c-myc, p53, and Bcl-2 expression and clinical outcome in uveal melanoma


Abstract

Aims—Overexpression of c-myc protein has independent prognostic significance in a variety of primary and metastatic cutaneous melanomas which suggests a possible role for this gene in melanoma-genesis. We have therefore examined the importance of this oncogene in uveal melanoma and studied the coexpression of two other gene products, Bcl-2 and p53, which might contribute to its effect.

Methods—The percentage of cells positive for nuclear c-myc expression was estimated by flow cytometric analysis of nuclei extracted from paraffin blocks. The expression of Bcl-2 and p53 protein was assessed by immunohistochemistry. A total of 71 tumours were studied and the results compared with survival with a mean follow up period of 6 years.

Results—c-myc was expressed in >50% of the cells by 70% of the tumours, and was independently associated with improved survival in a Cox multiple regression model. Although Bcl-2 was expressed by the majority of the cells in 67% tumours, it was without effect on prognosis. None of the cases studied showed convincing positivity for p53. Analysis of coexpression showed that the best survival was seen in c-myc+Bcl-2+ tumours and the worst in c-myc−Bcl-2− tumours.

Conclusion—The finding of improved rather than reduced survival in c-myc positive tumours is at variance with skin melanoma. There was no evidence to suggest that c-myc was modulated by upregulation of Bcl-2 or p53 inactivation/mutation. Although Bcl-2 is unlikely to have any effect on tumour growth or metastasis, it could contribute to the general lack of susceptibility to apoptosis in these tumours.

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Although uveal melanoma is the commonest tumour of the eye, it represents less than 1% of cancer registrations and is a good example of a “rare” tumour. However, it accounts for 13% of deaths from melanoma. Death is invariably due to metastatic disease.³ Local treatment involves radiotherapy or enucleation if the tumour is large.

Various chromosomal abnormalities have been found in uveal melanoma, notably loss of chromosome 3, which is associated with a poor prognosis.⁷ There is also loss of chromosome 6p with overrepresentation of chromosome 6q and chromosome 8. In contrast with skin melanoma, few uveal melanomas show karyotypic abnormalities of chromosomes 1 or 9.¹⁵ Little is known about the molecular pathogenesis of uveal melanoma. However, p53 abnormalities do not appear to be of major importance, despite early indications to the contrary¹⁷ and variable immunohistochemical results.¹⁶ Recent studies¹⁸ have implicated p16 in at least some uveal melanomas, although mutations have not yet been found.¹⁹

Overexpression of the c-myc oncogene is known to influence outcome in a number of tumours,¹²¹⁵ including skin melanoma in which FACS analysis of nuclear c-myc correlates with prognosis.¹⁴¹⁵ c-myc is expressed in uveal melanoma.¹⁶ Multiplication of chromosome 8 is common in uveal melanoma⁵¹¹¹¹⁷ and has been associated with poor prognosis.¹⁸ In addition, chromosome 8 multiplication has been found to correlate with growth in uveal melanoma.¹⁶ c-myc is in the amplified region of this chromosome and there is one report that cytoplasmic c-myc expression is a prognostic factor in uveal melanoma.¹⁷ The primary role of c-myc is involved in the regulation of cell proliferation, but it can also act with other genes to precipitate apoptosis via p53 dependent or independent mechanisms.²⁰ Rescue from this apoptotic pathway is often mediated by Bcl-2,²¹ which has been reported to be upregulated in most if not all uveal melanomas.²²²³ Since the effects of each of these proteins have not previously been studied in the same series of tumours, we have examined coexpression of Bcl-2 and p53 in the cohort of primary tumours in which c-myc was measured.

