Objective: To evaluate the role of immunohistochemical methods in the diagnosis of benign and malignant conjunctival melanocytic proliferations.

Design: Retrospective immunohistopathologic study.

Methods: Paraffin-embedded tissue sections from 20 conjunctival nevi and 15 invasive melanomas were immunoreacted with antibodies against cellular antigens S-100 protein, MART-1, HMB-45, CD-45, and Ki-67 nuclear proliferation protein.

Results: All nevi immunostained moderately to strongly for S-100 protein and MART-1. Results for HMB-45 were negative in the middle and lower subepithelial portions of 18 of 20 lesions; it was usually only weakly positive within the superficial junctional zone. Only 1 melanoma did not stain positively for S-100; MART-1 and HMB-45 were positive in all lesions at some level of intensity. Ki-67 positivity was restricted to the junctional zone of nevi and was diffuse in melanomas. The mean Ki-67 proliferation indices were 1.89% for the nevi and 17.3% for the melanomas. CD-45 can help to highlight lymphocytes that immunostain with Ki-67. Melanomas in situ and atypical primary acquired melanoses had more than twice the Ki-67 proliferation counts of intraepithelial junctional nevocytes (P < .001) and more intense HMB-45 cytoplasmic staining than junctional zone nevocytes.

Conclusions: S-100 and MART-1 were not useful in separating benign from malignant lesions. Results for nevus cells beneath the junctional zone were overwhelmingly negative for HMB-45 and Ki-67. Two nevi and all melanomatous nodules were positive for HMB-45 (P < .001). A higher Ki-67 proliferation index convincingly separated melanomas from nevi (P < .001). Immunostaining for HMB-45 and Ki-67 are valuable adjuncts to careful histopathologic evaluation in assessing benign and malignant conjunctival melanocytic tumors.


METHODS

From the regular and consultation files of the David G. Cogan Laboratory of Ophthalmic Pathology at the Massachusetts Eye and Ear Infirmary, lesions that carried the diagnoses of conjunctival nevi and malignant melanoma were retrieved from January 1, 2004, to February 28, 2009. Hematoxylin-eosin–stained slides were critically reexamined without knowledge of the clinical history. Twenty nevocytic lesions in 20 patients, 15 nodules of melanoma in 13 patients, and 2 melanomas in situ in 2 patients were judged appropriate for inclusion in this study and further evaluation. The Massachusetts Eye and Ear Infirmary Institutional Review Board considered this study exempt under local guidelines. Health Insurance Portability and Accountability Act compliance was maintained, and care of the patients in this study was in accordance with the Declaration of Helsinki and all federal and state laws.

Immunohistochemical staining using monoclonal antibodies with the immunoperoxidase method was performed with appropriate controls in the Immunodiagnostic Laboratory of the Department of Pathology at the Massachusetts General Hospital. Staining was performed retrospectively on all benign and malignant lesions with antibodies for the iden-
nents (intraepithelial and immediately subepithelial) of the
in the “Nevi” and “Intraepithelial Melanocytic Proliferations”
µm. Two separate counts of Ki-67–positive cells are provided
counts that were as free as possible of lymphocytes. A 10
richest in Ki-67–positive cells. Fields were selected for the cell
thelial basement membrane. The latter region in nevi was
positivity were located predominantly in the junctional
subepithelial nevi), nuclei exhibiting Ki-67 nuclear protein
pound nevi (as distinct from purely junctional nevi or entirely
isolated small cell clusters was considered focal. In the com-
(Ventana Medical Systems). Tissues were counterstained with
formed using UltraView and diaminobenzidine as the chromogen
bation with UltraView HRP-conjugated multimer antibody re-
primary antibody incubations. The tissue sections were washed
staining systems (Ventana Medical Systems) using validated pro-
67, rabbit monoclonal antibody (IgG, prediluted, Ventana
Laboratories Inc, Dedham, Massachusetts); HMB-45, mouse
monoclonal antibody (IgG1, 1:100 dilution, Dako Corpora-
tion, Carpenteria, California). CD-45, mouse monoclonal ant-
ibody (IgG1, prediluted; Ventana Medical Systems); and Ki-
67, rabbit monoclonal antibody (IgG, prediluted, Ventana
Medical Systems).

The staining was done on BenchMark XT automated tissue staining systems (Ventana Medical Systems) using validated protocols. Endogenous peroxidase activity was blocked by hydrogen peroxide before antibody incubation. A combination of EDTA and boric acid in TRIS buffer (CC1 reagent; Ventana Medical Systems) was applied to the tissue sections for antigen retrieval as needed, and the process was carried out before primary antibody incubations. The tissue sections were washed and incubated with the primary antibodies, followed by incubation with UltraView HRP-conjugated multimer antibody reagent (Ventana Medical Systems). Antigen detection was performed using UltraView and diaminobenzidine as the chromogen (Ventana Medical Systems). Tissues were counterstained with hematoxylin.

