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.**

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Uveal melanoma is the most common primary adult ocular malignancy with an incidence of 0.6 cases/100 000 inhabitants.[1] Their high ability to produce metastases is well recognised. Proton beam irradiation is currently used in the treatment of choroidal and ciliary body melanomas to destroy the tumour while preserving some vision and retaining the globe. The histological variables and the proliferation markers that predict the outcome of patients with uveal melanoma in terms of metastatic disease and tumour related death have been previously explored, using variance in nucleolar area,[2 3] inverse standard deviation of the nucleolar area,[2 4] mitotic index,[5 6] DNA or RNA ploidy status,[7–11] bromodeoxyuridine labelling,[6 12] and PCNA immunostaining.[6 13 14]

With the development of the proliferation associated monoclonal antibody Ki-67 by Gerdes *et al* in 1983,[15] identification of cells in mid-G1 (first gap), S (DNA synthesis), G2 (second gap), and M (mitosis) phases of the cell cycle on tissue sections has become possible.[16] Growth fraction assessed with Ki-67 is reported as an independent prognostic marker in several tumours,[17] such as non-Hodgkin's lymphomas, gliomas, soft tissue tumours, and non-irradiated uveal melanomas.[18]

The cellular proliferation of uveal melanoma after brachytherapy has been previously studied.[10 13 19–21] However, to our knowledge, the proliferation rate of uveal melanoma treated by proton beam irradiation has not been described using Ki-67 immunostaining. Since radiation induced damage within tumours seems to be responsible for tumour cell death and sterilisation of uveal melanoma cells, an immunohistochemical study using Ki-67 antigen detection appears to be an attractive approach to analyse the effects of proton beam irradiation on uveal melanomas. The purpose of the current study was to compare the proliferation rate of uveal melanomas irradiated by charged particles of proton with that of non-irradiated melanomas using immunohistochemical staining with a monoclonal antibody against Ki-67, and to correlate Ki-67 score with conventional clinical and pathological prognostic variables.

Patients and methods

Forty consecutive patients with uveal melanoma underwent enucleation between 1991 and 1996, either without adjunct 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 recur-

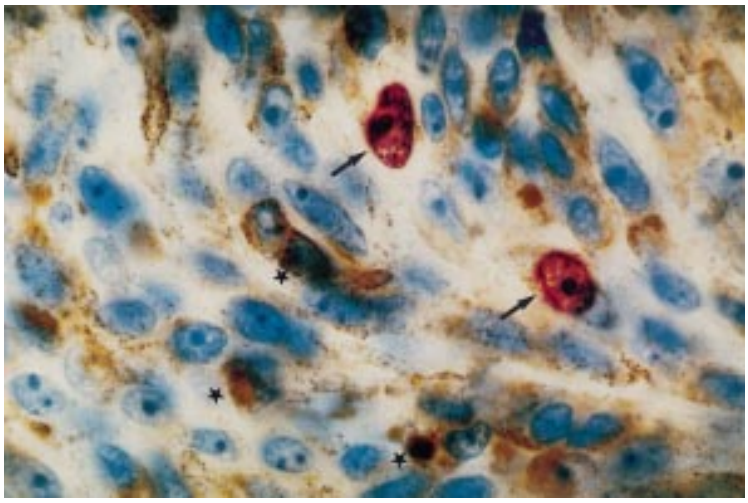

Figure 1 Ki-67 immunostaining in an irradiated choroidal spindle cell melanoma. Note the speckled red nuclear and nucleolar staining (arrows) readily distinguished from the brown endogenous melanin pigment (stars) (original magnification ×1000).

rence, 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, hyalinised wall, thrombosis), and tumour cell type according to the modified Callender classification.[22] 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.[23] 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 pretreated 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 χ^2 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 χ^2 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

