Cell Cycling and Prognosis in Uveal Melanoma

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ABSTRACT

Uveal melanoma cell cycling, quantified by bromodeoxyuridine (BrdUrd)-labeling index and mitotic index, is predictive of tumor-related mortality. Serial sections from 36 formalin-fixed melanoma specimens were labeled with BrdUrd and stained with hematoxylin and eosin. All tumors were assessed for the area of highest cell cycling activity and counts for mitotic figures and BrdUrd labeling were performed in these areas in a masked manner. The BrdUrd labeling index and mitotic index were calculated and analyzed in relation to tumor-related mortality and histopathological criteria (largest tumor diameter, cell type, extracoidal extension, ocular location). Cox multivariate analysis estimated an increased relative risk of tumor-related mortality of 2.32 (95% confidence interval, 1.22-4.41) per doubling of BrdUrd labeling index and 2.41 (95% confidence interval, 1.29-4.49) per doubling mitotic index. Larger tumors, nonspindle cell tumors, and anterior-located tumors tended to have higher cycling rates.

INTRODUCTION

The prognostic importance of variations in uveal melanoma cell cycling is uncertain. We and others have noted that the paucity of tumor cells are actively cycling in this malignancy (1). In previous studies with BrdUrd, a thymidine analogue incorporated into the melanoma nucleus only during DNA synthesis, <2% of uveal melanoma cells were in that phase of the DNA cell cycle (1). In comparative studies, we noted that this technique of enumerating actively cycling tumor cells was much more sensitive than counts of mitosis per HPF to detect cells with intact reproductive integrity (2).

The effect of cell cycling rates on metastases-free survival in uveal melanoma has not been well characterized. The majority of survival studies have not included any cell cycling measures. In these studies other factors shown to correlate with length of metastases-free survival include cell type, the number of epithelioid cells per HPF, tumor size, extracoidal extension, location of the anterior margin of the tumor, morphometric measures of nuclear or nucleolar size and variability, intratumor vascular pattern, and degree of pigmentation (3-12).

Some studies have shown that mitotic activity is a prognostic factor in uveal melanoma. By using linear discriminant analysis, the four best factors for predicting metastases were cell type, largest tumor diameter, scleral extension, and mitotic activity (13). Multivariate Cox model results have shown various ordered combinations of prognostic factors dependent on the selection of the patient population, prognostic variables included, and techniques of quantifying histopathological measures. Seddon et al. (14) found that the five best factors in their model included the number of epithelioid cells per HPF, largest tumor diameter, location of anterior margin, scleral extension, and pigmentation. Although the intratumor mitotic rate was a significant univariate predictor, it was not found to be a leading prognostic factor in multivariate analysis (14, 15). Folberg et al. (12) found the best stepwise model for their series of 234 patients with uveal melanoma involved vascular patterns, largest tumor diameter, mitotic rate, age, lymphocytic infiltration, and sex. Conditioning on their seven-factor model, mitotic rate was the most significant prognostic factor; however, surprisingly, 52% of the tumors had not mitoses noted (12).

BrdUrd labeling is a more sensitive technique to elucidate cycling cells than enumeration of the MI, and it has been our impression that patients with actively cycling tumors had a poor prognosis. We hypothesized that a stronger prognostic correlation might be present using BrdUrd labeling in uveal melanoma than previously observed with mitosis. Poorer prognosis in tumors with higher LIs has been found in primary cutaneous melanoma and cutaneous melanoma metastases (16, 17). In breast cancer, time to metastatic onset was shorter in the highest labeled group (18, 19). Poorer prognosis with the higher LI is also present in astrocytic tumors (20, 21).

The historical approach to assess cell cycling has been to count random or generalized mitoses per HPFs without considering the most active area of cycling. In human prostate adenoma, breast carcinoma, and pulmonary adenocarcinoma, BrdUrd labeling varied in different tumor areas (22-24). In gastric malignancies higher tumor cell labeling was noted at the site of invasion than in the central area of the tumor (25). It is likely that assessment of tumor cycling in the area of highest activity might have a stronger association with tumor-related mortality than shown previously.

