Immuno-phenotype of Conjunctival Melanomas
Comparisons With Uveal and Cutaneous Melanomas

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Objective: To characterize the immunophenotypic expression pattern of conjunctival melanomas, with the use of standard melanoma markers as well as microphthalmia transcription factor and p75 neurotrophin receptor.

Design: Eleven conjunctival melanomas, including 1 caruncular melanoma, were immunolabeled with a panel of antibodies that included S100, tyrosinase, melan-A, HMB-45 and HMB-50 combination, microphthalmia transcription factor, and p75 neurotrophin receptor. The results were tabulated on the basis of intensity and pervasiveness of labeling and compared with a previous study of uveal melanomas.

Results: Immunolabeling with S100 was at significantly higher levels in conjunctival melanomas than in uveal melanomas. Tyrosinase, HMB-45 and HMB-50 combination, melan-A, and microphthalmia transcription factor were expressed at high levels in conjunctival melanomas, whereas p75 neurotrophin receptor was not expressed.

Conclusions: Melanomas of the conjunctiva, including the caruncle, expressed S100, tyrosinase, melan-A, HMB-45 and HMB-50 combination, and microphthalmia transcription factor at high levels, suggesting that these are good markers for this melanoma subtype. Expression of S100 was significantly higher in conjunctival melanomas than in uveal melanomas. The immunophenotypic pattern of conjunctival melanomas is most similar to the epithelioid subtype of cutaneous melanomas.

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they may not necessarily be able to differentiate malignant from benign melanocytic lesions.\textsuperscript{1,17,18,21}

We have characterized the immunophenotypic expression pattern of 11 conjunctival melanomas, including 1 caruncular melanoma, with a panel of antibodies used previously to characterize uveal and cutaneous melanomas.\textsuperscript{14,22} The antibody panel included S100, HMB-45 and HMB-50 combination, tyrosinase, melan-A, microphthalmia transcription factor (MiTF), and p75 neurotrophin receptor (p75NTR). We believe this to be the first report of the expression levels of tyrosinase, MiTF, and p75NTR in conjunctival melanomas, as well as the first immunophenotypic characterization of a caruncular melanoma.

### METHODS

#### TISSUE

Ten conjunctival melanomas and 1 caruncular melanoma initially were retrieved from the archives of the University of Washington Pathology Department, Seattle, on the basis of pathology reports. The diagnoses were reconfirmed. Only 6 of the conjunctival melanomas and the single caruncular melanoma had sufficient tissue for analysis. Four conjunctival melanomas were retrieved from the archived tissue of Emory University, Atlanta, Ga. The diagnoses were reconfirmed. In addition, 5 uveal melanomas were retrieved from the archives of the University of Washington Pathology Department and stained in parallel with the conjunctival melanomas. The results were compared with a historical data set of 15 uveal melanomas stained previously.\textsuperscript{14}

#### IMMUNOLABELING

The immunocytochemical labeling was performed as described previously.\textsuperscript{14} Briefly, 3-µm sections were obtained from formalin-fixed, paraffin-embedded archival tissue blocks. Sections were dewaxed, rehydrated, and treated with 3% hydrogen peroxide in methanol to inhibit nonspecific peroxidase activity. Heat-induced epitope retrieval was performed for all sections other than for S100 labeling. For S100, sections were blocked for nonspecific protein binding by incubating slides in a blocking solution of 2% normal goat serum diluted in phosphate-buffered saline for 10 minutes. Slides were then incubated with primary antibody diluted in blocking solution for 40 minutes at room temperature. Primary antibody dilutions were as follows: p75NTR antibody (1:200) (mouse monoclonal MS-394-P1; Neomarkers Inc, Fremont, Calif); S100 (1:8000) (Z311; DAKO, Carpinteria, Calif); melan-A (1:50) (clone A103, M7196; DAKO); tyrosinase (1:50) ( monoclonal clone T311, NCL-TYROS; Novocastra/Vector Laboratories, Burlingame, Calif); HMB-45 and HMB-50 combination, also known as HMB-45/50 (1:1600/1:500) ( monoclonal antibodies HMB-45 and HMB-50, University of Washington); and MiTF (1:25) (a gift from David Fischer, MD, PhD, Dana Farber Cancer Institute, Boston, Mass). After several rinses with phosphate-buffered saline, slides were incubated with biotinylated secondary antibody diluted in blocking solution for 25 minutes. The slides were then rinsed in phosphate-buffered saline followed by incubation with a horseradish peroxidase–avidin complex (Vector Elite, Burlingame) for 25 minutes at room temperature. Antibody labeling was visualized with the use of 3,3′ diaminobenzidine as a chromogen, with nickel chloride enhancement. Slides were then counterstained with methyl green, dehydrated through a graded series of ethanol, and coverslipped with permanent mounting medium (Electron Microscopy Sciences, Fort Washington, Pa). Negative controls used a substitution of diluted normal rabbit serum in place of the primary antibody.