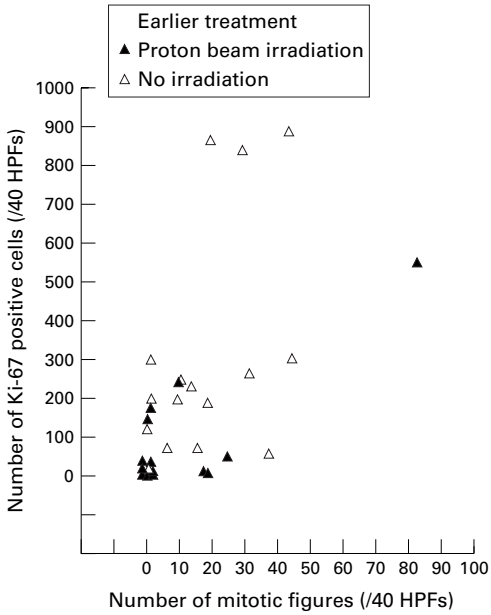

Figure 2 Mitotic figures plotted against Ki-67 positive cells for 30 eyes. Filled symbols represent data for tumours treated by proton beam irradiation and open symbols, data for non-irradiated tumours. The mitotic figures and Ki-67 immunoreactive cells were counted in 40 high power fields (HPFs).

months). In the control group, the mean interval between diagnosis and enucleation was 15.6 months (range 15 days–9 years).

For the overall population, the mean follow up periods after diagnosis and after enucleation were 2.5 years (range 4 months–12 years) and 1 year (range 4 months–3.5 years) respectively, and were similar in the two groups of patients (p = 0.7 and p = 0.5, respectively).

The melanoma was located on the posterior pole (nine eyes), in the equatorial posterior segment (nine eyes), the ciliary body (one eye), or the ciliary body and peripheral posterior segment (11 eyes). When classified according to the modified Callender classification, tumours were spindle type in 11 eyes, mixed type in 16 eyes, and epithelioid type in three eyes.

The two groups of patients (irradiated versus non-irradiated) were comparable, since no statistically significant difference in any of the histological prognostic factors (histological LTD, ciliary body involvement, optic nerve involvement, scleral extension, vortex vein invasion, cell type) was found between both groups. In irradiated eyes, the number of balloon cells was significantly higher, when compared with the number in non-irradiated eyes (p = 0.046).

Ki-67 immunoreactivity was revealed in all but two irradiated melanomas and in all non-irradiated tumours. The Ki-67 positive cells were diffusely distributed in non-irradiated melanomas, whereas a focal and patchy pattern of staining was observed in tumours after proton beam irradiation. The immunoreactive cells were predominantly located at the apical border of the irradiated tumours compared with the basal periscleral zones. No immunoreactivity was seen adjacent to the necrotic areas, and the staining increased in cells away from tumoral necrosis.

The Ki-67 score varied from 0 to 4.09 (median 0.47, in 20 HPFs) in the irradiated uveal melanomas, and from 0.15 to 7.05 (median 1.04) in the control group. The median score in the overall population was 0.8. In the irradiated group, 12 tumours displayed a score <1, one tumour a score between 1 and 3, and one tumour showed a score ⩾3. In comparison, seven non-irradiated melanomas showed a Ki-67 score <1, and three a score ⩾3. Figure 2 shows number of mitotic figures plotted against Ki-67 positive cells for 30 eyes (in 40 HPFs).

Table 1 presented relations between Ki-67 score and clinicopathological variables. Given the number of cases, the correlations were calculated on the overall population (30 patients)—that is, grouping irradiated with non-irradiated eyes. Ki-67 score was significantly higher in non-irradiated tumours, compared with irradiated melanomas. This score was significantly correlated with mitotic index and histological LTD, in the overall population. The extent of fibrosis was inversely proportional to the Ki-67 score. The absence of tumoral pigmentation was significantly correlated with a higher Ki-67 score, compared with the pigmented tumours. Moreover, Ki-67 score was significantly lower in uveal melanomas with thrombotic vessels. No significant correlation was found between Ki-67 score and cell type, presence of Bruch rupture, necrosis, number of balloon cells, and prevalence of tumour related death.

