Cell growth and p53 expression in primary acquired melanosis and conjunctival melanoma

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Abstract
Aims—To evaluate cell growth and the pattern of p53 suppressor gene expression in atypical primary acquired melanosis (PAM) and in recurrent conjunctival melanoma.

Methods—Eighteen specimens of PAM with atypia and 24 specimens, comprising early and late lesions, from 12 patients with conjunctival melanoma were stained for the proliferating cell nuclear antigen using the PC10 antibody, and for the p53 gene product using the BP53-12-1, 1801 and DO7 clones. The immunoreactive cells were counted manually and the data evaluated statistically.

Results—Seven of nine PAM specimens progressing to melanoma expressed PC10. None of these lesions expressed the p53 gene product. The number of proliferating cells was higher in the late than in the early lesions of conjunctival melanoma. Four of the 12 recurrent melanomas displayed focal, but minimal, p53 expression. The proliferating cell count in the p53 positive tumours was very similar to that of the p53 negative conjunctival melanomas.

Conclusion—Examination of the expression of proliferating cells in atypical PAM may be used as an adjunct to predict which lesions will progress to melanoma. The increase in the number of proliferating cells over time in recurrent conjunctival melanomas probably reflects more aggressive behaviour and may be used to monitor recurrence. The absence of p53 expression in PAM and minimal staining of conjunctival melanomas did not correlate with cell growth, suggesting that alterations in the p53 tumour suppressor gene are uncommon and late events in conjunctival melanoma, and that p53 expression is unlikely to be a useful prognostic indicator.

Keywords: p53 expression, recurrent conjunctival melanoma, primary acquired melanosis, malignant transformation, cell proliferation.

Conjunctival malignant melanoma is a rare tumour, with a 10 year, tumour related mortality rate of 30%. One 60% of patients with conjunctival melanoma experience recurrences.1 2 32% have more than one recurrence4 and many patients with multiple recurrences finally undergo orbital exenteration due to unresectable tumours.5 This suggests that recurrent conjunctival melanomas become more aggressive over time. Primary acquired melanosis (PAM) of the conjunctiva is a potential precursor lesion of conjunctival melanoma, and 50% of lesions with cytological atypia will ultimately progress to malignant melanoma.6

While the presence of epithelial cells and histological patterns other than basal hyperplasia may be more common in atypical PAM giving rise to melanoma, little is known of other predictors of malignant transformation.7 It is conceivable that cell proliferation is more marked in atypical PAM that progresses to melanoma than in lesions that remain confined to the epithelium. In addition, if the aggressive behaviour of recurrent conjunctival melanoma correlates with increased cell growth, recurrent disease may be monitored by assessing cell proliferation.

The proliferative compartment may be assessed by the fraction of cells expressing the proliferating cell nuclear antigen (PCNA), a 36 kD nuclear protein associated with the S phase of the cell cycle.8 Previous studies indicate that the PC10 monoclonal antibody directed against PCNA may be used to assess prognosis in conjunctival melanoma.9 The p53 tumour suppressor gene encodes a 53 kD nuclear phosphoprotein believed to be involved in the regulation of cell growth.10 p53 gene mutations were first reported in colorectal carcinoma,11 and have since been demonstrated in a variety of neoplasms.12 Wild-type p53 has a short half-life, five to 20 minutes, and is usually not detectable by immunohistochemical methods.13 In contrast, mutant p53 is much more stable and can be detected immunohistochemically.13 14 The development of antigen retrieval techniques15 16 and antibodies that recognise epitopes resistant to denaturation enables the BP53-12-1,15 1801,16 17 and DO718 19 clones to be used on formalin fixed, paraffin wax embedded specimens.

Methods
IMMUNOPATHOLOGY
A pilot study on the effect of prolonged fixation of tissue in 4% formaldehyde was undertaken initially. Briefly, tissue samples of a colorectal carcinoma were fixed in 4% formaldehyde for one, two, seven, and 14 days. These samples were then stained immunohistochemically using the protocol and antibodies outlined below. Positive p53 staining was detected after seven but not 14 days following pretreatment in a microwave oven and with all three p53 antibodies listed below. The BP53-12-1 antibody provided a more intense staining pattern, but all three clones produced satisfactory results. When pretreatment in a microwave oven was omitted, staining with PC10 produced...
a positive result after 14 days' fixation. The staining intensity was further improved on microwave pretreatment, even for those specimens fixed in formaldehyde for 14 days.

