as benign lentigo or melanotic macule of the nail unit, from melanoma in situ (MIS) relies primarily on assessing melanocyte density along the nail unit epithelial basal layer in close correlation with the clinical presentation. Intraepidermal melanocyte density of less than 9 cells per millimeter along the epithelial basal layer is usually considered to characterize BAM, while more than 30 cells per millimeter is considered definitive for MIS.[2,3] Sometimes, melanocyte cytologic atypia proves useful in distinguishing BAM from MIS. If the patient is older, it is more difficult to distinguish BAM from MIS clinically. However, the recent appearance of a new, darkly pigmented band in an older patient suggests that the process is MIS. Unfortunately, the density of melanocytes may vary widely in BAM and MIS, depending upon the skin type of the patient and upon the area sampled. Indeed, BAM and MIS may occasionally be almost identical histopathologically.

Identifying immunohistochemical (IHC) stains, especially newer ones such as p16, which might distinguish benign from malignant, could aid in these diagnostic impasses. Mutation and loss of function of the *p16* gene, in particular, has been identified as an important driver of melanoma.[4] Loss of IHC staining for the p16 protein, however, may or may not be a clue to an underlying mutation. Negative p16 IHC staining may simply represent a lack of identifiable

expression or downregulation of the p16 protein, with no underlying mutation being present. Currently, there is limited knowledge about the spectrum of p16 expression in the array of pigmented lesions, especially junctional melanocytic proliferations. Thus, characterization of the p16 IHC staining pattern in pigmented lesions, from benign lentigo to in situ, invasive or metastatic melanoma, may help to define the utility and the limits of the test in distinguishing benign from malignant. The objective of this study was to identify IHC stains, particularly p16, that could be of diagnostic utility specifically in distinguishing BAM from MIS of the nail unit.

2 | METHODS

2.1 | Inclusion criteria

The selected cases for BAM and MIS that were included in our study had to meet the following criteria: (a) a definitive diagnosis of BAM or MIS; (b) a clinical age between 21 and 100 years at the time of tissue sample submission; and (c) additional paraffin-embedded tissue available for additional IHC studies. Cases of melanoma in which dermal invasion was identified were excluded.

2.2 | Processing of specimens

Tissue blocks for each case were retrieved from tissue archival storage. IHC staining was performed on unstained, 5-μm sections placed on charged slides. The slides were processed in an automated BenchMark ULTRA staining module (Ventana Medical Systems Inc, Tucson, Arizona). An appropriate titer for the p16, HMB45 and two-color Ki-67/MelanA was predetermined using a College of American Pathologists-approved protocol, and a positive control was included on the same slide. The automated staining involved 74 steps beginning with deparaffinization at 72°C and ending with a final slide rinse and coverslipping. See staining protocols below for more information.

2.2.1 | MelanA, HMB45, and p16 staining protocol

Each slide is inserted into the automated BenchMark ULTRA staining module. The slide is first deparaffinized at medium temperatures and warmed to 72°C. The slide is then warmed to 95°C and incubated for 8 minutes. For MelanA, cell conditioner (Ventana Medical Systems Inc, Tucson, Arizona) is applied and the slide is then incubated in cell conditioner at 95°C for 56 minutes total. For HMB45, the slide is incubated at 95°C for 172 minutes and for p16 for 56 minutes. The slide is then cooled to 36°C and incubated for 4 minutes. 1:250 MelanA is then added to the slide, coverslip is applied, and the slide is incubated at 36°C for 40 minutes. For HMB45, 1:100 HMB45 is added, and for p16, a dilution of 1:50 is added. For HMB45 and p16, the incubation is at 36°C for 32 minutes. After incubation, the slide is rinsed with distilled water.

2.2.2 | Fontana-Masson staining protocol

Each slide is deparaffinized and rinsed with distilled water. Ammoniacal silver solution is prepared with Fontana silver solution in 27 mL of distilled water and concentrated ammonium hydroxide is added via drop titration until solution appears clear. The slide is then placed in ammoniacal silver solution and incubated in 58 to 60°C water bath until the slide becomes a golden brown color. The slide is then rinsed with distilled water and then placed in 0.1% gold chloride solution for 30 seconds and then rinsed. The slide is then placed in 5% sodium thiosulfate solution for 1 minute and rinsed for 2 to 3 minutes thereafter. The slide is then placed in Nuclear Fast Red solution for 5 minutes and rinsed. The slides are then dehydrated with two changes of 100% alcohol and cleared with two changes of xylene. A coverslip is applied.

