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Original contribution

Conjunctival nevi and melanoma: multiparametric immunohistochemical analysis, including p16, SOX10, HMB45, and Ki-67☆,☆☆

Tatyana Milman MD [a,b,*], Qiang Zhang PhD [a,c], SuMae Ang MD [d], David Elder MD [e], Cristiane M. Ida MD [f], Diva R. Salomao MD [f], Sara E. Lally MD [a,d], Jerry A. Shields MD [a,d], Rose A. Hamershock MA [a,c], Kareem Sioufi MD [d], Carol L. Shields MD [a,d,1], Ralph C. Eagle Jr MD [a,b,1]

[a] *Department of Ophthalmology, Sidney Kimmel Medical College of Thomas Jefferson University, Philadelphia, PA, 19107, USA*
[b] *Department of Pathology, Wills Eye Hospital, Philadelphia, PA, 19107, USA*
[c] *Biostatistics Consulting Core, Vickie and Jack Farber Vision Research Center, Wills Eye Hospital, Philadelphia, PA, 19107, USA*
[d] *Ocular Oncology Service, Wills Eye Hospital, Philadelphia, PA, 19107, USA*
[e] *Department of Pathology and Laboratory Medicine, Hospital of University of Pennsylvania, Perelman School of Medicine, PA, 19104, USA*
[f] *Department of Laboratory Medicine and Pathology, Mayo Clinic, Mayo Clinic College of Medicine and Science, Rochester, MN, 55905, USA*

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Summary The role of p16 in the diagnosis and prognosis of conjunctival melanocytic lesions in the context of other clinical and immunohistochemical parameters has not been systematically explored.

This study was conducted to determine whether p16 is a useful parameter in the diagnosis and prognosis of conjunctival melanocytic nevi and melanoma, either independently or as a component of immunohistochemical panels. Sixty-one patients underwent 61 biopsies for conjunctival melanocytic lesions between 2014 and 2018. Pathologic diagnoses were melanoma (n = 25, 41%), nevus (n = 21, 34%), and conjunctival melanocytic lesion of uncertain malignant potential (n = 15, 25%). The biopsies were assessed for expression of p16, SOX10, HMB45, and Ki-67. In a multivariable model, the parameters most predictive of melanoma versus nevus were diffuse HMB45 staining (odds ratio [OR] = 45, confidence interval [CI] = 4.4–457, P = .02] and p16 nuclear H-score≤115

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* Corresponding author. Department of Pathology, Wills Eye Hospital, 840 Walnut Street, Philadelphia, PA, 19107, USA
E-mail address: tmilman@willseye.org (T. Milman).
[1] The last authorship is shared between Dr. Carol L Shields and Dr. Ralph C Eagle, Jr.

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(OR = 9.5, CI = 1.2−77; P = .04). There was no association of p16 expression with melanoma thickness. Next-generation sequencing identified no *CDKN2A* mutations or copy number alterations in 12 conjunctival melanomas, including the tumors with absent p16 expression. This study demonstrates that p16 immunohistochemical stain is useful in distinguishing conjunctival melanocytic nevi from melanoma, particularly in combination with HMB45. P16 expression does not appear to correlate with *CDKN2A* status and melanoma thickness.

1. Introduction

Conjunctival melanocytic lesions as a group account for up to one half of all conjunctival tumors [1]. These lesions include benign, premalignant, and malignant conjunctival intraepithelial melanocytic proliferations, benign conjunctival melanocytic nevi, and malignant melanoma [2]. Accurate distinction between conjunctival nevi and melanoma has immense prognostic and therapeutic implications. While most conjunctival nevi follow a benign clinical course, conjunctival melanoma can invade the local tissues of the eye, recur in spite of treatment, and spread systemically through lymphatic drainage and hematogenously in 20−30% of patients, leading to significant morbidity and mortality [3].

Despite the extensive body of literature dedicated to delineation of the clinical and histopathologic criteria to accurately distinguish between conjunctival nevi and melanoma, these lesions continue to present a considerable diagnostic challenge. In fact, similar to the well-recognized subjectivity in interpretation of cutaneous melanocytic lesions by expert dermatopathologists, a subset of melanocytic proliferations of the conjunctiva exists that cannot be reproducibly classified by pathologists as benign, malignant, or indeterminate [4,5].

The limitations of morphologic evaluation of challenging conjunctival melanocytic lesions have led to incorporation of immunohistochemical and molecular genetic studies into our diagnostic arsenal. Integration of immunohistochemical panels that include either Melan-A, S100, MITF, or SOX10 (melanocytic and neural crest markers), HMB45 (activated melanocyte marker), and the Ki-67 proliferation marker have been shown to be helpful in distinction between conjunctival nevi and melanoma [6]. Additionally, molecular genetic studies targeting structural chromosomal and molecular genetic alterations, analogous to those performed on cutaneous melanocytic lesions, have been found helpful in distinguishing between conjunctival nevi and melanoma [7,8]. Although these ancillary studies can improve diagnostic accuracy, they have limitations. The aforementioned immunohistochemical panels are imperfect, and molecular genetic studies of conjunctival specimens frequently are limited by the small quantity of lesional tissue, cost, turnaround time, and the availability of a molecular pathologist [4].

Recent studies on cutaneous melanocytic lesions have documented the usefulness of the p16 immunohistochemical stain, both as a single stain and as a component of an immunohistochemical panel in distinguishing between nevi and melanomas [9−11]. P16INK4a, encoded by the *CDKN2A* gene on chromosome 9p21, belongs to the protein family of cyclin-dependent kinase inhibitors and is an important negative regulator of the cell cycle [12]. P16 has been shown to play a critical role in melanocyte senescence, an important barrier for tumorigenesis or progression to melanoma [13]. Germline *CDKN2A* mutations have been implicated in patients with familial melanoma, whereas acquired *CDKN2A* mutations and *CDKN2A* loss have been documented in a subset of sporadic melanomas [12]. In addition to its value in discrimination between cutaneous melanoma and nevi, loss of p16 nuclear expression has been shown in some studies to be associated with tumor progression, unfavorable prognosis, and nodal metastasis [14−17].

There are limited and controversial data on the role of p16 in conjunctival melanocytic lesions. Pache et al. found no association between p16 expression and lesion type in their analysis of 76 conjunctival melanocytic tumors. However, that study was limited by the absence of detailed methodology to substantiate its conclusions [18]. Conversely, Zoroquiain et al examined 9 melanomas and 19 nevi and demonstrated decreased p16 expression in melanoma when compared with nevi, analogous to cutaneous melanocytic tumors [19]. While providing valuable data supported by meticulous assessment of p16 expression, that analysis was limited by a small sample size, lack of detailed correlation with clinical, other immunohistochemical, and molecular genetic parameters, and the absence of outcome data [19].

