Immunohistochemical Profiling of Conjunctival Melanocytic Intraepithelial Lesions, Including SOX10, HMB45, Ki67, and P16

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• PURPOSE: To determine the usefulness of melan-A, SOX10, HMB45, and p16 immunohistochemical stains in the distinction between the low-grade and high-grade conjunctival melanocytic intraepithelial lesions, either independently or as components of an immunohistochemical panel.
• DESIGN: Retrospective observational case series.
• METHODS: Institutional pathology records between 2014 and 2018 were searched for all patients with conjunctival melanocytic intraepithelial lesions. Biopsies without supporting clinical history or tissue available for review and immunohistochemical analysis were excluded. Clinical, histopathologic, and immunohistochemical (p16, SOX10, HMB45, and Ki-67) findings were recorded.
• RESULTS: Thirty-one patients underwent 47 biopsies for conjunctival melanocytic lesions between 2014 and 2018. Pathologic diagnoses were low-grade conjunctival melanocytic intraepithelial lesion (n = 18, 38%) and high-grade conjunctival melanocytic intraepithelial lesion/melanoma in situ (n = 29, 62%). The addition of melan-A and SOX10 immunohistochemical stains resulted in an upgrade of conjunctival melanocytic intraepithelial lesion from low-grade to high-grade in 2 (4%) of 47 cases. The addition of melan-A and SOX10 immunohistochemical stains did not downgrade any of the histomorphologically high-grade lesions. In a clinical-pathologic multivariable model, the parameters most predictive of high-grade melanocytic intraepithelial lesion/melanoma in situ were involvement of the caruncle (odds ratio [OR] = 19, confidence interval [CI] 1.6-212; $P = .02$] and p16 cytoplasmic H-score >30 (OR = 81, CI 2.7 to >999; $P = .01$)
• CONCLUSION: Although the stains for melanocytic markers melan-A and SOX10 facilitate assessment of melanocytic intraepithelial lesions, the current immunohistochemical panels have limited value in distinction between the low-grade and high-grade intraepithelial melanocytic proliferations and need to be used judiciously. (Am J Ophthalmol 2021;222:148–156. © 2020 Elsevier Inc. All rights reserved.)

CONJUNCTIVAL MELANOCYTIC INTRAEPITHELIAL LESIONS include conjunctival epithelial hypermelanosis and benign, premalignant, and malignant conjunctival melanocytic intraepithelial proliferations.[1–6] The accurate distinction between conjunctival melanocytic intraepithelial lesions has important prognostic and therapeutic implications. While a subset of conjunctival melanocytic intraepithelial proliferations follow a benign clinical course, others can behave in a malignant fashion, progressing to conjunctival melanoma, a locally aggressive and potentially lethal neoplasm.[1–6]

The 2 most commonly used histopathologic classification systems, the primary acquired melanosis (PAM) and the conjunctival melanocytic intraepithelial neoplasia (C-MIN) systems, provide histomorphologic criteria that serve to distinguish between benign, low-risk, and high-risk conjunctival melanocytic intraepithelial lesions.[4,5] The PAM classification system stratifies conjunctival melanocytic intraepithelial lesions into prognostically distinct categories: (1) PAM without atypia, (2) PAM with mild atypia, and (3) PAM with moderate and severe atypia.[4] The C-MIN classification adopts a scoring system that stratifies conjunctival melanocytic intraepithelial lesions from a score of 0 (conjunctival epithelial hypermelanosis) to a score of 10 and recommends that the term melanoma in situ be applied to C-MIN scores of 5 or greater.[5] A more recently proposed classification system in the fourth edition of the WHO Classification of Tumours of the Eye was designed to simplify the terminology and allow comparison between the PAM and C-MIN classification systems using the following stratification: (1) low-grade conjunctival melanocytic intraepithelial lesion (CMIL), corresponding

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to PAM without or with mild atypia and C-MIN scores 1-2; (2) high-grade CMIL, corresponding to PAM with moderate to severe atypia and C-MIN scores 3-5; and (3) melanoma in situ, corresponding to PAM with severe atypia involving >75% of the epithelium and a C-MIN score >5.[6]