2.2.3 | Ki-67/MelanA staining protocol

Each slide is inserted into an automated BenchMark ULTRA staining module. The slide is first deparaffinized at medium temperatures and warmed to 72°C. The slide is then warmed to 95°C and incubated for 8 minutes. Cell conditioner is applied and the slide is incubated in cell conditioner at 95°C for 56 minutes. The slide is cooled to 37°C and incubated for 4 minutes. 1:250 Ki-67 is then added to the slide, a coverslip is applied, and the slide is incubated at 36°C for 32 minutes. The slide is washed and incubated for 4 minutes at 37°C. 1:250 MelanA is then added to the slide, a coverslip is applied, and the slide is incubated at 37°C for 48 minutes. The slide is then washed in distilled water.

2.3 | Examination of specimens

The diagnostic (H&E, Fontana-Masson, and MelanA) and the IHC study slides (p16, HMB45, and Ki-67/melanA) were screened by one nail expert dermatopathologist (C.T.T.). Because of light, variable staining of the p16 slides, a second nail expert dermatopathologist (J.A.) reviewed the diagnostic and the p16 slides. Positive controls for each IHC study were also examined.

3 | RESULTS

Clinical information and diagnostic histologic features are reported in Table 1. Figure 1 shows an example of H&E (hematoxylin and eosin) stain in an MIS case. The p16, HMB45, and Ki-67/MelanA IHC staining results are presented in Table 2. Of note, all cases of BAM and MIS were positive for HMB45 and two cases of MIS showed positive nuclear staining for Ki-67 in MelanA-positive melanocytes. Figure 2 shows positive staining of HMB45 in a BAM (Figure 2A) and MIS (Figure 2B) case. Figure 3 shows a positive Ki-67/MelanA staining in an MIS case. With p16, all cases of BAM were negative, and two cases of MIS showed positive nuclear staining (Figure 4), but neither of these cases was positive for Ki-67/MelanA staining. Cytoplasmic staining for p16 was not definitely present.

4 | DISCUSSION

We have demonstrated that p16, HMB45, and Ki-67/MelanA IHC studies are not reliable in distinguishing benign from malignant pigmented nail lesions (BAM and MIS). It is not surprising that the

TABLE 1 Patients diagnosed with BAM and MIS with demographics, affected nail, staining results, and H&E diagnostic features

Group	Case	Age	Sex	Affected nail (lateral/digit)	MelanA	Fontana-Masson	Epithelial pigment	Melanophages	Obvious melanocytes
BAM	1	33	F	L/thumb	+	+	+	–	–
	2	52	F	L/unknown	+	+	+	–	–
	3	38	F	R/third finger	+	+	+	+	–
	4	32	F	L/second toe	+	+	–	–	–
	5	23	F	R/thumb	+	+	+	–	–
	6	46	F	L/thumb	+	+	+	–	–
	7	72	F	L/great toe	+	+	+	–	–
	8	71	M	L/fifth finger	+	+	+	–	–
	9	58	M	R/great toe	+	+	+	–	–
	10	45	F	R/second finger	+	+	+	+	–
MIS	11	69	F	L/great toe	N/A	N/A	+	–	+
	12	70	F	L/second toe	N/A	N/A	+	–	+
	13	72	M	R/second finger	+	N/A	+	+	+
	14	95	F	R/great toe	+	N/A	+	+	+
	15	67	F	R/second toe	+	N/A	–	+	+
	16	64	F	L/thumb	+	N/A	+	+	+
	17	76	M	R/second finger	+	N/A	–	+	–
	18	70	F	L/second toe	N/A	N/A	+	+	+

Abbreviations: BAM, benign activation of junctional melanocytes; H&E, hematoxylin and eosin; MIS, melanoma in situ; N/A, not performed.