In this current study, we evaluate the usefulness of p16 and other immunohistochemical parameters (Melan-A, SOX10, HMB45, and Ki-67) in distinguishing between conjunctival nevi and melanoma. We correlate p16 expression in a subset of conjunctival melanomas with *CDKN2A* gene status. We further evaluate in a multivariable model which pathologic and clinical parameters most accurately discriminate between conjunctival nevi and

melanoma. Finally, we assess the performance of our diagnostic algorithm on a group of challenging melanocytic lesions (melanocytic lesions of uncertain malignant potential/indeterminate melanocytic proliferations).

2. Materials and methods

2.1. Case selection and review

A retrospective review of medical records at a single center between March 1, 2014 and March 1, 2018 was conducted to identify all patients with conjunctival nevi and melanoma who underwent biopsy and had sufficient clinical information and tissue available for pathologic evaluation. Lesions with no tissue available for pathologic evaluation or the biopsies without supporting clinical history were excluded from the study. Normal conjunctival map biopsies from a patient without conjunctival disease were used as controls.

Clinical data collected included patient age at the time of surgery, sex, clinical diagnosis, clinical features of the tumor (location, extent), history of prior biopsy, intervention, outcome (recurrence, metastasis), and length of follow-up. The study followed the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board.

2.2. Histopathology and immunohistochemistry

Routine sections stained with hematoxylin-eosin were prepared from paraffin-embedded, formalin-fixed tissues. Immunostaining was performed with the following primary antibodies: monoclonal mouse anti-human SOX10 (prediluted; Biocare, Pacheco, CA), monoclonal mouse anti-human MART-1 (Melan-A) (diluted 1:50; DAKO, Carpinteria, CA), monoclonal mouse anti-human HMB45 (diluted 1:40; Thermo Fisher Scientific, CA), monoclonal mouse anti-human Ki-67 (prediluted, DAKO), and monoclonal mouse anti-human p16 (prediluted, Ventana, Tucson, AZ) using standard immunohistochemical techniques. All immunohistochemical stains were prepared with a Leica autostainer BOND III using the Bond Polymer Refine Red Detection Kit in accordance with the manufacturers' instructions. Sections were counterstained with a modified Mayer's hematoxylin, dehydrated, cleared, and mounted. Appropriate positive and negative controls were included in all protocols. Additionally, reliability of staining in the study tissues was assessed by evaluation of internal controls: basal epithelial melanocytes in appropriate samples (Melan-A and SOX10), basal epithelial cells and/or stromal inflammatory cells (Ki-67), and macrophages (p16).

Histopathologic diagnoses were rendered independently by 2 ophthalmic pathologists, and in discordant cases, a consensus diagnosis was reached. Malignant melanoma was staged in accordance with AJCC 8th edition pathologic staging guidelines for conjunctival melanoma [20]. Conjunctival melanocytic nevi were classified as junctional, compound, and subepithelial in accordance with WHO 4th edition terminology [21].

Immunohistochemical staining for Melan-A and SOX10 was performed to highlight the distribution of melanocytes in the tissue for accurate interpretation of other immunohistochemical stains and was reported as positive or negative. The tissues with suboptimal staining for both Melan-A and SOX10 were excluded from the study. Variability in nuclear size was evaluated by SOX10 immunohistochemistry, which was scored as follows: none = no appreciable nuclear size variation, mild = two-fold nuclear size variation, moderate = three-fold nuclear size variation, severe = four-fold or greater nuclear size variation. HMB45 expression was recorded as negative, diffuse (entire lesion staining), stratified (only superficial component of the lesion staining, applied only to nevi and melanoma), and patchy (nodular foci of staining, applied only to nevi and melanoma). Ki-67 immunostaining was assessed in "hot spots" with a 40x objective in the epithelial/junctional and stromal components of the lesion separately (when applicable) and expressed as the percentage of nuclear-stained cells relative to the total number of tumor cells in the "hot spot". The small size of the lesions precluded assessment of proliferative index within 1 mm^2 field. P16 immunoreactivity was assessed by scoring separately the intraepithelial/junctional and stromal components of the lesion (when applicable) and also by evaluating the expression in the entire lesion (total score). Because the methodology of p16 scoring varies among the published studies, we incorporated several of the most comprehensive scoring systems to evaluate their reproducibility and ability to discriminate between various melanocytic lesions. In addition, similar to the methodology of Mihic-Probst et al, we evaluated both nuclear and cytoplasmic p16 expression in melanocytes [16]. P16 expression was assessed separately in the nucleus and cytoplasm of melanocytes and scored for staining intensity (none, weak, moderate, and strong) and for percentage of immunoreactive cells. These data were then used to calculate the H-score values for nuclear and cytoplasmic p16 expression, defined as H-score = 1 × (% cells with weak staining intensity) + 2 × (% cells with moderate staining intensity) + 3 × (% cells with strong staining intensity), with the scores ranging from 0 to 300. Additionally, p16 staining was expressed as the percentage of immunoreactive cells with nuclear expression only,

cytoplasmic expression only, and with any nuclear or cytoplasmic expression. Because most conjunctival melanomas in our cohort demonstrated a diffuse and "checkerboard"/mosaic patterns of p16 expression (similar to nevi) without zonal or complete loss of p16 expression, we did not specifically assess these patterns in our lesions.

2.3. *CDKN2A* gene status analysis

Molecular genetic studies on all conjunctival invasive melanomas with sufficient tumor cellularity and tumor quantity (constituting 12 specimens) were performed at CARIS Life Sciences laboratory (Irving, TX) in accordance with established protocols. Briefly, one hematoxylin and eosin−stained section and 10 unstained, 5-μm-thick tissue sections were prepared on glass slides from the paraffin-embedded, formalin-fixed blocks. When necessary, the tissue was microdissected for tumor enrichment (minimum acceptable tumor cellularity 20%). Next-generation sequencing was performed on the extracted tumor DNA using the Illumina NextSeq platform. An Agilent custom designed SureSelect XT assay was used to enrich 592 whole-gene targets, including the *CDKN2A* gene (the complete list of genes and amino acids evaluated by this platform can be found at www.carislifesciences.com). All variants reported by this assay are detected with >99% confidence based on the frequency of the mutation present and the amplicon coverage with an average depth of coverage >750x. This assay was documented to have sensitivity of >95% for base substitutions at ≥5% of mutant allele frequency and sensitivity of >90% for copy number alterations (amplifications ≥6 copies).

2.4. Interphase fluorescence in situ hybridization studies

Because fluorescence in situ hybridization (FISH) with commercially available probes assessing copy numbers of RREB1 (6p25), MYB (6q23), and CCND1 (11q13) genes compared with CEP6 (a chromosome 6 centromeric reference point) was documented to be useful in classifying equivocal conjunctival melanocytic lesions [7] and because of our interest in evaluating CDKN2A copy number status, we adopted the expanded FISH probe set with the commercially available probes currently used for evaluation of cutaneous melanocytic tumors [22].

