Comparative Analysis of Proliferating Cell Nuclear Antigen, Bromodeoxyuridine, and Mitotic Index in Uveal Melanoma

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Purpose. Recent production of a monoclonal antibody, PC10, against proliferating cell nuclear antigen (PCNA) makes it possible to evaluate cell cycling in formalin-fixed tissues. In this study, the authors quantitatively evaluated the relationship between PCNA expression and two other measures of cell cycling, bromodeoxyuridine labeling index (BrdU LI) and mitotic index (MI), in archival uveal melanomas. The authors also examined the relative prognostic importance of each measure.

Methods. Serial sections from 35 formalin-fixed, paraffin-embedded uveal melanomas were immunostained with PC10 and BrdU antibody using a standard avidin-biotin-peroxidase method. A quantitative scoring system was used to evaluate the fraction of cells that were positive for PCNA, BrdU, and mitotic figures in the regions of high cycling. The LIs of the different markers were compared, and their prognostic importance was evaluated.

Results. The median PCNA LI was 3.05% compared to the median BrdU LI of 0.94% and the median MI of 0.034%. The PCNA LI was more variable in replicate sections than either the MI or the BrdU LI. The correlation between PCNA LI and BrdU LI was 0.58, between PCNA LI and MI it was 0.46, and between BrdU LI and MI it was 0.81. The relative risk of tumor-related mortality per doubling of BrdU LI was 2.35, and of MI it was 2.34. Although these were significant, PCNA LI of 1.08 was not.

Conclusions. Proliferating cell nuclear antigen immunostaining did not demonstrate a strong relationship with either BrdU LI or MI. Unlike MI and BrdU LI, PCNA LI was not correlated with tumor-related mortality. Caution is warranted in the interpretation of PCNA immunostaining in uveal melanomas. Invest Ophthalmol Vis Sci. 1995;36:2762–2767.

The cell proliferation rate of solid tumors is an important parameter for survival, choice of optimum therapy, and treatment response.1–4 In uveal melanoma, we previously have used both mitotic counts in the area of highest tumor proliferation and detection of in vivo incorporated BrdU with appropriate monoclonal antibody to measure tumor cell proliferation.1 We have noted that although both of these techniques correlate well, the latter is a more sensitive method to detect low levels of cycling.5 The rate of tumor cell proliferation of uveal melanoma has been shown to be predictive of survival and of local tumor response.1,6

Several techniques, including immunohistologic assessment of tumor cell proliferation, can be used to measure tumor cell growth rates. Immunostaining for intrinsic cellular proliferation markers represents a straightforward, simple technique that can be performed routinely on formalin-fixed, paraffin-embedded specimens and allows simultaneous assessment of standard morphology.

Proliferating cell nuclear antigen (PCNA), a 36-kDa nuclear protein that functions as an auxiliary protein to DNA polymerase delta, is a marker of cell proliferation.7 Elevated nuclear levels of this protein are maximum during late G1 and S-phase.8 Several antibodies recognizing PCNA, including PC10, are available commercially and are reactive in formalin-fixed, paraffin-embedded tissues.9 In contrast to BrdU label-
ing, PCNA studies do not require in vivo administration of the thymidine analog, and they have been described as a sensitive technique for the assessment of tumor cell proliferation in archival specimens. 10

In this study, we assessed, in a masked manner, a cohort of uveal melanomas with known outcome using PCNA, BrdU, and mitosis counts to determine the reproducibility of each proliferation measure, their relationship with one another, and their association with patient survival.

MATERIALS AND METHODS

Sample Selection
All 35 study patients with uveal melanoma underwent primary enucleation between December 1985 and March 1992. Each patient received a 15-minute intravenous infusion of BrdU (200 mg/m²) 1 hour before tumor removal. Informed consent was obtained after an explanation of the nature and possible consequences of the study, which adhered the tenets of the Declaration of Helsinki. Permission to administer BrdU was obtained from the Human Experimentation Committee at the University of California, San Francisco and from the National Cancer Institute. Follow-up was available on all 35 patients through August 1994. Tumors selected were either amelanotic or lightly pigmented because bleaching tumors with more melanin had a negative effect on specific staining. Heavily pigmented tumors, patients receiving any form of radiotherapy, and tumors with inadequate representative sections were excluded from the study. One patient in whom mitotic and BrdU indices were calculated was excluded because of insufficient specimen for PCNA labeling.

The median largest tumor diameter of these melanomas was 17 mm (range, 6 to 24 mm), and the median height determined from A-scan ultrasonography was 11.5 mm (range, 3.3 to 20 mm). The median patient age was 61 years (range, 23 to 83 years). Histologically, the melanoma cell type was spindle in six patients, predominantly spindle in 12 patients, mixed in 16 patients, and epithelioid in 1 patient. Tumors in 12 of 35 patients involved the ciliary body, and in 5 patients there was extracocular extension. Metastases were detected in 15 patients, with the median time to detection 2.6 years (range, 0.1 to 6.3 years). The overall median follow-up was 3.4 years; 15 of 20 patients without detectable metastases were followed for at least 3 years.

