EXTENDED REPORT

Immunophenotypic markers to differentiate between benign and malignant melanocytic lesions

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Background/aims: The authors investigated the expression of S100A1, S100A6, S100B, MelanA, and CEA in conjunctival naevi, primary acquired melanosis (PAM), conjunctival melanoma, and uveal melanoma in order to assess their potential usefulness in the pathological differential diagnosis of these entities.

Methods: Paraffin embedded sections of 18 conjunctival naevi, 14 PAM, 16 conjunctival melanomas, and 20 uveal melanomas were immunostained for S100A1, S100A6, S100B, MelanA, and CEA, and expression was scored semiquantitatively.

Results: Expression of S100A1 differed significantly between conjunctival naevi and conjunctival melanoma, with percentages of positive cells of 30.6% and 71.4%, respectively. Conjunctival melanomas had high average scores for S100A1 and S100B (71.4%, 62.9%, respectively), while uveal melanomas also had high S100A1 but low S100B scores (88.5%, 18.5%, respectively). MelanA was highly variable; naevi and uveal melanoma had higher average scores than conjunctival melanoma. CEA was hardly detectable in all four groups.

Conclusion: S100A1 seems to be a possible candidate to differentiate conjunctival naevi from conjunctival melanoma. S100B seems to differentiate between uveal melanoma and conjunctival melanoma. However, the study size was small and therefore the data have to be confirmed by others.

T he most common melanocytic lesions of the conjunctiva include conjunctival naevus, primary acquired melanosis (PAM), and conjunctival melanoma. Clinically, their distinction may be difficult,[1] and in case of doubt, histological investigation is required. PAM is subdivided histologically into a typical and an atypical form, and especially the latter, as well as conjunctival melanoma, has a high tendency for local recurrence after treatment.[2 3] The mortality rate of patients with a conjunctival melanoma is about 30% in 10 years.[2–10]

Immunophenotypic markers might be of help in distinguishing between benign and malignant melanocytic lesions of the conjunctiva. Previous studies addressed S100, MelanA, and HMB45 expression in conjunctival melanoma,[11–17] and a few studies compared expression patterns on conjunctival naevus or PAM with conjunctival melanoma.[14–17] Although most markers have proved to be of value in the establishment of the melanocytic origin of various lesions, they are of much less help in the distinction between a melanocytic naevus and a melanoma.[14–16]

Calcium binding proteins like S100 have been implicated in establishing the malignant and metastatic phenotype of various tumours.[18–20] The S100 protein family consists of over 20 members. The expression of S100A1, S100A2, S100A3, S100A4, S100A6, and S100B has been studied previously in cutaneous melanoma.[21–25] S100A6 seems of some use in the distinction between a Spitz-naevus and a cutaneous melanoma.[25] The S100B level in serum is of considerable interest as a prognostic marker in cutaneous melanoma, and has been used to monitor patients with metastatic cutaneous melanoma.[23]

In this study we stained histological samples of conjunctival naevus, PAM, conjunctival melanoma, and uveal melanoma for S100A1, S100A6, S100B, MelanA, and CEA.

MATERIALS AND METHODS

Patients and tissues

Eighteen conjunctival naevi, 14 PAM (one with no atypia, four with mild atypia, six with moderate atypia, and three with severe atypia), 16 conjunctival melanomas, and 20 uveal melanomas (six epithelioid, eight spindle, and six mixed cell type) from different patients were collected from the pathology archives of the Leiden University Medical Centre, Leiden, the Netherlands. Patient records were used to obtain information on local or distant recurrence, and to investigate whether patients with PAM or naevus subsequently developed conjunctival melanoma.

