Usefulness of a Red Chromagen in the Diagnosis of Melanocytic Lesions of the Conjunctiva

Kailun Jiang, MD; Seymour Brownstein, MD, FRCSC; Kay Lam, MD; Bruce Burns, MD, FRCPC; James Farmer, MD, FRCSC, FRCPC

IMPORTANCE Immunohistochemical analyses may assist in the diagnosis of precancerous and cancerous conjunctival lesions.

OBJECTIVE To use Vector Red (VR) to identify an immunologic marker that is sensitive for all melanocytes and another that is sensitive and specific for activated and/or atypical conjunctival melanocytic lesions (MLs).

DESIGN, SETTING, AND PARTICIPANTS Eight specimens each of control lesions (normal conjunctiva and normal uvea as well as choroidal melanoma) and 8 from the diagnostic categories (conjunctival nevus, primary acquired melanosis with mild or no atypia, primary acquired melanosis with moderate to severe atypia, and conjunctival melanoma) that provided sufficient quantity and quality of tissue were available for processing. The specimens were obtained from the Ophthalmic Pathology Laboratory, The Ottawa Hospital, from 2005 to 2013. The specimens were immunolabeled with human melanoma black 45 (HMB45), melanoma antigen recognized by T cells 1 (Melan-A), S100, and Ki67 using VR and a double panmelanoma cocktail (dPANMEL) using 3,3′-diaminobenzidine (DAB) and VR. The HMB45-immunolabeled specimens were additionally developed with DAB, with and without overnight bleaching with hydrogen peroxide, 4%. Data were collected by 2 pathologists who were masked to sample grouping.

MAIN OUTCOMES AND MEASURES Differentiation between benign and malignant MLs based on immunomarker profile.

RESULTS Immunoreactivity was best visualized in specimens with VR. Melan-A labeled all melanocytes (100% sensitivity; panmelanocyte marker) without discriminating between benign and malignant lesions (0% specificity). Atypical melanocytes were most specifically labeled with HMB45 (96% specificity, 97% sensitivity; atypia marker). In primary acquired melanosis specimens, we found that the percentage of HMB45 ($P < .001$), S100 ($P < .001$), and Ki67 ($P \leq .02$) positivity increased significantly with worsening atypia.

CONCLUSIONS AND RELEVANCE We recommend VR, which rarely requires specimen bleaching, as the standard substrate for immunohistochemical analysis of conjunctival MLs. We found Melan-A and HMB45 to best characterize MLs. In conjunctival MLs, the use of VR with Melan-A and HMB45 provides substantial sensitivity for all melanocytes and for atypical melanocytes, respectively, and reduces specimen-processing time for laboratories performing immunohistochemistry on MLs.

Author Affiliations: Department of Ophthalmology, University of Ottawa, The Ottawa Hospital, Ottawa, Ontario, Canada (Jiang, Brownstein, Lam, Farmer); Department of Pathology and Laboratory Medicine, University of Ottawa, Ottawa Hospital, The Ottawa Hospital, Ottawa, Ontario, Canada (Jiang, Brownstein, Lam, Burns, Farmer), Ottawa Hospital Research Institute, Ottawa, Ontario, Canada (Jiang, Brownstein, Lam).

Corresponding Author: Seymour Brownstein, MD, FRCSC, Department of Ophthalmology, The Ottawa Hospital–General Campus, Ste W6213, 501 Smyth Rd, Ottawa, ON K1H 8L6, Canada (sbrownstein@ohri.ca).