The immunohistochemical staining intensity was graded as negative (−), weak/mild (+), moderate (+ +), or strong (+ + +). If a large majority of cells stained, then the pattern was regarded as diffuse; staining of scattered single cells or isolated small cell clusters was considered focal. In the compound nevi (as distinct from purely junctional nevi or entirely subepithelial nevi), nuclei exhibiting Ki-67 nuclear protein positivity were located predominantly in the junctional region. This zone was subdivided into 2 parts: intraepithelial nevocytic nests and an immediately subepithelial population of nevocytes located in the most superficial conjunctival substantia propria within a vertical depth of 75 µm from the epithelial basement membrane. The latter region in nevi was richest in Ki-67–positive cells. Fields were selected for the cell counts that were as free as possible of lymphocytes. A 10 × 10 graticule at ×400 magnification was used to assist in accurate subepithelial cell counts in 4 different high-power fields, each encompassing a 100-µm horizontal span and a depth of 75 µm. Two separate counts of Ki-67–positive cells are provided in the “Nevi” and “Intraepithelial Melanocytic Proliferations” subsections of the “Results” section for the 2 different components (intraepithelial and immediately subepithelial) of the junctional zone. For the invasive nodules of melanoma, Ki-67 nuclear protein staining was reported as a proliferation index (ie, the percentage of cells with positive nuclear staining out of the total number of cells counted in four ×400 microscopic fields), again using a 10 × 10 graticule. The melanomas in situ were examples of the most severe intraepithelial atypical melanocytic proliferations diagnosed in 2 patients, that is, markedly atypical primary acquired melanosis (PAM).

Because of the frequent and often prominent presence of a lymphocytic infiltrate in the nevi and melanomas, care had to be taken to distinguish the deposition of the Ki-67 immuno-
product in the nuclei of tumor cells from that in lymphocytes in S-phase, which was helped by CD-45 immunolabeling to re-
veal the extent of the lymphocytic infiltrate. The immuno-
stained nuclei were largest in the melanoma cells, smaller and often ovoid in the junctional nevus cells, and smallest and rounder in the lymphocytes. Large, germinial center cells of fol-

licles also stained for Ki-67, but little confusion was encoun-
tered because of their architecture.

Ki-67 positivity was compared among the intraepithelial junc-
tional nests of nevi, the overlying or lateral (radial) primary ac-
quired melanoses within the epithelium associated with nod-
ules of melanoma, and the melanomas in situ in 4 high-power (×400) microscopic fields. The nuclear outlines of positively stained basal germinal epithelial cells could not be reliably ex-
cluded from those of the melanocytes. The results were there-
fore scored as the mean of the total number of immunoreac-
tive cells counted in 4 high-power fields, which commingled
the nuclei of melanocytes and a subpopulation of basal germi-
nal epithelial cells. The latter were presumed to be similar in number from case to case. Furthermore, the discernment of the cellular borders between melanocytes and keratinocytes was difficult in the immunolabeled and hematoxylin-eosin counterstained slides, thereby preventing a meaningful denomina-
tor from being determined.

Clinical information, including location, ocular histories, and clinical photographs, when available, were reviewed and ana-
lyzed after the histomorphologic and immunohistochemical find-
ings were determined. The immunohistochemical data were com-
pared with those previously published in the ophthalmic and dermatologic literatures by conducting a PubMed search based on the following keywords: nevi, melanoma, skin, conjunctiva, immunohistochemistry, S-100, MART-1, HMB-45, and Ki-67.

The bulk of the findings are summarized in Table 1 and
Table 2. For comparative purposes, Ki-67 immunolabeling was eval-
uated separately for the intraepithelial melanocytes in nevi, atypical PAM, and melanomas in situ, and the results are pre-
sented in Table 3. For the nevi, Ki-67–positive neocytes in the most superficial stromal component of the junctional zone of the substantia propria (75 µm in depth) were also counted and are reported in the “Nevi” subsection of the “Results” section. Analyses using the Fischer exact test for HMB-45 positive stain-
ing and the Mann-Whitney test for the Ki-67–positive staining were performed to determine the statistical significance of dif-
erential immunostaining between the nevi with a subepithelial junctional component and the nodules of melanoma. With re-
spect to the HMB-45 analysis, any degree of positivity from weak to strong, whether diffuse or focal, was considered positive; sta-
tistical correlations of the varying degrees of intensity were not attempted. Statistical evaluation of S-100 and MART-1 immuno-
staining was not performed because of the overwhelming preva-
lence of positive results in the benign and malignant lesions.