However, despite the extensive body of literature dedicated to delineating the clinical and histopathologic criteria that serve to accurately distinguish between prognostically disparate conjunctival melanocytic intraepithelial proliferations, these lesions continue to present a significant diagnostic challenge.[7] The limitations of the morphologic evaluation of challenging conjunctival melanocytic lesions have led to incorporation of immunohistochemical studies into our diagnostic arsenal.[8–11] It has been suggested that the addition of immunohistochemistry can greatly facilitate assessment and improve diagnostic accuracy when conjunctival melanocytic intraepithelial lesions that are prone to evolve into melanomas are examined histopathologically.[9]

In this study, we evaluate the usefulness of immunohistochemical stains melan-A, SOX10, HMB45, Ki-67, and p16 in distinguishing between the low-grade and high-grade conjunctival melanocytic intraepithelial lesions.

METHODS

THE WILLS EYE HOSPITAL INSTITUTIONAL REVIEW BOARD approved this study. The study was performed in compliance with HIPAA guidelines and with the tenets of the Declaration of Helsinki.

• **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 melanocytic intraepithelial lesions who underwent biopsy and had sufficient clinical information and tissue available for pathologic evaluation. Lesions with no tissue available for pathologic evaluation or 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 [clock hours of conjunctiva in relationship to limbus and greatest diameter/base]), history of prior biopsy, intervention, outcome (recurrence, local disease progression from low-grade to high-grade melanocytic intraepithelial lesion and from melanocytic intraepithelial lesion to invasive melanoma), and length of follow-up.

• **HISTOPATHOLOGY AND IMMUNOHISTOCHEMISTRY:** Consecutive sections were prepared from paraffin-embedded, formalin-fixed tissues and stained with hematoxylin-eosin stain and immunohistochemical stains. Immunostaining was performed with the following primary antibodies: monoclonal mouse anti-human SOX10 (prediluted; Biocare, Pacheco, California, USA), monoclonal mouse anti-human MART-1 (melan-A) (diluted 1:50; DAKO, Carpinteria, California, USA), monoclonal mouse anti-human HMB45 (diluted 1:40; Thermo Fisher Scientific, Waltham, Massachusetts, USA), monoclonal mouse anti-human Ki-67 (prediluted, DAKO), and monoclonal mouse anti-human p16 (prediluted; Ventana, Tucson, Arizona, USA) using standard immunohistochemical techniques. All immunohistochemical stains were prepared with a Leica autostainer BOND III using the Bond Polymer Refine Red Detection Kit (Leica Biosystems, Wetzlar, Germany.) 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 (T.M. and R.C.E.) and a consensus diagnosis was reached in discordant cases. The histopathologic diagnoses initially were rendered on hematoxylin-eosin-stained preparations and then modified, when necessary, based on the results of melan-A and SOX10 immunohistochemistry. Conjunctival melanocytic intraepithelial lesions were classified in accordance with 3 established classification systems: (1) PAM, as described by Folberg and associates[4]; (2) C-MIN, as described by Damato and Coupland[5]; and (3) WHO Classification of Tumours of the Eye, 4th edition.[6] Supplemental Table 1 (Supplemental Material available at AJO.com) provides a summary of the 3 classification systems.

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 = 2-fold nuclear size variation, moderate = 3-fold nuclear size variation, severe = 4-fold or greater nuclear size variation (Supplemental Figure; Supplemental Material at AJO.com). HMB45 expression was recorded as absent or present. Ki-67 immunostaining was assessed in "hot spots" with a 40× objective 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 a 1 mm^2 field. Because the methodology of p16 scoring varies among

the published studies and is not well documented for intraepithelial/intraepidermal melanocytic lesions, we incorporated several of the most comprehensive scoring systems to evaluate their reproducibility and ability to discriminate between conjunctival melanocytic intraepithelial lesions. In addition, similar to the methodology of Mihic-Probst and associates, we evaluated both nuclear and cytoplasmic p16 expression in melanocytes.[12] 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, with cytoplasmic expression only, and with any nuclear or cytoplasmic expression.

• **STATISTICAL ANALYSIS:** Summary statistics are reported for demographic, clinical, and pathologic characteristics on a patient and biopsy level. In patient-level comparisons, the Fisher exact test was used to determine a difference between groups for categorical variables, while *t* tests (2 groups) or ANOVA (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 recategorized as dichotomous variables and tested with logistic regression. Logistic regression with stepwise selection was used to determine potential predictors of various diagnosis groups 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 intraclass correlations. All analyses were performed in SAS V9.4 (SAS Institute Inc, Cary, North Carolina, USA) and 2-sided $P < .05$ was considered to be statistically significant.