Published online March 13, 2014.
Although most melanocytic lesions (MLs) of the conjunctiva are benign and thus do not progress to melanoma, certain types are prone to becoming malignant and need to be readily identified. Of the various melanocytic conjunctival lesions, the most concerning is conjunctival melanoma. Conjunctival melanoma is associated with an estimated mortality rate of 30% in 10 years. It has been reported 2-3 that 50% to 75% of conjunctival melanomas develop from primary acquired melanosis (PAM), which is a frequent lesion of the conjunctiva consisting of melanin accumulation in squamous cells or melanocyte hyperplasia. 2, 4 It is present in 36% of the white population and is subcategorized as with or without atypia. 4 PAM with atypia has a 13% to 46% chance of progressing to melanoma, whereas PAM without atypia is a benign lesion. 5, 6 The rate of transformation into melanoma increases with the degree of atypia, leading some groups 7 to propose that PAM with severe atypia be classified as melanoma in situ. Distinction between benign and malignant MLs can be difficult clinically and generally requires histologic examination for diagnosis. In our experience, an accurate diagnosis can be best obtained by identifying a sensitive and specific immunohistologic marker of all melanocytes and another marker that is sensitive and specific for atypia.

In most centers in North America, immunohistochemical analysis of heavily pigmented MLs of the conjunctiva relies almost exclusively on 3,3′-diaminobenzidine (DAB) as a brown substrate. Use of DAB may require bleaching of samples to remove the confounding melanin pigment, but bleaching can affect cellular antigenicity and may be incomplete. We evaluated an alternative substrate, Vector Red (VR; Leica Biosystems, Inc), whose vibrant red color is distinct from that of melanin pigment. Therefore, we also assessed the samples for the presence of VR, which generally renders bleaching unnecessary.

### Methods

Retrospective and prospective case series were conducted. Methods for securing human tissue were humane and included proper written informed consent in adherence to the Declaration of Helsinki. The participants did not receive financial compensation. The protocol was approved by the University of Ottawa Hospital Research Ethics Board. This case series was conducted using the resources of the Ophthalmic Pathology Laboratory, University of Ottawa Eye Institute, and the Department of Pathology in The Ottawa Hospital.

This study included a minimum of 8 specimens of each diagnostic category, including nevus, PAM with atypia separated into mild and moderate to severe, PAM without atypia, and conjunctival melanoma (Table 1). Only the PAM specimens showing melanocyte hyperplasia were included in the present study. Atypia involving less than one-third of the epithelial thickness is classified as mild, less than two-thirds is considered moderate, and close to full thickness is considered severe. Specimens used in this study were collected from January 1, 2005, to March 31, 2013. We included consecutive paraffin-embedded specimens of a given diagnostic category. The specimens that did not have a sufficient amount or quality of tissue for processing were excluded.

We examined the histochemical and immunohistochemical profiles of these specimens and compared them with those of control specimens consisting of normal conjunctiva and normal uvea as well as choroidal melanoma in enucleated globes.

On each specimen, the following melanocytic immunolabelings were performed using antibodies against the following antigens (Supplement eTable): double panmelanoma cocktail (dPANMEL), S100, human melanoma black 45 (HMB45), melanoma antigen recognized by T cells 1 (Melan-A), and Ki67. We hereinafter refer to these antibodies by the antigen that they are raised against. We also stained for hematoxylin-eosin with hydrogen peroxide, 4% (H2O2), bleaching and with potassium permanganate/oxalic acid bleaching. The specimens immunolabeled with HMB45 were processed separately with the DAB substrate and the VR substrate methods, as well as with 4% H2O2 bleach with the DAB substrate. The dPANMEL was developed concurrently with the DAB and Fast Red (Biocare Medical) substrates, hence it is referred to as a “double” panmelanoma cocktail. The remaining markers were developed with only the VR substrate.

Immunoreactivity and analysis were undertaken in a masked manner by 2 separate pathologists (S.B. and B.B.) and scored semiquantitatively using brightfield microscopy (original magnification ×400). Mean values of the 2 scores were used. Staining for dPANMEL, S100, HMB45, and Melan-A were graded on a scale of 0 to 4+, with 0 denoting 0% of staining present in melanocytic cells, 1+ representing less than 25%, 2+ indicating less than 50%, 3+ showing less than 75%, and 4+ representing 100% of the cells. Staining

