

EXTENDED REPORT

The relation between *c-myc* expression and interferon sensitivity in uveal melanoma

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Background/aim: Interferons (IFN) are currently being used to treat melanoma, including some patients with uveal melanoma. IFN is thought to inhibit tumour growth through downregulation of the *c-myc* oncogene; the overexpression of which has been shown to be associated with resistance in cell lines. The aim of this study was to investigate the relation between *c-myc* gene expression and IFN sensitivity in a series of uveal melanomas in a short term chemosensitivity assay.

Methods: Tumours from 45 patients with uveal melanoma who had undergone enucleation were studied. The ATP chemosensitivity assay was used to study sensitivity to IFN- α -2b in freshly isolated cells from each tumour. Flow cytometry was used to assess *c-myc* expression in formalin fixed material from the primary specimens.

Results: There was a wide range of IFN sensitivity between the specimens whereas *c-myc* expression was universal and present in 80% of the tumour cells in 80% of the specimens. Higher *c-myc* expression was associated with IFN- α resistance as measured by the maximum percentage of inhibition ($p=0.05$) and there was a trend with the IFN sensitivity index ($p=0.07$).

Conclusions: These results demonstrate that tumours with high *c-myc* expression are also associated with IFN resistance. Future research is required to explore the potential of *c-myc* gene manipulation combined with IFN therapy.

The clinical course of uveal melanoma is unpredictable. Most patients with uveal melanoma have no demonstrable evidence of metastatic disease at the time of initial diagnosis. However, up to 50% of patients will develop secondary disease. Systemic chemotherapy that is effective in cutaneous melanoma has failed to show activity in uveal melanoma.^{1,2} The BOLD chemotherapy regimen (dacarbazine, lomustine, vincristine and bleomycin) combined with interferon alfa has been shown to have modest activity against metastatic uveal melanoma in hepatic and extrahepatic sites.^{3,4} Recent results suggest that fotemustine and GeT (gemcitabine + treosulfan) may be effective.⁵

Controversy still surrounds the use of interferon alfa in cutaneous melanoma^{6,7} but it remains the subject of clinical investigation. In uveal melanoma, experimental studies *in vitro*⁸ and *in vivo*⁹ have also indicated a potential role for interferons. Interferon alfa and interferon gamma treatment inhibited cell growth and upregulated the expression of class I and II histocompatibility antigen (HLA) antigens in two primary uveal melanoma cell lines⁸ while treatment with IFN- α -2b resulted in decreased hepatic metastases from intraocular melanoma in a murine model.⁹

The mechanisms by which interferon alfa exerts its anti-tumour effect have not been fully established. Several lines of evidence have suggested that *c-myc* has an important role in the action of interferon alfa. Administration of interferon alfa to tumours has been shown to lead to downregulation of *c-myc* oncoprotein expression and upregulation of p21/WAF gene expression, together leading to G1/S phase delay and a reduction in tumour proliferation.^{10,11} Studies in Balb/c 3T3 fibroblasts demonstrated that parental cells were sensitive to the antimitogenic effects of interferon alfa and beta but that cell cycle arrest was abolished by the transfection of a construct that linked the two coding exons of *c-myc* to the long terminal repeat (LTR) of Ha-MS virus producing steady state levels of exogenous *c-myc* mRNA which were 3–10-fold higher than the endogenous mRNA levels.¹² A similar effect was noted in M1 myeloblastic cells transfected with an SV-40

driven *c-myc* plasmid.¹³ These studies suggest that overexpression of the *c-myc* gene, and the subsequent failure to downregulate its expression, is associated with interferon resistance.

IFN mediated downregulation of *c-myc* has been shown to occur by a reduction in the half life of its mRNA and not through an effect on transcription rate.¹⁴ Further evidence has suggested that the selective reduction in the half life of *c-myc* mRNA occurs through an increase in the activity of the 2',5'-oligoadenylate synthetase/RNase L (2',5'-oligo (A) synthetase) pathway.¹⁵

Another pathway linking IFN and *c-myc* is the modulation of HLA expression. Interferon is known to increase the level of class I HLA mRNA¹⁰ and surface expression of HLA molecules.⁸ Conversely, overexpression of *c-myc* oncogene expression is associated with decreased HLA expression on the surface of tumour cells^{16,17} reducing their antigenicity.