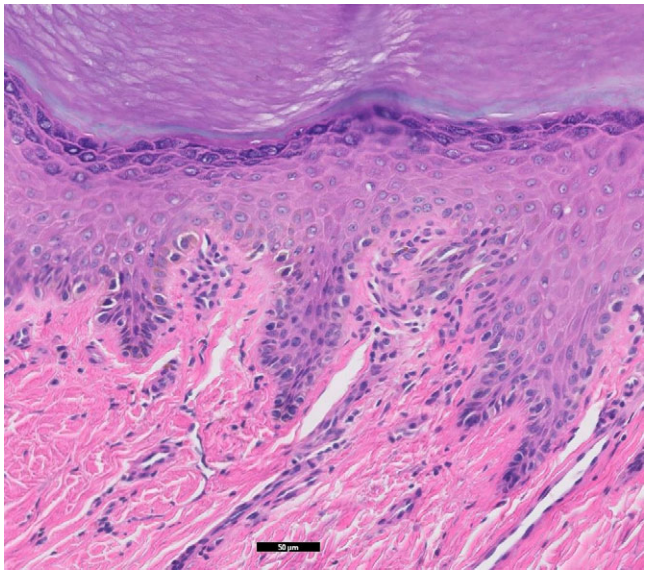

FIGURE 1 Melanoma in situ (H&E) showing proximal nail fold epithelium with a proliferation of large, single junctional melanocytes irregularly scattered across the junction (×400)

HMB45 is positive in MIS, but it is interesting that it is positive in BAM, suggesting that clinicopathologically BAM is a new and proliferative lesion. In fact, perhaps BAM should indeed be considered a neoplasm rather than simple activation. However, all cases of BAM had negative staining for p16, which could suggest that loss of p16 expression is an early event and that there are other redundant cell cycle regulatory mechanisms present that prevent progression from benign to malignant cells. It is also possible that the negative p16 staining of BAM cases was a result of the hypocellularity of the lesions and that the positive p16 staining in the two MIS cases was the result of the hypercellularity of the tumor.

TABLE 2 Patients diagnosed with BAM and MIS with staining results and melanocyte density

Group	Case	p16	HMB45	Ki-67/MelanA	Melanocyte density (cells/mm^2)
BAM	1	–	+	–	8
	2	–	+	–	8
	3	–	+	–	5
	4	–	+	–	1[a]
	5	–	+	–	7
	6	–	+	–	8
	7	–	+	–	3
	8	–	+	–	6
	9	–	+	–	10
	10	–	+	–	8
MIS	11	–	+	–	>50
	12	–	+	+	>50
	13	–	+	–	23
	14	–	+	–	>50
	15	+	+	–	>50
	16	–	+	–	46
	17	–	+	+	20
	18	+	+	–	>50

Abbreviations: BAM, benign activation of junctional melanocytes (of nail); MIS, melanoma in situ.
[a]Remaining tissue sample had very little epithelium remaining.

The absence of Ki-67/MelanA IHC staining, a proliferative marker, in BAM could be credited to the paucity of melanocytes, which is generally <9 melanocytes per millimeter of length along the nail matrix or bed basal epithelium. As mentioned above, the presence of melanocytic atypia is another important consideration, as significant cytologic atypia with mitotic figures are never observed in BAM, in contrast to MIS.

