Published in final edited form as:

J Cutan Pathol. 2019 May ; 46(5): 310–316. doi:10.1111/cup.13424.

Expression of p15 in a spectrum of spitzoid melanocytic neoplasms

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Abstract

Background: Accurate classification of spitzoid melanocytic lesions is difficult due to overlapping clinical and histopathologic features between Spitz nevi, atypical Spitz tumors (ASTs), and spitzoid melanomas. Expression of p16 (*CDKN2A*) has been used as a marker of spitzoid lesions. However, its expression may be variable. p15 is a tumor suppressor encoded by *CDKN2B*, loss of which has been recently shown to promote transition from nevus to melanoma. We sought to determine whether p15 is a useful immunohistochemical marker to distinguish Spitz nevi from spitzoid melanomas, and to compare p15 and p16 staining in this population.

Methods: Immunohistochemistry for p15 and p16 was performed on Spitz nevi (n=19), ASTs (n=41), and spitzoid melanomas (n=17). Immunoexpression was categorized by a four-tiered system: 0 (negative), 1+ (weak), 2+ (moderate), 3+ (strong).

Results: 3+/strong p15 staining was observed in 68.4% of Spitz nevi, 34.2% of ASTs, and 17.7% of spitzoid melanomas. By contrast, we observed 3+ p16 staining in roughly equivalent percentages of Spitz nevi (57.9%), ASTs (56.1%), and spitzoid melanomas (58.8%).

Conclusion: These data illustrate that p15 may be more useful than p16 as a biomarker to help distinguish benign from malignant spitzoid lesions.

Keywords

Spitz nevi; melanoma; atypical Spitz tumor; p15; p16; biomarker

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Conflicts of Interest: J.T. receives a research grant (to the Trustees of the University of Pennsylvania) from Pfizer Inc. for work that is unrelated to this manuscript and received payment for continuing medical education work related to psoriasis that was supported indirectly by Eli Lilly and Novartis. S.A.M., C.P.O., T.D., T.W.R., J.T.S., and E.Y.C. disclosed no relevant conflicts of interest.

INTRODUCTION

Spitzoid melanocytic lesions (Spitz nevi, atypical Spitz tumors, and spitzoid melanomas) display overlapping clinical and histopathologic patterns, and are therefore difficult to classify, even for experienced dermatopathologists.^{1,2} Spitz nevi were first described in 1948 by Sophie Spitz, and are now known to be benign lesions which occur predominantly in children and young adults^{3–5}. Clinically, these lesions occur throughout the body with a slight predominance in the head and neck for children and extremities for adults.^{3,6} The typical histopathologic characteristics of Spitz nevi include: vertical symmetry, nests of epithelioid and/or spindled melanocytes in the epidermis with clefting around the nests, presence of multiple Kamino bodies, dispersion of dermal melanocytes, and mitoses $< 2/\text{mm}^2$.^{2,5,7} In contrast, spitzoid melanomas classically display vertical asymmetry, epithelioid and/or spindled melanocytes with high nuclear:cytoplasmic ratios, absent or few Kamino bodies, lack of dispersion of melanocytes in the dermis, atypical mitotic figures, deep dermal mitoses, and mitotic rate $> 2/\text{mm}^2$.^{8,9} Spitzoid malignant melanomas have the capacity to metastasize, although cases occurring in young children tend to have better prognosis compared to those occurring in adults.^{10,11} Because the histopathologic appearance of Spitz nevi and spitzoid melanomas can be similar, spitzoid melanomas are on occasion only diagnosed retrospectively after patients present with a metastasis.^{10,12}

An intermediate category of spitzoid lesion, “atypical Spitz tumors”, show histological features of classic Spitz nevi with one or more of the concerning histopathologic features of spitzoid melanomas.^{1,2,12–14} Atypical Spitz tumors (ASTs) have a fairly high incidence of sentinel lymph node positivity, estimated to be approximately 39% aggregating data from several studies, although the rate of distant metastasis seems to be low and the overall prognosis appears to be better than for patients with unambiguous melanomas.^{15,16}

