Effects of proton beam irradiation on uveal melanomas: a comparative study of Ki-67 expression in irradiated versus non-irradiated melanomas

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Abstract
Aims—To assess the cellular proliferation using the monoclonal antibody Ki-67, in paraffin embedded uveal melanomas irradiated by proton beam, as well as in non-irradiated uveal melanomas.

Methods—30 enucleated eyes were included for histopathological study and Ki-67 immunostaining. Patients were enucleated between 1991 and 1996 for uveal melanoma, 14 after proton beam irradiation and 16 without treatment (control group). The mean follow up period was 2.5 years after diagnosis and 1 year after enucleation.

Results—A significant relation was found between Ki-67 score and mitotic index ($r = 0.56, p = 0.001$), histological largest tumour diameter ($r = 0.38, p = 0.03$), fibrosis ($r = -0.35, p = 0.05$), absence of tumoral pigmentation ($p = 0.05$), and presence of vascular thrombosis ($p = 0.03$). The Ki-67 score was significantly higher in the non-irradiated group ($p = 0.01$) and in the group of patients whose cause of enucleation was tumoral evolution ($p = 0.005$) compared with the group of patients enucleated after neovascular glaucoma. The Ki-67 score was very high in a case of orbital recurrence of uveal melanoma and metastatic death. 70% of metastasised tumours showed a Ki-67 score higher than the median value.

Conclusion—Ki-67 labelling is a reliable method of estimating the proliferative activity in uveal melanomas after proton beam irradiation. The Ki-67 score is significantly correlated with prognostic variables (mitotic index and histological largest tumour diameter), and with radiation effects after proton beam irradiation.

Patients and methods
Forty consecutive patients with uveal melanoma underwent enucleation between 1991 and 1996, either without adjuvant therapy (17 patients) or after proton beam irradiation (23 patients). Proton beam irradiation (60 Gy) had been used in the treatment of uveal melanomas for 198 patients at the Nice Biomedical Cyclotron between June 1991 and August 1996. In the control group, eyes were enucleated if the tumour size was excessive (maximum tumour height >10 mm) or in the case of neovascular glaucoma. In the irradiated group, enucleation was performed in cases of continued tumour growth or tumour recurr-
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Figure 1. Ki-67 immunostaining in an irradiated choroidal spindle cell melanoma. Note the speckled red nuclear and nucleolar staining (arrows) readily distinguished from the brown endogenous melanin pigment (stars) (original magnification ×1000).

ence, or in cases of neovascular glaucoma. The cause of enucleation was classified as “neovascular glaucoma” or “tumoral evolution” (large melanoma, continued tumour growth, or recurrence).

Nine irradiated eyes were excluded from this study for the following reasons: association of brachytherapy before proton beam irradiation (three cases), technically unsatisfactory samples (paraffin blocks not available for the preparation of histological sections, four cases), or extensively necrotic tumours (two cases). Moreover, one non-irradiated eye was excluded as it was a technically unsatisfactory sample. All iris melanomas were excluded from this study. All enucleations were carried out after consultation in our institution. Follow up data were available for all patients and ranged from 4 months to 12 years (mean 27.5 months) after diagnosis and from 4 months to 41 months after enucleation (mean 12 months).

The following pretreatment clinical data included: age and sex of the patient, delay of the irradiation after diagnosis, echographic maximum tumour height (MTH) and largest tumour diameter (LTD), and the location of the tumour. Post-treatment examination evaluated MTH and LTD measured by ultrasonography before enucleation, the time of metastasis, or death.

HISTOPATHOLOGY

The paraffin blocks of 30 primary ocular melanomas were obtained from the files of the Croix-Rousse Hospital (Lyons, France). All tumours were Bouin fixed and routinely processed between 1991 and 1996. For each case, two to 10 (median four) haematoxylin and eosin and safran stained slides were reviewed. The following criteria were recorded: LTD, scleral extension, rupture of Bruch’s membrane, degree of pigmentation, extent of necrosis (estimated as percentage of tumour area), tumour blood vessels damage (none, hyalised wall, thrombosis), and tumour cell type according to the modified Callender classification. Histological LTD was determined as the largest tumour diameter in contact with the sclera. The mitotic figures were counted in 40 high power fields (HPF) and a quantitative evaluation of balloon cells was performed in 10 HPFs.