#### SCORING

The immunolabeled specimens were interpreted by evaluation of both the intensity and the pervasiveness (the extent or proportion of immunolabeling) of the immunocytochemical labeling as described previously.\textsuperscript{16} The intensity was ranked on a scale of 0 to 3 as follows: 0, absence of labeling; 1, low; 2, moderate; and 3, high. Pervasiveness was ranked on a scale of 0 to 3 as follows: 0, absence of labeling; 1, local, or less than 10% of cells; 2, patchy, or 10% to 75% of cells; and 3, diffuse, or more than 75% of cells. Scores were rated independently by at least 2 authors. Agreement of scores was reached in all cases. The mean value was calculated as the average of scores and reported as ±SEM.

#### STATISTICAL ANALYSIS

Data were expressed graphically as the mean ±SEM. Statistical comparisons were made between the current data set for conjunctival melanomas and a historical data set of uveal melanomas processed in an identical fashion to the tissue in the current study.\textsuperscript{14} Data sets were compared with an unpaired 2-tailed t test in Microsoft Excel (Microsoft Corp, Redmond, Wash).

#### PHOTOGRAPHY

Images for photomicrographs were collected with a digital camera (CoolSnap; R. S Photometrics, Tucson, Ariz) attached to a microscope (Leitz Orthoplan; Leica Microsystems Inc, Bannockburn, Ill) (magnification to the CCD chip, ×27.5). Images were saved as 1392×1040 twenty-four-bit RGB TIFF files. The figure was assembled in Adobe Photoshop 5 (Adobe Inc, Seattle) with minimal contrast and brightness enhancement.

### RESULTS

The immunophenotypic expression pattern of the single caruncular melanoma was consistent with the expression patterns of the conjunctival melanomas with each of the antibodies examined and thus was included in the statistical analysis of the conjunctival melanomas (n = 11). A photomicrograph showing the typical expression pattern detected for each of the antibodies in the conjunctival melanomas is shown in Figure 1. S100 labeled 10 of 11 conjunctival melanoma specimens, with an average intensity score of 1.9±0.3 and a pervasiveness score of 2.3±0.2. HMB-45/50 labeled all 11 conjunctival melanoma specimens, with an average intensity score of 2.6±0.2 and a pervasiveness score of 2.7±0.1. Melan-A labeled all 11 conjunctival melanoma specimens, with an intensity score of 3.0±0.2 and a pervasiveness score of 2.4±0.3. MiTF labeled 10 of 11 conjunctival melanoma specimens, with an intensity score of 2.4±0.3 and a pervasiveness score of 2.4±0.3. The p75NTR was not detected in any of the conjunctival melanomas.
Figure 1. Photomicrographs of a conjunctival melanoma illustrating the immunophenotypic labeling patterns. Note the absence of labeling by p75 neurotrophin receptor and the clear labeling by S100, HMB-45/50, tyrosinase, melan-A, and microphthalmia transcription factor (MiTF).