RESULTS

NEVI

Twenty discrete nevi were studied in 20 patients. Ten nevi were located on the epibulbar surface away from the lim-
bus, 4 at the limbus, 2 in the caruncle, and 1 in the plica. Three were at the eyelid margin and involved the adja-
cent palpebral conjunctiva. Clinically, 16 nevi were pig-
mented, and, in the remaining 4, no pigment could be visualized. There were 10 males and 10 females with a mean age of 40.1 years (median, 35 years; range, 6-76 years) (Table 1).

Twelve nevi possessed variably prominent intraep-
ithelial junctional activity (Table 1). Two were purely junc-
tional and composed of conspicuous large intraepithe-

celial nests of polygonal nevocytes (ie, theques) that were clearly demarcated from the surrounding keratinocytes (Figure 1A). A variable band of subepithelial lympho-

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Light Microscopy

Junctional 2
Junctional 1
Junctional 3
Compound-Junctional 2
Compound-Junctional 3
Predominantly junctional
Compound-junctional

Among the 16 microscopically pigmented nevi, 10 had a predominantly junctional component and the remaining nevi were purely subepithelial (Table 1). Pigmented cells were concentrated in the superficial region of the subepithelial component; maturation into smaller cells usually occurred toward the base (Figure 1B). Among the 10 compound nevi (predominantly junctional, balanced, and predominantly subepithelial types), there was no tendency for individual cell invasion of the epithelium (ie, pagetoid spread) nor a prominent lateral or radial intraepithelial extension beyond the subepithelial component. Multinucleated nevus giant cells were present subepithelially in 8 lesions; there was no topographic predilection of the lesions with this feature. Other histomorphologic characteristics are described in Table 1.

The immunohistochemical staining of the lesions was monotonously repetitive and consistent (Table 1). We use the term junctional zone to include intraepithelial and superficial stromal nevocytic components, the latter constituted by the 75 µm of stroma immediately beneath the epithelium into which the intraepithelial nevocytes first drop off (ie, abtropfung). All melanocytic cells were immunolabeled for S-100 and MART-1 (Figure 1D, bottom-left inset, and F); staining was diffuse and not focal or sectoral in the subepithelial cells of the nevi. The multinucleated nevus cells stained in the same fashion as the mononucleated cells. In 10 of 12 lesions with a junctional component, HMB-45 was typically lightly and spottily positive in some of the cells in the junctional nests and immediately subepithelially (Figure 1F); in 1 case, it was only faintly positive in the cells of the middle and