In this study we have assessed, in the areas of highest cycling within a tumor defined by BrdUrd uptake, the association between BrdUrd LI, MI, and tumor-related mortality. We note a strong correlation between cell cycling and uveal melanoma-associated metastases.
MATERIALS AND METHODS

Experimental Design. Written and oral informed consent was obtained from all patients. Approximately 1 h before tumor removal all patients received a 15-min i.v. infusion of 200 mg/m² BrdUrd. All uveal melanomas were treated with primary enucleation, by the same surgeon, between December 1985 and March 1992. Follow-up was available on all 36 patients through August 1993. Tumors with heavy pigment were excluded from the study because required bleaching of sections adversely affects BrdUrd staining.

Immunohistochemistry. After enucleation, specimens were immediately placed in 10% buffered formalin. Eyes were processed in a standard manner in the eye pathology laboratory. Contiguous 10 μm serial sections were obtained. Two sections were stained with hematoxylin and eosin. Two other sections were labeled with BrdUrd using a modified technique that we have described previously (1).

Briefly, we exposed the slides for 45 min to a monoclonal anti-BrdUrd antibody (Caltag, South San Francisco) diluted 1:2500 in calcium- and magnesium-free phosphate-buffered saline containing 10% horse serum. The slides were rinsed with phosphate-buffered saline and the secondary antibody, biotinylated anti-mouse IgG (Vector, Burlingame, CA) diluted 1:200 in calcium- and magnesium-free phosphate-buffered saline containing 10% horse serum was applied. Endogenous peroxidases were quenched in 3% hydrogen peroxide for 30 min. BrdUrd labeling was detected using an alkaline phosphatase system. We incubated the slides with avidin-biotin complex elite (Vector) for 30 min and developed the color with Vector Violet (Vector).

Cell Counting. All slides were read in a masked manner. Sketches were made of all tumors from histopathological sections. By scanning sections for mitotic figures and BrdUrd labeling, the area of highest cycling activity was visually assessed and noted on each sketch. The counting was begun in one corner of the predetermined area of highest cycling activity and a grid of 5 × 4 HPFs (×400) was counted. The number of positive cells in each field was recorded for analysis. Fields with <70% tumor cells, usually due to the presence of large vessels, were avoided. Nontumor cells (lymphocytic infiltrate, vessel endothelium) were not included in counts.

On the BrdUrd-stained sections, tumor cells with positive nuclei (detected by a violet color) were counted. On the hematoxylin and eosin sections, mitotic figures were counted according to the following criteria: (a) absence of a nuclear membrane and (b) presence of hairy projections of nuclear material. Cell density was estimated from five HPFs on the hematoxylin and eosin sections in representative fields from the same area of highest activity. The BrdUrd LI was calculated as the number of BrdUrd-labeled cells per HPF divided by the cell density. Similarly, the MI was calculated as the number of mitoses per HPF divided by the cell density.

Of 36 tumors, 28 showed agreement across BrdUrd replicates. For those 8 tumors where the replicates did not agree, a third section was stained and counted. In all cases the additional count was closer to the higher of the two originals. The median BrdUrd count was used in the analysis.

RESULTS

Table 1 summarizes the patient data. Subjects ranged in age from 23 to 83 years old, with a median age of 62 years. The majority (30/36) of the melanomas were diagnosed just prior to their treatment. Six cases had clinically detected growth prior to enucleation, two of these after failure of laser therapy. Tumors ranged in diameter from 6.0 to 24.0 mm. Twelve tumors involved the ciliary body and 29 spanned across the equator. Five tumors showed extrascleral extension. Follow-up ranged from 0.25 to 7.5 years, with a median of 2.9 years and a minimum of 1.3 years. Twenty-three patients remained disease free at an average of 3.4 years. One of 7 patients with spindle cell tumors died of metastases, 4 of 12 patients with predominantly spindle tumors died of metastases, and 8 of 16 patients with mixed tumors died of widespread melanoma. The one patient with an epithelioid tumor had no evidence of systemic disease at 2.2 years of follow-up. The time from metastatic detection to death from metastases was often rapid. In 10 of the 13 metastatic cases this time interval was less than 6 months.