### Table 1. Patient Demographics

<table>
<thead>
<tr>
<th>Category</th>
<th>Sample Size, No.</th>
<th>Female/Male</th>
<th>Age, Mean (SD), y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal conjunctiva</td>
<td>8</td>
<td>5/3</td>
<td>47 (18)</td>
</tr>
<tr>
<td>Normal choroida</td>
<td>19</td>
<td>6/13</td>
<td>60 (19)</td>
</tr>
<tr>
<td>Uveal melanoma</td>
<td>10</td>
<td>4/6</td>
<td>67 (12)</td>
</tr>
<tr>
<td>Conjunctival nevus (compound)</td>
<td>11</td>
<td>6/5</td>
<td>37 (8)</td>
</tr>
<tr>
<td>PAM with no or mild atypia</td>
<td>10</td>
<td>3/7</td>
<td>47 (17)</td>
</tr>
<tr>
<td>PAM with moderate to severe atypia</td>
<td>12</td>
<td>8/4</td>
<td>76 (13)</td>
</tr>
<tr>
<td>Conjunctival melanoma</td>
<td>8</td>
<td>4/3</td>
<td>82 (8)</td>
</tr>
</tbody>
</table>

Abbreviation: PAM, primary acquired melanosis.

* Ten of the 19 normal choroid samples were from nondiseased sections of uveal melanoma–enucleated globes.

b Six of the 12 PAM samples with moderate to severe atypia were associated with a conjunctival melanoma.
PAM specimens with mild to no atypia, 6 PAM specimens with moderate to severe atypia, and 8 conjunctival melanomas, 6 of which also showed moderate to severe atypia (Table 1). Control tissue specimens were obtained from 27 individuals; 10 of these were uveal melanoma with large adjacent areas of normal choroid, 9 were choroid from phthisical eyes, and the remaining 8 were conjunctiva with benign pterygiums.

Pan melanocytic Marker

In a noninferiority comparison, antigenicity for Melan-A was similar to that of the dPANMEL cocktail in all benign and malignant melanocytic cells (Figure 1), showing 100% sensitivity (Table 2). However, neither the dPANMEL cocktail nor Melan-A was able to distinguish between benign and malignant cells (0% specificity). For a given lesion, reactivity against the dPANMEL cocktail was present in more than 75% of melanocytic cells. Similarly, in all but 2 specimens, Melan-A was present in more than 75% of melanocytic cells. In those 2 specimens, Melan-A antigenicity was positive in more than 50% of melanocytic cells.

Marker for Atypia

Atypical melanocytes were most specifically labeled with HMB45 (96% specificity, 97% sensitivity) (Table 2). The percentage of HMB45- and Ki67-positive cells increased significantly with worsening atypia (Figure 2). Negative staining for S100 was displayed by 38% of normal conjunctival melanocytes and 45% of PAM specimens. Based on control values, Ki67 of 3% or lower was deemed negative. Because only melanocytic cells were studied in this project, specificity for stains for all melanocytes could not be meaningfully calculated. Antigenicity against all 3 markers increased with increasing atypia (S100 and HMB45, \( P < .001 \); Ki67, \( P \leq .02 \)). However, S100 was extensively positive in benign melanocytes as exemplified in specimens of conjunctival nevi and in the control samples of normal conjunctiva and choroid.

Bleaching and Usefulness of VR Substrate

Staining with 4% \( \text{H}_2\text{O}_2 \) generally had no significant effect on the HMB45 cellular antigenicity. Of the 62 distinct specimens examined, only 5 samples (8%) had discrepancies between slides treated with or without 4% \( \text{H}_2\text{O}_2 \). The slides treated with 4% \( \text{H}_2\text{O}_2 \) tended to have slightly diminished antigenicity (up to 50% reduction). Furthermore, it was difficult to interpret the degree of reactivity because bleaching with 4% \( \text{H}_2\text{O}_2 \) was at times sporadic. Of the 51 pigmented specimens, 7 (13.7%) had incomplete and patchy bleaching with 4% \( \text{H}_2\text{O}_2 \). The degree of immunoreactivity in specimens developed with VR was similar to those treated with or without 4% \( \text{H}_2\text{O}_2 \) and developed with DAB (Figure 3). In 62 samples developed with VR, 3 displayed reduced antigenicity (5%) and 2 showed increased antigenicity (3%) (up to 50% different in immunoreactivity). For slides developed with VR, additional bleaching was not required in any of the 51 pigmented melanocytic lesions.

