PC-10 as a Predictor of Prognosis After Antigen Retrieval in Posterior Uveal Melanoma

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Purpose. The immunoexpression of the PC-10 monoclonal antibody for the proliferating cell nuclear antigen is claimed to have prognostic value in diverse tumors, but previous data on posterior uveal melanoma are conflicting. The aim of the current study was to investigate further the potential value of the PC-10 antibody in predicting tumor-related death after enucleation for posterior uveal melanoma.

Methods. One observer calculated the number of cells after antigen retrieval that showed immunoreactivity for PC-10 in the high expression areas of 212 specimens containing posterior uveal melanomas. Survival data for all patients were entered into stepwise multivariate Cox regressions that included other potential prognostic covariates. The prognostic accuracy was assessed by receiver operating characteristic curve analysis.

Results. The only covariates of statistically significant prognostic value were the number of cells featuring immunoreactivity for PC-10 and the largest tumor diameter. When using the median PC-10 count as the cutoff, the cumulative 10-year survival proportion was 84% for the low PC-10 count group and 40% for patients harboring tumors with high PC-10 counts. Those with tumors featuring high PC-10 counts had a 5.8 times greater risk to die of metastatic melanoma. However, the prognostic accuracy of the PC-10 count was not significantly better than that of the largest tumor diameter, presumably because of insufficient statistical power.

Conclusions. The number of cells showing immunoreactivity for the PC-10 antibody may be used to assess prognosis in posterior uveal melanoma, provided that antigen retrieval is performed. Additional work using a larger sample size is warranted for better comparison of the predictive accuracy with that of other prognostic markers. Invest Ophthalmol Vis Sci. 1996;37:1451-1458.

Posterior uveal melanoma is the most common primary intraocular malignancy, with a long-term, tumor-related mortality rate of 40% to 50%. Often, parameters that may be assessed clinically, such as the largest tumor dimension, are used to predict outcome. However, when tissue is available for histopathologic examination, the cell type may be graded according to the modified Callender classification. Although cytologic features appear to carry prognostic information, the subjective Callender grading system suffers from insufficient reproducibility. Various cytomorphometric measurements have been introduced to provide more objective assessments, and a simplified technique based on the mean of the 10 largest nucleoli appears to correlate with prognosis. Furthermore, other workers recently correlated the intratumoral vascular pattern with outcome; however, they also challenged the prognostic value of cytomorphometry. Cell proliferation is a basic concept of most biologic processes and may be of interest in a diversity of conditions, including tumors and inflammatory disease. In neoplasia, a general concept has evolved that the rate of growth of any tumor will reflect its subsequent behavior pattern. Although the actual proliferative rate is difficult to assess in vivo, diverse techniques may be used to study the size of the proliferative compartment. Mitosis is the only phase in the cell cycle that may be identified by a simple morphologic examination, and mitotic counts consequently are convenient for a gross examination of the proliferative
compartment. Indeed, mitotic activity has been correlated with adverse prognosis in uveal melanoma, suggesting that cell proliferation is important\(^{11,12}\), recognizing mitotic figures, however, may be difficult, and several reports emphasize the poor intraobserver reproducibility.\(^ {13-15}\) In addition, mitotic cells represent a minority of cycling cells, and, by assessing only mitotic cells, the proliferative compartment is likely to be underestimated. However, cell proliferation antibodies that detect a larger proportion of cycling cells are now available for the study of archival tissue, and antibodies that recognize the Ki-67 antigen have been used to detect the proliferative compartment in posterior uveal melanomas of eyes enucleated after external beam radiotherapy.\(^ {16}\) Similarly, the PC-10 monoclonal antibody has been applied to study cycling cells in uveal melanomas of eyes enucleated after failed plaque radiotherapy, and 3 of 4 tumors that caused metastatic death were found to have high PC-10 scores.\(^ {17}\) However, in posterior uveal melanoma, the prognostic value of both these markers remains unclear. The current study was designed to evaluate the potential benefit of the PC-10 antibody when predicting outcome of patients enucleated for posterior uveal melanoma.