Immunohistochemistry

Specimens were fixed in 4% neutral buffered formaldehyde and embedded in paraffin. Immunohistochemical reactions were performed using the streptavidin-biotin method. Sections were cut at 4 μm and mounted on glass slides, and deparaffinised in xylene (four times, 5 minutes each) and ethanol 99% (twice, 5 minutes each). The endogenous peroxidase activity was blocked by incubating the slides with methanol/H_2O_2 0.3% for 20 minutes. After the slides were washed, antigen retrieval was performed by boiling in citrate buffer (Dako, Glostrup, Denmark) for 10 minutes. Slides were washed again in phosphate buffered saline (PBS), and they were incubated overnight with the first antibody at 4°C. Rabbit anti-S100A1 polyclonal (dilution 1:100) (A5109, Dako), mouse anti-S100A6 mAb clone CACY-100 (dilution 1:4000) (S-5049, Sigma-Aldrich, Steinheim, Germany), mouse anti-S100B mAb clone DAK-S100B/2 (dilution 1:100) (M7221, Dako), mouse anti-MelanA mAb clone A103 (dilution 1:100) (M7196, Dako), and mouse anti-CEA mAb clone 11-7 (dilution 1:50) (M7072, Dako) were used. As negative control, the primary antibody was replaced by PBS. Slides were then incubated with biotinylated anti-mouse anti-rabbit Ig (Dako) for 30 minutes. After washing, the slides were labelled with Streptavidin-HRP (Dako) for

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Abbreviations: DAB, 3,3 diaminobenzidine; PAM, primary acquired melanosis; PBS, phosphate buffered saline

30 minutes; hereafter the labelling was made visible with a 30 minute incubation in AEC (3-amino-9-ethylcarbazole) or DAB (3,3 diaminobenzidine). Slides were counterstained with Mayer's haematoxylin and finally embedded in Kaiser's glycerine. For the CEA antibody, sections of colon carcinoma were used as a positive control; for S100A1, S100A6, S100B, and MelanA, sections of cutaneous melanoma were used.
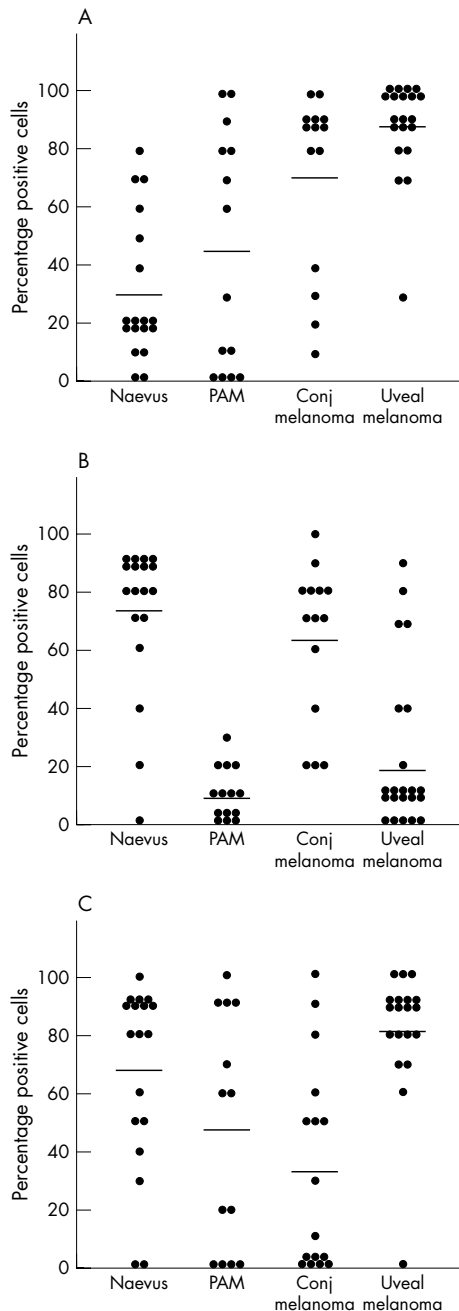

Figure 1 Percentage positive cells after staining for antibodies against S100A1 (A), S100B (B), and MelanA (C) in conjunctival naevus, PAM, conjunctival melanoma, and uveal melanoma specimens. Each dot represents a single case, the line represents the average score for each group of melanocytic lesions. (S100A1: naevus versus conjugated melanoma p = 0.001, Mann-Whitney U test) (S100B: PAM versus naevus p<0.0001, PAM versus conjugated melanoma p<0.0001, conjugated melanoma versus uveal melanoma p<0.0001, Mann-Whitney U test).