We have previously shown that *c-myc* overexpression predicts for poor outcome in a series of cutaneous melanomas^{18,19} and other variants including subungual²⁰ and acral lentiginous melanoma.²¹ Interestingly, overexpression of *c-myc* oncoprotein was associated with better outcome in uveal melanoma.²² This study was undertaken to directly relate *c-myc* oncoprotein expression with sensitivity of primary uveal melanomas to interferon alfa assessed using an *ex vivo* ATP chemosensitivity assay.

MATERIALS AND METHODS

Tumour material

The surgical specimens were obtained from patients who had undergone enucleation for uveal melanoma at Moorfields Eye Hospital and St Bartholomew's Hospital, London.

Abbreviations: CAM, complete assay medium; CTL, cytotoxic T lymphocyte; FITC, fluorescein isothiocyanate; HLA, histocompatibility antigen; IFN, interferons; LTR, long terminal repeat; MI, maximal inhibitor; NK, natural killer; PBS, phosphate buffered saline; RLU, relative light units

Uveal melanomas were detected in patients presenting with reduced visual acuity, a field defect, or by an associated retinal detachment or vitreous haemorrhage. All patients were treated by surgical enucleation of the affected eye performed as an in-patient procedure under general anaesthesia. All patients presented with only primary disease as confirmed by a preoperative metastatic evaluation consisting of clinical examination, routine liver function tests, and a liver ultrasound test. Patients with tumours demonstrating extrascleral extension at the time of resection and histopathological diagnosis were treated subsequently with orbital radiotherapy. Those developing distant metastases at the time of this study were treated with chemotherapy as for metastatic cutaneous melanoma. Each uveal melanoma specimen was divided into two portions under sterile conditions. One underwent conventional histopathological examination and the other was used to assess its sensitivity to interferon. Measurement of the *c-myc* oncogene expression in each tumour was subsequently performed from material obtained from the paraffin blocks.

ATP chemosensitivity assay

The sensitivity of each uveal melanoma specimen to interferon alfa was assessed using the ATP chemosensitivity assay, which we have previously reported for uveal melanoma.²³ A portion of each fresh uveal melanoma specimen was removed under sterile conditions. The samples were then cut into small cubes, 10 ml of collagenase blend H (0.375 mg/ml Sigma) was added, and the mixture incubated overnight to allow digestion to occur. The digest was washed with 10 ml of PBS, centrifuged at 1100 rpm for 5 minutes and the pellet resuspended in 10 ml of complete assay medium (CAM-DCS Innovative Diagnostik Systeme, Hamburg, Germany). The live cell count was assessed using a trypan blue exclusion method. A volume of 5×10^3 live cells were subsequently added to each well of a 96 well plate in 100 μ l of CAM. The cells were incubated for 6 hours to allow them to attach to the plate.

Intron-A (IFN α -2b, Schering Plough) was then added to each well at a number of concentrations corresponding to 6.25%, 12.5%, 25%, 50%, 100%, or 200% of the standard therapeutic dose (100% = 1000 IU/ml). Each combination was tested in triplicate. One row of 12 wells received CAM only, and no interferon, and was termed M0 as the negative control. One further row of 12 wells in each plate acted as the positive control (M1) and received the maximal inhibitor of cell survival (MI; DCS Innovative Diagnostik Systeme). The plate was incubated for 6–7 days at 37°C in 5% CO₂ and high humidity. The cells were checked every 2–3 days for infection or overgrowth by microscopy. Following the incubation, ATP was extracted from the cells using 50 μ l of TCER (DCS Innovative Diagnostik Systeme). A volume of 50 μ l from each well was transferred to another 96 well plate and 55 μ l of luciferin-luciferase (DCS Innovative Diagnostik Systeme) added to each well to hydrolyse the ATP. The plate was loaded into a Dynatech ML1100 luminometer and the light emitted from each well measured and expressed as relative light units (RLU). This measure of total ATP has been shown to be a surrogate for a clonogenic assay²⁴ and superior to assays based on MTT (3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide).²⁵ The mean percentage inhibition of cell growth/survival for the triplicate wells was calculated using the formula:

$$\text{Mean \% inhibition of cell growth} = \frac{1 - (\text{Test} - \text{M1}) \times 100}{(\text{MO} - \text{M1})}$$

The IFN sensitivity index was calculated by adding the mean percentage growth inhibition, for all six drug concentrations, together and subtracting this figure from 600. A sensitivity index of less than 600 corresponds to tumour sensitivity to IFN and above 600 to resistance. The maximum percentage inhibition of IFN for each tumour was the maximum inhibitory percentage produced on the dose-response curve at any dose.