**METHODS**

The specimens used for this study were from 340 paraffin blocks of consecutively obtained globes with melanomas derived from the choroid or ciliary body. All globes initially were examined and sectioned by one pathologist, and care was taken to include the largest dimension of each tumor in the line of sectioning. Recently, these specimens were reviewed and reclassified according to the modified Callender classification.\(^ {2}\) The 340 patients did not undergo any treatment other than enucleation, and no patient was lost to follow-up.\(^ {1}\)

Thirty-two paraffin blocks no longer contained tissue for sections to be made, but two sections were cut at 4 μm from each of the remaining 308 paraffin blocks. The tissue sections were deparaffinized, rehydrated, and blocked with endogenous peroxidase with H\(_2\)O\(_2\) for 30 minutes. One tissue slide of each specimen was then treated with microwave heating at 780 W for 5 + 5 minutes in a microwave oven, operating at 2450 MHz (Miele, Gutersloh, Germany). During microwave processing, the tissue slides were covered by an antigen retrieval solution of 0.01 M citrate buffer, pH 6.\(^ {18}\) The remaining section of each specimen was left untreated. Both tissue slides of all specimens were then covered with nonimmune serum for 30 minutes and incubated overnight with the monoclonal PC-10 antibody in a working dilution of 1:20 (Dakopatts, Glostrup, Denmark). The next morning, tissue slides were incubated with a secondary antibody. Finally, all sections were treated with avidin-biotin complexes for 30 minutes, stained with 3-amin-9-ethylcarbazole, and counterstained with Mayer's hematoxylin. Sections were not bleached. Positive controls were obtained from sections cut from paraffin blocks of a colorectal carcinoma with a PC-10 labeling index of 40% to 50%. Before paraffin embedding, three small tissue pieces had been allowed to fix in formaldehyde for periods of 2, 7, and 14 days, respectively. Each positive control included three tissue sections fixed for these periods of time. Negative controls were provided by using a nonimmune serum.

After tissue processing, all slides were examined by a Zeiss Axioscope microscope (Carl Zeiss, Oberkochen, Germany) equipped with a 10 × 10 square eyepiece graticule. All cells containing distinct nuclear immunoreactivity were considered positive irrespective of staining intensity. Microwave-processed tissue provided the overall strongest immunoreactivity, and in some pairs only the microwave-heated section showed detectable staining. Only the microwave-processed sections were used for further assessment, and, in these sections, the immunopositive cells of 10 high-power fields of 0.0625 square mm each from areas of maximum immunoreactivity were counted manually by one observer. Ninety-six specimens completely lacked immunoreactivity for the PC-10 antibody, even after antigen retrieval. Some of these tumors were largely necrotic, whereas others were assumed to be overfixed inadvertently by formaldehyde. Consequently, all these specimens were excluded from further study. At a later stage, and without knowledge of the initial result, the same observer repeated the counts of 30 randomly selected sections. The observer did not have access to survival data during the counting process.

The tenets of the Declaration of Helsinki were followed, and adequate survival data without loss to follow-up were obtained for all patients from the Swedish National Causes of Death Registry. The survival time from the date of enucleation to death or to the end of 1991 was considered censored if the patient was alive at the end of the study or if the patient had died of any cause that was not melanoma related. Multivariate proportional hazard regressions were modeled based on the backward stepwise elimination of variables with a significance of 0.1 or less. In view of the repeated hypothesis testing, the significance level was set at 0.01. Finally, the prognostic accuracy of the PC-10 count was compared to that of the largest tumor diameter using receiver operator characteristic (ROC) curve analysis. For this part of the study, only data from patients who died of tumor or who were alive at the 10-year follow-up were accessed. The assumption that patients who are alive at a 10-year fol-
FIGURE 1. Microphotographs of posterior uveal melanomas featuring only a few cells with PC-10 immunoreactivity (A) and extensive PC-10 positive immunostaining (B). The staining intensity is weaker in mitotic cells (arrow). Bar = 50 μm.

low-up are unlikely to die of metastatic melanoma allowed for sensitivity and specificity calculations and for the construction of ROC curves. The areas under the ROC curves were then calculated using Wilcoxon statistics based on pairwise comparisons. In comparing the respective areas, correction was made for the between-area correlation. Calculations were computer based, using CSS:STATISTICA (StatSoft, Tulsa, OK) and Statgraphics 5.0 (STSC, Rockville, MD) as software packages.