Scoring
The immunolabelled slides were interpreted by determining the percentage positively staining cells, scored on a scale of 0% to 100%, in steps of 10%. Slides were independently scored by at least two people; in all cases agreement was reached.

Statistics
For statistical analysis, the mean (SEM) was used. Data were analysed in SPSS 11.0 (SPSS Inc, Chicago, IL, USA). A one way ANOVA test was used to calculate significance between the groups. A two tailed non-parametric Mann-Whitney U test was used to compare two variables.

RESULTS
Of a total of about 400 slides, six were lost because of technical problems or lack of residual tissue in the blocks. As a consequence, only 13 of 14 PAM specimens could be stained with S100A6 and MelanA, 14 of 16 conjunctival melanoma were stained with S100A1 and S100B. Figure 2 shows the staining results for S100A1, S100A6, S100B, and CEA staining in representative sections of conjunctival naevi, PAM, conjunctival melanoma, and uveal melanoma.

S100A1
S100A1 positively stained more than 10% of the lesional cells of 16 of the 18 conjunctival naevi (89%), of 10 of 14 PAM (71%), of all conjunctival melanomas (n = 14, 100%), and all uveal melanoma (n = 20, 100%) (fig 2). Significantly more cells were positive in conjunctival melanoma compared to conjunctival naevi (71.4%, and 30.6%, respectively) (p = 0.001 Mann-Whitney) (fig 1), while the PAM reacted intermediately (45.0% positive cells).

S100A6
In all uveal melanomas more than 10% of the cells stained positively; the average percentage of positive cells was very high (72.1%). All conjunctival tissues showed severe non-specific staining, and could therefore not be scored: most epithelial cells stained positive, which has been described previously.[21] [22]

S100B
More than 10% of the cells scored positively with S100B of 17 of the 18 conjunctival naevi (94%), of eight of 14 PAM (57%), of all conjunctival melanoma (n = 14, 100%), and of 15 of 20 uveal melanoma (75%). Comparing the average scores for S100B, naevus and conjunctival melanoma scored significantly higher than PAM (p<0.001) (fig 1). The average percentage positive cells in conjunctival melanoma was significantly higher than in the uveal melanoma (62.9%, 18.5%, respectively) (p<0.0001, Mann-Whitney) MelanA
    MelanA stained more than 10% of the cells in 16 of the 18 naevi (89%), nine of 13 PAM (69%), nine of 16 conjunctival melanoma (56%), and 19 of 20 uveal melanoma (95%). Most naevi and uveal melanoma had a good expression of MelanA, while in PAM and conjunctival melanoma expression was very variable (fig 1).