Flow cytometric analysis of *c-myc* oncoprotein

Measurement of the *c-myc* oncogene expression in each tumour was subsequently performed from material obtained from the paraffin blocks as described previously.²⁰ Two 35 μ m sections were cut from the block used for diagnosis, dewaxed in xylene, and rehydrated through a series of alcohols. Nuclei extraction was achieved by incubation with pepsin solution (4 mg/ml in 0.1 M HCl, Sigma Chemicals, Poole, UK) for 45 minutes at 37°C. The extracted nuclei were filtered through a 35 μ m mesh and divided into two samples, one of which acted as a control while the other was used for *c-myc* staining. The nuclei concentration was adjusted to 10^6 cells/ml in phosphate buffered saline (PBS) and stained for *c-myc* using a rabbit polyclonal antibody to the human oncoprotein (Cambridge Research Biomedicals Ltd, Cambridge). A pellet of each test sample was incubated in a volume of 100 μ l of dilution buffer (PBS + 0.5% normal goat serum + 0.5% Tween 20) containing 4 μ l of the *c-myc* antibody (final dilution 1:25) for 1 hour at room temperature. The control sample was incubated with the corresponding rabbit immunoglobulin fraction as negative control and baseline for flow cytometry. After washing in PBS, both samples were incubated with a 1:20 dilution of the secondary fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG antibody (Sigma) for 45 minutes at room temperature. The samples were washed in PBS and resuspended in 1 ml PBS containing 1 mg/ml ribonuclease A (Sigma), to which 20 μ l of propidium iodide (Sigma) was then added. Stained samples were analysed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) by assessment of 10 000 events from each sample. The percentage of cells expressing *c-myc* protein was defined by setting a region on the control sample containing less than 1% of the events. This region was then superimposed on the *c-myc* stained sample to define the percentage positivity.

Data analysis

Clinicopathological features including age, sex, and histological parameters of the primary tumour were obtained from

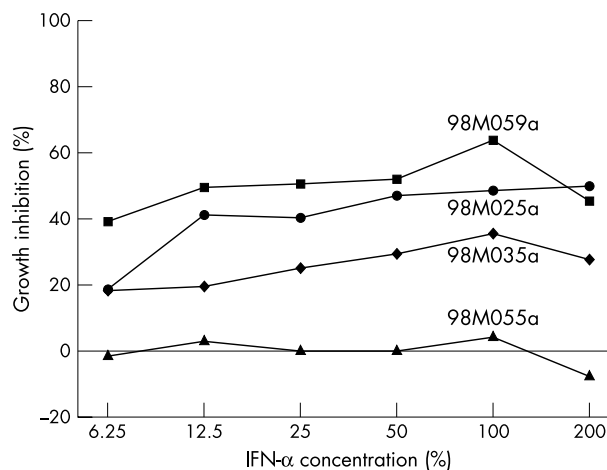


Figure 1 Typical dose-response curves of uveal melanoma cells to interferon alfa (IFN- α).

each patient's records. The histological parameters of the tumours available were the largest tumour diameter, the mitotic index, and the predominant cell type (spindle cell, epithelial or mixed). Data were analysed using JMP (SAS Institute) and the statistical relations between parameters assessed using the Anova *t* test and the Spearman rank correlation.

RESULTS

Patient characteristics

The median age at presentation was 59.5 years (range 25–85 years). Twenty six patients were male and 19 female. All patients in the study group presented with only primary disease at the time of enucleation. The mean largest tumour diameter, measured from the histological section, was 13.8 mm (range 8–22 mm). Sixteen tumours were of spindle cell morphology, 19 tumours were of mixed cell type, and nine tumours exhibited epithelioid cell type. Three tumours were necrotic and therefore unclassifiable.