Eighteen archival specimens of atypical PAM, including nine cases which progressed to melanoma, and 24 specimens, comprising early and late lesions, from 12 patients with recurrent conjunctival melanoma were retrieved from the files of the St Erik's Eye Hospital, Stockholm. Pertinent clinical data were obtained from the hospital records and from the Swedish National Causes of Death Registry (table 1).

Sections, 3 μm thick, were cut from each paraffin wax block, deparaffinised and rehydrated. Endogenous peroxidase was blocked by immersing the slides in 3% H2O2 for 30 minutes. The tissue sections were then covered with 0-01 M citrate buffer, pH 6-0, and were heated in a microwave oven (Miele M720, operating at 2450 MHz (Miele, Gütersloh, Germany)) at 780 W for 10 minutes, with a short pause after the first five. Four slides from each specimen were then incubated overnight with, respectively, PC10 (Dako, Glostrup, Denmark), diluted 1 in 20, DO7 (Dako), diluted 1 in 25, BP53-12-1 (Biogenex, San Ramon, California, USA), diluted 1 in 80, and 1801 (Biogenex), diluted 1 in 40. The slides were then exposed to avidin-biotin complexes for 60 minutes, stained with 3-amino-9-ethylcarbazole and counterstained with Mayer's haematoxylin.

Table 1. Clinical data

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*Age at diagnosis of first lesion; ‡ Follow up (months) after diagnosis of first lesion until end of study or death from any cause.

Positive controls for all primary antibodies were obtained from paraffin wax blocks of a colorectal carcinoma with previously recorded expression of the p53 protein in approximately 60–70% of all tumour cells and a PC10 labelling index of about 40–50%. Negative controls were provided by omitting the primary antibodies from the protocol.

ASSESSMENT OF IMMUNOSTAINING

Eight high power fields (0-0625 mm²) from areas of maximum immunoreactivity were assessed using a Zeiss (Carl Zeiss, Oberkochen, Germany) Axioskop microscope with a 10× eyepiece graticule. All cells containing distinct nuclear immunoreactivity were regarded as positive irrespective of the staining intensity. To estimate intra-observer reproducibility, all PC10 counts for the early lesions were repeated blindly. As the BP53-12-1 clone provided the most distinct staining pattern of the antibodies recognising p53, this clone was used for the quantitative assessment of p53 immunoreactivity.

DATA ANALYSIS

Data were analysed using following software: CSS:STATISTICA (StatSoft, Tulsa, Oklahoma, USA), Statgraphics 5.0 (STSC, Rockville, Maryland, USA), and Confidence Interval Analysis 1.1 (BMJ, London, UK). Normality was assessed by frequency histograms, and two tailed t tests for paired and unpaired data were then used as appropriate. Non-normal continuous data were analysed by the Mann-Whitney U test; Fisher's exact test was used for proportions. Statistical significance was set at the 5% level and hypothesis testing was supplemented by the calculation of 95% confidence intervals (CI).

Results

Nine of the 18 patients with atypical PAM eventually developed conjunctival melanoma. The median follow up was 46 months (range 21–233 months) for those patients who did not develop melanoma and was 36 months (range 7–125 months) for those who underwent malignant transformation. This difference was not significant (p = 0.12). All patients were treated with a combination of surgery and cryotherapy,

Figure 1. Atypical PAM that subsequently progressed to melanoma (patient 2). A single intraepithelial melanocyte displays strong intranuclear staining for PCNA (arrows). The section was counterstained with Mayer's haematoxylin. Original magnification × 910. Bar = 20 mm.
and all completed the study. The median interval between early and late recurrences was 538 days (10th and 90th centiles, 227 and 1407 days, respectively).

IMMUNOSTAINING
A strong granular staining pattern was evident in the nuclei of the PCNA positive tumours. In PAM lesions these cells appeared singly (fig 1); however, in melanomas PCNA positive cells appeared as small clusters (figs 2A and 2B). A large proportion of cells also displayed weaker immunoreactivity, particularly cells undergoing mitosis, possibly due to dissolution of the nuclear membrane. Although considerable intra-tumoural heterogeneity was a common feature, PC10 intra-nuclear immunoreactivity was present in all conjunctival melanomas.