Table 1. Conjunctival Nevomelanocytic Nevi

<table>
<thead>
<tr>
<th>Patient No./ Sex/Age, y</th>
<th>Location</th>
<th>Lesion Diagnosis</th>
<th>Nevus Giant Cells</th>
<th>Pigment</th>
<th>Cysts</th>
<th>Lymphocytes</th>
<th>S-100</th>
<th>MART-1</th>
<th>HMB-45</th>
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<tbody>
<tr>
<td>1/M/10 Bulbar OD</td>
<td>Pure junctional</td>
<td>Neg</td>
<td>2+</td>
<td>Neg</td>
<td>1+</td>
<td>2+</td>
<td>3+</td>
<td>1+</td>
<td></td>
</tr>
<tr>
<td>2/M/6 Limbal OS</td>
<td>Pure junctional</td>
<td>Neg</td>
<td>2+</td>
<td>Neg</td>
<td>1+</td>
<td>3+</td>
<td>2+</td>
<td>3+</td>
<td>1+</td>
</tr>
<tr>
<td>3/F/9 Bulbar OD</td>
<td>Predominantly junctional, 1+</td>
<td>Neg</td>
<td>1+</td>
<td>1+</td>
<td>1+</td>
<td>2+</td>
<td>3+</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>4/M/16 Bulbar OS</td>
<td>Compound-junctional</td>
<td>Neg</td>
<td>1+</td>
<td>3+</td>
<td>3+</td>
<td>2+</td>
<td>3+</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>5/M/15 Bulbar OS</td>
<td>Compound-junctional</td>
<td>1+</td>
<td>3+</td>
<td>Neg</td>
<td>Neg</td>
<td>3+</td>
<td>3+</td>
<td>3+</td>
<td></td>
</tr>
<tr>
<td>6/F/32 Bulbar OS</td>
<td>Compound-junctional</td>
<td>1+</td>
<td>3+</td>
<td>Neg</td>
<td>3+</td>
<td>2+</td>
<td>3+</td>
<td>1+</td>
<td></td>
</tr>
<tr>
<td>7/F/60 Plica OD</td>
<td>Junctional</td>
<td>1+</td>
<td>2+</td>
<td>3+</td>
<td>2+</td>
<td>2+</td>
<td>3+</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>8/M/36 Limbal OS</td>
<td>Predominantly subepithelial</td>
<td>Neg</td>
<td>1+</td>
<td>2+</td>
<td>Neg</td>
<td>3+</td>
<td>3+</td>
<td>1+</td>
<td></td>
</tr>
<tr>
<td>9/M/17 Limbal OS</td>
<td>Predominantly subepithelial</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>3+</td>
<td>3+</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>10/M/32 Bulbar OD</td>
<td>Predominantly subepithelial</td>
<td>1+</td>
<td>1+</td>
<td>1+</td>
<td>1+</td>
<td>2+</td>
<td>3+</td>
<td>Neg</td>
<td></td>
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<tr>
<td>11/F/34 Bulbar OD</td>
<td>Predominantly subepithelial</td>
<td>1+</td>
<td>1+</td>
<td>2+</td>
<td>Neg</td>
<td>2+</td>
<td>3+</td>
<td>1+</td>
<td></td>
</tr>
<tr>
<td>12/M/21 Bulbar OS</td>
<td>Predominantly subepithelial</td>
<td>Neg</td>
<td>2+</td>
<td>Neg</td>
<td>Neg</td>
<td>1+</td>
<td>3+</td>
<td>Neg</td>
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<tr>
<td>13/M/52 Bulbar OD</td>
<td>Pure subepithelial</td>
<td>Neg</td>
<td>2+</td>
<td>Neg</td>
<td>Neg</td>
<td>2+</td>
<td>3+</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>14/F/76 Limbal OS</td>
<td>Pure subepithelial</td>
<td>Neg</td>
<td>2+</td>
<td>3+</td>
<td>1+</td>
<td>2+</td>
<td>3+</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>15/F/70 Caruncle OD</td>
<td>Pure subepithelial</td>
<td>1+</td>
<td>1+</td>
<td>2+</td>
<td>Neg</td>
<td>2+</td>
<td>3+</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>16/F/54 Bulbar OD</td>
<td>Pure subepithelial</td>
<td>Neg</td>
<td>1+</td>
<td>2+</td>
<td>Neg</td>
<td>1+</td>
<td>3+</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>17/M/54 Caruncle OD</td>
<td>Pure subepithelial</td>
<td>Neg</td>
<td>1+</td>
<td>3+</td>
<td>Neg</td>
<td>3+</td>
<td>3+</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>18/F/72 Micocutaneous junctional OD</td>
<td>Pure subepithelial</td>
<td>2+</td>
<td>1+</td>
<td>Neg</td>
<td>Neg</td>
<td>3+</td>
<td>3+</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>19/F/68 Micocutaneous junctional OD</td>
<td>Pure subepithelial</td>
<td>1+</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>3+</td>
<td>3+</td>
<td>Neg</td>
<td></td>
</tr>
<tr>
<td>20/F/68 Micocutaneous junctional OD</td>
<td>Pure subepithelial</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>3+</td>
<td>3+</td>
<td>Neg</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: Neg, not present or negligible.

a For light microscopic analysis, 1+ indicates present; 2+, mild to moderate; 3+, conspicuous.
b For immunohistochemical analysis, 1 indicates light; 2 moderate; 3 strong; generally diffuse unless focal (f) is specified.
deeper layers of the substantia propria. In 2 nevi, neither the junctional nor subepithelial regions stained for HMB-45. Intraepithelial dendritic melanocytes, when present, were variably decorated with all 3 markers. Ki-67 positivity was restricted to nevus cells scattered within the epithelial-stromal junctional zone (Figure 1F, inset). In 9 of the 10 compound nevi, the total number of cells with Ki-67 nuclear immunostaining in the most superficial zone of the substantia propria (evaluated in each case for a distance of 100 µm horizontally and a vertical depth of 75 µm beneath the epithelium) was 30 out of a total of 1583 cells counted in 4 high-power fields (mean, 3.33 cells; median, 3 cells; range, 0-7 cells), or an overall mean proliferation index of 1.89%. One lesion did not allow an accurate subepithelial junctional cell count because of the paucity of cells in the superficial stroma (ie, very early colonization). Ki-67 immunostaining was not identified in the deeper stromal region beneath the superficial stromal junctional zone, as defined herein, except for reactivity detected in dispersed lymphocytes or small lymphoid aggregates. Widely separated epithelial basal germinal cells immunoreacted with Ki-67, including those within epithelial inclusion cysts.