HE indicates hematoxylin-eosin.
The expression patterns of the 5 uveal melanomas immunolabeled in the current study together with the conjunctival melanomas (data not shown) were compared with the historical data set of 15 uveal melanomas labeled previously. No differences were detected between the uveal melanoma samples stained in the current study and those of the historical data set. Thus, we compared the results of the conjunctival expression patterns with those from the historical data set (Figure 2). The uveal melanoma data set includes spindle (n = 5), epithelioid (n = 5), and mixed (n = 5) uveal melanomas. When the conjunctival melanoma expression pattern was compared against each group separately, the intensity and pervasiveness of S100 expression were significantly higher in the conjunctival melanomas than in the uveal spindle melanomas (P = .004 and P = .01, respectively). HMB-45/50 and MiTF both showed significantly higher intensity values in the mixed uveal samples than in the conjunctival melanomas (P = .01 and P = .045, respectively). When the subclasses of uveal melanomas were grouped together and compared as a whole (n = 15) against the conjunctival melanomas (n = 11), only the expression of S100 was found to be statistically different between the uveal and conjunctival melanomas, for both the intensity (P = .02) and pervasiveness (P = .04).

The purpose of this study was to determine the pattern of immunophenotypic expression of conjunctival melanomas and a caruncular melanoma. Because of the rarity of conjunctival and caruncular melanomas, our sample size was small. However, all 11 specimens of conjunctival melanomas, including the single specimen of caruncular melanoma, showed high expression of S100, tyrosinase, melan-A, HMB-45/50, and MiTF, suggesting that these antibodies are useful markers for these 2 types of melanoma. The p75NTR was expressed at minimal levels in both the conjunctival and caruncular melanomas.

Conjunctival melanomas expressed S100 at significantly higher levels than uveal melanomas. Our previous studies showed that cutaneous melanomas also expressed S100 at higher levels when compared with uveal melanomas. The more intense S100 immunolabeling of conjunctival melanomas compared with that of uveal melanomas was also found in our own retrospective examination of the results of Heegaard et al. Their results showed that all of their 13 conjunctival melanomas expressed S100, of which most (12 of 13) were intensely labeled. In contrast, in their uveal melanoma samples, most of their samples were expressed at lower levels of intensity.

Although we did find some differences between uveal and conjunctival melanomas, these differences were largely related to the intensity and pervasiveness of the staining as opposed to the presence or absence of the particular antigen. The relevance of the similarity in immunophenotype may reflect the fact that both tumors are melanomas. In fact, the immunophenotypic pattern seen in conjunctival melanomas in the current study is very similar to that seen in cutaneous epithelioid melanomas, but highly distinct from that of cutaneous spindle melanomas. The high expression of all of the standard melanoma antibodies (S100, melan-A, tyrosinase, HMB-45/50, MiTF) and minimal expression of p75NTR is an immunophenotypic pattern that characterizes cutaneous epithelioid melanomas. In this study, we found that an identical pattern characterizes conjunctival melanomas and the caruncular melanoma. The conjunctival and caruncular melanoma pattern is in sharp contrast to the pattern of cutaneous spindle melanomas, where the standard melanoma markers (with the exception of S100) are poorly expressed, and where p75NTR is highly expressed. The Table is a simplified summary that compares the immunophenotypic patterns of conjunctival (including caruncular) melanomas with uveal and cutaneous melanoma subtypes.

The conjunctiva, including the caruncle, shares certain embryonic similarities with the skin, as both are of surface ectodermal origin. The results of this study show that conjunctival and cutaneous melanomas also share...
Immunophenotypic Expression Patterns of Cutaneous, Uveal, and Conjunctival Melanomas*

<table>
<thead>
<tr>
<th>Type of Melanoma</th>
<th>S100</th>
<th>Tyrosinase</th>
<th>HMB-45/50</th>
<th>Melan-A</th>
<th>MITF</th>
<th>p75NTR</th>
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<tbody>
<tr>
<td>Skin†</td>
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<td>High</td>
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<tr>
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<tr>
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<tr>
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<tr>
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<td>High</td>
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</table>

*MITF indicates microphthalmia transcription factor; p75NTR, p75 neurotrophin receptor; high, expressed in most specimens at moderate to high intensity and pervasiveness; 0+, expressed in a minority of specimens at low intensity; +, expressed in many specimens but at low intensity; and 0, no expression.

†Data from Iwamoto et al.‡ (MITF labeling from Granter and King et al.§).

‡Data from Iwamoto et al.**

similarities of their immunophenotypic expression patterns that contrast with those of the uveal tract.

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