CEA
CEA was completely negative in most lesions, but in one naevus , two conjunctival melanomas, and 15 uveal melanomas, groups of non-melanocytic cells were positive (fig 2). The total number of CEA positive cells was very low in all categories.
    The atypia stage of the PAM lesions did not correlate with the expression levels of the tested antibodies. No significant difference in expression was seen between uveal or conjunctival melanomas in patients with or without metastasis, although for S100A1 and S100A6 there was a trend towards
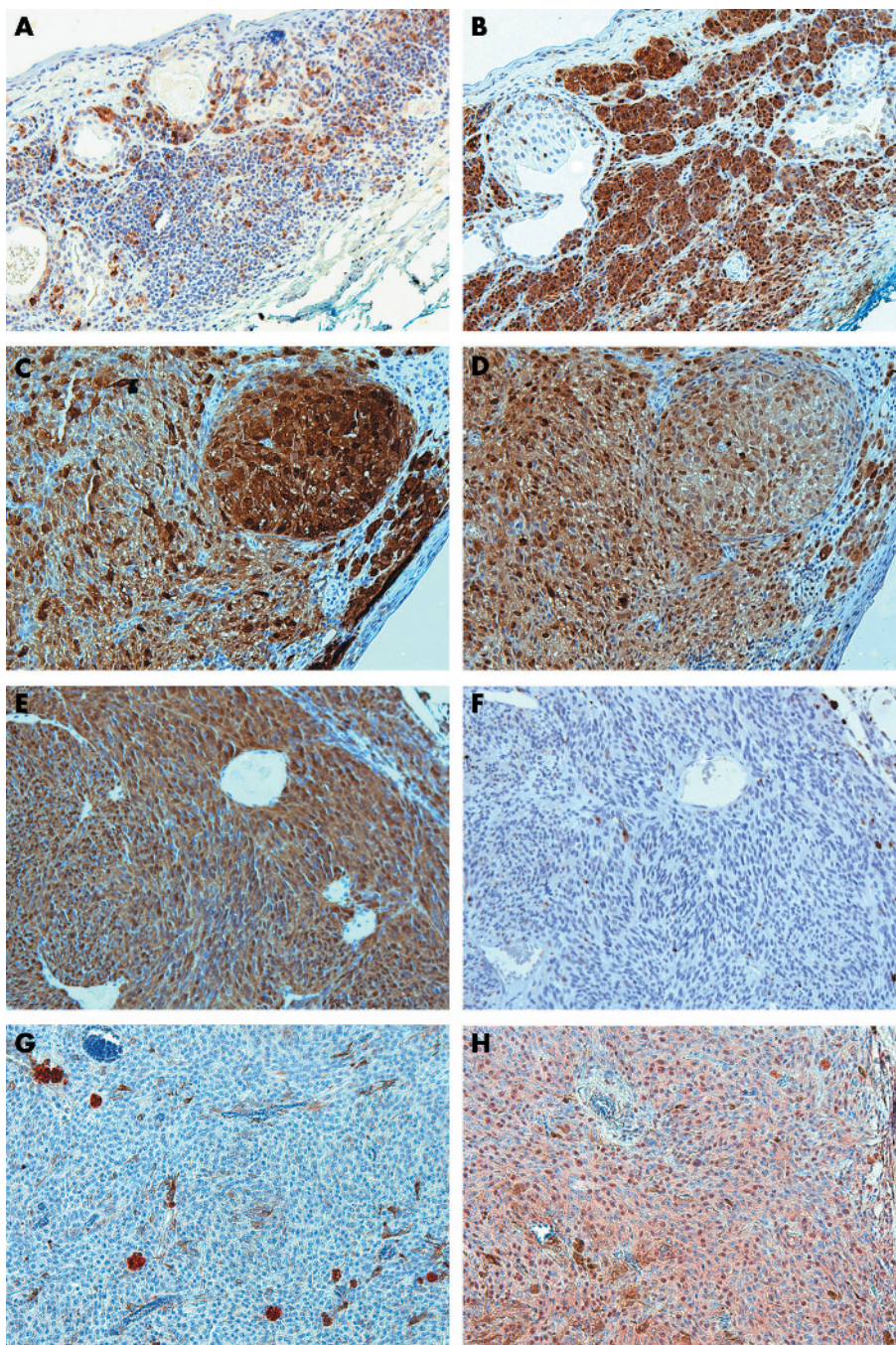
Figure 2 (A) and (B) represent the S100A1 and S100B expression on conjunctival naevi, respectively. (C) and (D) show the expression of S100A1 and S100B on conjunctival melanoma, respectively. (E–H) represent the S100A1, S100B, CEA, and S100A6 expression on uveal melanoma, respectively. Mark the difference in S100A1 expression between conjunctival naevi and conjunctival melanoma, also the S100B expression between uveal and conjunctival melanoma is remarkably different. All figures are with a magnification of ×200.

higher expression in uveal melanoma patients with metastatic disease.