**MELANOMAS**

Thirteen patients had 15 nodules of conjunctival melanoma (2 displayed 2 separate, nonoverlapping nodules). There were 2 additional patients who were diagnosed with melanoma in situ with almost complete melanocytic replacement of conjunctival epithelium, the most severe degree of preinvasive, atypical PAM, analo-
Acquired Melanoses Associated With Melanoma In Situ, and Primary Acquired Melanoses Associated With Melanoma

Table 3. Intraepithelial Ki-67–Positive Cells in Junctional and Compound Nevi, Melanomas In Situ, and Primary Acquired Melanoses Associated With Melanoma

<table>
<thead>
<tr>
<th>No. of Patients</th>
<th>Diagnosis</th>
<th>Mean No. of Ki-67–Positive Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Pure junctional nevus</td>
<td>7.8</td>
</tr>
<tr>
<td>1</td>
<td>Predominantly junctional nevus</td>
<td>9.0</td>
</tr>
<tr>
<td>3</td>
<td>Balanced compound nevus</td>
<td>8.3</td>
</tr>
<tr>
<td>6</td>
<td>Predominantly subepithelial nevus</td>
<td>6.0</td>
</tr>
<tr>
<td>2</td>
<td>Melanomas in situ</td>
<td>34.5</td>
</tr>
<tr>
<td>9</td>
<td>PAM with melanoma nodules</td>
<td>19.7</td>
</tr>
</tbody>
</table>

Abbreviation: PAM, primary acquired melanosis.

- a Mean absolute counts of positively stained nuclei of intraepithelial cells in 4 high-power fields.
- b Entirely intraepithelial nevocytic nests.
- c Small subepithelial component.
- d Equally prominent junctional and subepithelial components.
- e Small, persisting junctional component.
- f Replacement of most of conjunctival epithelium by severely atypical melanocytes without nodule formation.

The spindle-cell lesions displayed some desmoplastic features, relative hypocellularity and a collagenous matrix, and an especially pronounced lymphocytic inflammation, well demonstrated by CD-45 immunostaining.

Nine nodules of melanoma had associated microscopic PAM; in the remaining 6 nodules, PAM was not detected in the excised specimens but was noted in the clinical evaluation in all but 1 case (patient 15; Table 2).

The results from immunolabeling of the cells in the nodules and of those responsible for the atypical intraepithelial melanoses were the same for each lesion, including the 2 completely spindle-cell lesions. Fourteen of 15 invasive nodules and the 2 melanomas in situ stained for S-100 diffusely or focally; results for 1 were negative. All 15 nodules and the 2 melanomas in situ stained for MART-1 with variable intensities in a diffuse pattern. HMB-45 diffusely immunostained the cytoplasm of the cells of 13 of the 15 nodules and was marked in any associated PAM (Figure 2C and D) and typically had a granular character; it was focal in 2 nodules (Figure 2C, inset). Staining was diffuse and strongly positive among the cells constituting the melanomas in situ (Figure 2D, inset bottom right).

Ki-67 staining was distributed throughout the nodules, sometimes in a somewhat uneven pattern (Figure 2E); no differences were noted among the lesions composed of pure epithelioid cells, mixed epithelioid-spindle cells, or spindle cells (Figure 2F). The spindle-cell lesions had a prominent lymphocytic infiltrate as demonstrated by CD-45 immunostaining (Figure 2F, inset). The Ki-67–positive cells counted in 4 high-power (×400) fields for each nodule (Table 2) ranged from 24.5 to 77.2 cells among all lesions (mean, 59.3 cells; median, 58.3 out of a mean total cell count of 343 cells). The overall mean Ki-67 proliferation index for the nodular melanomas was 17.3% (median, 17%; range, 7.1%-22.5%) (Table 2).

Comparison of the HMB-45–positive immunostaining between the proportion of melanoma nodules (15 of 15) vs the proportion of nevi (2 of 18) using Fisher exact test revealed a P value of <.001. Comparison of the Ki-67–positive immunostaining between the percentage of cells in melanoma nodules vs the percentage of cells in the junctional zone of nevi using the Mann-Whitney test revealed a P value of <.001. The differences in both categories of staining therefore proved to be statistically significant.