FISH studies were performed at Mayo Clinic (Rochester, MN) in 15 histomorphologically challenging lesions (conjunctival melanocytic lesion of uncertain malignant potential/indeterminate melanocytic proliferation) using 3 commercially available enumeration strategy probes sets (Abbott Molecular, Abbott Park, IL) to interrogate the copy number state of a) 6p25 region by RREB1 probe labeled in SpectrumRed [control CEP6 (D6Z1) labeled in SpectrumAqua]; b) 6q23 region by MYB labeled in SpectrumGold; c) 11q13 region by CCND1 labeled in SpectrumGreen; d) 8q24.1/D8Z2 region by MYC labeled in SpectrumOrange and CEP8 (D8Z2) labeled in SpectrumGreen; and e) 9p21/D9Z1 region by CDKN2A labeled in SpectrumOrange and CEP9 (D9Z1) labeled in SpectrumGreen. Areas containing the melanocytic cells of interest were delineated on an H&E-stained tissue section and included at least 25 cells for scoring. Using the hematoxylin-eosin−stained slide as reference, the targeted areas were etched with a diamond-tipped etcher on the back of the unstained slide to be tested. Probe sets were applied to the etched areas, hybridized, and washed using in-house validated protocols. Twenty-five to 50 nonoverlapping nuclei within the areas of interest were scored by a technologist with the results expressed as the percent of abnormal nuclei using the following clinically validated cut-off values: a) % of cells RREB1 >2 = cut-off <14%, b) % of cells CCND1 >2 = cut-off <14%, c) % of cells MYC >2 = cut-off <26%, and d) % of cell CDKN2A = 0 = cut-off <9%. Clinical validation studies were performed using 55 skin melanocytic lesions (melanoma and nonatypical nevi), and the clinical sensitivity of this assay was determined to be 86% when at least 1 of the probes tested is abnormal and at least 50% of the nuclei exhibit the abnormality with a clinical specificity of 100%.

2.5. Statistical analysis

Summary statistics are reported for demographic, clinical, and pathological characteristics on a patient and biopsy level. In patient-level comparisons, the Fisher's exact test was used to determine a difference between groups for categorical variables, while t-tests (2 groups) or analysis of variance (3 groups or more) were used for normally distributed continuous variables, and rank sum (2 groups) or Kruskal-Wallis (3 groups or more) tests were used for non-normally distributed continuous variables. In biopsy-level comparisons, non-normally distributed continuous variables were tested with Clustered Wilcoxon Rank Sum, ordinal variables were tested with cumulative logistic regression (accounting for correlated data), and dichotomous variables were tested with logistic regression (accounting for correlated data). Categorical variables without an order to their levels were re-categorized as dichotomous variables and tested with logistic regression. Logistic regression with stepwise selection was used to determine potential predictors of conjunctival nevi and melanoma and disease recurrence. Effects of all factors were also modeled at biopsy level. The agreement between the 2 observers for interpretation of p16 expression was assessed by the intraclass correlations. All analyses were performed in SAS