Immunohistochemical stains are frequently employed to help differentiate spitzoid melanocytic lesions, but all are imperfect discriminators. The melanocyte markers S100, MART-1/Melan-A, and SOX10 are positively expressed in both benign and malignant spitzoid neoplasms. HMB-45 staining is usually diminished in the deep component of benign melanocytic nevi including Spitz nevi, and more homogenous/diffuse staining in melanomas, although there is variability in these patterns.¹⁰ Ki-67 stains are used to assess proliferative indices in melanocytic lesions (often combined with MART-1 to more precisely label proliferating melanocytes). While increased Ki-67 double staining is expected in malignant melanomas, melanomas may also show low Ki-67 expression.^{17,18} Several molecular genetic studies using comparative genomic hybridization (CGH) and fluorescence in situ hybridization (FISH) have been promising, but availability and cost limit the clinical utility of these techniques.^{19–22}

Although imperfect, p16 immunohistochemical staining is commonly employed in the evaluation of spitzoid melanocytic lesions.¹³ p16 protein is encoded by the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene located on chromosome 9p21, and functions as a tumor suppressor by inhibiting the CDK4/cyclin D complex at the G1/S cell cycle checkpoint.^{23,24} While early studies suggested that p16 expression is largely retained in

benign Spitz nevi and lost in spitzoid melanomas^{25–27}, a later study determined that p16 expression did not vary significantly between benign and malignant spitzoid neoplasms.²⁸

Simultaneous deletion of the closely positioned *CDKN2A* and *CDKN2B* genes on chromosome 9p21 is among the most common genetic events in all of human cancer. Loss of the corresponding p16 and p15 proteins are both functionally significant. In melanocytes, p15 loss is now known to directly promote the transition from benign nevus to melanoma.²⁹ Moreover, p15 expression has emerged as a promising diagnostic marker to distinguish benign nevi from melanomas, as p15 immunohistochemical staining is positive in most nevi and absent in many melanomas.^{29,30} Intriguingly, p16 is retained in some cases of melanoma in which p15 is lost, raising the possibility that immunohistochemistry labeling of p15 may be a more specific diagnostic tool than p16.^{29,30} In this study, we examined the utility of p15 as well as p16 immunohistochemical staining in a cohort of 77 of spitzoid melanocytic neoplasms, including Spitz nevi, atypical Spitz tumors, and spitzoid melanomas.

MATERIALS AND METHODS

Specimen selection

Use of specimens in this study was provided through an Institutional Review Board (IRB) approved protocol at the University of Pennsylvania (#808225). Seventy-seven cases from 2013 to 2016 with diagnoses of Spitz nevi (n=19), atypical spitzoid tumors (n=41), and spitzoid melanomas (n=17) were identified from the NewPath database in the Division of Dermatopathology, Department of Dermatology at the Hospital of the University of Pennsylvania. At the time of original diagnosis, each case was reviewed at the daily consensus conference within the dermatopathology division, with accompanying clinical information and immunohistochemical stains including MART-1/Ki67 double stains and p16 stains. The original glass slides and paraffin blocks were retrieved. The median ages at the time of diagnosis of patients with Spitz nevi, ASTs, and spitzoid melanomas were 19, 32, and 39, respectively. For the spitzoid melanomas, 15/17 were thin lesions measuring 1 mm in Breslow depth or less, with the other 2 melanomas measuring 1.5 mm and 2.1 mm in thickness. Five of the 17 melanomas were managed with sentinel lymph node biopsy in addition to wide local excision, with 2 of the 17 melanomas revealing micrometastases in lymph nodes. All of the melanoma patients are alive at the time of this writing, with a mean of 48 months of follow up time. By contrast, only 1/41 of the patients diagnosed with ASTs underwent a sentinel lymph node biopsy, which was negative.

Immunohistochemical staining

Immunohistochemical studies were performed on 5-um-thick paraffin sections using a Bond-3 immunostainer (Leica Microsystems, Bannockburn, IL), with a primary antibody against p16 (1:4, CINtec, Basel, Switzerland). Both diaminobenzadine and Fast Red substrates were used to visualize the antigen-antibody complexes, yielding brown and red chromogens, respectively.

p15 immunohistochemical staining was completed manually using peroxidase/diaminobenzadine complex methods (EXPOSE biotin free detection kit, Abcam,

Cambridge, MA). Specifically, tissue sections were briefly deparaffinized and rehydrated. Heat induced antigen retrieval was performed at sub-boiling temperature for 10 minutes using 10mM citrate buffer pH 6.0 to quench endogenous peroxidase. The tissue sections were incubated in 3% H₂O₂ for 14 minutes at room temperature. The tissue sections were then incubated with the primary antibody to p15 (1:200, Abcam, Cambridge, UK) overnight at 4°C. The secondary antibody was a goat anti-rabbit horseradish peroxidase conjugate, supplied as part of the detection kit. The tissue sections were incubated for 20 minutes at room temperature with the secondary antibody. Following multiple washes in tris-buffered saline, the antigen-antibody complex was visualized with diaminobenzadine solution. The tissues were then counterstained with hematoxylin, dehydrated, and then cover slipped.