INTRAEPITHELIAL MELANOCYTIC PROLIFERATIONS

Ki-67 immunoreactivity within the junctional nests (Figure 1F, inset) of 2 purely junctional, 1 predominantly junctional, 3 balanced compound, and 6 predominantly subepithelial nevi (a total of 12 lesions) was compared with that of the intraepithelial melanocytic proliferations (Figure 2E, inset) diagnosed either as melanomas in situ without nodules (2 cases) or PAM with atypia (a total of 11 lesions) accompanying 9 nodules of melanoma (Table 3). The mean absolute Ki-67–positive cell counts of the intraepithelial junctional nevus cells in the 12 nevi with such components ranged from 5.75 to 9 (mean, 7.5) in 4 high-power fields. The mean positive cell counts obtained in the 2 melanomas in situ were 25 and 44. In the atypical PAM components accompanying the 9 nodules of melanoma, the mean positive cell
Figure 1. Conjunctival nevi. A, Numerous, well-defined intraepithelial nevocytic nests (arrows) in a completely junctional nevus with a surface covering of keratinocytes. Note the subepithelial band of chronic inflammation (arrowheads) (hematoxylin-eosin, original magnification ×200). B, Compound nevus with junctional nests of cohesive nevocytes present in clear-cut intraepithelial cavities. There are subepithelial nests and sheets of stromal nevus cells, some of which are pigmented superficially toward the upper left (hematoxylin-eosin, original magnification ×200). C, Nests and diffuse collections of subepithelial nevus cells. Note the entrapped epithelial inclusion cysts and the total absence of intraepithelial nevocytic junctional nests (hematoxylin-eosin, original magnification ×100). D, Intraepithelial nests of nevocytes in a pure junctional nevus are uniformly and diffusely immunolabeled for S-100 protein. Bottom-left inset, MART-1 intensely stains nevocytes; bottom-right inset, HMB-45 also immunostains nevocytes in a lighter, more granular fashion (immunoperoxidase reaction, original magnification ×200 for panel D and inset). E, Compound nevus displays positive staining for S-100 in both junctional and subepithelial components. Nonstaining cells are inflammatory. Inset, positive S-100 immunostaining of a completely subepithelial nevus with uninvolved overlying epithelium (original magnification ×200). F, HMB-45–positive immunolabeling of a compound nevus is confined to the intraepithelial junctional nests and only the most superficial stromal nevus cells (arrows). Nonstaining nevus cells and inflammatory cells are present in the bottom half of the photomicrograph. The arrowhead identifies melanin-bearing cells, probably macrophages. The inset displays moderate Ki-67 positivity of dark-staining nuclei limited to the intraepithelial nevocytes within junctional nests; staining is absent from subepithelial nevocytic clusters (original magnification x200).
Both benign and malignant melanocytic proliferations of the skin\textsuperscript{20-22} and conjunctiva\textsuperscript{3-26} are diagnostically challenging because of their protein histomorphologic appearances; immunohistochemical markers can offer additional and supportive diagnostic information.\textsuperscript{16-20,27,28} With the application of monoclonal antibodies against the nuclear proliferation protein Ki-67, which is more sensitive than counting mitotic figures for determining cellular proliferative activity (ie, identifying nuclei in late G1, S, M, and G2 phases of the cell cycle), the ability to diagnose cutaneous benign and malignant disorders has been further improved.\textsuperscript{3-15} This probe, however, has yet to be systematically used in studies of common conjunctival melanocytic lesions. In this article, our aim was to resolve inconclusive results from earlier studies in an ongoing search for a diagnostically serviceable immunohistochemical approach to conjunctival melanocytic lesions.\textsuperscript{29} One facet of our study that distinguishes it from ongoing melanocytic investigations with discrepant results have explored the diagnostic utility of the HMB-45 and MART-1 genes coding for melanosome-related cytoplasmic antigens.\textsuperscript{14-21,30} A clear-cut set of results that can be used in light microscopic diagnosis has not yet been successfully elucidated. To date, immunohistochemical techniques are not routinely used in the analysis and diagnosis of conjunctival melanocytic lesions.\textsuperscript{29}

Ki-67 immunolabeling has been attempted once before with 69 conjunctival nevi and 2 melanomas, but only parsimonious descriptions of the results were provided.\textsuperscript{31} That particular study concluded that benign nevi expressed nuclear proliferation marker Ki-67 at low levels and did not supply either quantitative or semiquantitative information nor venture any comments regarding the 2 melanomas. A case report of a conjunctival Spitz nevus demonstrated low Ki-67 immureactivity confined to the junctional zone but in none of the cells constituting the middle and lower levels of the lesion in the substantia propria,\textsuperscript{32} which is comparable to what has been found in cutaneous Spitz and dysplastic nevi.\textsuperscript{7,8,10,11} Ki-67 has been profitably applied in a single study\textsuperscript{33} to the evaluation of cases of PAM, with and without atypia. Nevi and invasive melanomatos nodule were not included in that study. The only other class of conjunctival lesions that has been productively assessed immunohistochemically with the Ki-67 marker is the spectrum of squamous cell dysplasias and carcinomas.\textsuperscript{34}

Our study establishes that reproducible immunohistochemical staining differences exist between common conjunctival nevi and invasive melanomas with select markers. With respect to the 18 of 20 nevi in this series with a subepithelial stromal component, the nevocytes of this region were positive for S-100 and MART-1, as were the nevocytes in the junctional nests, when present. There was, however, a characteristic immunopattern exhibited by HMB-45. Patchy immunostaining was generally restricted to the junctional zone, composed of intrapapillary nevocytic nests and nevocytes in the immediately subepithelial substantia propria to a depth of 75\textmu m, in 10 of 12 lesions with this feature (2 lesions were entirely negative); the intrapapillary dendritic melanocytes were also lightly stained. The nonjunctional nevocytes in the deeper substantia propria were negative in all but 2 cases, in which only light staining was detected in small foci; these lesions were nevertheless categorized as HMB-45 positive according to the design of this study. Our results also included findings derived from 2 purely junctional nevi. Both arose in boys aged 6 and 10 years and had identical mild diffuse intraepithelial HMB-45–positive staining. A gradient or zonal pattern for HMB-45 has been well described in common cutaneous junctional and compound nevi\textsuperscript{11,33} and in Spitz nevi.\textsuperscript{3} It has been commented upon by some ophthalmic investigators, but less attention than it deserves has been paid to this aspect of conjunctival nevus in differential diagnosis.\textsuperscript{5,10,11}