RESULTS

• **CLINICAL CHARACTERISTICS:** *Low-grade vs high-grade conjunctival melanocytic intraepithelial lesions/melanoma in situ*. Clinical characteristics of the patients with low-grade conjunctival melanocytic intraepithelial lesions and high-grade conjunctival melanocytic intraepithelial lesions/melanoma in situ are summarized in Table 1 and documented in the Figure.

At the time of initial encounter, there were 16 patients with low-grade conjunctival melanocytic intraepithelial lesions and 15 patients with high-grade conjunctival melanocytic intraepithelial lesions and melanoma in situ. Of the 31 patients, 16 (52%) had more than 1 biopsy. A comparison (low-grade vs high-grade melanocytic intraepithelial lesions/melanoma in situ) revealed high-grade melanocytic intraepithelial lesions/melanoma in situ in older patients (65 vs 53 years, $P = .02$), more often in whites (14/15, 93% vs 6/16, 37%; $P < .001$), larger size (median 6 vs 2 clock hours; $P = .02$), and involving nonbulbar conjunctiva, such as fornix and tarsus (54% vs19%; $P = .06$) and the caruncle (39% vs 6%, $P = .06$). Over median follow-up (10 vs 1.3 months, $P = .02$), high-grade melanocytic intraepithelial lesions/melanoma in situ demonstrated greater recurrence rate (40% vs 6%; $P = .04$). One (3%) high-grade melanocytic intraepithelial lesion progressed to invasive melanoma 26 months after diagnosis.

Control tissues. Three control conjunctival biopsies were obtained from the clinically unremarkable nasal bulbar, temporal bulbar, and inferior tarsal conjunctiva of the left eye of a 77-year-old man who had an upper eyelid circumscribed well-differentiated sebaceous carcinoma, without intraepithelial pagetoid spread (Figure).

• **HISTOPATHOLOGY AND IMMUNOHISTOCHEMISTRY:** *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 (Figure).

Low-grade vs high-grade conjunctival melanocytic intraepithelial lesions/melanoma in situ. There were 18 low-grade conjunctival melanocytic intraepithelial lesions and 29 high-grade conjunctival melanocytic intraepithelial lesions/melanoma in situ. Addition of melan-A and SOX10 immunohistochemical stains has resulted in revision of histopathologic diagnosis of conjunctival hypermelanosis/PAM without atypia to C-MIN = 1/PAM without atypia in 2 (4%), from C-MIN = 1/PAM without atypia to C-MIN = 2/PAM with mild atypia in 4 (9%), from C-MIN = 2/PAM with mild atypia to C-MIN = 3/PAM with moderate-to-severe atypia (nonbasilar hyperplasia) in 2 (4%), and from C-MIN = 2/PAM with mild atypia to C-MIN = 1/PAM without atypia in 1 (2%) of 47 cases. Collectively, the addition of melan-A and SOX10 immunohistochemical stains resulted in an upgrade of conjunctival melanocytic intraepithelial lesion from low-grade to high-grade in 2

TABLE 1. Comparison of Clinical Characteristics of Patients With Low-Grade and High-Grade Conjunctival Melanocytic Lesions