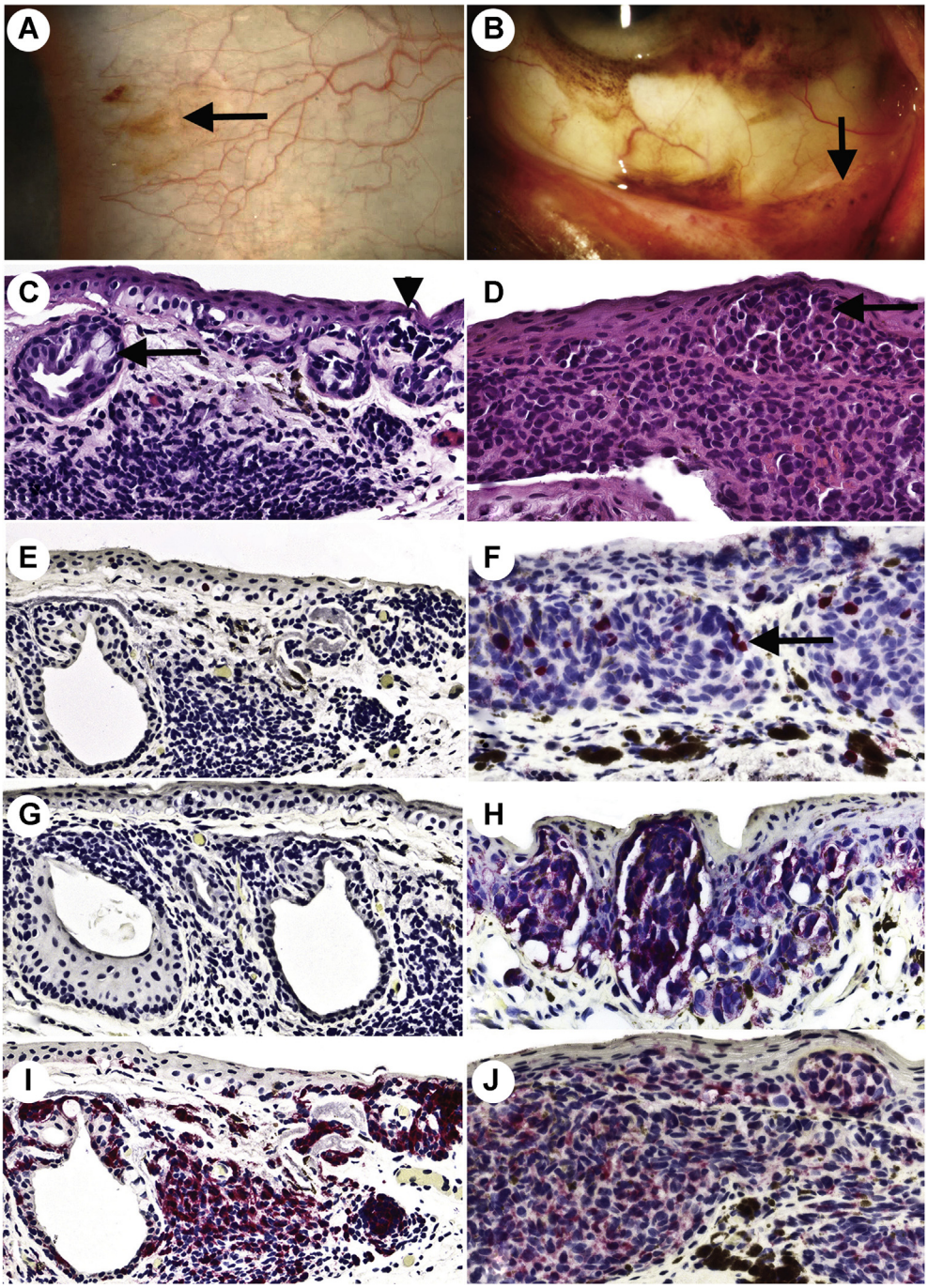

Fig. 1 Conjunctival melanocytic nevi and melanoma: clinical and pathologic features. A. Conjunctival melanocytic nevus: Circumscribed, variably lightly pigmented juxtalimbal conjunctival nodule with several translucent cysts (arrow). B. Conjunctival melanoma: Multifocal diffuse pigmentation (overall at least 6 clock hours) with focal nodularity involves the limbal and bulbar conjunctiva, the adjacent cornea, and extends into the forniceal conjunctiva (arrow). C. Conjunctival melanocytic nevus: Nested proliferation of melanocytes at the epithelial-substantia propria junction (arrowhead) and in the stroma, where melanocytes surround cystic and solid rests of conjunctival epithelium (arrow) (stain, hematoxylin-eosin; original magnification ×75). D. Conjunctival melanoma: Diffuse involvement of conjunctival epithelium by confluent epithelioid melanocyte nests, which focally replace nearly entire epithelial thickness (arrow) and the invasion of the underlying substantia propria (stain, hematoxylin-eosin; original magnification ×100). E. Conjunctival melanocytic nevus: No proliferative activity is evident with Ki-67 immunostain (stain, Ki-67; original magnification ×75). F. Conjunctival melanoma: Ki-67 labels cycling nuclei in the tumor (arrow) (stain, Ki-67; original magnification ×100). G. Conjunctival melanocytic nevus: HMB45 is negative in the lesion (stain, HMB45; original magnification ×75). H. Conjunctival melanoma: HMB45 is diffusely expressed in the neoplastic cells (stain, HMB45; original magnification ×100). I. Conjunctival melanocytic nevus: P16 is expressed strongly and diffusely in the nuclei of junctional melanocytes and in a speckled or "checkerboard" pattern in the stromal component of the lesion (stain, p16; original magnification ×75). J. Conjunctival melanoma: P16 shows weak staining in occasional neoplastic nuclei and more diffuse weak staining in the cytoplasm (stain, p16; original magnification ×100).

V9.4 (SAS Institute Inc., Cary, NC), and two-sided $P < .05$ was considered to be statistically significant.

3. Results

3.1. Clinical characteristics

3.1.1. Conventional conjunctival nevi and melanoma

Clinical characteristics of the patients with conjunctival malignant melanoma and conjunctival melanocytic nevi are summarized in Supplemental Table 1 and documented in Fig. 1.

There were 25 patients with invasive melanoma and 21 patients with conjunctival melanocytic nevi. A comparison (conjunctival melanoma versus nevus) revealed patients with melanoma were older (mean age 67 versus 21, $P < .001$) with larger tumors (mean 4 versus 1 clock hour, $P < .001$) and more often in the nonbulbar conjunctiva (36% versus 29%; $P = .001$). A comparison revealed no difference in sex, race, or prior biopsy elsewhere. Over median follow-up (10 versus 1 month, $P < .001$), those with melanoma were more likely to experience local tumor recurrence (72% versus 5%; $P < .001$).

3.1.2. Melanocytic lesions of uncertain malignant potential

There were 15 patients with melanocytic lesions of uncertain malignant potential (Table 1), 8 females (53%) and 7 males (47%), with a mean age of 50 (median 49, range 17–85), who underwent 16 biopsies (1 patient had 2 biopsies). The clinical impression was nevus in 9 patients (60%) and melanoma in 6 patients (40%). Of 4 patients with an extended follow-up (mean 16 months, range 6–32 months), 2 lesions recurred with the diagnosis of melanoma rendered in recurrent lesions.

3.2. Histopathology and immunohistochemistry

3.2.1. Control tissues

The 3 control conjunctival tissues contained small, basally distributed melanocytes, averaging 1 per every 3–4 basal epithelial cells, highlighted with the SOX10 and Melan-A stains. The melanocytes did not express HMB45, p16, and Ki-67.

3.2.2. Conventional conjunctival nevi and melanoma

Of 21 melanocytic nevi, 1 (5%) was predominantly junctional (>90% junctional component), 9 (43%) were compound, 8 (38%) were predominantly subepithelial (>90% subepithelial component), and 3 (14%) were subepithelial. Twenty-five malignant melanomas had the following pathologic T designations: T1a, 10 tumors (40%); T1b, 1 tumor (4%); T2a, 5 tumors (20%); T2b, 3 tumors (12%); T3b, 2 tumors (8%); and T3c, 4 tumors (16%). Sentinel lymph node biopsy was performed in 4 (16%) patients and was positive (N1) in 2 (8%) tumors. One of these tumors was also associated with pathologically confirmed ipsilateral parotid metastasis.

The pathologic features of conjunctival melanocytic nevi and malignant melanoma are summarized in Table 2 and illustrated in Fig. 1. A comparison (melanoma versus nevus) revealed that melanoma demonstrated mitotic activity (median 1 mitotic figure/1 mm^2 [average 3, range 0–30] versus 0), diffuse HMB45 expression (80% versus 5%, $P < .001$), three-fold or greater nuclear size variation with SOX10 immunostain (63% versus 0%, $P < .001$), Ki-67 proliferative activity >5% in both intraepithelial/junctional (48% versus 0%, $P < .001$) and stromal components (71% versus 0%, $P < .001$), lower nuclear p16 expression in both intraepithelial/junctional (mean H-score = 53 versus 153, $P < .001$; mean % positive nuclei = 20 versus 52, $P < .001$) and stromal components (mean H-score = 43 versus 132, $P < .001$; mean % positive nuclei = 16 versus 44, $P < .001$), lower cytoplasmic p16 expression in both intraepithelial/junctional (mean H-score = 80 versus 153, $P = .002$) and stromal components (mean H-score = 68 versus 133, $P < .001$), and lower p16 expression overall in the entire lesion (mean nuclear H-score = 40 versus 127, $P < .001$; mean % positive nuclei = 15 versus 45, $P < .001$; and mean cytoplasmic H-score = 80 versus 127, $P = .03$). Clonal loss of p16, commonly seen in cutaneous melanomas, was not observed in these conjunctival lesions.

3.2.3. Immunohistochemical parameters and conjunctival malignant melanoma thickness

We found no association of nuclear and cytoplasmic p16 expression with pathologic T category in malignant melanoma. Paired comparison of intraepithelial and stromal p16 values and nonparametric correlation of p16 values with melanoma thickness revealed no association of p16 expression with tumor thickness. Similarly, there was no association between T category and other immunohistochemical parameters (SOX10, HMB45, and Ki-67).

3.2.4. Assessment of concordance for p16 expression interpretation by 2 observers

The intraclass correlation coefficients between the 2 observers were 0.91 (nuclear H-score), 0.92 (cytoplasmic H-score), and 0.92 (total % of positive cells).