BrdUrd LI ranged from 0.01 to 6.97%, with a median of 1.08%. Only six tumors had a BrdUrd LI over 2.00%. MI ranged from 0.003 to 0.27%, with a median of 0.04%.

Marked regional variation of cell cycling was noted in many tumors. Table 2 shows the location of highest cycling activity in all tumors subgrouped by tumor shape. Tumors with collar button formation due to a break in Bruch’s membrane tended to have highest cycling toward the apex. Tumors without Bruch’s membrane penetration tended to have highest cycling in the center of the tumor. In six of seven tumors with segmented areas of pigmentation, the highest cycling was in the area of lighter pigment.

Sequential sections were stained and counted. In 28 of the 36 patients, the pairs of BrdUrd measurements varied within an expected range as determined by pairwise binomial tests for equal counts across sections (26). In the latter eight cases in which multiple observations did not agree, an additional section was stained. In all of these latter cases, the last BrdUrd replicate was closer to the higher of the two original observations. The median relative difference between observations was 20% (IQR, 7–42%). In cases with triplicates, dropping the low count, the median was 10% (IQR, 5–20%). All 36 mitotic measures varied within the expected range. Their median relative difference was 20% (IQR, 5–42%).

Multivariate analysis was performed to determine variables that were independently related to outcome. Cox multivariate analyses and likelihood ratio statistics for testing against the null model of no covariate effects are shown in Table 3 separately for LI and MI (27, 28). A doubling of LI had an increased relative risk estimate of 2.32 (95% confidence interval, 1.22–4.41). A doubling of the MI had an increased relative risk estimate of 2.41 (95% confidence interval, 1.29–4.49). Additional significant multivariate prognostic factors included cell type, defined as mixed versus nonmixed, and largest tumor diameter. Although not statistically significant, ciliary body involvement was also associated with increased relative risk. Kaplan-Meier survival curves for BrdUrd LI and MI are illustrated in Figs. 1 and 2 (29). Fig. 1 shows the probability of not detecting melanoma metastases after stratifying on the BrdUrd...
LI. Patients with a LI above 0.80% had a worse prognosis. The precipitous drop in the survival curve reflects that the patient with the most follow-up had detectable metastases. Fig. 2 shows the probability of not detecting melanoma metastases after stratifying on the MI. Patients with a MI >0.04% had a worse prognosis.

Table 4 summarizes the factors correlated with both the LI and MI. Although none of the relationships reached statistical significance, the data are consistent with larger tumors, nonspindle cell tumors, and anteriorly located tumors having higher cycling indices. Each of these three variates is known to be associated with a poorer prognosis.

A plot of the BrdUrd index versus MI is given in Fig. 3. There is general agreement between the two measures. The univariate distributions each appear to be log Normally distributed. One outlier in which the tumor had both a low MI and very low LI is evident. We have noted that the length of S phase in uveal melanomas is on average 13.7 h long.4 If one LI. Patients with a LI above 0.80% had a worse prognosis. The precipitous drop in the survival curve reflects that the patient with the most follow-up had detectable metastases. Fig. 2 shows the probability of not detecting melanoma metastases after stratifying on the MI. Patients with a MI >0.04% had a worse prognosis.

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4 S. Kroll and D. H. Char, unpublished observations.
Table 3  Summary of Cox model analyses

<table>
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<tr>
<th>Variable</th>
<th>Scale</th>
<th>Relative Risk</th>
<th>95% Confidence Interval</th>
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<td>0.97, 1.08</td>
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Likelihood ratio test = 23 on 5 df, P = 0.0003

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Likelihood ratio test = 23.7 on 5 df, P = 0.0003

Fig. 1  Kaplan-Meier metastases-free time interval estimates stratified by BrdU LI. Hatch marks, censored observations (i.e., length of follow-up for those patients without melanoma metastases).