Chemosensitivity profiles

Examples of typical interferon alfa dose-response curves for four uveal tumours are displayed in figure 1. There was a range of responses from specimens that were relatively resistant to the action of interferon alfa such as one case that had a maximum percentage of inhibition of 5.5% and an interferon alfa sensitivity index of 593.8. In contrast another specimen was relatively sensitive to interferon alfa with a maximum percentage of inhibition of 64.1% and an interferon sensitivity index of 296.8. The dose-response curves tended to be quite shallow such as seen in figure 1C.

The distributions of maximum percentages of inhibition and interferon alfa sensitivity indices for the series of tumours are shown in figure 2. The mean maximum percentage of inhibition by interferon alfa was 17.3% (SD 36.3%) and ranged from –97.9% (that is, growth) to 99.8%. There were 10 tumours which showed apparent stimulation of growth and this was particularly evident in four patients

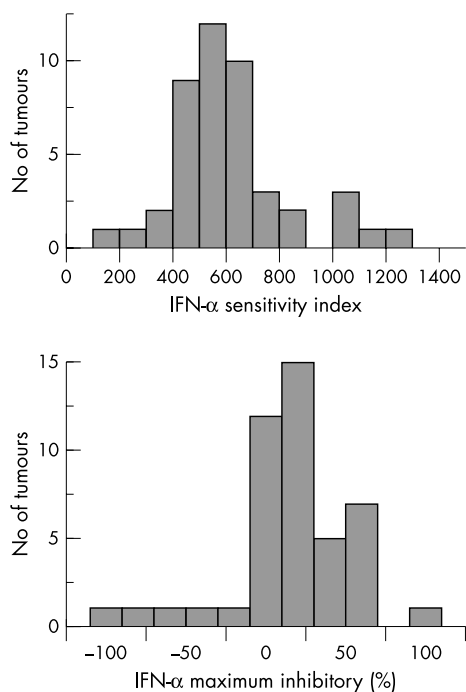


Figure 2 The distribution of (top) sensitivity index to interferon alfa (IFN- α) and (bottom) maximal percentage inhibition among the 45 uveal melanoma tumours in this study.

which had a more than 40% increase in ATP levels. The mean interferon sensitivity index for the series of tumours was 617.5 (224.5) and ranged from 133 to 1269. Using the criterion of a score of 600 as the cut off between more and less sensitive, 56% were classified into the sensitive group. As expected there was a strong correlation ($r^2 = 0.84$, $p > 0.0001$) between the two parameters of sensitivity.

Chemosensitivity profiles and clinicopathological parameters

There was a statistically significant difference ($p = 0.048$) in maximum inhibitory index between the younger patients (mean 30.8%) and the older patients (mean 11.3%) when the patients were stratified according to age, above and below the median for the whole group (61 years). A similar trend ($p = 0.058$) was seen with the interferon alfa sensitivity index being lower in the younger patients (mean 527.7) than the older patients (mean 643.5)

Although the trend was for larger tumours to be less sensitive (interferon alfa sensitivity index 582.9, maximum inhibition 16.9%) than smaller tumours (546.9 and 28.6%), this did not reach statistical significance.

There was no difference in response to interferon alfa when comparing the tumours between male and female patients or between tumours with high or low mitotic indices using either of the interferon alfa sensitivity indices.

Tumours of mixed cell type tended to be more resistant (630.8, 8.5%) than epithelial (567.6, 24.2%) and spindle tumours (574.5, 28.6%) but this was not statistically significant. These differences reached statistical difference ($p = 0.041$) if maximal growth inhibition was compared between the mixed cell type and the other two histologies.

C-myc oncoprotein and clinicopathological features

All 45 uveal melanomas analysed in this study showed evidence of staining for the c-myc oncoprotein. The median positivity for the series was 92.0% and the mean 85.1% (range 11.8–100%). The distribution of c-myc positivity in the series of tumours is shown in figure 3. The variation in c-myc between tumours was relatively small (coefficient of variation 25%) as 80% of specimens had a positivity rate of greater than 80%.