Clinical Characteristic	LGCMIL[a] (N = 16)	HGCMIL[b]/MIS[c] (N = 15)
Sex, n (%)		
Female	9 (56)	9 (60)
Male	7 (44)	6 (40)
	$P = .83$	
Age in years		
Mean ± SD (CI)	53 ± 17 (44-62)	65 ± 11 (59-71)
Median (min, max)	56 (19-82)	69 (35-79)
	$P = .02$	
Race, n (%)		
White	6 (37)	14 (93)
African American	7 (44)	0
Asian	3 (19)	0
Hispanic	0	1 (7)
Other	0	0
	$P < .001$	
Laterality, n (%)		
Right	5 (31)	10 (67)
Left	11 (69)	5 (33)
	$P = .05$	
Lesion epicenter, n (%)		
Limbus/bulbar	14 (88)	10 (67)
Plica	0	0
Caruncle	0	0
Tarus/fornix	2 (13)	5 (33)
Eyelid margin	0	0
	$P = .22$	
Total clock hours, n (%)		
Mean ± SD (CI)	4 ± 4 (2-7)	7 ± 4 (5-9)
Median (min, max)	2 (1-12)	6 (2-12)
	$P = .02$	
Nonbulbar involvement, n (%)		
Fornix/tarsus	3 (19)	7 (54)
	$P = .06$	
Plica	4 (25)	5 (39)
	$P = .69$	
Caruncle	1 (6)	5 (39)
	$P = .06$	
Prior biopsy elsewhere, n (%)		
No	14 (88)	9 (60)
Yes	2 (13)	6 (40)
	$P = 0.11$	
Recurrence at any point, n (%)		
No recurrence	15 (94)	9 (60)
Recurrence	1 (6)	6 (40)
	$P = .04$	
Length of follow-up (months), n (%)		
Mean ± SD (CI)	4.7 ± 9.9 (−0.6 to 10)	36 ± 64 (1-72)
Median (min, max)	1.3 (0-36)	10 (0-229)
	$P = .02$	

HGCMIL = high-grade conjunctival melanocytic intraepithelial lesion; LGCMIL = low-grade conjunctival melanocytic intraepithelial lesion; MIS = melanoma in situ.

All comparisons are performed for patient clinical characteristics of the lesions at the time of initial encounter.

[a]Primary acquired melanosis (PAM) without atypia, PAM with mild atypia, conjunctival melanocytic intraepithelial neoplasia (C-MIN) score = 1 and 2.[4–6]

[b]PAM with moderate or severe atypia, C-MIN score = 3-5.[4–6]

[c]C-MIN score >5.[4–6]