Fig. 2  Kaplan-Meier metastases-free time interval estimates stratified by MI. Hatch marks, censored observations (i.e., length of follow-up for those patients without melanoma metastases).

| Table 4  Cell cycling by tumor subgroups |
|---------|-----------------|-----------------|
|         | n | Mean LI (%) | Mean MI (%) |
| Largest tumor diameter (mm) |     |               |
| <15     | 11 | 0.85          | 0.037        |
| 15–18   | 14 | 1.54          | 0.053        |
| 18.5–    | 11 | 1.45          | 0.062        |
| Location of anterior tumor margin |
| Post equator      | 7  | 0.76          | 0.028        |
| Anterior equator, invasive choroid | 17 | 1.49          | 0.058        |
| Ciliary body      | 12 | 1.31          | 0.054        |
| Cell type         |     |               |
| Spindle           | 7  | 0.75          | 0.032        |
| Predominantly spindle | 12 | 1.27          | 0.046        |
| Mixed             | 16 | 1.63          | 0.066        |
| Epithelioid       | 1  | 0.13          | 0.010        |

neoplasm (30). For all tumors combined the ratio of S phase to mitosis was 24:1. This suggests that the mitosis detection time is about 35 min. A χ² test for equality of the ratio between mitotic figures and BrdUrd-labeled cells across tumors demonstrated significant tumor to tumor variability (χ² = 167.0, degrees of freedom = 35, P < 0.0001). This may be caused by tumor to tumor variability of either the length of S phase or the length of mitosis, diurnal cell cycling variations, and measurement error.

We also examined the regional homogeneity of cell cycling within the sampled area of 2.3 mm². If the number of cells per HPF is constant and the cycling behavior of cells is locally stationary over the 20 HPFs, then the distribution of counts per HPF should follow a Poisson distribution. For the Poisson distribution the variance is equal to the mean. Fig. 4 is a scatter plot of the variance of each 20 HPF region versus the mean of each region on a log-log scale. Although the majority of the sections did not exhibit extra Poisson variability, Poisson overdispersion is evident (variance greater than the mean) in higher labeled tumors. This suggests that the excessive cell cycling rate variability noted on the gross tumor sections is also evident in the localized sampled areas. The average variance to mean ratio was 1.63. Possible reasons for the excess variability include cell cycle synchronicity, variable cell density, vascular heterogeneity, or other cell cycle-dependent variables exhibiting within tumor heterogeneity.
DISCUSSION

We observed a significant correlation between uveal melanoma cell cycling and metastases. Previous studies have found that random mitoses per HPF were a prognostic sign; however, in most series analyzed with multivariate analysis it was not as important as largest tumor diameter, cell type, ciliary body involvement, or scleral extension (13, 14, 31). A major difference in our methods was the strategy to count areas of highest cell cycling instead of random fields. Analysis of BrdUrd LI and MI from areas of highest cycling activity may result in the evaluation of more biologically and prognostically relevant tumor cell populations.

Tumor heterogeneity has been previously reported in uveal melanomas as well as other solid tumors (22–25). Intratumor cell proliferation has significant regional differences, often with higher cycling activity at the advancing border of the tumor (22, 25, 32). We used a principal analogous to analyzing the most histologically malignant areas to assess maximal cell cycling. The standard procedure for assessment of random mitotic counts has been criticized for poor reproducibility due to observer bias, failure to locate active regions, and difficulty in differentiating mitotic figures from pyknotic nuclei and other artifacts (33–35). Results from the multicenter morphometric mammary carcinoma project showed that in a routine setting, mitosis counting can be learned, within a reasonable time, with highly reproducible results if a strict protocol is carefully followed (36). We observed high correlation between BrdUrd LI and MI replicates.

Scanning BrdUrd-labeled sections at lower power enabled us to identify areas of highest cycling. These areas were confirmed on sections stained with hematoxylin and eosin by enumeration of mitotic figures. In some sections it was difficult to assess the area of highest cell-cycling activity solely from mitotic figures on the sections stained with hematoxylin and eosin due to the low numbers of cycling cells in uveal melanomas. Other cell cycle markers such as proliferating cell nuclear antigen may assist in characterizing intratumor cell cycling patterns. In preliminary studies we have not noted a vast discrepancy in terms of high cycling region delineated by either BrdUrd or proliferating cell nuclear antigen.