There was no significant difference between oncoprotein positivity in the younger (<61 years) patients (84.8%) compared with the older patients (80.5%). However, there was a trend ($p = 0.074$) towards higher c-myc positivity in uveal melanomas from male patients (88.5%) compared with those from females (76.3%). Similarly, there was a trend ($p = 0.073$) towards higher oncoprotein positivity levels in the larger tumours (89.7%) compared to the smaller tumours (75.7%) using a stratification above and below the median diameter for the group as a whole (13 mm). There was no

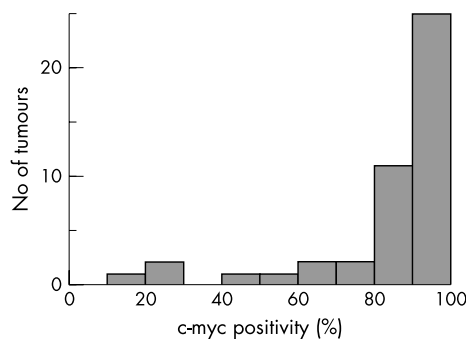


Figure 3 The distribution of c-myc oncoprotein positivity within the 45 tumours in this study.

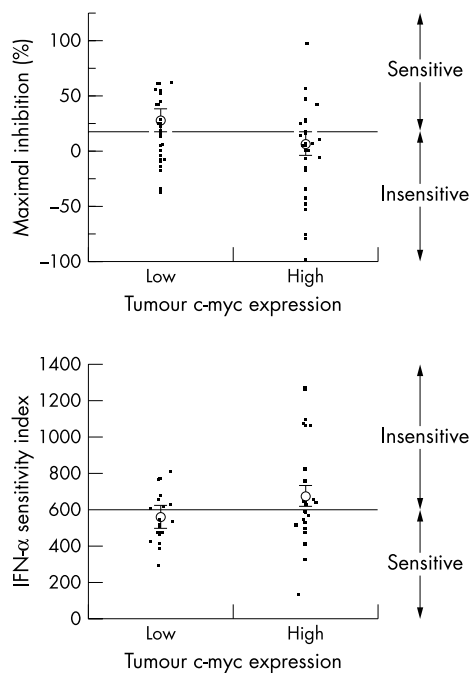


Figure 4 The association between *c-myc* positivity and maximal percentage growth inhibition (top) and interferon alfa sensitivity index (bottom). The symbol represents the mean value in each group while the bars are the standard deviation.

difference in *c-myc* positivity between those tumours with a high mitotic index compared to those with a low index. Unlike the interferon alfa sensitivity, there was no significant difference in *c-myc* positivity between tumours demonstrating a predominantly spindle cell, mixed, or epithelial cell type.

Interferon alfa sensitivity and *c-myc* expression

Correlation analysis revealed no direct correlation between interferon alfa sensitivity and *c-myc* oncoprotein expression. However, this may be because of the preponderance of high *c-myc* expressing tumours. When the series of tumours were divided into two groups above and below the median *c-myc* positivity value (92%) some associations were observed. Figure 4 (top) shows that the mean maximal inhibitory percentage for the low *c-myc* expression group was 28.1%. The mean maximal inhibitory percentage for the high *c-myc* expression group was 7.0% and this just reached significance ($p = 0.049$). Correspondingly (fig 4, bottom), the mean interferon sensitivity index for the lower *c-myc* expression group was 553.4 (in the sensitive range) while the mean interferon sensitivity index for the high *c-myc* expression group was 673.5 (in the resistant range); this did not quite reach significance ($p = 0.073$).

DISCUSSION

Irrespective of primary treatment with eye conserving therapies or enucleation, up to 50% of uveal melanomas will develop metastases during the next decade. Uveal melanoma is notable for its haematogenous dissemination and its tendency to metastasise into the liver.¹⁻²⁶ Once metastasised, life expectancy is short because of the refractory nature of the disease to conventional chemotherapeutic approaches.²⁶ Evidence from tumour doubling times suggests that micro-metastases lie dormant in many cases and do not progress until after removal of the primary lesion and that treatment can impact on the growth of the secondary lesion.²⁷ There is a clear need for an effective adjuvant therapy for patients at

high risk of metastasis from ocular melanoma as there is also for patients with other high risk melanoma types.