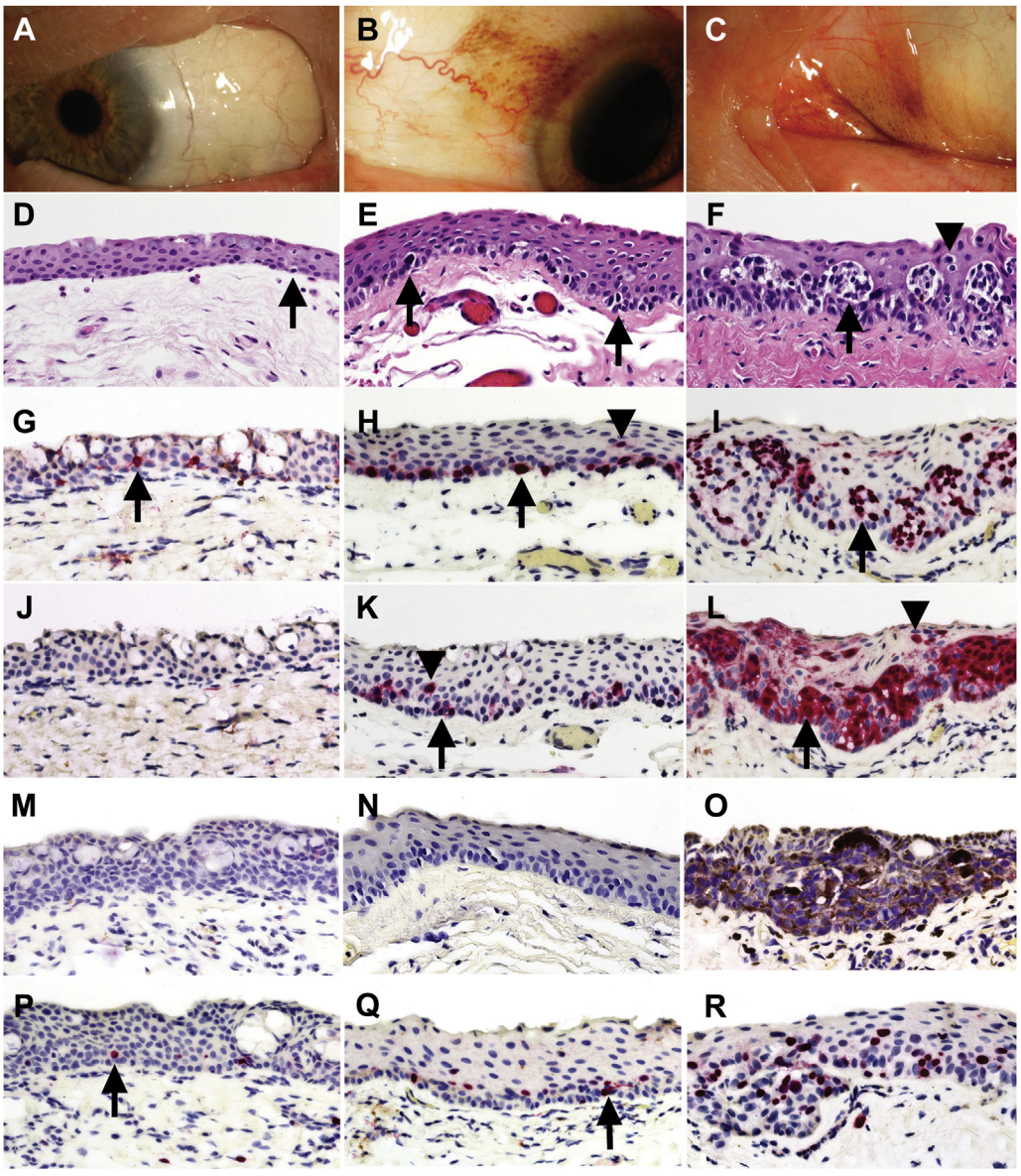

FIGURE 1. Conjunctival melanocytic intraepithelial lesions: clinical and pathologic features. (A) Normal conjunctiva (control). (B) Low-grade conjunctival melanocytic intraepithelial lesion manifests as a speckled pigmentation with poorly defined borders involving the limbal and bulbar conjunctiva for approximately 2 clock hours. (C) High-grade conjunctival melanocytic intraepithelial lesion presents as a poorly defined area of pigmentation involving the bulbar conjunctiva, plica semilunaris, and the adjacent caruncle. (D) Normal (control) temporal bulbar conjunctiva demonstrates rare basally distributed melanocytes with small nuclei and inconspicuous cytoplasm (arrow) [stain, hematoxylin-eosin; original magnification×100]. (E) Low-grade conjunctival melanocytic intraepithelial lesion features increased in density, predominantly basally distributed melanocytes with occasional mildly enlarged nuclei (arrows) and inconspicuous cytoplasm [stain, hematoxylin-eosin; original magnification ×100]. (F) High-grade conjunctival melanocytic intraepithelial lesion features nests of melanocytes with mildly pleomorphic, focally enlarged nuclei and abundant cytoplasm (arrow) and occasional intraepithelial single cell migration/pagetoid scatter (arrowhead) [stain, hematoxylin-eosin; original magnification ×100]. (G) Normal conjunctiva: melan-A immunostain highlights the small basally distributed melanocytes with dendritic cytoplasmic processes (arrow), separated by 3-5 basal epithelial cells [stain, melan-A; original magnification ×100]. (H) Low-grade conjunctival melanocytic intraepithelial lesion: SOX10 highlights increased in density, predominantly basally distributed melanocytes with focal mild nuclear enlargement (arrow) and occasional intraepithelial migration (arrowhead) [stain, SOX10; original magnification ×100]. (I) High-grade conjunctival intraepithelial melanocytic lesion: SOX10 highlights confluent melanocyte nests (arrow) [stain, SOX10; original magnification ×100]. (J) Normal conjunctiva: The melanocytes are negative for p16 [stain, p16; original magnification ×100]. (K) Low-grade conjunctival melanocytic intraepithelial lesion: P16 strongly labels the nuclei of basally distributed melanocytes (arrow) and highlights rare cells with intraepithelial migration (arrowhead) [stain, p16; original magnification ×100]. (L) High-grade conjunctival melanocytic intraepithelial lesion: P16 is strongly expressed in the nucleus and cytoplasm of

(4%) of 47 cases. The addition of melan-A and SOX10 immunohistochemical stains did not downgrade any of the histomorphologically high-grade lesions.