3.3. Logistic regression for clinical and pathologic features that enable distinction between conjunctival melanoma and nevi

In a multivariable model, the parameters most predictive of a malignant melanoma versus nevi were diffuse HMB45 staining (odds ratio [OR] = 45, confidence interval [CI] 4.4–457, $P = .01$) and p16 nuclear H-score ≤115 (OR = 9.5, CI 1.2–77; $P = .04$). The probability of a melanoma was 96% (CI 76–99%; $P = .002$) for lesions with diffuse HMB45 staining and a nuclear H-score ≤115

and 5% (CI 0.5−31%, $P = .007$) for lesions with absent HMB45 staining and a nuclear H-score >115.

When applying these diagnostic parameters to 15 conjunctival melanocytic lesions of uncertain malignant potential (Table 1), the algorithm confirmed the histopathologic impression of conjunctival melanocytic nevus in 2 cases (Patients 1 and 2) and the histopathologic impression of nevoid malignant melanoma in 4 cases (Patients 5, 7, 9, and 11). In 3 nevoid lesions with significant atypia, where the histopathologic diagnosis was equivocal (Patients 4, 8 and 10), the algorithm supported the diagnosis of malignant melanoma. In 4 lesions concerning for melanoma evolving in a pre-existing melanocytic nevus, the algorithm confirmed the presence of a nevoid component adjacent to melanoma in 3 lesions (Patients 12, 14 [Fig. 2], and 15) and was supportive of the entire lesion being nevoid melanoma in Patient 13. The algorithm was unable to accurately distinguish a nevus with granular cell features from melanoma (Patient 6). When combining the algorithm with the follow-up and FISH data, the algorithm prediction of a nevus was compatible with FISH-intact and nonrecurrent status in 3 of 3 (100%) lesions (Patients 1, 2, and 3), and the algorithm prediction of melanoma was compatible with FISH-copy number altered or melanoma recurrent status in 5 (Patients 5, 7, 11, 12, 13) of 12 (42%) lesions. Of note, the algorithm predicted melanoma in all 3 lesions with chromosomal copy number alterations by FISH and in 2 of 4 FISH-intact lesions, which recurred as melanoma. Also of note, none of the chromosomal copy number alterations by FISH involved *CDKN2A* locus.

3.4. P16 expression and *CDKN2A* gene status

Next-generation sequencing was performed on 12 conjunctival invasive melanomas with sufficient tumor cellularity and tumor quantity with pathogenic mutations identified in *NRAS* (4/12, 33%), *NF1* (4/12, 33%), *BRAF* (3/12, 25%), *ATRX* (2/12, 17%), *ATM* (2/12, 17%), *SED2* (1/12, 8%), *TP53* (1/12, 8%), and *SF3B1* (1/12, 8%) genes and amplification in *FGF3* (2/12, 17%), *FGF4* (2/12, 17%), and *CCND1* (1/12, 8%) genes [the detailed molecular genetic data will be documented in a separate report]. *CDKN2A* mutations and copy number alterations were not identified in any of these 12 tumors. Expression of p16 protein was low in these tumors, with the median p16 nuclear H-score of 9 (mean 40, range 0−160). Three of 12 tumors (25%) had completely absent p16 expression (Fig. 3).

4. Discussion

Conjunctival melanoma is a rare but potentially deadly ocular malignancy, with high propensity for regional recurrence and up to 30% risk of metastasis [3]. According to a recent epidemiologic study, conjunctival melanoma incidence increased by 5.5% biannually from 1973 to 1999, and this increase was particularly pronounced in white men and patients aged 60 years and older [23]. Thus, timely and accurate identification of conjunctival melanoma is essential.

Conjunctival melanoma can resemble melanocytic nevus and can arise in a nevus, prompting biopsy [24,25]. Although the distinction between conventional conjunctival nevus and melanoma is generally histomorphologically straightforward, some conjunctival nevi, particularly in young patients, can be dominated by an extensive junctional component, confluence of junctional melanocytic nests, absence of apparent maturation, and cytomorphologic atypia raising concern for melanoma. Analogously, some conjunctival melanomas are composed of bland melanocytes with a nevoid appearance. This morphologic overlap between a subset of conjunctival melanomas and nevi presents a diagnostic challenge, necessitating ancillary studies [7,26].

Our study results suggest that p16 may be a valuable adjunct to our diagnostic arsenal, when morphologic distinction between conjunctiva melanocytic nevus and melanoma is not straightforward. Similar to prior studies, we demonstrated that a diffuse pattern of HMB45 expression in conjunction with a high Ki-67 proliferative index help distinguish melanoma from nevi [6]. Interestingly, 12% of the melanomas in our study demonstrated some stratification of HMB45 expression, a pattern that is typically seen in nevi [6]. However, most nevi in this study lacked HMB45 expression entirely. We noted higher variability in nuclear size using SOX10 immunohistochemistry in melanoma, when compared with the nevi. Importantly, we demonstrated significantly increased nuclear p16 expression in both junctional and stromal components of the nevi when compared with the intraepithelial and stromal components of melanoma. In a multivariable analysis, incorporating all independently significant clinical and pathologic parameters, only HMB45 and nuclear p16 expression were found to be significant factors that serve to distinguish conjunctival melanoma from nevus.

Several studies of cutaneous melanoma documented an association between loss of p16 nuclear expression and vertical growth phase, stage, progression, and metastasis [14−17]. Zoroquiain et al. also noted decreased nuclear p16 expression in their cohort of 9 conjunctival melanomas when compared with 2 cases of primary acquired melanosis with atypia lesions and lower p16 expression in melanomas greater than 2 mm in thickness [19]. In our study, we observed no association between p16 nuclear or cytoplasmic expression with tumorigenic stage of melanoma, recurrence, or disease progression. Although limited by relatively small sample size, predominance of thin tumors, and lack of long-term follow-up, our data suggest that p16 may not be a useful prognostic biomarker in conjunctival melanoma.

Table 1 Conjunctival melanocytic lesions of uncertain malignant potential: clinical and pathologic characteristics.