Despite some controversy regarding its efficacy in cutaneous melanoma, chemoimmunotherapy has been used in the treatment of disseminated uveal melanoma.⁴⁻²⁸ A key component of these schedules is the inclusion of interferons. However, uveal melanoma presents a paradox in that, unlike cutaneous melanoma, lack of expression of MHC class I on primary uveal melanoma has been found to be correlated with a better patient survival and, conversely, overexpression with poorer survival.²⁹⁻³⁰ MHC class I upregulation appears to be one of the mechanisms involved in IFN activity.¹⁰ In cutaneous melanoma, downregulation of HLA class I in primary lesions is associated with increased thickness of the lesion, tumour progression, and reduced survival. Cutaneous melanoma metastases have been shown to exhibit a lower HLA class I expression than primary tumours³¹ and this is associated with a worse survival.³¹⁻³² This evidence presents a clear rationale to pursue immunotherapeutic approaches in cutaneous melanoma but is counterintuitive for uveal melanoma.

However, the biology of uveal melanoma is complex and represents a distinct entity from its cutaneous relative in many aspects even though they share a common cell of origin; our studies on *c-myc* are an example of this paradigm. In cutaneous melanoma we have found overexpression of the proto-oncogene to be associated with poor survival in primary, secondary, and variant lesions of this disease¹⁸⁻²¹⁻³³ and like others¹⁶ to be associated with downregulation of class I HLA expression.¹⁷ In contrast, *c-myc* overexpression in uveal melanoma predicts for better survival,²² yet the inverse relation between *c-myc* and class I HLA expression is similar to cutaneous melanoma.³⁴ In a further study we explored the relation between *c-myc*, *bcl-2*, and *p53* in uveal melanoma and speculated that overexpression of *c-myc* in the presence of an intact *p53* pathway would render the tumour cells susceptible to apoptosis and possibly less capable of metastasis, resulting in a better prognosis. In cutaneous melanoma, *p53* mutation is a late event³⁵ and may predispose to metastasis. Tumours would have a higher mitotic and apoptotic rate than uveal melanoma, but their *p53* mutation may make apoptosis during metastasis less likely. High *c-myc* in such cases would result in faster growth and metastasis, resulting in a poor prognosis.

The paradox of class I HLA overexpression being associated with poor outcome in uveal melanoma has also been the subject of speculation.²⁹⁻³⁰⁻³⁶ HLA class I molecules are essential for cytotoxic T lymphocyte (CTL) responses against malignant cells, and loss of HLA class I expression may lead to immune silent tumour cells. However, loss of HLA class I antigens makes cells more sensitive to natural killer (NK) cell mediated lysis. This might suggest that CTL mediated control of tumour growth does not have a major role in the clinical course of uveal melanoma and that the data are compatible with a potential role of NK cell mediated control of haematogenous metastatic spread. Another tantalising aspect of interferon alfa activity is a potential anti-angiogenic effect at repeated low doses,³⁷ which might be particularly effective considering the mode of spread of uveal melanoma.

The data presented in this study show a modest overall response, although with considerable inter-tumour variation, of uveal melanoma cells to interferon alfa; this is similar to a previous study in cutaneous melanoma.³⁸ The tumours expressing the highest levels of *c-myc* were the least sensitive to interferon. These results support the concept that *c-myc* downregulation is associated with the cell growth inhibition produced by interferon alfa and that resistance is associated with tumour *c-myc* overexpression.

This has important implications for the use of interferon as adjuvant therapy in melanoma. The patient groups which would gain most from effective adjuvant therapy—that is, those with the worst prognosis and the highest c-myc expression levels when considering cutaneous melanoma, are the least sensitive to interferon. This may explain why the efficacy of interferon as an adjuvant agent in melanoma has been questioned. It suggests that the use of interferon alone as adjuvant therapy in cutaneous melanoma is unlikely to be beneficial as has been demonstrated by the majority of clinical trials.^{39–42}

The link between c-myc overexpression and interferon alfa resistance supports the use of therapeutic strategies to block the effects of the c-myc gene, such as antisense gene therapy,⁴³ to overcome tumour resistance to interferon alfa. The combination of these two therapies may therefore offer a potential method to increase the efficacy of interferon and offer the prospect of more successful adjuvant therapy in the future. The caveat would be whether modulation of c-myc expression in uveal melanoma would lead to a phenotype that may be intrinsically less susceptible to apoptosis. The interplay between interferon alfa, c-myc, and biological processes is clearly complex in uveal melanoma and a fertile and a challenging area for future research.

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