Immunohistochemistry
After enucleation, specimens were placed in 10% buffered formalin and processed in a standard manner in the eye pathology laboratory. Contiguous 10-μm serial sections were obtained. Sections were stained with hematoxylin and eosin, BrdU, and PC10 in duplicate.

Immunohistochemical detection of PCNA and BrdU was performed using a standard avidin–biotin–peroxidase method. 11 Briefly, sections were deparaffinized by passage through xylene and graded alcohols. After blocking for at least 8 hours in 10% horse serum at 4°C, anti-PCNA monoclonal antibody (Boehringer Mannheim, Indianapolis, IN) was applied in a dilution of 1:200. At this concentration, overnight incubation at 4°C gave consistent nuclear immunostaining patterns across replicate sections. A secondary antibody, biotinylated anti-mouse immunoglobulin G (Vector Laboratories, Burlingame, CA) was applied in a dilution of 1:200. Endogenous peroxidases were quenched with 3% hydrogen peroxide for 30 minutes. Proliferating cell nuclear antigen was detected using an avidin–biotin–complex method (ABC Elite, Vector) and Vector Violet (Vector) as the chromogen. Negative controls were established by staining normal pineal gland and substituting mouse immunoglobulin for primary antibody in the protocol.

Immunohistochemical detection of BrdU was performed as previously described. 1 Briefly, slides were treated with 2 N HCl for 60 minutes at room temperature, followed by treatment with 0.1% protease (Sigma, St. Louis, MO) before they were placed in blocking serum. Anti-BrdU monoclonal antibody (Cal-tag, San Francisco, CA) was applied to sections in a dilution of 1:2500 and incubated for 45 minutes at room temperature. Detection of antigen was performed as described above.

Cell Counting
All slides were read in a masked manner. The staining pattern of PCNA antigen and the BrdU labeled cells showed considerable regional variation within tumors. Diagrams of each tumor were drawn from histopathologic sections. The region of highest activity was determined by scanning full tumor sections for the intensity of BrdU staining and mitotic figure presence. The area of highest cycling activity was noted on each sketch. Counting was begun in one corner of the predetermined area of highest cycling, and 20 high-power fields (×400) were counted. The number of positive cells in each field was recorded for analysis. All cells displaying nuclear PCNA staining (light or heavy) were counted as positive. Similarly, all cells displaying nuclear BrdU staining were counted as positive. The mitotic index was determined by counting mitotic figures according to criteria of absence of a nuclear membrane and presence of hairy projections of nuclear material.

Uveal melanomas demonstrate heterogeneity of cell size across tumors. To control for variations in the
number of cells per high-power field, cell densities were estimated from five high-power fields in the representative fields from the same area of highest activity on the hematoxylin and eosin sections. This was used to calculate the percent of tumor cells positive for each of the proliferation markers as opposed to indices per millimeters. The PCNA and BrdU LIs and MI were calculated as the percent of positive cells.

**Statistical Techniques**

Each of the proliferation markers was transformed to a log scale. This transformation symmetrized the distribution of each marker across tumors. The univariate distributions each appeared to be log normally distributed (see Figs. 1 to 3). Parametric and nonparametric correlation measures were used. The effect of each cell proliferation marker on tumor-related survival was analyzed using univariate and multivariate Cox proportional hazards models. Multivariate models included patient age, tumor involvement of the ciliary body, cell type, largest tumor diameter, and the respective cell proliferation measures.

**RESULTS**

Multiple observations from sections taken within 0.3 mm of each other were separately counted for mitoses, BrdU uptake, and PCNA labeling. The PCNA index demonstrated the greatest variability, defined as the standard deviation of counts divided by the mean of the counts across tumor sections (Table 1). The variability in mitotic counts was caused by the low prevalence of mitosis. Similarly, variability in BrdU counts was infrequent and usually was related to occasional low counts. Although a similar pattern was evident in the PCNA counts, this was not always the case. TriPLICATE PCNA observations with two sections stained on the same day and one section stained on another day were obtained in 13 tumors. The ratio of the relative within-day variance to the relative across-day variance was equal to 0.37. Thus, the variability across batches studied on different days was greater than those on the same day. However, some tumors with multiple measurements on the same day also demonstrated excessive variability. To minimize the effects of outliers, the median value of each cycling measure was used for all analyses.

The median MI was 0.034% (interquartile range [IQR], 0.018% to 0.071%). The median BrdU LI was 0.99% (IQR, 0.98% to 1.80%). The median PCNA index was 3.05% (IQR, 1.45% to 4.80%). Bivariate plots of each of the three paired cycling measures are given in Figures 1 to 3. All plots exclude one tumor that had both BrdU and PCNA indices less than 0.01%. The BrdU and mitosis measurements correlated better than either of the PCNA correlations. On average, the BrdU LI was approximately 25 times larger than the MI. Therefore, the length of S-phase
is approximately 25 times longer than mitosis. The plot of PCNA with BrdU had slightly more scatter, and the PCNA with mitosis plot had the poorest fit. High PCNA indices in low mitotic tumors and low PCNA indices in high mitotic tumors were present. In general, however, PCNA staining could be used to delineate the intratumor area of highest cell proliferation.