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,

Table 1 Correlations between Ki-67 score and clinicopathological findings in the overall population

Factors	*Correlation coefficient*	*p Value*
Mitosis	0.562	0.001
Histological LTD	0.38	0.038
Fibrosis	−0.35	0.05
	Mean (SD) of Ki-67 score	*p Value*
Tumoral pigmentation		0.048
Absence	3.1 (2.6)	
+	0.8 (0.9)	
++	1.5 (1.1)	
+++	0.62 (0.6)	
Vascular lesions		0.03
Absence	1.49 (1.9)	
Hyalinised vessel wall	1.31 (1.1)	
Thrombosis	0.09 (0.15)	
Treatment		0.013
Control group	1.7 (1.7)	
Proton beam	0.7 (1)	
Cause of enucleation		0.005
Neovascular glaucoma	0.68 (1.1)	
Tumoral evolution	1.61 (1.6)	

LTD = largest tumour diameter.

aged 50 and 66 years, died because of their disease, both with metastasis. The first patient was enucleated with no previous irradiation, related to a spontaneous neovascular glaucoma, 9 years after the diagnosis. The patient developed bone and lung metastasis 3 years after enucleation and died 4 months later. Histopathological status included posterior pole localisation, histological LTD 9 mm, scleral invasion (1/3 outer), spindle cell type, a low mitotic count (1/40 HPF, median value in the overall population = 9), and a low Ki-67 score (0.15; median value in the overall population = 0.8). The second patient was rapidly irradiated after the diagnosis of a choroidal melanoma. Four years later, this patient was enucleated for a tumour regrowth, then developed bone metastasis and orbital recurrence 3 months after enucleation. Histopathological examination showed: LTD 21 mm, a neovascular glaucoma, a scleral invasion (1/3 inner), a mixed cell type, a high mitotic count (82/40 HPF), and a high Ki-67 score (4.09). The patient died 3.5 years after the diagnosis.

Seven patients developed metastasis, including hepatic (five cases), bone, and bone-lung localisation, with a mean delay of 20 months (range 0–39 months). Ki-67 score was greater than the median value in five of these seven cases and was closely correlated with mitotic index ($p = 0.001$). Two of these seven patients died of metastatic disease. Seventy per cent of the tumours with metastasis were associated with a Ki-67 score higher than the median value.

Discussion

We undertook an immunohistochemical study using anti-Ki-67 antibody to assess the proliferation rate of a series of uveal melanomas treated by proton beam irradiation, and we compared our findings with the Ki-67 immunoreactivity of non-irradiated uveal melanomas. The assessment of proliferation activity, predictive of survival, and local tumour response cannot be determined by conventional light microscopy. Although measurements of DNA content by thymidine or bromodeoxyuridine labelling have been found to be much more sensitive than mitotic count,[24] 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.[6 13 14 18 25] Since PCNA is detectable in almost quiescent cells adjacent to some tumours[26] presumably because of its long half life, and since its prognostic value is contested,[6 18 25] we have studied the proliferation activity in irradiated and non-irradiated uveal melanomas using the Ki-67 immmunostaining.

Consistent with previous reports,[19 27 28] 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 tumoral 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.[29] 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,[18] and in contrast with other reports,[12 27] Ki-67 score was correlated with histological LTD in our series. LTD is thought to represent a reliable prognostic factor in uveal melanomas[30] 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.[28]

Balloon cells are observed in uveal[31] and cutaneous melanomas. Ballooning degeneration is more common in irradiated tumours[32] and could possibly fit with a radiobiological effect.[33] Blood vessel damage leading to necrosis and later to fibrosis has been described as being related to irradiation effects.[32 34] 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.[19] Irradiated melanomas were found to be significantly more often aneuploid than non-irradiated melanomas.[10] The reduced proliferative activity of uveal melanomas was also observed after brachytherapy, using the Ki-67[19 21] or PCNA reactivity.[13 20] These effects was reported in patients enucleated for tumour regrowth when

compared with patients enucleated because of adverse treatment effects or personal preference.[20] 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[35] or flow cytometry[36] 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).

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