The pathologic features of conjunctival melanocytic intraepithelial lesions are summarized in Table 2 and illustrated in the Figure. A comparison of low-grade vs high-grade conjunctival melanocytic intraepithelial lesions/melanoma in situ revealed that high-grade lesions more often were from nonbulbar conjunctiva (85% vs 56%; $P = .02$), with frequent HMB45 expression (44% vs 0%), 2-fold nuclear size variation with SOX10 immunostain (50% vs 0%), Ki-67 proliferative activity ≥1% (58% vs 0%), and higher cytoplasmic p16 expression (mean H-score = 82 vs 0, $P < .001$). A comparison showed no difference in nuclear p16 expression.

• **LOGISTIC REGRESSION FOR CLINICAL AND PATHOLOGIC FEATURES THAT ENABLE DISTINCTION BETWEEN LOW-GRADE AND HIGH-GRADE CONJUNCTIVAL MELANOCYTIC INTRAEPITHELIAL LESIONS:** In a multivariable model, the parameters most predictive of a high-risk conjunctival melanocytic intraepithelial lesion vs low-risk conjunctival melanocytic intraepithelial lesion were involvement of the caruncle (odds ratio [OR] = 19, confidence interval [CI] 1.6-212; $P = .02$) and p16 cytoplasmic H-score >30 (OR = 81, CI 2.7 to >999; $P = .01$) (Supplemental Table 2, Supplemental Material at AJO.com).

DISCUSSION

CONJUNCTIVAL MELANOMA IS A RARE AND POTENTIALLY deadly ocular malignancy, with high propensity for regional recurrence and up to 30% risk of metastasis.[3,13] According to a recent epidemiologic study, conjunctival melanoma incidence has been increasing and this increase was particularly pronounced in white men and patients aged 60 years and older.[14] Conjunctival melanocytic intraepithelial neoplasia is the most common precursor of conjunctival melanoma.[15] Conjunctival melanocytic intraepithelial lesions can be subdivided into prognostically distinct low-grade (PAM with no or mild atypia, low-risk PAM) and high-grade (PAM with moderate-to-severe atypia, high-risk PAM) categories.[4,16,17]

The clinical risk factors associated with high-grade conjunctival melanocytic intraepithelial proliferation and melanoma, and the risk factors for disease recurrence, progression, and metastasis, are well established and include older age, white race, nonbulbar location, extent of clock-hour involvement of the conjunctiva, and incisional diagnostic biopsy prior to referral.[3,15,17] Our patients with low-grade and high-grade conjunctival melanocytic intraepithelial lesions had clinical characteristics similar to those previously reported in the literature, supporting the conclusion that our study was conducted on a representative population.

Although clinical diagnostic parameters greatly influence our management of patients with conjunctival melanocytic intraepithelial lesions, histopathologic diagnosis of biopsy material remains the gold standard, particularly in the differentiation between the benign and premalignant conjunctival melanocytic intraepithelial proliferations. The distinction between these 2 disease processes is primarily cytomorphologic and architectural. The conjunctival melanocytic intraepithelial lesions with low likelihood of progression to melanoma (conjunctival hypermelanosis, PAM without or with mild atypia/conjunctival melanocytic intraepithelial proliferation/neoplasia without or with mild atypia) are characterized by cytomorphologically normal or minimally atypical melanocytes with small, condensed nuclei and inconspicuous cytoplasm, which are distributed predominantly along the basal epithelial layer. In contrast, higher-grade melanocytic proliferations (PAM with moderate-to-severe atypia, conjunctival melanocytic intraepithelial proliferation/neoplasia with moderate-to-severe atypia, melanoma in situ) frequently feature melanocytes with enlarged nuclei, occasional nucleoli, and conspicuous cytoplasm (epithelioid morphology), which are arranged in nests or singly with frequent intraepithelial migration and pagetoid scatter. While in principle these histopathologic parameters are simple to follow, in practice many intraepithelial melanocytic proliferations tread the gray line between benign-appearing and frankly atypical lesions. As a result, pathologists rely heavily on integration of clinical information with histopathologic findings and, often, on ancillary studies.