The mitotic counts obtained in this study are significantly higher than those reported by other authors. There is contradictory evidence on the effect of delayed fixation on the MI; while one early study demonstrated no significant decrease in the MI with delayed fixation, a later study found a rapid decline in the MI over a 2-h delayed fixation time (37, 38). Fixation of uveal melanoma may be delayed by poor scleral penetration of the fixative agent. In our study the majority of the eyes had prefixation scleral removal so fresh tumor tissue could be obtained for other studies. This maneuver increased rapidity of fixation. The agreement of the ratio of cells in DNA synthesis phase to those in mitosis would mitigate the likelihood of a fixation artifact. The entrance criteria of the tumors in this study may limit the generalization of the conclusions. All tumors were treated by primary enucleation and the majority were too large to be treated by either $^{125}$I-labeled brachytherapy, helium ion irradiation, or eye wall resection. Thus, the study cohorts were of poorer prognosis than a typical subset of our uveal melanomas. The other entrance criteria of light or absent pigmentation have been suggestive of improved survival relative; however, the higher cycling noted in the lighter pigmented areas in the majority of tumors with segmented pigmentation was indicative of a progression toward lighter pigmentation with increased cycling rates (4–6, 14).
Our conclusions are somewhat limited by the small sample size of the cohort. The Cox model, which relates prognostic factors only at each observed metastatic event, is estimated from the 13 metastatic events. Various approaches were used to test the robustness and sensitivity of our results. The jackknife resampling technique (39) resulted in decreases in the relative risk estimates as well. Since this technique does not correct for multiple outliers, the three cases with greatest distance from normal (the lowest and highest LI cases and the most rapid metastatic onset case) were dropped and the model was refit. This resulted in a drop in relative risk from 2.32 to 1.76 for the LI and a drop from 2.41 to 1.89 for the MI. This suggests that although the estimates are influenced by specific subsets of observations, the overall conclusion that cell cycling is an additional independent prognostic factor for time to metastatic onset is maintained. In addition the strong evidence linking cycling rates and prognosis in a wide variety of tumors (40) suggests that these findings are to be expected and the differences between our results and those of previous studies is in part due to a different sampling technique and greater concern for accurate cell cycling estimates.

The systematic selection of the high region for each tumor violates some sampling principles because the selected region does not characterize the full tumor cell population. Accordingly, tumor growth rates based on these LIs will be overestimated and tumor potential doubling times will be underestimated. The 20 HPFs viewed on each section encompass a surface area of 2.268 mm$^2$ (radius of each HPF, 0.19 mm). If cell cycling homogeneity varies independently of direction, the sampled area can be thought to represent a sphere with a volume of 2.57 mm$^3$. This volume encompasses at least one million cells based on our observed cell densities. Hence, under these assumptions the cell population we are characterizing is of substantial size. The mildly overdispersed Poisson counts from the counting region and the similarity for both mitotic and labeling indices from sections over 0.2 mm apart also suggest that the sampled cell population characterizes a sizable tumor population.

Further studies to examine the cell proliferation rates across more distant sections are needed to fully assess the reliability of the sampling technique. Choroidal melanomas demonstrate a variety of cell population compositions. We have noted that tumors demonstrating homogeneous cell morphology tend to have a uniform cycling rate across the tumor. Tumors composed of clonal components, some spindle, some mixed, and some epithelioid cells, tend to have a much greater variability of cycling rates within the tumor. We have also noted that the region of high proliferation was dependent on the tumor shape as shown in Table 2. In preliminary studies of global cell proliferation we have noted that sections from the margins of the tumor have lower cycling rates than those from the thicker, center sections. Thus, sections taken close to the tumor edges should be avoided.

We have observed a strong correlation between uveal melanoma cell cycling and tumor-related mortality. We believe our results have shown a stronger correlation than previous studies because we assessed cell cycling in the area of highest proliferative activity. MI in conjunction with BrdUrd-labeled tumor sections can be used to prognostically assess uveal melanoma.

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