In our study, Ki-67 immunolabelling was consistently negative among the middle and lower subpapillary nevus cells, but was mildly positive within the intrapapillary junctional nests and sporadically positive among the most superficial stromal nevocytes (ie, to a depth of 75\textmu m just beneath the epithelium). A mean proliferation index of 1.89% was discovered in this subepithelial zone, which is comparable to a 1.7% to 2.0% finding in cutaneous nevi.\textsuperscript{6,11} In the skin, dysplastic nevus (a class of lesion not included in this study because of their rarity in the conjunctiva) display an intermediate proliferation index of 2.9\%,\textsuperscript{11} which is much closer to benign nevi and the melanomas. The mean Ki-67 proliferation indices of 1.89% for the nevi and 17.3\% for the melanomas in the present study were statistically significant ($P<.001$). The mean Ki-67 index was 47\% in an analogous study of cutaneous melanomas,\textsuperscript{13} which, at the time of clinical discovery, are typically larger and more advanced tumors than conjunctival melanomas. In a meta-analysis reviewing many publications, a Ki-67 threshold index as low as 10\% was considered sufficient to diagnose a cutaneous melanoma.\textsuperscript{8}

Of the 15 melanomatos nodules in 13 patients described herein, S-100 immunostaining was moderately to strongly positive in 13 cases, mildly positive in 1, and negative in 1. In the lesion negative for S-100, weak MART-1 positivity and strong HMB-45 positivity were identified (patient 5; Table 2). MART-1 was vividly
Figure 2. Conjunctival melanomas. A, Subepithelial nodule of melanoma composed of epithelioid cells and interspersed lymphocytes. Inset displays a mixed spindle-epithelioid cell melanoma (hematoxylin-eosin, original magnification ×200). B, One of the 2 melanomas in situ in this study manifests a nonnested continuous sheet of atypical medium-sized dyscohesive epithelioid melanocytes residing above the epithelial basement membrane. There is only a thin umbrella of covering squamous epithelial cells (hematoxylin-eosin, original magnification ×200). C, Uniform and intense HMB-45 immunoreactivity of large nests of mixed epithelioid and spindle melanoma cells. Note the prominent intraepithelial, nonnesting component of atypical melanocytes that is also strongly HMB-45 positive (Immunoperoxidase reaction, original magnification ×100). The inset (original magnification ×200) demonstrates an unequivocally positive but more erratic and modest cytoplasmic staining pattern in another case of pure epithelioid cell melanoma. D, The superficial epithelioid and deeper spindle-cell (arrows) populations of this nodule are strongly HMB-45 positive (original magnification ×100). The inset, bottom left, shows positive staining in a pure spindle-cell melanoma. The inset, bottom right, highlights HMB-45 cytoplasmic staining in a melanoma in situ that is far more intense than that observed in a junctional nevus (compare with Figure 1D, bottom-right inset) (both insets, original magnification ×200). A similar degree of staining is exhibited in primary acquired melanosis (PAM) with atypia. E, Ki-67 prominently stains the nuclei of the intraepithelial atypical melanocytes and those of many of the invasive melanoma cells. Inset shows dramatically dense Ki-67 nuclear positivity of PAM with atypia associated with a nodule of melanoma (original magnification ×200 for panel E and inset). F, Melanomatous spindle cells are admixed with a prominent inflammatory cell infiltrate. Ki-67 positively staining nuclei of the melanoma cells are larger than the smaller, round positive nuclei of the lymphocytes (original magnification ×200). The inset (original magnification ×400) reveals the CD-45–positive cell membrane staining of lymphocytes with their smaller nuclei among the tumor spindle cells.
positive in all but 1 lesion (also in patient 5). These data are in keeping with those reported for cutaneous and nonconjunctival mucosal melanomas.\(^1,2\) Whether of pure epithelioid or mixed epithelioid-spindle cell composition, all of the melanomatous nodules in this series were HMB-45 positive, most often diffusely but rarely focally. If not helpful in differential diagnosis, as a secondary practical consideration, MART-1 staining offers the clearest and most reliable definition in conjunctival specimens of normal dendritic melanocytes and of intraepithelial melanocytic proliferations, as well as of the cells comprising subepithelial portions of benign and malignant lesions. It reveals the presence of melanocytes to an extent that cannot be fully appreciated in hematoxylin-eosin–stained sections.