Several investigators demonstrated the usefulness of immunohistochemical panels in distinguishing between

melanocytes and highlights focally confluent melanocyte nests (arrow) and the pagetoid scatter (arrowhead) [stain, p16; original magnification ×100]. (M) Normal conjunctiva: The melanocytes are negative for HMB45 [stain, HMB45; original magnification ×100]. (N) Low-grade conjunctival melanocytic intraepithelial lesion: The melanocytes are negative for HMB45 [stain, HMB45; original magnification ×100]. (O) High-grade conjunctival melanocytic intraepithelial lesion: HMB45 is weakly expressed in the intraepithelial melanocyte nests [stain, HMB45; original magnification ×100]. (P) Normal conjunctiva: Ki-67 labels occasional basal epithelial cells [stain, Ki-67; original magnification ×100]. (Q) Low-grade conjunctival melanocytic intraepithelial lesion: Ki-67 labels occasional basal epithelial cells and rare mildly enlarged melanocytes (arrow) [stain, Ki-67; original magnification ×100]. (R) High-grade conjunctival melanocytic intraepithelial lesion: Ki-67 demonstrates increased proliferative activity in the enlarged intraepithelial melanocyte nuclei (proliferative index of approximately 20%) [stain, Ki-67; original magnification ×100].

TABLE 2. Comparison of Pathologic Characteristics of Low-Grade and High-Grade Conjunctival Melanocytic Lesions

Pathologic Parameter	LGCMIL[a] (N = 18)		HGCMIL[b]/MIS[c] (N = 29)
Biopsy location, n (%)			
Bulbar and limbus	8 (44)		4 (15)
Nonbulbar	10 (56)		23 (85)
		$P = .02$	
Variability in nuclear size using SOX10 immunohistochemistry,[d] n (%)			
None	12 (100)		13 (50)
Low	0 (0)		13 (50)
Moderate	0 (0)		0 (0)
High	0 (0)		0 (0)
		P not available	
HMB45 staining pattern, n (%)			
Negative	4 (100)		9 (56)
Positive	0 (0)		7 (44)
		P not available	
Ki67 index – epithelium/E-S junction,[e] n (%)			
<1%	4 (100)		5 (42)
1%-5%	0 (0)		4 (33)
6%-10%	0 (0)		1 (8)
11%-20%	0 (0)		1 (8)
>20%	0 (0)		1 (8)
		P not available	
P16, n (%)			
% positive nuclei[f]			
Mean ± SD (CI)	8 ± 18 (−1 to 17)		25 ± 29 (14-36)
Median (min, max)	0 (0-75)		10 (0-90)
		$P = .10$	
Nuclear H-score[g]			
Mean ± SD (CI)	24 ± 55 (−3 to 52)		71 ± 86 (38-104)
Median (min, max)	0 (0-225)		30 (0-270)
		$P = .11$	
Cytoplasmic H-score[h]			
Mean ± SD (CI)	0 (0)		82 ± 88 (49-116)
Median (min, max)	0 (0)		60 (0-270)
		$P < .001$	

HGCMIL = high-grade conjunctival melanocytic intraepithelial lesion; LGCMIL = low-grade conjunctival melanocytic intraepithelial lesion; MIS = melanoma in situ.

All comparisons are performed for patient clinical characteristics of the lesions at the time of initial encounter.

[i]Melanocytes with p16-positive cytoplasmic staining/total number of melanocytes.

[a]Primary acquired melanosis (PAM) without atypia, PAM with mild atypia, conjunctival melanocytic intraepithelial neoplasia (C-MIN) score = 1 and 2.[4–6]

[b]PAM with moderate or severe atypia, C-MIN score = 3-5.[4–6]

[c]C-MIN score >5.[4–6]

[d]None = no appreciable nuclear size variation; mild = 2-fold nuclear size variation; moderate = 3-fold nuclear size variation; severe = 4-fold or greater nuclear size variation.

[e]Ki-67-positive melanocyte nuclei/total melanocyte nuclei.

[f]p16-positive nuclei/total number of melanocytes.

[g]3× % of strongly staining nuclei + 2× % of moderately staining nuclei + % of weakly staining nuclei (range 0-300).

[h]3× % of cells with strongly staining cytoplasm + 2× % of cells with moderately staining cytoplasm + % of cells with weakly staining cytoplasm (range 0-300).

nonatypical and atypical intraepithelial melanocytic proliferations.[8,9,18,19] Melanocyte and neural crest lineage markers melan-A, S100, MITF, and SOX10 can highlight the nonbasilar and nested distribution of melanocytes.[18] HMB45 expression, typically associated with activated melanocytes, has been found to be useful in distinguishing

melanosis with atypia from melanosis without atypia.[19] Melan-A and HMB45 cytoplasmic stains can highlight the abundant cytoplasm and epithelioid morphology of atypical melanocytes, distinguishing them from morphologically normal dendritic melanocytes with scant cytoplasm.[9,18,19] Additionally, melanoma in situ has been shown to have a higher Ki-67 proliferative index, when compared to PAM.[20] Our study results support these observations.