Expression of p53 protein in melanomas was limited to small nodules or even single cells, which were surrounded by tumour cells without apparent immunoreactivity (fig 2C). However, the staining pattern of the small number of p53 positive cells was almost always strong and granular. The BP53-12-1 antibody showed a more distinct staining pattern than the DO7 or 1801 clones. In two conjunctival melanomas, only the BP53-12-1 antibody invoked a positive response. Staining with 3-amino-9-ethylcarbazole produced a red colour that could be easily differentiated from melanin.

REPRODUCIBILITY
Repeated PC10 counts for early recurrent lesions indicated good intra-observer agreement between the two counts. The mean (SD) of the difference between the paired counts was $-4.8 (16.5)$ and a graphical display (fig 3) suggested that the difference was independent of the average PC10 count. The two tailed $t$ test for paired data confirmed the absence of bias ($p = 0.33$). The 95% CI of the difference in counts was $-15.3$ to $5.7$.

PRIMARY ACQUIRED MELANOSIS
Of the nine specimens of atypical PAM that progressed to melanoma, seven contained PC10 positive melanocytic cells. The immunopositive cells often appeared in the middle or superficial part of the epithelium (fig 1). By contrast, only two specimens from patients who did not undergo subsequent malignant transformation contained a few PC10 positive cells in the basal layer. The remaining seven specimens in this group did not contain PC10

Figure 2  Recurrent conjunctival melanoma in patient 3 with PCNA immunoreexpression in both the early (A) and late (B) lesions. Note the increased number of immunoreactive cells in the late lesion. The staining pattern is variable and some intensely stained cells can be seen (closed arrow). Some minimal and focal staining for the p53 gene product (open arrows) was present in both the early (not shown) and late (C) lesions. All sections were counterstained with Mayer’s haematoxylin. Original magnification × 650. Bar = 50 mm.

Figure 3  Assessment of reproducibility showing the difference between the repeated PC10 counts for each conjunctival melanoma (early lesions) plotted against the average of the two counts. The differences are small and seem to be independent of the average counts. The broken lines indicate the 95% limit of agreement defined as the mean difference ±2 SD.
positive cells. This difference is statistically significant (p = 0.028), but the sample was too small for life table based analysis. There was no difference between the survival times of the two groups when the event of interest was defined as the development of invasive melanoma (p = 0.12). In contrast to PC10, none of the 18 PAM specimens contained p53 positive cells.

**CELL PROLIFERATION OVER TIME**

The PC10 counts for the two specimens of each conjunctival melanoma are presented in fig 4. The mean of the PC10 counts in early lesions was 260, whereas the mean of the PC10 counts in late lesions was 632. The mean (SD) of the differences between the PC10 counts of early and late lesions was 371 (342). A hypothesis test of the paired data indicated a significant increase in PC10 counts (p = 0.003), with a corresponding 95% CI of 154–588.

**p53 IMMUNOREACTIVITY**

In four cases the conjunctival melanoma specimens contained some degree of detectable p53 immunoreactivity. In the early lesions these counts were 15, 25, 36, and 133 immunopositive cells per measured area as defined above. In the late lesions the comparative scores were 7, 45, 43, and six cells. The fractions of p53 immunoreactive cells to PC10 immunopositive cells in the late lesions were 1–4%, 1–8%, 5–5%, and 7–9%, respectively. In the late lesions of the four cases with p53 positive conjunctival melanoma the mean (SD) PC10 count was 546 (199), whereas the eight patients with immunonegative tumours had a mean corresponding PC10 count of 675 (405). This difference was not significant (p = 0.57; t test for independent data; 95% CI 357–615).

**Discussion**

Folberg et al demonstrated that 50% of atypical PAM give rise to melanoma, and that tumour progression was more common in the presence of epitheloid cells and in lesions with a predominant pattern other than basilar hyperplasia. Furthermore, these data suggest that the detection of proliferating melanocytes by staining with PC10 may be used to predict which lesions that are likely to undergo malignant transformation. In this study the median time interval between a confirmed diagnosis of atypical PAM and subsequent development of melanoma was three years; this interval was 2-5 years in a previous report. In the present study patients with atypical PAM who did not develop melanoma were followed for four years and it is unlikely that these patients will develop melanoma.