Pt N	Age sex	Clinical history	Pathologic diagnosis	HMB45	P16	Probability of MM (CI)	Interpretation of HMB45-P16 algorithm	FISH	Outcome, Follow-up
1	52 F	Lesion for many years with recent growth	Compound nevus with mild cytologic atypia in epithelial component	Negative	>115	5% (0.6−31)	Data strongly support diagnosis of nevus	Intact	N/A, 1 month
2	17 F	Lesion for several years with growth	Juvenile (inflamed) compound nevus with atypia	Negative	>115	5% (0.6−31)	Data strongly support diagnosis of nevus	Intact	N/A, 1 month
3	72 F	Lesion for many years with recent growth	Concern for melanoma in situ arising in compound nevus	Negative	≤115	32% (9−70)	Data support diagnosis of nevus	Intact	N/A, 1 month
4	17 M	Lesion for several years with growth	Compound nevus with diffuse severe cytologic atypia	Patchy	≤115	66% (3−99)	Data support diagnosis of MM	Intact	N/A, 1 month
5	68 F	Pigmented lesion for several months	Atypical melanocytic tumor, favor nevoid MM	Patchy	≤115	66% (3−99)	Data support diagnosis of MM	8q24.1+ 6p25+	N/A, 1 month
6	29 F	Lesion for many years with recent growth	Compound nevus with granular cell features	Patchy	≤115	66% (3−99)	Data support diagnosis of MM	Intact	N/A, 1 month
7	63 M	Pigmented lesion for several months	Atypical melanocytic tumor, favor nevoid MM	Diffuse	>115	69% (19−95)	Data support diagnosis of MM	6p25+ 6q23-	N/A, 1 month
8	47 F	Recently noted tarsal conjunctival lesion	Melanocytic tumor of uncertain malignant potential, favor blue nevus	Diffuse	≤115	96% (76−99)	Data strongly support diagnosis of MM	Intact	No recurrence, 19 months
9	78 F	Pigmented lesion for several months	Nevoid melanoma	Stratified	≤115	65% (0.23−92)	Data support diagnosis of MM	Intact	No recurrence, 8 months
10	23 M	Pigmented lesion for several months	Concern for melanoma in situ, arising in a nevus with minor stromal component	Stratified	≤115	65% (0.23−92)	Data support diagnosis of MM	Intact	N/A, 1 month
11	70 M	Pigmented lesion for several months	Atypical melanocytic tumor, favor nevoid MM	Stratified	≤115	65% (0.23−92)	Data support diagnosis of MM	Intact	MM recurrence x2, 32 months
12	49 M	Lesion for many years with recent growth	MM arising in a nevus (favored) *vs*. nevus with atypia: MM component	Diffuse	≤115	96% (76−99)	Data support diagnosis of MM arising in a nevus	Intact	MM recurrence x1, 6 months
			MM arising in a nevus (favored) *vs.* nevus with atypia: Nevus component	Negative	>115	5% (0.6−31)		Intact	
13	43 M	Lesion for several years with growth	Nevoid MM (favored) *vs.* MM arising in a nevus: MM component	Stratified	≤115	65% (0.23−92)	Data support diagnosis of nevoid MM	6p25+ 6q23-	N/A, 1 month
			Nevoid MM (favored) *vs.* MM arising in a nevus: Nevus component	Stratified	≤115	65% (23−92)		6p25+ 6q23-	

14	32 F	Lesion for many years with recent growth	MM arising in a nevus (favored) *vs.* nevoid MM: MM component	Diffuse	≤115	96% (76–99)	Data support diagnosis of MM arising in a nevus	N/A, 1 month
			MM arising in a nevus (favored) *vs.* nevoid MM: Nevus component	Negative	>115	5% (0.6–31)	Intact	
15	85 M	Lesion for many years with recent growth	MM arising in a nevus (favored) *vs.* nevoid MM: MM component	Patchy	≤115	66% (3–99)	Data support diagnosis of MM arising in a nevus	N/A, 1 month
			MM arising in a nevus (favored) *vs.* nevoid MM: Nevus component	Negative	>115	5% (0.6–31)	Intact	

Abbreviations: N, Number; P16, P16 nuclear H-score; MM, malignant melanoma; CI, confidence interval; F, female; M, male; FISH, fluorescence in situ hybridization study; N/A, not available.

The data on morphologically straightforward conjunctival nevi and melanoma provide a benchmark for p16 interpretation. However, the actual value of p16 study is determined by its usefulness in interpretation of challenging melanocytic lesions. Although increasingly common in the modern era, molecular genetic studies are still not widely used for diagnosis of ambiguous melanocytic conjunctival lesions because of their small size and also because of lack of access to molecular genetic testing and the high cost of these studies. To overcome these difficulties, we selected FISH as molecular diagnostic modality to evaluate individually the epithelial/junctional and various stromal components of relatively small melanocytic lesions of uncertain malignant potential and for correlation with clinical, morphologic, and immunohistochemical data. Our results highlight the limitation of molecular genetic studies in evaluation of melanocytic conjunctival lesions as, for most cases, FISH testing was noninformative. Although shown by several groups to be highly sensitive and specific in the diagnosis of conjunctival and cutaneous melanocytic lesions, melanoma FISH cumulative sensitivity and specificity values range from 43% to 100% (86% in our lab) and 29%−80% (100% in our lab), respectively [7,22]. As a result, clinical, histomorphologic, and immunohistochemical parameters remain the cornerstone for diagnosis. When applying our diagnostic algorithm to a set of conjunctival melanocytic lesions of uncertain malignant potential, several observations emerge. While a panel comprising p16 and HMB45 appears to be useful in confirming the morphologic impression in lesions with uniform morphologic features, it may have limitations in lesions where there is concern for occult intraepithelial colonization by melanoma in pre-existing nevus and in lesions with an atypical HMB45 staining pattern (including granular cell nevus and blue nevus) [26]. Evaluation of the efficacy of p16 staining in a larger group of conjunctival melanocytic lesions with long-term follow- up data and, when available, molecular genetic data, is necessary to conclusively establish its utility in the distinction of challenging conjunctival melanocytic nevi from melanoma. Data from more patients will help the multivariable model building with improved parameter estimates and confidence intervals, which in turn will help predict outcome for future challenging cases.

When comparing our study results to the p16 data in cutaneous melanocytic lesions, we noted both similarities and differences, which may be influenced by the underlying molecular genetics. P16, a gene product of *CDKN2A*, plays a critical role in melanocyte senescence [13]. Studies on cutaneous melanocytic lesions document absent p16 expression in normal melanocytes and an increase in nuclear p16 expression in sun-damaged skin, melanoma in situ, and nevi, suggesting that p16 pathway activation is an important barrier to tumorigenesis or progression to melanoma [13,27,28]. Loss of p16 nuclear expression in cutaneous melanoma has been correlated with underlying

Table 2 Comparison of pathologic characteristics of conjunctival nevi and melanoma[a].