Scoring of p15 and p16 immunohistochemical staining and determination of mitotic rate

Immunoexpression for p15 and p16 were categorized by a four-tiered scale: 0 (negative), 1+ (weak), 2+ (moderate), 3+ (strong), as depicted in Figure 1. For negative cases, there was a complete loss of expression by the tumor cells. For tumor to be considered a 3+/strong expresser, the immunoexpression was diffuse and strong in both nuclei and cytoplasm of the lesional cells. The 2+/moderate expressing cases had diffuse cytoplasmic staining but only 5–50% of lesional cells retained nuclear expression. The 1+/weak expressing specimens showed only retained weak expression within the cytoplasm and had <5% nuclear staining. Mitotic rates were determined through the “hot spot” method³¹ and compared to the staining intensities of both p15 and p16. Scoring of the immunohistochemical stains was performed by two of the investigators in a blinded fashion (S.A.M. and E.Y.C.).

Statistical analysis

Fisher’s exact test was used to evaluate differences in proportions of Spitz/spitzoid lesions across different categories of p15 or p16 staining intensities. A non-parametric test for trends in p15 and p16 staining intensity across Spitz/spitzoid lesion groups was performed. Fisher’s exact statistical test was used to examine the significance of the association between of p15 and p16 immunostaining scores and lesion type. Spearman’s rank correlation was used to examine the association between mitotic count and p15 or p16 staining intensity. A 2-tailed P value of less than 0.05 was considered to be statistically significant. All statistical analyses were performed with Stata (Version 14, StataCorp, College Station, TX, USA).

RESULTS

Nineteen Spitz nevi, 41 ASTs, and 17 spitzoid melanoma specimens were examined for expression of p15 and p16. Qualitative staining scores were given to each specimen, independent of previously rendered histopathologic diagnosis. In general, we found that Spitz nevi displayed diffuse, strong nuclear and cytoplasmic expression of both p15 and p16, while spitzoid melanomas often showed loss of both nuclear and cytoplasmic p15 and p16 (Figure 2).

Table 1 shows that 3+ p15 staining was much more frequently observed in Spitz nevi (68.4%) compared to ASTs (34.2%), which in turn showed more frequent 3+ staining than Spitzoid melanomas (17.7%). In contrast, roughly equivalent percentages of Spitz nevi

(57.9%), ASTs (56.1%), and spitzoid melanomas (58.8%) showed 3+ p16 staining. A trend test illustrated that a p15 staining intensity was more likely to be more intense in more benign lesions ($p<0.001$). In contrast, no trend in staining intensity for p16 was observed across the spectrum of benign to malignant lesions ($p=0.309$).

ASTs showed an intermediate degree of immunoexpression of both p15 and p16, more intense than spitzoid melanomas and less intense than Spitz nevi overall (Table 1). For p15, the staining intensity of ASTs was evenly distributed between the scores of 1+, 2+ and 3+, as 34.2% of the ASTs were graded as 1+, 31.7% were graded 2+, and 34.2% were graded 3+. p16 staining revealed 2 AST cases that were entirely negative, 12.2% scored at 1+ intensity, 26.8% scored at 2+ intensity, and 56.1% scored at 3+ intensity.

We also examined proliferation rates (mitoses per mm²) in Spitz nevi, ASTs, and spitzoid melanomas in relationship to p15 and p16 staining intensities. Using the Spearman's correlation, there was a moderate inverse relationship between mitotic count and p15 staining intensity (Spearman's $\rho = -0.45$) that was statistically significant ($p=0.003$), shown in Figure 3. However, the p16 staining intensity and mitotic counts were only weakly inversely related (Spearman's $\rho = -0.30$), and the relationship is not statistically significant ($p=0.05$).

DISCUSSION

In this study, we used immunohistochemistry to determine whether p15 expression was a useful adjunct diagnostic marker to help distinguish lesions across the continuum of spitzoid melanocytic tumors including true Spitz nevi, atypical Spitz tumors, and spitzoid melanomas. We found that p15 staining was more likely to be positive in Spitz nevi compared to spitzoid melanomas. Atypical Spitz tumors showed an intermediate staining pattern with lower p15 intensity than the wholly benign Spitz nevi but greater than frankly malignant spitzoid melanomas.