RESULTS
At the time of enucleation, the median age of the 212 patients was 64 years (range, 23 to 87 years; 10th to 90th percentile, 47 to 77 years). Ninety-nine patients were women, and 113 were men. Tumors were from the ciliary body in 17 patients, the anterior choroid in 73 patients, and the posterior choroid in 122 patients. Invasion was smaller than one third of scleral thickness in 107 tumors and larger than one third of scleral thickness but intrasclerally confined in 67 tumors, and it included extrascleral extension in 38 tumors. Melanoma cells were classified as spindle cells in 123 tumors and as epithelioid cells in 45 tumors. Thirty-seven tumors featured a mixed-cell pattern, whereas seven tumors were necrotic. The largest tumor diameter ranged from 5 to 23 mm (median, 11 mm; 10th to 90th percentile, 8 to 15 mm). The maximum tumor height ranged from 1 to 20 mm (median, 6 mm; 10th to 90th percentile, 3 to 10 mm). Follow-up periods ranged from 6 to 22 years. During follow-up, 71 patients (33.5%) died of metastatic disease whereas 141 patients (66.5%) either died of other causes or were alive at the end of study. Melanoma-related deaths occurred from day 101 to day 6848 (18.1 years) after enucleation. The 5-year cumulative survival proportion based on melanoma-related deaths only was 76%, and the corresponding 10-year survival proportion was 62%.

Positive PC-10 controls retained an acceptable staining pattern for a maximum of 7 days of formaldehyde fixation without microwave processing, which increased to a maximum of 14 days after antigen retrieval preparation that included microwave heating. In positive controls and in sections included in the study, most tumor cells displaying immunoreactivity for the PC-10 antibody revealed an intense granular staining of the nuclei. However, a proportion of cells also displayed a weaker staining pattern, in particular mitotic cells. In the sections included in the study, extreme variations in the staining pattern between various tumors were noted (Fig. 1). Tumors with a large proportion of immunopositive cells also showed a considerable intratumor heterogeneity. In particular, areas in the tumor base and center showed fewer im-
multivariate analysis, only the PC-10 counts, the largest tumor with substantial changes of the covariate values. By univariate analysis, only the PC-10 counts, the largest tumor with substantial changes of the covariate values. By univariate analysis, only the PC-10 counts, the largest tumor with substantial changes of the covariate values.

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Scoring</th>
<th>P Value</th>
<th>Hazard Ratio</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-10 count</td>
<td>Quartiles; cutoff = 34, 78, 176 cells</td>
<td>$&lt;10^{-10}$</td>
<td>2.38</td>
<td>2.13–2.63</td>
</tr>
<tr>
<td>Tumor diameter</td>
<td>Quartiles; cutoff = 9, 11, 13 mm</td>
<td>$&lt;10^{-5}$</td>
<td>1.75</td>
<td>1.52–1.98</td>
</tr>
</tbody>
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**DISCUSSION**

The PC-10 monoclonal antibody detects an epitope on the proliferating cell nuclear antigen, a 36-kDa...
nuclear protein associated with the cell cycle. The concentration of the proliferating cell nuclear antigen increases during the S-phase of the cell cycle, and flow cytometry studies confirm that the PC-10 clone may be used as an S-phase marker. This is supported by work using bromodeoxyuridine labeling, indicating that the PC-10 clone may be used as an operational marker for the growth fraction of neoplastic rat tissue. PC-10 immunoreactivity has been detected in diverse human tumors, and recently the number of cells staining for the PC-10 antibody was shown to have prognostic significance in conjunctival melanomas. Similarly, another clone, which also recognizes the proliferating cell nuclear antigen, appears to provide a useful marker for the progression of skin melanomas. In diverse tumors of nonmelanocytic lineage, PC-10 immunostaining appears to carry prognostic in-

![Figure 3](image1.png)

**FIGURE 3.** The respective cumulative survival proportion for the two groups of patients featuring tumors with PC-10 counts below and above the median value. Death by any cause other than metastatic melanoma was considered a censored event.