Pathologic parameter	Melanoma (N = 25)	Nevi (N = 21)
Biopsy location: (%)		
Bulbar and limbus	13 (54)	15 (71)
Nonbulbar	11 (46)	6 (29)
	$P = .23$	
Variability in nuclear size using SOX10 immunohistochemistry[b]*: N (%)*		
None	2 (8)	15 (79)
Low	7 (29)	4 (21)
Moderate	13 (54)	0 (0)
High	2 (8)	0 (0)
	$P < .001$	
HMB45 staining pattern: N (%)		
Negative	2 (8)	14 (67)
Diffuse	20 (80)	1 (5)
Patchy	0 (0)	2 (9)
Stratified	3 (12)	4 (19)
	$P < .001$	
Ki67 index—epithelium/E−S junction[c]*: N (%)*		
<1%	2 (9.5)	17 (85)
1−5%	9 (43)	3 (15)
6−10%	5 (24)	0 (0)
11−20%	2 (9.5)	0 (0)
>20%	3 (14)	0 (0)
	$P < .001$	
Ki67 index—stroma: N (%)		
<1%	4 (17)	18 (90)
1−5%	5 (21)	2 (10)
6−10%	5 (21)	0 (0)
11−20%	4 (17)	0 (0)
>20%	6 (25)	0 (0)
	$P < .001$	
P16—epithelium/E−S junction: N (%)		
%positive nuclei[d]		
Mean ± SD (CI)	20 ± 17 (13−28)	52 ± 23 (40−64)
Median (min, max)	20 (0−50)	55 (2−90)
	$P < .001$	
Nuclear H-score[e]		
Mean ± SD (CI)	53 ± 49 (30−76)	153 ± 70 (116−191)
Median (min, max)	50 (0−150)	163 (5−270)
	$P < .001$	
Cytoplasmic H-score[f]		
Mean ± SD (CI)	80 ± 64 (50−110)	153 ± 63 (120−187)
Median (min, max)	77 (0−190)	162 (5−250)
	$P = .002$	
P16—stroma: N (%)		
%positive nuclei		
Mean ± SD (CI)	16 ± 20 (8−25)	44 ± 17 (36−52)
Median (min, max)	10 (0−60)	40 (2−80)
	$P < .001$	
Nuclear H-score		
Mean ± SD (CI)	43 ± 56 (20−66)	132 ± 50 (109−156)
Median (min, max)	12 (0−160)	120 (5−210)
	$P < .001$	
Cytoplasmic H-score		
Mean ± SD (CI)	68 ± 61 (43−93)	133 ± 48 (110−155)
Median (min, max)	75 (0−160)	120 (5−210)
	$P < .001$	
P16—total: N (%)		
%positive nuclei		
Mean ± SD (CI)	15 ± 17 (8−22)	45 ± 18 (37−54)
Median (min, max)	5 (0−50)	50 (2−70)
	$P < .001$	
Nuclear H-score		
Mean ± SD (CI)	40 ± 46 (20−59)	127 ± 60 (98−155)
Median (min, max)	9 (0−160)	135 (5−210)
	$P < .001$	
%positive cytoplasm[g]		
Mean ± SD (CI)	36 ± 31 (23−49)	49 ± 20 (39−58)
Median (min, max)	30 (0−90)	50 (2−90)
	$P = .08$	
Cytoplasmic H-score		
Mean ± SD (CI)	80 ± 69 (51−109)	127 ± 56 (101−153)
Median (min, max)	80 (0−210)	120 (5−210)
	$P = .03$	

Abbreviations: N, number; SD, standard deviation; CI, confidence interval.

[a] All comparisons are performed for pathologic characteristics of the lesions at the time of initial encounter.

[b] Variability in nuclear size using SOX10 immunohistochemistry (none = no appreciable nuclear size variation, mild = two-fold nuclear size variation, moderate = three-fold nuclear size variation, severe = four-fold or greater nuclear size variation).

[c] Ki-67 proliferative index = Ki-67 positive melanocyte nuclei/total melanocyte nuclei.

[d] P16 %positive nuclei = p16 positive nuclei/total number of melanocytes.

[e] P16 nuclear H-score = 3x % of strongly staining nuclei + 2x % of moderately staining nuclei + % of weakly staining nuclei (range 0−300).

[f] P16 cytoplasmic H-score = 3x % of cells with strongly staining cytoplasm + 2x % of cells with moderately staining cytoplasm + % of cells with weakly staining cytoplasm (range 0−300).

[g] P16 %positive cytoplasm = melanocytes with p16 positive cytoplasmic staining/total number of melanocytes.

CDKN2A mutations and copy number variations in a subset of tumors [11,29]. Our study similarly documented an increase in p16 nuclear expression in melanoma in situ and nevi, compatible with p16 pathway activation. However, we did not observe an association of p16 expression with vertical growth phase of conjunctival melanoma and tumor stage. These findings may be, in part, determined by the underlying molecular genetic landscape of conjunctival melanoma, a mucosal melanoma that shares some similarities with cutaneous melanoma but also has some