We also compared p15 and p16 expression in the same tissues in our study, as p16 is frequently used in the evaluation of benign vs malignant spitzoid lesions.³² In our present study, we found through that p16 staining intensity was not significantly different among the different categories of spitzoid melanocytic lesions. This is consistent with a prior study by Mason and colleagues which also showed that p16 expression did not vary consistently across the spitzoid continuum.²⁸ It should be noted, however, that in their study of pediatric spitzoid neoplasms, Al Dhaybi *et al.* found that p16 was strongly expressed in Spitz nevi and absent in spitzoid melanomas.²⁵ More recently, Harms and colleagues observed that p16 expression was significantly higher in Spitz nevi relative to ASTs or spitzoid melanomas using H scores.³³ While our study did not reveal a significant difference in p16 staining intensity in Spitz nevi versus ASTs versus spitzoid melanomas, we did observe that p15 expression was positively correlated with benign lesions. Our findings therefore suggest that p15 immunohistochemical staining may be a better diagnostic discriminator of spitzoid melanocytic lesions than p16 staining. Using the two stains in combination with one another may also yield improved diagnostic accuracy.

Complete loss of p16 immunohistochemical staining has been suggested as a specific, although not sensitive, marker of borderline and malignant spitzoid neoplasms.³³ Since *CDKN2B* (which encodes p15) is adjacent to *CDKN2A* (which encodes p16) on chromosome 9, we were interested in how well absence of p15 staining correlated to absence of p16 immunohistochemical staining. A total of 5 cases of spitzoid melanoma showed absent p15 staining; of those 4 cases also demonstrated absent p16 staining. The remaining melanoma case with absent p15 staining showed 3+ p16 staining. Interestingly, the case was also sent for Myriad MyPath testing, yielding a score of 4.7, in the “likely malignant” range of scores. One melanoma case demonstrated negative p16 staining but 2+ p15 staining. None of the ASTs in our cohort showed absent p15 staining. Two cases showed absent p16 staining, and corresponded to 1+ and 2+ p15 staining.

Increased dermal mitotic rate has been traditionally used by dermatopathologists as a marker of atypia.¹⁴ An inverse correlation between mitotic rate in our cohort of spitzoid lesions and p15 staining intensity was also observed in this study, with decreased p15 intensity observed in lesions with high mitotic rate. It should be noted too that p16 staining did not show a statistically significant correlation with mitotic activity.

Our study confirms an observation from prior studies, that p15 is expressed not only in melanocytes but in epithelial tissues (Figure 1A-C, and Figure 2C, F). We observed higher levels of p15 than p16 expression in epidermal keratinocytes, outer root sheath keratinocytes and adnexal epithelium in this study as well as in prior studies.^{29,30} Previous studies have indicated that p15 mRNA and protein expression are higher in epithelial cells than p16 under most experimental conditions.^{34,35} Therefore, the increased epidermal and adnexal epithelial staining may represent the fact that p15 is more highly expressed in epithelial cells than p16. We do not think that this epithelial p15 staining is primarily due to background but rather reflects biological differences in p15 and p16 expression in epithelial tissues.

One of the limitations of this study is that it is retrospective in nature, and conducted at a single institution. While the gold standard for diagnosis of spitzoid lesions in this study was histopathology with consensus opinion, it would be useful in the future to correlate clinical outcomes of these spitzoid lesions to p15 expression, especially since p16 staining has been shown to be a useful surrogate marker for loss of *CDKN2A* in prognostication of atypical Spitz tumors.³²

Another limitation of our study is the limited correlation with molecular analyses, since most of the specimens included in this study were not originally sent for other molecular work up (CGH, FISH, or Myriad MyPath testing). CGH was performed on one of the melanoma specimens, which had a positive lymph node biopsy. CGH was negative in that specimen, which revealed 2+ p15 staining and absent p16 staining in our study. As previously mentioned, Myriad MyPath testing was performed on one melanoma specimen which showed absent p15 staining and 3+ p16 staining, yielding a score of 4.7, in the “likely malignant” range of scores. Additional studies examining the correlation of p15 immunohistochemical staining with molecular tests would be useful going forward.