IMMUNOHISTOCHEMISTRY

For each case, one representative tissue section was chosen for immunohistochemical study, using avidin-biotin-peroxidase complex technique, as previously described. Immunohistochemical detection of Ki-67 was performed using the monoclonal antibody MIB-1 (Ki-67) (Immunotech, Marseille, France, diluted 1:20). To enhance immunoreactivity with MIB-1 antibody, tissue sections were pre-treated with 0.05% trypsin digestion (0.05 mg/100 ml citrate buffer, pH = 6, 37°C, ICN Biomedical, Orsay, France) in two 5 minute cycles. The slides were rinsed in distilled water, placed in the preheated solutions (citrate buffer 10 mM, pH 6), and treated for the microwave procedure (740 W, 4 minute cycles with an interval of 1 minute between cycles to check on the fluid in the jars).

A negative control was run using the same technique and omitting the primary antibody. Bouin fixed paraffin embedded sections of a Burkitt lymphoma served as positive control. We used haematoxylin, eosin, and safran stained slides to identify representative tumour areas. Cells were considered Ki-67 stained if any reddish nuclear staining, either focal or diffuse, could be identified (Fig 1).

Ki-67 positive and negative cells were evaluated at high power magnification (×40). For overall proliferation assessment, a Ki-67 score was calculated as: Ki-67 score (%) = (PCtotal/ Ntotal) × 100, where Ntotal is the total number of counted cells and PCtotal is the total number of Ki-67 positive cells. Each stained section was evaluated on 20 random HPFs, with count repeated three times and averaged.

STATISTICAL ANALYSIS

Comparisons on clinical and pathological features between the control and the irradiated group were performed using non-parametric tests (Pearson or Fisher’s exact χ² test, Kruskall–Wallis and Mann–Whitney test). The relations between clinicopathological variables and Ki-67 score were studied on the overall population using the Spearman test, Mann–Whitney test, and Pearson χ² test. Statistical analysis was performed using the Statistical Package for the Social Science program (SPSS), with p value below 0.05 being regarded as significant.

Results

Nineteen of the 30 patients were male (63%). The mean age at diagnosis was 59 years (range 31–86). Proton beam treated patients had been irradiated between 1991 and 1996, within 3 months of diagnosis in 93% of the cases. The mean interval between proton beam irradiation and enucleation was 16 months (range 4–41
Table 1: Correlations between Ki-67 score and clinicopathological findings in the overall population

<table>
<thead>
<tr>
<th>Factors</th>
<th>Correlation coefficient</th>
<th>p Value</th>
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</thead>
<tbody>
<tr>
<td>Mitosis</td>
<td>0.562</td>
<td>0.001</td>
</tr>
<tr>
<td>Histological LTD</td>
<td>0.38</td>
<td>0.038</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>−0.35</td>
<td>0.05</td>
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<tr>
<td>Mean (SD) of Ki-67 score</td>
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</table>

Among patients enucleated because of tumoral evolution (n = 14 in the control group and n = 5 in the irradiated group), Ki-67 score and mitotic index were significantly higher in the non-irradiated group (p = 0.05 and 0.06, respectively) compared with the group treated by proton beam irradiation. In the overall population, the Ki-67 reactivity was higher in the group of patients enucleated for tumoral evolution (n = 19) than in the group of patients enucleated for neovascular glaucoma (n = 11) (p = 0.005).