Univariate and multivariate results of each of the cycling measures are given in Table 2. The multivariate results include adjustment for tumor cell type, ciliary body involvement, patient age, and largest tumor diameter. Each of these is a known prognostic factor in uveal melanoma. Although both the MI and the BrdU LI were significant prognostic factors of tumor-related survival, the PCNA index was not prognostic in either univariate or multivariate analyses.

In light of the prognostic importance of both the MI and the BrdU LI, as well as the moderate correlation between the PCNA index and each of the other indices, we explored the failure for PCNA to have prognostic importance. Patients whose tumors metastasized were highlighted in each of the bivariate plots. Although the length of follow-up is ignored, high ratios of PCNA to either mitosis or BrdU were associated with better outcomes. Similarly, low ratios of PCNA to either BrdU or mitosis were associated with poorer outcome. Inclusion of PCNA into a multivariate model with either the BrdU LI or the MI resulted in better prognosis with higher values of the PCNA index. The latter prognostic results with PCNA were not statistically significant.

**DISCUSSION**

Although PC10 immunoreactivity has been shown to correlate with BrdU labeling and with mitosis in several tumors, our results demonstrated distinct discordant features. In contrast to measurements of either BrdU or mitosis, PCNA did not correlate with prognosis in uveal melanoma. We have noted previously that BrdU LI and MI in areas of highest cycling activity within uveal melanoma correlated strongly with outcome. We did not find PCNA LI to be a good quantitative measure of cell cycling or outcome compared to either BrdU LI or MI. Evans and coworkers found a similar paradoxical correlation between high PCNA staining and good outcome in cutaneous melanomas. Woosley and colleagues noted that PCNA LI in cutaneous malignant melanomas had no prognostic significance, whereas MI in the regions of high activity did. Davidsson and coauthors also found that PCNA was not of prognostic importance in malignant melanoma of the ear. Similar findings have been reported in other tumors.

The reasons for the disparity between these several cell cycling measures and prognosis is uncertain. Both the MI and the BrdU LI are prevalence measures. Each represents the percent of cells in a specific phase of the cell cycle (DNA synthesis phase or mitosis) at a specific point in time. The PCNA index is not...
were smaller paired stained nuclei in addition to were in S-phase at the time of fixation. Hence, in some between the PCNA index and the cell proliferation factors may play a role in the accumulation of PCNA activity in nonneoplastic tissues is greatly reduced after 48 hours of fixation in formalin and virtually is abolished after 72 hours. In our study, fixation times were approximately 24 hours; it is unlikely that variable fixation intervals were an important factor in this study.

BrdU uptake shows distinct, reproducible, nuclear immunolabeling across serial sections, whereas PCNA immunostaining had a greater staining variability across serial sections. This variability in PCNA intratumor staining intensity may have resulted in greater subjective interpretation of positive cells. Because PC10 antibody dilution was controlled, it is doubtful that this parameter was responsible for the variable staining, as suggested.

Several other investigations have shown potential problems with PCNA data. Proliferating cell nuclear antigen staining was shown to overestimate the growth fraction of LoVo cell line xenografts when compared to mitotic counts. Coltara and Gown observed that PCNA staining defined different cell subpopulations compared to those that either were incorporating BrdU or were Ki-67 positive. Finally, Hall and coauthors noted that some neoplasias displayed deregulated PCNA expression not correlated with cell kinetics.

In conclusion, PCNA immunostaining did not appear closely related either to the S-phase fraction or to the Mi, and it was not an important prognostic factor in uveal melanoma. This is possibly the result of the complex nature of PCNA and its half-life. In view of the myriad factors known to influence PCNA labeling, caution is warranted in its interpretation. Proliferating cell nuclear antigen staining was useful, however, for purposes of rapidly identifying regions of high tumor cell proliferation. Further studies are warranted to elucidate the paradoxic correlation between PCNA staining and outcome.

**Key Words**

bromodeoxyuridine (BrdU), mitotic index, prognosis, proliferating cell nuclear antigen, uveal melanoma

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**TABLE 2. Summary of Cox Model Analyses of Cell Cycling Measures**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Scale</th>
<th>Relative Risk</th>
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<td></td>
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<td>Mitotic index</td>
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<td>(1.36, 4.55)</td>
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<td>BrdU labeling index</td>
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<td>(1.16, 3.44)</td>
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<td>PCNA index</td>
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<td><strong>Multivariate Analyses</strong></td>
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<tr>
<td>Mitotic index</td>
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<td>(1.31, 4.18)</td>
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<td>BrdU labeling index</td>
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* Multivariate models each included largest tumor diameter, patient age, and indicator variables for mixed-cell type and involvement of the ciliary body.
References