Most conjunctival melanomas with multiple recurrences ultimately require orbital exenteration, and may become more aggressive over time. As a high PC10 count in conjunctival melanomas correlates with an adverse prognosis, it is likely that the increase in PC10 counts over time in recurrent conjunctival melanoma reflects more aggressive behaviour. Four recurrences from two cases of conjunctival melanoma were available for study by immunohistochemistry. The limited data from these two tumours suggest that the PC10 count increases sharply at exenteration and while further studies are needed to elucidate the clinical importance of the increase in cell proliferation over time, PC10 counts may be used to monitor recurrent conjunctival melanoma. In the event of a significant increase in the proliferating cell count, aggressive therapy may have to be undertaken.

The epitope recognised by PC10 is stable for most fixation periods used for routine specimens. However, archival material may have been fixed for prolonged periods of time, which may result in a curtailed staining pattern. Recent data indicate that heating tissue sections in a microwave oven in an antigen retrieval solution containing heavy metal salts or distilled water results in consistent staining patterns in specimens fixed in formalin for up to 24 months. These findings were confirmed by our pilot study, using fixation periods of up to 14 days. We found that citrate buffer produced staining as satisfactory and reproducible as that obtained using a commercially available antigen retrieval solution (Biogenex).

In this study p53 positive and negative conjunctival melanomas had similar PC10 counts. This is in agreement with a study by Barbareschi et al who found that p53 expression is not associated with the percentage of PC10 labelled cells in neoplasms of the central nervous system. However, studies on oesophageal carcinomas and lung carcinomas suggest that in these tumours the p53 tumour suppressor gene may upregulate cell proliferation.

Data on the pattern of p53 expression in cutaneous malignant melanomas is limited and contradictory. Several studies indicate that many cutaneous melanomas and melanoma cell lines exhibit p53 immunoreactivity. By contrast, an additional report only observed immunoreactivity in 5% of 61 cutaneous malignant melanomas. Recently, Platz et al failed to detect p53 expression in cutaneous dysplastic naevi and only recognised immunoreactivity in 17% of invasive cutaneous melanomas.
These findings parallel those of the present study. Tobal et al.28 studied expression of p53 in freshly sampled ocular melanomas and found a positive staining pattern in 12 of 18 cases of choroidal melanoma. Exons 5 to 8 of the p53 gene of two of these melanomas were sequenced and point mutations were found at codons 238 and 253. A study of a family with the Li-Fraumeni syndrome also documented the presence of p53 immunoreactivity in two historical specimens of uveal melanoma.31 However, positive p53 immunostains should not be regarded as unequivocal evidence of p53 gene mutations. Although several studies have reported an excellent correlation between positive immunostains and missense mutations detected by DNA sequencing,32 33 some authors reported missense mutations in the p53 gene of tumours lacking p53 immunoreactivity.34 Others have been unable to detect mutations in cases with positive p53 immunostains.35

The findings presented here suggest that p53 alterations are not significant events in the tumorigenesis of conjunctival melanoma. Furthermore, if p53 mutation is indeed a significant event in the pathogenesis of conjunctival melanoma this would be expected to correlate with cell growth.36 The results of this study indicate that p53 positive conjunctival melanomas do not contain more proliferating cells than tumours completely lacking p53 immunoreactivity.

In conclusion, examination of the expression of proliferating cells in atypical PAM may be used as an adjunct to predict which lesions will progress to melanoma. The increase in the number of proliferating cells over time in recurrent conjunctival melanomas probably reflects more aggressive behaviour and may be used to monitor recurrence. The absence of p53 expression in PAM and minimal staining of conjunctival melanomas did not correlate with cell growth, suggesting that alterations in the p53 tumour suppressor gene are uncommon and late events in conjunctival melanoma, and that p53 expression is unlikely to be a useful prognostic indicator.

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36 Kennedy SM, MacGeogh C, Jaffe R, Spruck CH, et al. Overexpression of the oncprotein p53 in primary hepatic tumors of childhood does not correlate with gene muta-