HMB-45 positivity was uniformly detected throughout all levels of the melanomatous lesions without a gradient diminishing from superficial to deeper layers. Based on our results, HMB-45 negativity in the subepithelial, nonjunctional component of a conjunctival melanocytic lesion (observed in 18 of our 20 nevi) tends to indicate a benign proliferation, whereas HMB-45 positivity throughout the entire extent of the subepithelial lesion should heighten suspicion of a malignant proliferation (\(P < .001\)). Two extremely rare exceptions to this rule are granular and clonal (ie, inverted) nevi.\(^35-37\)

There were 2 spindle-cell lesions in this series with a suggestion of desmoplastic melanomatous features: 1 (patient 10) stained strongly for S-100, moderately for MART-1, and lightly for HMB-45, whereas the second (patient 15) stained lightly for S-100, strongly for MART-1, and moderately for HMB-45 (Table 2). Classic desmoplastic melanomas in the skin and other mucous membranes may be only positive for S-100.\(^1,2,22,27,28\)

Although statistically significant, positive, Ki-67 nuclear-staining differential counts have been established in PAM with and without atypia.\(^33\) The quantitative Ki-67 results in our study should facilitate the distinction between active junctional nests in a nevus (low counts) and atypical PAM (high counts); they were also statistically significant at \(P < .001\). Purely junctional conjunctival nevi are encountered in the first decade of life when conjunctival melanomas and PAM occur only as curiosities.\(^38-40\) PAM with atypia as a precursor to the development of nodules of melanoma is a disease of the sixth or seventh decade or later.\(^23,25,26\) Although the patients' ages establish a guideline, pathologists have relied chiefly on histomorphologic criteria to separate atypical PAM from junctional nevi.\(^23,25,38-44\) This subject has been eclipsed by the debate about whether PAM with atypia is identical to melanoma in situ.\(^42-44\)

We propose that, in cases of PAM, immunohistochemical staining for the presence of HMB-45 and the detection of Ki-67 positivity should be routinely used when tissue is available (Table 3). HMB-45 cytoplasmic positive staining is much more intense in melanoma in situ and in PAM with atypia when compared with the staining of intraepithelial junctional nevocytes. Ki-67 positivity is at least 2 to 3 times as great in the first 2 categories as in the last. Especially in childhood melanocytic lesions with florid junctional components, these results ought to be diagnostically insightful. These additional findings could also enhance the prediction of which cases of PAM with atypia have the potential to evolve into invasive melanoma.

A cautionary word must be added regarding the grading of Ki-67 nuclear positivity, which readily includes lymphocytes. The presence of inflammatory cells is often prominent in childhood nevi owing to their mucosal milieu and was detected frequently in the nevi and melanomas in this series\(^24,35,46\) (Tables 2 and 3). Ki-67–positive Langerhans cells, germinal basal cells in the surface epithelium and in epithelial inclusion cysts, connective tissue histiocytes/macrophages, and vascular endothelial cells in the S-phase of the cell cycle must also be considered and excluded. Unfortunately, the immunodiagnostic laboratory at the Massachusetts General Hospital does not perform double immunohistochemical staining. The outline of the nuclear immunoreactant in lymphocytes is usually smaller and rounder than that of melanoma cells but sometimes approximates that of nevus cells. CD-45 staining of a sequential tissue section in a high Ki-67–positive zone can sometimes assist in resolving this issue by immuno-staining lymphopoietic and hematopoietic cells (which are also S-100 and MART-1 negative).

In summary, although careful light microscopic analysis of conjunctival melanocytic lesions remains the mainstay for diagnosis, not all ophthalmic pathologists and general pathologists have had enough experience to diagnose these lesions comfortably.\(^29\) Childhood lesions, in which the junctional component and an inflammatory infiltrate are often pronounced, can be disquieting. Therefore, our findings regarding HMB-45 and Ki-67 immunostaining should provide an additional level of confidence in separating benign from malignant conditions. We believe that our data further support the proposition that conjunctival melanomas are more akin to cutaneous than uveal melanomas; this topic continues to receive serious consideration.\(^20,38\) There is no doubt that other markers will emerge that will play an enhanced role in differential diagnosis. For example, Wilms tumor gene (\(WT1\)) has recently been discovered to support the diagnosis of conjunctival melanoma along with HMB-45.\(^45\) We should point out that we did not attempt to assess the predictive value of our data regarding ultimate behavior and the possible development of metastases.

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Correspondence: Frederick A. Jakobiec, MD, DSc, David D. Cogan Laboratory of Ophthalmic Pathology, Massachusetts Eye and Ear Infirmary, 243 Charles St, Ste 321, Boston, MA 02114 (fred_jakobiec@meei.harvard.edu).

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REFERENCES