![Figure 4](image2.png)

**FIGURE 4.** The receiver operating characteristic (ROC) curves for the PC-10 count and the largest tumor diameter. The accuracy of a parameter may be estimated by the area under the ROC curve, and, as a comparison, chance alone will provide an area of 0.5. The area under the PC-10 count ROC curve is 0.78 (standard error = 0.0424), whereas the largest tumor diameter provides an area of 0.70 (standard error = 0.0484). This suggests that the PC-10 count has a slightly better prognostic accuracy; however, the difference between the two areas is not statistically significant ($P = 0.12$).

**TABLE 3.** Prognostic Accuracy Assessment* of PC-10 Counts† When the Respective Tenth Percentile is Used as the Cutoff

<table>
<thead>
<tr>
<th>Cutoff (Percentile)</th>
<th>PC-10 Count</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>10th</td>
<td>18</td>
<td>1.00</td>
<td>0.17</td>
</tr>
<tr>
<td>20th</td>
<td>29</td>
<td>0.93</td>
<td>0.30</td>
</tr>
<tr>
<td>30th</td>
<td>38</td>
<td>0.89</td>
<td>0.40</td>
</tr>
<tr>
<td>40th</td>
<td>53</td>
<td>0.84</td>
<td>0.51</td>
</tr>
<tr>
<td>50th</td>
<td>78</td>
<td>0.80</td>
<td>0.65</td>
</tr>
<tr>
<td>60th</td>
<td>104</td>
<td>0.70</td>
<td>0.73</td>
</tr>
<tr>
<td>70th</td>
<td>148</td>
<td>0.57</td>
<td>0.82</td>
</tr>
<tr>
<td>80th</td>
<td>191</td>
<td>0.39</td>
<td>0.90</td>
</tr>
<tr>
<td>90th</td>
<td>367</td>
<td>0.27</td>
<td>0.97</td>
</tr>
</tbody>
</table>

* Based on 132 patients that either died of tumor or were alive at a follow-up of 10 years.
† The PC-10 counts are calculated for 10 high power fields of maximum expression.

In contrast, other reports claim that PC-10 labeling indices have poor prognostic value in gastric carcinomas and breast carcinomas. Also, one recent preliminary study on formaldehyde-fixed, paraffin-embedded tissue from uveal melanoma failed to correlate PC-10 immunolabeling with prognosis, whereas another established an inverse relationship between the PC-10 immunostaining of the primary tumor and the survival times of patients who were not cured. We think these conflicting results depend, in part, on different techniques for tissue sampling, on the use of labeling indices rather than the total number of cells per square unit, and on fixation artifacts that may be alleviated by microwave processing.