Within the follow-up period, uveal melanomas metastasised in 23% of the patients and led to death in two cases. These two patients,
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almost quiescent cells adjacent to some we have studied the proliferation in conventionally fixed histological material. Since PCNA is detectable in DNA content by thymidine or bromodeoxyuridine labelling have been found to be much more sensitive than mitotic count, this technique does not allow the use of archival material with a long term follow up period. Monoclonal antibodies against PCNA (PC-10) have been reported to assess cell proliferation in conventionally fixed histological material. Since PCNA is detectable in almost quiescent cells adjacent to some tumours presumably because of its long half life, and since its prognostic value is contested, we have studied the proliferation activity in irradiated and non-irradiated uveal melanomas using the Ki-67 immunostaining. Consistent with previous reports, we found that the Ki-67 score was correlated with the mitotic index. However, results were discrepant in two patients with a melanoma of low mitotic rate (<5/40 HPF) and a Ki-67 score higher than the median value. The first patient was treated by proton beam irradiation and enucleated because of tumoural recurrence and the second patient was treated primarily by enucleation, owing to the large tumoral size and a total retinal detachment. Comparisons between mitotic count per 40 HPFs (14.4 (SD 18.5)) and number of Ki-67 immunoreactive cells per 40 HPFs (203.46 (256.9)) showed an overestimation of proliferating cells using Ki-67 immunostaining (p < 0.001), related to the expression of the nuclear antigen in most of the phases of the cell cycle, whereas mitotic count reflects only the M phase. Immunohistochemical detection of Ki-67 antigen is more sensitive than mitotic count to identify cycling cells. Indeed, the mitotic rate has been described as an insensitive measure of reproductive integrity of treated cells. Measurements can be high owing to a prolonged M phase of the cell cycle rather than a high proliferation rate of the lesion.

In irradiated or non-irradiated tumours, as previously reported, and in contrast with other reports, Ki-67 score was correlated with histological LTD in our series. LTD is thought to represent a reliable prognostic factor in uveal melanomas and the strong relation between this factor and Ki-67 score in our study is of particular interest. Our results suggest a relation between Ki-67 score and prognosis since 70% of the tumours with metastasis were associated with a Ki-67 score higher than the median value. Given the prevalence of death in our series (2/30 patients) and the relatively short follow up period in some patients, correlation between Ki-67 score and survival could not be analysed. Further clinical trials on a larger scale are needed to draw a definitive conclusion regarding the independent prognostic value of the Ki-67 labelling.

Balloon cells are observed in uveal and cutaneous melanomas. Ballooning degeneration is more common in irradiated tumours and could possibly fit with a radiobiological effect. Blood vessel damage leading to necrosis and later to fibrosis has been described as being related to irradiation effects. The association of a significantly lower Ki-67 score with the extend of thrombotic vessels within irradiated tumours reflects the effects of proton beam irradiation. Moreover, the Ki-67 score was significantly lower in the irradiated group compared with the control group and was significantly higher in irradiated melanomas enucleated after tumour regrowth compared with those enucleated for neovascular glaucoma. Therefore, Ki-67 immunostaining seems to be a useful adjunct to the assessment of tumoral response to proton beam irradiation in uveal melanomas. A correlation between Ki-67 reactivity, ploidy, and radiation induced changes has also been reported in uveal melanomas after brachytherapy. Irradiated melanomas were found to be significantly more often aneuploid than non-irradiated melanomas. The reduced proliferative activity of uveal melanomas was also observed after brachytherapy, using the Ki-67 or PCNA reactivity. These effects was reported in patients enucleated for tumour regrowth when...
compared with patients enucleated because of adverse treatment effects or personal preference. We could confirm this difference between tumours with tumoral evolution and tumours with neovascular glaucoma.

In the future, the evaluation of cell proliferation by Ki-67 score using immunocytochemistry or flow cytometry on fine needle biopsy of uveal melanomas before irradiation could influence the clinical and therapeutic management of selected patients. A more accurate dose of irradiation for each patient could be applied on the basis of the Ki-67 score with less resultant ocular morbidity.

In conclusion, we have demonstrated that Ki-67 score in uveal melanomas after proton beam irradiation was significantly correlated with two major prognostic factors (histological LTD, mitotic index). Moreover, a higher Ki-67 score was found in uveal melanomas with metastasis compared with tumours without metastatic evolution. The Ki-67 immunostaining was significantly associated with radiation effects after proton beam irradiation. Further studies are needed to confirm the prognostic significance of Ki-67 immunostaining in uveal melanomas treated by proton beam irradiation.

This study was presented at the XXIst meeting of the Club Jules Gonin, 31 August 1998, Edinburgh, Scotland (paper).