In addition to the aforementioned panels, recent studies 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 cutaneous and conjunctival nevi and melanomas.[11,20–25] 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. It has been shown to play a critical role in melanocyte senescence, acting as a barrier for tumorigenesis or progression to melanoma.[20,24] There are limited data on the usefulness of p16 in discrimination between nonatypical and atypical conjunctival melanocytic intraepithelial lesions.[11] In our comparison of low-grade and high-grade conjunctival melanocytic intraepithelial proliferations we noted increased p16 cytoplasmic expression in high-grade melanocytic intraepithelial lesions, contrasting with absent cytoplasmic p16 expression in low-grade lesions.

Notably, in a multivariable model, incorporating all independent statistically significant clinical and immunohistochemical parameters, only caruncular location and p16 cytoplasmic H-score were found to be strongly associated with high-grade conjunctival melanocytic intraepithelial proliferation. However, these findings have to be interpreted in the morphologic context of conjunctival melanocytic intraepithelial lesions. The overlap in nuclear p16 expression between the low-grade and high-grade conjunctival melanocytic intraepithelial lesions limits meaningful usefulness of this stain. Careful assessment of histomorphology will likely yield information on nuclear size variability and cytoplasm abundance in atypical melanocytes, indirectly assessed by SOX10 and p16, respectively. While HMB45 expression and Ki-67 proliferative activity were more frequent in high-grade lesions in our study, approximately half of high-grade lesions were negative for these markers. Additionally, the relatively small sample size in our study limits the power of our logistic regression analysis. Therefore, although limited by a small sample size, our multivariable analysis results suggest that when attempting to distinguish between low-grade and high-grade conjunctival melanocytic intraepithelial lesions, clinical context (location) and cytomorphology (nuclear and cytoplasmic enlargement) remain the key diagnostic parameters.

Our study results support the judicious use of immunohistochemical stains, such as SOX10, melan-A/MART, HMB45, Ki-67, and p16, in the assessment of conjunctival melanocytic intraepithelial lesions. Although they admittedly change the light microscopic diagnosis of grade in relatively few cases, they do make assessment of these frequently challenging lesions much easier for the pathologist. In practice it often is difficult to determine if melanocytic hyperplasia is present when routine sections stained with hematoxylin-eosin are examined, and its degree if present. In such cases, immunohistochemical stains for melanocytic markers SOX10 and melan-A readily disclose the presence, number, and location of melanocytes within the epithelium, greatly facilitating interpretation. In addition, these immunohistochemical stains provide information about atypia such as nuclear size and cytoplasm characteristics. Although HMB45, Ki-67, and p16 occasionally are helpful, they generally have limited value in distinguishing between the low-grade and high-grade conjunctival melanocytic intraepithelial lesions beyond careful morphologic analysis. The emerging molecular genetic data on conjunctival melanoma and its precursor lesions[26–28] may in time yield practically useful laboratory assays that will assist in identifying the conjunctival melanocytic intraepithelial lesions with potential to progress to melanoma.

CRediT AUTHORSHIP CONTRIBUTION STATEMENT

TATYANA MILMAN: METHODOLOGY, DATA COLLECTION, Data analysis and interpretation, Writing - original draft, Writing - review & editing, Grant and laboratory support. **Qiang Zhang:** NA. **SuMae Ang:** NA. **David Elder:** NA. **Sara E. Lally:** Data collection, Data analysis and interpretation, Writing - original draft, Writing - review & editing. **Jerry A. Shields:** Data collection, Data analysis and interpretation, Writing - original draft, Writing - review & editing. **Rose A. Hamershock:** NA. **Kareem Sioufi:** NA. **Carol L. Shields:** Data collection, Data analysis and interpretation, Writing - original draft, Writing - review & editing. **Ralph C. Eagle:** Methodology, Data collection, Data analysis and interpretation, Writing - original draft, Writing - review & editing, Grant and laboratory support.

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