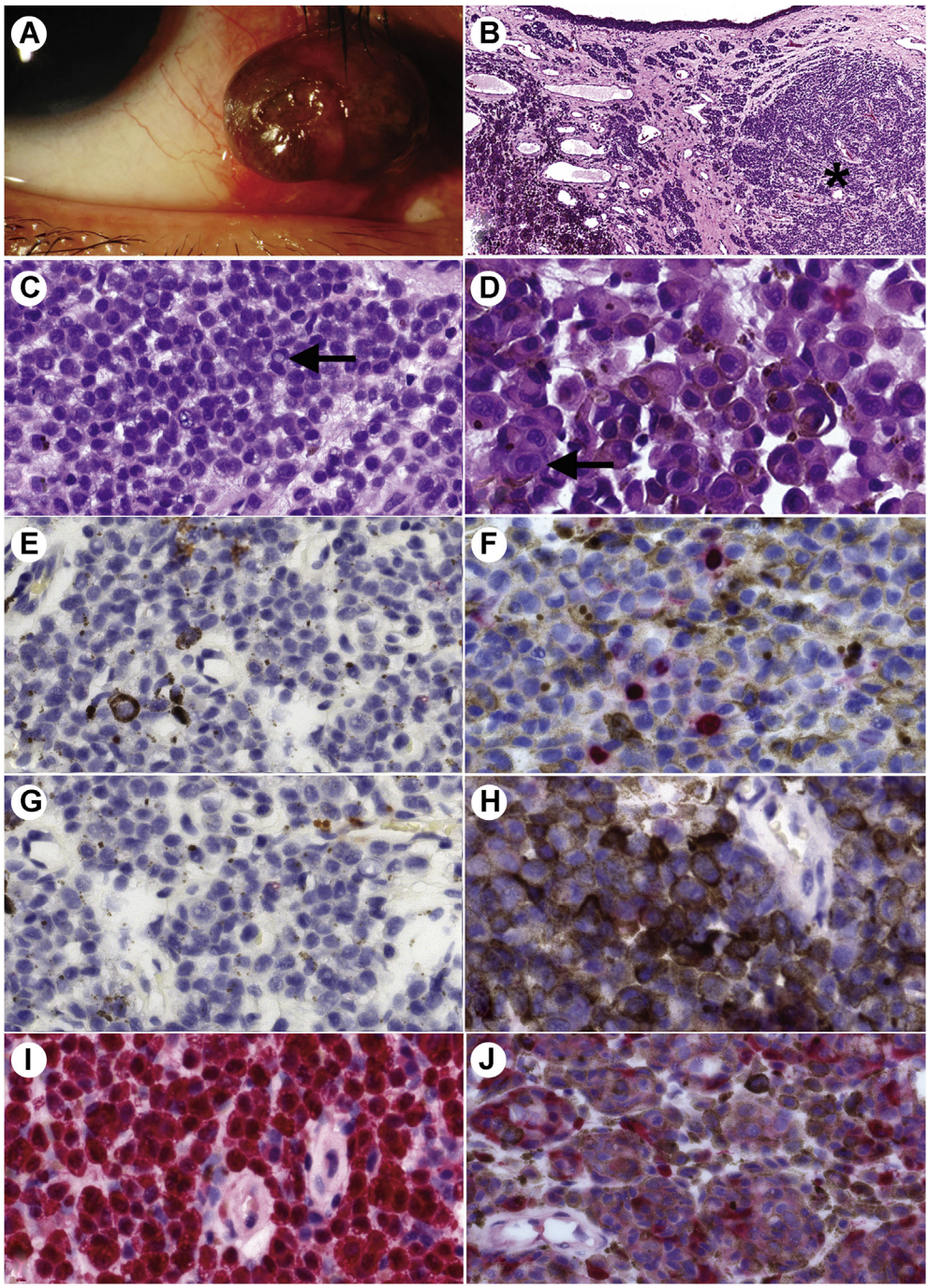

Fig. 2 Conjunctival melanoma arising in a nevus. A. Large pigmented mass with central ulceration in the region of plica semilunaris and caruncle, B. At scanning magnification, the lesion has 2 components: nests of amelanotic cells with uniform nuclei, suggestive of a nevus (asterisk), and more irregular nests of cells with larger nuclei, suggestive of melanoma (stain, hematoxylin-eosin; original magnification ×25). C. At higher magnification, the nevoid component of the lesion features uniform small nuclei without distinct nucleoli, with occasional intranuclear cytoplasmic pseudoinclusions (arrow) and scant cytoplasm (stain, hematoxylin-eosin; original magnification ×200). D. The melanoma component features large nuclei with prominent nucleoli (arrow) and abundant cytoplasm (stain, hematoxylin-eosin; original magnification ×200). E. Ki-67 is negative in the nevoid component of the lesion (stain Ki-67; original magnification ×200). F. Ki-67 highlights cycling cells in the melanoma component of the lesion (stain, Ki-67; original magnification ×200). G. HMB45 is negative in the nevoid component of the lesion (stain, HMB45; original magnification ×200). H. HMB45 is weakly expressed in the cytoplasm of the melanoma (stain, HMB45; original magnification ×200). I. P16 is strongly expressed in the nuclei and cytoplasm of the nevoid component of the lesion (stain, P16; original magnification ×200). J. P16 is weakly expressed in most melanoma nuclei (all stains, original magnification ×200).

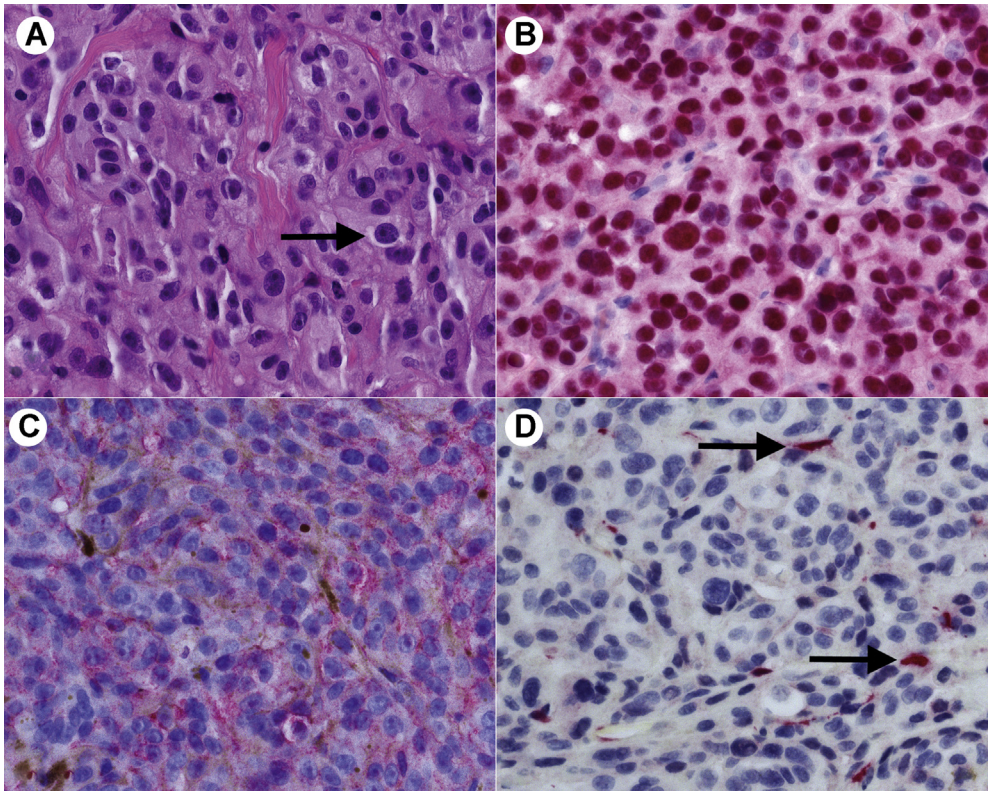

Fig. 3 Conjunctival melanoma with wild-type CDKN2A and absent p16 expression. A. Nests of pleomorphic epithelioid melanoma cells with enlarged nuclei, focally prominent nucleoli and abundant cytoplasm (arrow) (stain, hematoxylin-eosin; original magnification ×100). There was no mutation or copy number variation for *CDKN2A* by NGS (data not shown). B. SOX10 highlights four-fold variation in nuclear size (stain, SOX10; original magnification ×100). C. The neoplastic cells diffusely express HMB45 (stain, HMB45; original magnification ×100). D. P16 is negative in the neoplastic nuclei and is expressed in the infiltrating macrophages (internal positive control, arrows)(stain: P16; original magnification ×100).

recently recognized differences [3,30]. Unlike cutaneous melanoma, none of the conjunctival melanomas in our study demonstrated mutations or copy number variations in *CDKN2A*, including the tumors with absent p16 expression. These results suggest that other molecular genetic or epigenetic events influence p16 status in conjunctival melanoma, which can be explored in future studies.

5. Conclusions

In summary, our study demonstrated usefulness of immunohistochemical markers in distinguishing conjunctival melanocytic nevi from melanoma, using p16 in combination with HMB45. Additional larger studies with long-term follow-up are required to assess the usefulness of these markers in morphologically challenging conjunctival nevi and melanoma. Unlike in many cutaneous melanomas, loss of p16 expression in conjunctival melanoma is not induced by *CDKN2A* mutation and copy number alterations and does not appear to correlate with melanoma stage.

Appendix ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humpath.2020.07.020.

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