DISCUSSION

In this study, we set out to determine whether expression patterns of various markers may differentiate between benign and malignant conjunctival pigmented lesions, as previous studies did not yield a positive result.[14–17] In our study, S100A1 staining differed between a naevus and a conjunctival melanoma. The naevi had low S100A1 expression (mean 30.6%), while in most conjunctival melanomas expression was strong (mean 71.4%) (figs 1 and 2). It must be noted that because of the rarity of histological sections of conjunctival naevi and conjunctival melanomas, the sample size is small. Since no other studies have been published

about S100A1 staining of melanocytic conjunctival lesions, confirmation by others will be awaited. S100B staining did not differ between conjunctival naevi and conjunctival melanoma, however, a lower staining was seen in the PAM lesions (fig 1). Others also found no difference in S100 expression between conjunctival naevi and conjunctival melanoma.[16 17 26] Steuhl *et al* did also find slightly lower expression of S100 in epithelial melanosis than in conjunctival naevi or conjunctival melanoma.[16] However, Hitzer *et al*, and Sharara *et al* did not find any difference between PAM and conjunctival naevi , and conjunctival melanoma.[17 26] The former studies used the "general" S100 antibody which probably represents the S100B used in our study.

To our surprise MelanA was not present in seven of the 16 conjunctival melanomas, while the expression in PAM and

Table 1 Expression of S100A1, S100A6, and S100B of conjunctival melanoma, uveal melanoma, and cutaneous melanoma

	S100A1	S100A6	S100B
Conjunctival melanoma †	+++	*	++
Uveal melanoma †	+++	+++	+
Cutaneous melanoma ‡	0	++	++

*S100A6 stained very non-specific on the conjunctival melanomas and could therefore not be scored.
†Data from this study.
‡Data from literature.[21] [22] [25]

conjunctival naevi was higher. Others found higher expression of MelanA in conjunctival melanoma,[12] [13] although Heegaard et al found weak staining of the MelanA in most of the conjunctival melanomas.[12] Our findings indicate that MelanA is not a good marker for conjunctival melanoma.

Whether conjunctival melanoma is biologically more closely related to the skin melanoma or the uveal melanoma is still a topic of discussion. We included uveal melanoma samples as a control and to compare expression of the conjunctival lesions with this tumour. The S100B was as abundantly expressed in conjunctival melanomas as the S100A1, while in uveal melanomas the expression of S100A1 was also high, but S100B was low. Others also found higher expression of S100B in conjunctival melanoma than in uveal melanoma.[12] [13] Iwamoto claims that conjunctival melanoma is most similar to the epithelioid phenotype of the cutaneous melanoma.[13] Table 1 compares the S100A1, S100A6, and S100B data from this study with data on cutaneous melanoma in the literature. All lesions have similarities for one or two antibodies, but one cannot conclude that conjunctival melanoma resembles cutaneous melanoma more closely than uveal melanoma. Perhaps it is better to see them as three separate identities, although clinically the conjunctival melanoma has more similarities with cutaneous melanoma, since both have the tendency to metastasise to the regional lymph node first.

The tested antibodies may not only be used for differentiation between lesions but may also be potential serum metastases markers. Especially molecules with a high expression in the malignant lesion may be good candidates. Serum levels of S100B have proved to be reliable serum markers to follow metastasis in cutaneous melanoma.[23] For both uveal and conjunctival melanoma good serum markers may also be of help in detecting and following primary and metastatic disease. For uveal melanoma, S100A1 and S100A6 could be candidate serum markers, since they are abundantly expressed on uveal melanoma, for conjunctival melanoma S100A1 and S100B could be good candidates to test in the serum. Van Ginkel already implied that S100A6 could be of influence on malignant transformation of the uveal melanoma.[27] Serum S100B was not able to detect metastatic disease in patients with uveal melanoma,[28] probably since S100B is not well expressed in uveal melanoma, but serum S100A1b was able to detect metastatic disease in uveal melanoma patients (GS Missotten et al, submitted). As far as we know, no serum markers have yet been analysed in conjunctival melanoma. In 1976 Michelson et al reported a slightly elevated blood CEA levels in 45% of the uveal melanoma patients, although the increase of CEA levels was only marginal.[29] We also investigated the CEA expression of conjunctival and uveal lesions, but could not find any tumour labelling, although in some tumours we did find staining of a few non-melanocytic cells (fig 2), which were microscopically identified as melanophages. As CEA is not expressed, using it as a serum marker seems inefficient.

In conclusion, we think that of those we studied S100A1 is the best marker to differentiate between a naevus and conjunctival melanoma, although it does not provide an absolute cut-off value. Because of the small sample size, further studies will be needed to confirm these findings.

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