Results

Sample Population

Apart from controls, 35 specimens from 32 individuals were included in our study, consisting of 11 compound nevi, 10

A. A case of primary acquired melanosis (PAM) with moderate atypia containing extensive melanin pigmentation (hematoxylin-eosin, original magnification \( \times 400 \)). B. Components of double pan melanoma cocktail (dPANMEL) can be developed only with 3,3′-diaminobenzidine (DAB) (dark brown chromagen), which can be easily confused with melanin pigmentation (original magnification \( \times 400 \)). C, Staining of melanoma antigen recognized by T cells 1 (Melan-A) developed with Vector Red is readily distinguishable from the melanin pigmentation in the same cells (original magnification \( \times 400 \)). D, In a noninferiority comparison, antigenicity for Melan-A is similar to that of dPANMEL (antibodies against tyrosinase, S100, and MART-1) in all benign and malignant melanocytic cells. The extent of staining is indicated as 1+ representing less than 25%, 2+ indicating less than 50%, 3+ showing less than 75%, and 4+ representing 100% of the cells. The limit lines indicate SE; limit lines are not shown where SE values were too small.

intensities of 1+ or less were considered as negative. Ki67 was reported as a percentage of positive cells. The immunoreactivity data were analyzed using 2-tailed Kruskal-Wallis and Mann-Whitney tests, assuming a nonparametric population (GraphPad, version 5.0c; GraphPad Software, Inc). Significance was set at \( P < .05 \).
gator in one study, contested classifying these MLs as PAM progressing to melanoma.

Histologically, PAM had atypical histologic features, with 46% of the cases showing benign acquired melanosis and cancerous acquired melanosis. However, more recently, Ackerman et al17,18 in their review of more than 10 000 ocular specimens, found that 39% of 85 melanocytic conjunctival specimens were PAM, with 21% showing atypia.

Sugira et al19 further echoed the proposal of Ackerman et al20 as to classifying these lesions as befitting of their pathologic potential for malignancy. Extrapolating from these clinical and histologic findings, Damato and Coupland21 concluded that a broad clinical/pathologic entity such as PAM cannot convey the appropriate clinical prognosis for a given histologic finding in that it included benign lesions, such as hyperpigmentation, and also potentially malignant lesions with severe atypia. To this effect, Damato and Coupland proposed to limit PAM as a clinical diagnosis and introduced conjunctival melanocytic intraepithelial neoplasia as the counterpart pathological diagnosis, with a grading system based on both the cytologic and architectural characteristics of a given melanocytic proliferation. At present, most ophthalmic pathologists in North America, including our group, follow the histologic classification proposed by Zimmerman of PAM occurring without or with atypia, which may be mild, moderate, or severe.22,23

Immunohistochemical Characterization of Conjunctival Lesions
Conjunctival melanoma arises primarily from PAM in 50% to 75% of the cases.2-3 The remaining cases develop de novo, with a small percentage arising from nevi, usually with active junctional components.12,16 As discussed above, malignant potential thus far has been identified primarily in PAM with histologic atypia. It is therefore paramount to accurately identify these lesions. Ideally, one would prefer to use 2 single antibodies that display high sensitivity and specificity for staining either all or only atypical, potentially malignant melanocytic lesions. Several markers have been shown to label melanomas, but the relative performance of each marker in the conjunctiva remains unclear. A particularly, these markers have yet to be evaluated in terms of their useful for distinguishing between degrees of atypia in PAM. To identify immunohistochemical markers for the characterization of these conjunctival melanocytic lesions, we examined a candidate marker list including melanoma cocktail (anti-Melan-A and HMB45), melanoma cocktail (anti-Melan-A, HMB45, and antityrosinase), dPANMEL cocktail reacting with all melanocytes cannot be meaningfully calculated. Staining intensities of 0 to 1+ were considered negative. Based on control values, Ki67 of 3% or lower was deemed negative.