In our experience, PC-10 immunostaining is prone to considerable intratumoral heterogeneity. Assuming the proliferative compartments are responsible for tumor growth and possibly for metastatic spread, it seems logical to restrict evaluation to these areas. Possibly, such subpopulations carry more meaningful information than crude assays that use all cells in the sample as the denominator. In contrast, some argue that the assessed areas should include both those with the highest and those with the lowest per-
The potential role of fixation artifacts will be addressed in brief. Formaldehyde is the common fixative for histopathologic specimens, but it causes radical changes in the molecular organization of cells and tissues, any or all of which may affect detrimentally the accessibility or structure of the epitopes that antibodies recognize on their antigens. Microwave-based techniques were devised for histologic fixation, but subsequent findings indicated that microwave processing provides excellent staining results by some antibodies that usually are unreactive with tissue fixed by formaldehyde. Although it appears that the PC-10 antibody is superior to others in detecting epitopes on the proliferating cell nuclear antigen, microwave processing results in a considerably enhanced staining pattern for PC-10 by the "unmasking of antigens." Obviously, this is of great importance because fixation time rarely may be controlled retrospectively. In our previous work on conjunctival melanoma, fixation time was not a major problem, but in the current study, PC-10 immunoreactivity was not revealed before antigen retrieval processing in the majority of specimens. Even after microwave treatment, a substantial proportion of processed slides did not feature a single PC-10 immunopositive cell. Some of these tumors were largely necrotic, but histopathologic examination showed others to be viable. From the early to mid 1970s, these apparently viable, but negative, immunostaining tumors almost exclusively arrived at the laboratory during the summer. In those days, globes would have remained immersed in formaldehyde during summertime vacations. Thus, some of these specimens may have been fixed for as many as five weeks, a period presumably long enough to abolish completely any trace of PC-10 immunoreactivity.

Any histologic section from the center of a uveal melanoma includes a large number of cells, and it is highly probable that any such section would feature at least some cells that entered the cell cycle. Consequently, a complete absence of immunoreexpression probably depends on overfixation of the specimen or on large necrotic areas. For this reason, tumors that completely lacked immunoreexpression, even after microwave processing, were excluded from the current study. Indeed, a large proportion of excluded melanomas showed necrotic tumor areas. Similarly, previous workers using other techniques, had to discard a large proportion of necrotic tumors. Using the same criteria, our total rejection rate of 128 of 340 specimens (38%) compares with the 147 of 381 specimens (39%) excluded by Folberg and associates for a number of reasons. Although it is recognized that patients with necrotic tumors are more likely to die of metastatic disease than are other patients harboring posterior uveal melanomas, the current method, like others, unfortunately appears less suited for patients with this particular type of tumor. Still, the hazard ratio calculated in the current study suggests that patients with tumors featuring PC-10 counts above the median value have a 5.8 times greater risk of dying of metastatic melanoma than patients with tumors with lower PC-10 counts. To the best of our knowledge, this relative risk is among the highest ever recorded for a subset of patients with posterior uveal melanoma.

The ROC curve may be used to test the accuracy of diagnostic or prognostic systems that are based on continuous parameters, and the area under the ROC curve measures the goodness of the model. Values between 0.50 and 0.70 are considered to represent a low accuracy, whereas values of more than 0.90 suggest a high accuracy. Thus, the current data indicate that the largest tumor diameter has a low predictive accuracy, whereas the PC-10 count is moderately accurate in predicting outcome (Fig. 4). However, the difference between the areas under the ROC curves failed to reach statistical significance, presumably because of the small sample. In fact, to detect a similar, statistically significant difference with a power of 80% would require a minimum of 286 patients who died of tumor-related and nontumor-related causes. Although it appears that the prognostic accuracy of either the largest tumor diameter or the PC-10 count is far less than desirable, the ROC areas of these two parameters compare with those recently reported for two prognostic models predicting survival in cutaneous melanoma.

Immunohistochemistry is a routine procedure available in nearly all surgical pathology laboratories, and antigen retrieval techniques are no more difficult to master than immunostaining. Furthermore, the facilities needed are not considerably expensive, and prognostic immunostaining for the proliferating cell nuclear antigen could be introduced easily in the diagnostic routine. Possibly, formal counting may not be necessary because the slide could be scanned at low power, and the number of cells could be scored as
few or many. Care should be taken not to include necrotic tumors or potentially overfixed tissue that completely lack immunoreactivity for the PC-10 marker. In this context, microwave processing to enhance the pattern of immunoreactivity appears to constitute an essential part of the technique elaborated in the current study. However, additional work using a larger sample size is warranted to increase statistical power when comparing the current technique with other prognostic indicators.

Key Words
PC-10 antibody, posterior uveal melanoma, predictive accuracy, prognosis, receiver operator characteristic (ROC) curve

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