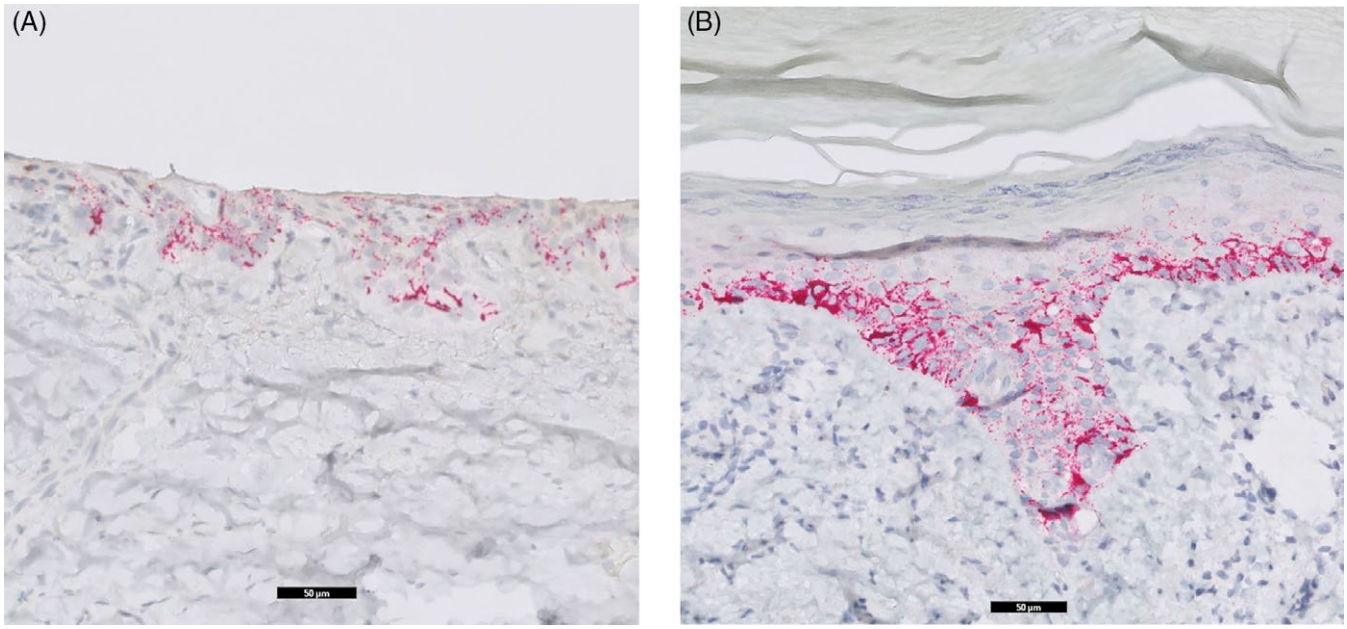

FIGURE 2 HMB45 immunohistochemical staining. (A) Benign activation of junctional melanocytes with a low density of junctional melanocytes (<9 per millimeter of length along the epithelial basal layer). (B) Melanoma in situ with a high density of confluent junctional melanocytes (>50 per millimeter of length along the epithelial basal layer; ×400)

The p16 IHC staining findings are significant because they add to the characterization of p16, as both IHC staining and assessment for mutations of the tumor suppressor gene *CDKN2A*, which codes for the p16 protein, are becoming important in the evaluation and diagnosis of pigmented lesions. Of particular interest in this study is that the positive p16 IHC staining was seen in two cases of malignancy (MIS) but in none of the benign lesions (BAM). This is in contrast to what has been observed in invasive melanoma, in which p16 IHC staining is often lost. The *CDKN2A* gene, which regulates the cell cycle at the G1/S checkpoint, has been shown to be important in a variety of cancers, including melanoma, and loss of p16 in primary invasive melanoma correlates with overall disease prognosis and metastasis-free survival.[5]

It is important to note that loss of p16 IHC staining may or may not represent mutation or loss of function of the *CDKN2A* gene. Absence of staining may be because of a lack of sensitivity of the p16 IHC study itself or because of downregulation of the p16 protein. Other mechanisms that may lead to a loss of p16 include reduced mRNA and/or protein expression, which is seen in primary prostate carcinomas.[6] Epigenetic downregulation of the 5′ CpG island of *p16* is also a well-known mechanism that leads to loss of p16 protein and is associated with many cancers.[7] Homozygous deletion of *p16* also plays a role in many cancers, including bladder, breast, and prostate.[8] In addition, p16 expression may be impacted by other genes in the regulatory pathway, including *CDK4* and the *Rb* (retinoblastoma) genes in the so-called "p16-CDK4-Rb axis." Although probably not of import in these pigmented nail lesions, human papillomavirus (HPV) may also impact p16 expression. Overexpression of p16 has been in HPV-related cervical squamous cell carcinoma and oropharyngeal tumors.[9,10] Finally, p16 expression may be related to an acute cellular response in neoplastic change in which the production of a non-functional p16 protein or mutation in the CDK4 protein leads to dysregulation by p16.

Limitations of our study lie largely in the small number of cases examined in this study and the demographics of our cases. First, we examined only ten cases of BAM and eight cases of MIS, which limits the power of this study. While we would have enjoyed a larger sample size, the number of available MIS cases was largely the limiting factor in increasing the power of our study. Cases were excluded because of limited tissue in the small biopsies available for the IHC stains. Second, it should be noted that in our cases of BAM, many were of an older age, which makes differentiation between BAM and MIS or other

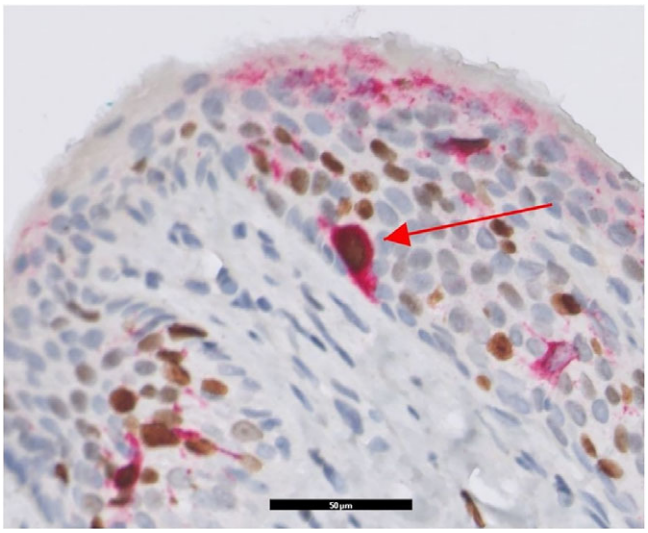

FIGURE 3 A dual-antibody Ki-67/MelanA immunohistochemical staining showing positive Ki-67 nuclear staining (brown) of a large, MelanA-positive melanocyte (red) in the nail unit epithelium in a melanoma in situ case (×400)