Table 2. Summary of Sensitivity and Specificity of Selected Immunolabels

<table>
<thead>
<tr>
<th>Immunolabels in Melanocytes</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
<td>Benign</td>
</tr>
<tr>
<td>dPANMEL</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Melan-A</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>S100</td>
<td>77</td>
<td>74</td>
</tr>
<tr>
<td>HMB45</td>
<td>41</td>
<td>4</td>
</tr>
<tr>
<td>Ki67</td>
<td>34</td>
<td>17</td>
</tr>
</tbody>
</table>

Abbreviations: dPANMEL, double panmelanoma cocktail; HMB45, human melanoma black 45; Melan-A, melanoma antigen recognized by T cells 1.
Tail (S100, antityrosinase, and anti-MART-1 [melanoma antigen recognized by T cells 1]), anti-Melan-A, antityrosinase (T311), Ki67 (anti–MIB-1), and antimicrophthalmia-associated transcription factor. From the conclusions of previous studies19,20 and the preliminary results of the present study, we found dPANMEL, S100, Melan-A, HMB45, and Ki67 to be especially informative, thus making these markers the focus of this study.

Several studies18,21-24 have been conducted to assess melanocytic markers in conjunctival tissue. However, many of these did not make direct contrast comparisons or they made nonstatistically powered comparisons between PAM with or without atypia and conjunctival melanoma. To our knowledge, ours is the first study to assess the usefulness of Ki67 in such context.

Panmelanocytic Marker

The dPANMEL cocktail is used for the identification of melanocytic lesions. The cocktail is a mixture of antibodies against tyrosinase (T311), MART-1 cocktail (M3-7C10 and M29E3), and S100.25 The dPANMEL cocktail analysis is performed in a manner similar to that of Melan-A in melanocyte
detection. However, the reliability of the dPANMEL cocktail as a diagnostic tool is in doubt because of nonmelanocytic staining. In particular, its S100 component stains not only for melanocytes but also for other cell types in the conjunctiva, such as benign and malignant cells derived from cells of neural crest origin (including Schwann and glial cells), antigen-presenting cells, macrophages, and metastatic breast carcinoma.26 Also, in the context of the conjunctiva, melanocytic cells display diminished and variable S100 antigenicity.27

Conversely, of particular interest is Melan-A, which labels a cytoplasmic antigen present in melanosomes (Supplement [eTable]).18 In cutaneous melanomas, S100 has been claimed by some28 to be more sensitive than Melan-A for the detection of melanocytic cells. In ocular tissues, the usefulness of Melan-A is controversial.24 Melan-A shows superior reactivity in choroidal melanoma compared with S100.29,30 However, in the conjunctiva, Melan-A immunoreactivity is variable. In a study involving 13 conjunctival melanoma specimens, all tumor samples reacted to Melan-A, but the reactions were much weaker than reactivity to S100. In a subsequent study, Keijser et al24 concluded that Melan-A was a poor marker for conjunctival melanocytes, with 44% of conjunctival melanoma and 30% of PAM specimens showing negative antigenicity. Conversely, other studies involving similar sample sizes concurred with our findings showing that both the intensity and the pervasiveness of Melan-A staining are stronger in conjunctival melanoma, PAM, and nevi than is S100 immunoreactivity.

Marker for Atypia
Although S100 does not appear to be a panmelanocytic stain in conjunctival tissue, antigenicity against S100 increases with worsening atypia. Historically, S100 had been the only marker available to assess the degree of progression of atypia in PAM and conjunctival melanoma.17,21,23,31 However, S100 is not an ideal marker for atypia because a large proportion of nonhyperproliferative and histopathologically normal conjunctival melanocytes, along with benign nevus cells, stain positive for S100.