It should also be noted that interpretation of p15 staining should be made in the context of other available histopathologic and clinical information for individual cases, as with any ancillary test. While p15 staining was a superior discriminator of Spitz nevi, ASTs, and spitzoid melanomas in our study compared to p16, 3+/strong positive p15 staining was observed in each of these categories (albeit much more frequently in Spitz nevi and ASTs than spitzoid melanomas). Complete loss of p16 staining was observed only in melanomas and ASTs in this study, and complete loss of p15 staining was observed only in melanomas. Therefore, as a practical point, complete loss of either p15 or p16 may justifiably be interpreted as an atypical finding in a spitzoid neoplasm.

In summary, our study demonstrates that p15 is a useful marker to distinguish between Spitz nevi and spitzoid melanomas, and also suggests that p15 may be a superior marker to p16 in this population. ASTs demonstrate an intermediate intensity of p15 staining, falling between the lower intensity staining for spitzoid melanomas and higher intensity staining for Spitz nevi. Finally, we observed a trend showing that higher mitotic counts, a worrisome feature in spitzoid melanocytic lesions and one that may portend poor prognosis, is associated with loss of p15 expression. Additional studies in the future will be useful in further confirming the utility of p15 as a diagnostic and potentially a prognostic marker for spitzoid melanocytic lesions.

Acknowledgments

Funding sources: This study was supported by a Dermatology Foundation Dermatopathology Career Development Award to E.Y.C., and in part by NIH RO1 CA-165836 to J.T.S. and the University of Pennsylvania Skin Biology and Diseases Resource-based Center P30-AR069589 (J.T.S., Core Director). J.T. is supported by National Institute of Arthritis and Musculoskeletal and Skin Diseases grant K23-AR068433.

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Figure 1: Qualitative assessment of p15 and p16 immunohistochemical (IHC) expression. A-C, p15 IHC staining (original magnification x50): A – 1+ (weak) expression, B – 2+ (moderate) expression, C – 3+ (strong) expression. D-F, p16 IHC staining (original magnification x50): D – 1+ (weak) expression, E – 2+ (moderate) expression, F – 3+ (strong) expression.

Figure 2: Comparison of p15 and p16 immunohistochemical expression in a Spitz nevus and spitzoid melanoma.

A-C, Spitz nevus (original magnification x200): A – hematoxylin and eosin stain, B – 3+/strong p16 staining, C – 3+/strong p15 staining. D-F: spitzoid melanoma with associated melanin pigmentation (original magnification x50): D – hematoxylin and eosin stain, E – Absent p16 staining, F – Absent p15 staining.

Figure 3: Relationship of mitotic count in ASTs to IHC expression.

Mitotic count and immunohistochemical (IHC) staining in both p15 and p16 shows an inverse relationship. p15 staining intensity and mitotic count are moderately inversely related, and the relationship is statistically significant; while p16 staining intensity and mitotic count are weakly inversely related, and the relationship is not statistically significant.

Table 1:
Comparing immunoeexpression of p15 and p16 with histologic characterization.

Spitz nevi, atypical Spitz tumors (ASTs), and spitzoid melanomas were stained with p15 and p16 stains. 3+ (strong) p15 staining was much more frequently observed in Spitz nevi compared to ASTs, which in turn showed more frequent 3+ staining than spitzoid melanomas. Roughly equivalent percentages of Spitz nevi, ASTs, and spitzoid melanomas showed 3+ p16 staining.

p15 Staining Intensity by Lesion Type					
Lesion	0	1+	2+	3+	Total
Spitz nevus (% cases)	0 0.00	1 5.26	5 26.32	13 68.42	19 100.00
AST (% cases)	0 0.00	14 34.15	13 31.71	14 34.15	41 100.00
Spitzoid melanoma (% cases)	5 29.41	5 29.41	4 23.53	3 17.65	17 100.00
Total (% cases)	5 6.49	20 25.97	22 28.57	30 38.96	77 100.00
$z = -3.98$, Probability $> z = 0.000$					
p16 Staining Intensity by Lesion Type					
Lesion	0	1+	2+	3+	Total
Spitz nevus (% cases)	0 0.00	1 5.26	7 36.84	11 57.89	19 100.00
AST (% cases)	2 4.88	5 12.20	11 26.83	23 56.10	41 100.00
Spitzoid melanoma (% cases)	6 35.29	1 5.88	0 0.00	10 58.82	17 100.00
Total (% cases)	8 10.39	7 9.09	18 23.38	44 57.14	77 100.00
$z = -1.02$, Probability $> z = 0.309$					