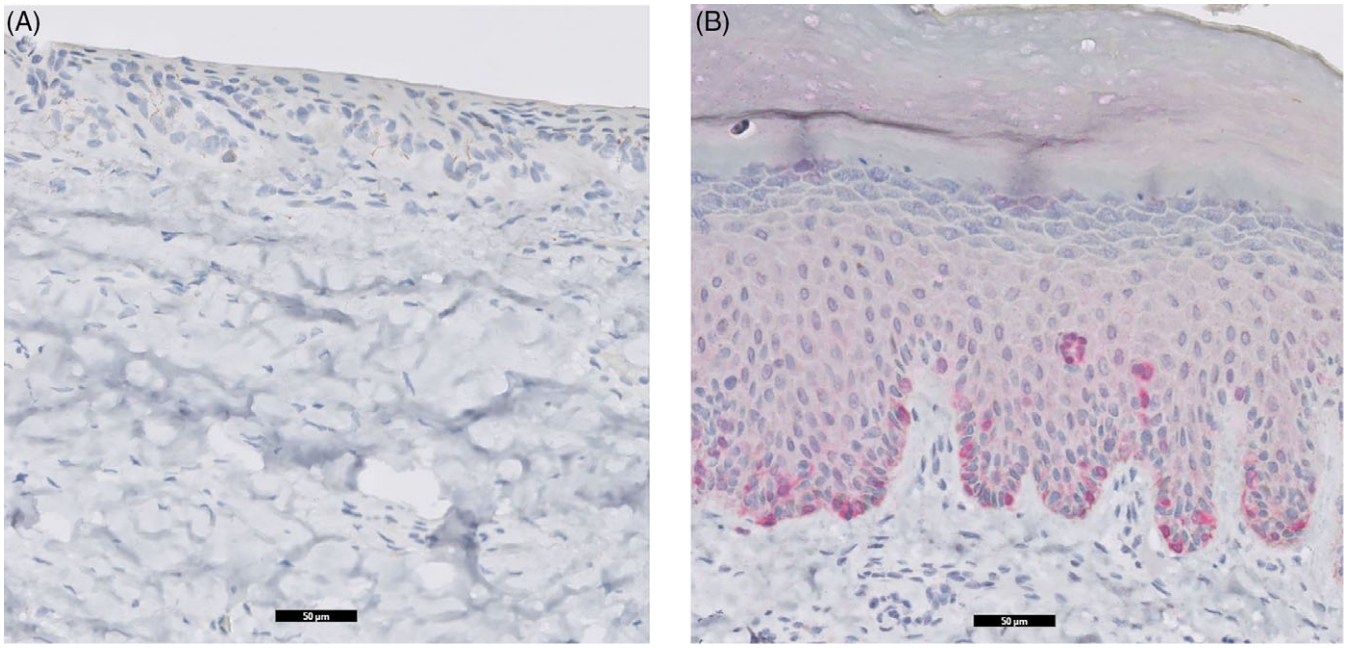

FIGURE 4 p16 immunohistochemical staining. (A) Benign activation of junctional melanocytes showing no staining of junctional melanocytes. (B) Melanoma in situ with positive nuclear staining of large, junctional melanocytes in nail unit epithelium (×400)

malignant lesion difficult. Overall, our demographics were skewed towards older individuals and female gender, which may make the results of this study less applicable to cases involving younger ages and/or male sex. Despite this, we are hopeful that this study contributes to the body of literature on the use of IHC for the differentiation between BAM and MIS and encourages further research to one day identify a quantitative method to distinguish between the two clinical entities in the spectrum of nail melanoma.

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How to cite this article: Chu A, André J, Rich P, Leachman S, Thompson CT. Immunohistochemical characterization of benign activation of junctional melanocytes and melanoma in situ of the nail unit. *J Cutan Pathol*. 2019;1–5. https://doi.org/10.1111/cup.13457