Since the early 1990s, HMB45 had been assessed for its ability to identify malignant melanocytes.21,31 It labels a cytoplasmic protein that is present in fetal melanocytes but is absent in normal adult melanocytes, and the reappearance of this antigen signals cellular dysregulation (Supplement [eTable]).31 Those studies found the usefulness of HMB45 as a marker of atypia to be questionable. However, upon reevaluation in the 2000s, the immunoreactivity of HMB45 was observed by several groups21,18,23 to correlate strongly with a worsening degree of atypia in the conjunctival MLs. Of these 3 studies, only the investigation conducted by Sharara et al17 assessed PAM in terms of with and without atypia in sufficient numbers for statistical analysis.

Another marker of cellular proliferation, Ki67, identifies an nuclear antigen that is present in non–gap 0 stage cells.22 In previous studies involving 13 conjunctival melanoma specimens, all tumor samples reacted to Melan-A, but the reactions were much weaker than reactivity to S100. In a subsequent study, Keijser et al24 concluded that Melan-A was a poor marker for conjunctival melanocytes, with 44% of conjunctival melanoma and 30% of PAM specimens showing negative antigenicity. Conversely, other studies involving similar sample sizes concurred with our findings showing that both the intensity and the pervasiveness of Melan-A staining are stronger in conjunctival melanoma, PAM, and nevi than is S100 immunoreactivity.

Marker for Atypia
Although S100 does not appear to be a panmelanocytic stain in conjunctival tissue, antigenicity against S100 increases with worsening atypia. Historically, S100 had been the only marker available to assess the degree of progression of atypia in PAM and conjunctival melanoma.17,21,23,31 However, S100 is not an ideal marker for atypia because a large proportion of nonhyperproliferative and histopathologically normal conjunctival melanocytes, along with benign nevus cells, stain positive for S100.

Since the early 1990s, HMB45 had been assessed for its ability to identify malignant melanocytes.21,31 It labels a cytoplasmic protein that is present in fetal melanocytes but is absent in normal adult melanocytes, and the reappearance of this antigen signals cellular dysregulation (Supplement [eTable]).31 Those studies found the usefulness of HMB45 as a marker of atypia to be questionable. However, upon reevaluation in the 2000s, the immunoreactivity of HMB45 was observed by several groups21,18,23 to correlate strongly with a worsening degree of atypia in the conjunctival MLs. Of these 3 studies, only the investigation conducted by Sharara et al17 assessed PAM in terms of with and without atypia in sufficient numbers for statistical analysis.

Another marker of cellular proliferation, Ki67, identifies a nuclear antigen that is present in non–gap 0 stage cells.22 In previous studies involving 13 conjunctival melanoma specimens, all tumor samples reacted to Melan-A, but the reactions were much weaker than reactivity to S100. In a subsequent study, Keijser et al24 concluded that Melan-A was a poor marker for conjunctival melanocytes, with 44% of conjunctival melanoma and 30% of PAM specimens showing negative antigenicity. Conversely, other studies involving similar sample sizes concurred with our findings showing that both the intensity and the pervasiveness of Melan-A staining are stronger in conjunctival melanoma, PAM, and nevi than is S100 immunoreactivity.
of Ki67 is confounded because it is not melanocyte specific in that it is present in any non–gap 0 stage cells and shows nonspecific staining in active basal epithelium, infiltrating lymphocytes and nevus cells, especially in the junctional region.

**Bleaching and Usefulness of VR Substrate**

More than 60% of MLs are pigmented; many of these require extensive bleaching for histologic and immunologic characterization.\(^1\)\(^2\) In PAM, where the degree of atypia has been shown\(^5\) to be predictive of its malignant potential, the cellular findings alone may not be sufficiently prognostic. In such cases, immunophenotypes can provide further diagnostic support. However, regardless of the specificity and sensitivity of a given antibody, the interpretation of our immunohistochemical results may often be confounded by the use of DAB, a common substrate for developing immunologic stains. Such cases, immunophenotypes can provide further diagnostic information.**

Conclusions

In conjunctival tissue, Melan-A has superior sensitivity and specificity for both benign and malignant melanocytic tumors.**

## REFERENCES

20. Faraji H, Brownstein S, Dorey MW, Jordan DR, Robertson S. Immunohistochemical analyses of...