Since previous work used FACS analysis to measure nuclear c-myc expression, we chose to use the same method here. However, Bcl-2 and p53 were assessed by immunohistochemistry to conserve tissue and allow comparison with similar studies.
Materials and methods

TUMOUR MATERIAL

Archival, paraffin embedded tumour specimens were obtained from the histopathology files of the Department of Pathology, Institute of Ophthalmology from a series of patients treated at Moorfields Eye Hospital from 1979–86. This series has been used for prognostic studies previously and is well documented with a median 6 year follow up. All specimens were obtained from previously untreated cases of large melanomas which were considered unsuitable for radiotherapy and were treated by enucleation; they therefore form a high risk group. There was a total of 71 cases with sufficient tumour material for analysis. This series had a median age at presentation of 60 years (range 12–81 years) with 34 female and 37 male patients. The largest tumour diameter (LTD) was 12 mm (histological section) with ciliary body (CB) involvement in 17 cases and extraocular extension (EOE) in seven tumours. The majority of tumours were of mixed (33) or spindle cell (31) type with two epithelioid tumours. Seven tumours were necrotic and not classifiable.

IMMUNOHISTOCHEMISTRY

Paraffin sections of 5 µm were cut from the formalin fixed block used for diagnostic histopathology. In all cases, an avidin-biotin complex (ABC) method was used to demonstrate binding of the primary antibodies to p53 (DO7, M7001) and Bcl-2 (M887) which were obtained from Dako Ltd, High Wycombe. All incubations were carried out at room temperature. Antigen retrieval was performed by pressure cooking the sections in TRIS-HCl buffer, pH 9.5, for 2 minutes: 5% urea was added to the buffer used for Bcl-2 antigen retrieval. Following washing, non-specific antibody binding was blocked by the addition of 5% dried milk in TRIS buffered saline (TBS), pH 7.6 for 25 minutes. The primary antibody was diluted 1:25 in TBS + 5% milk protein and left on the slide at room temperature for 60 minutes in a humidified covered tray. The slides were washed three times over 15 minutes in TBS and the second antibody, a biotinylated rabbit anti-mouse antibody (E0354, Dako), was added at 1 in 300 dilution in TBS and incubated for 45 minutes 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 in 20 in dilution of the secondary fluorescein isothiocyanate conjugated goat anti-rabbit IgG antibody (Sigma, Poole, Dorset) 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 µl of propidium iodide was then added. Stained samples were analysed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) and data analysed for 10 000 events from each sample. The percentage of cells expressing c-myc was defined by setting 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.

FLOW CYTOMETRY

Flow cytometry for c-myc was performed using a modified version of the method originally described by Watson et al. Two 35 µm sections were cut from the block used for diagnosis, dewaxed in xylene (2 × 10 minutes), and rehydrated through a series of alcohols. The tumour was separated from the surrounding normal tissue and nuclear extraction performed by incubation with pepsin solution (4 mg/ml in 0.1 M HCl) for 45 minutes at 37°C. The extracted nuclei were filtered through a 35 µm 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 µl dilution buffer (PBS + 0.5% normal goat serum + 0.5% Tween 20) containing 4 µl 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 in 20 in dilution of the secondary fluorescein isothiocyanate conjugated goat anti-rabbit IgG antibody (Sigma, Poole, Dorset) 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 µl of propidium iodide was then added. Stained samples were analysed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) and data analysed for 10 000 events from each sample. The percentage of cells expressing c-myc was defined by setting 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

The percentage of c-myc positive nuclei, p53, and Bcl-2 scores were entered into an ACCESS 2.0 database (Microsoft) for analysis together with the individual clinical data values.

Figure 1 Kaplan–Meier curve for c-myc showing improved survival in the tumours with higher c-myc positivity (n = 71, log rank test p <0.01). (a) High c-myc, (b) low c-myc.
previously recorded for these patients. Query derived subsets of data were imported into SPSS for Windows and analysed by Kaplan–Meier and log rank methods. A multivariate Cox proportional hazards regression model was used to compare those factors exhibiting prognostic associations and linear regression used to examine possible correlation between individual factors.

### Results

#### C-MYC POSITIVITY

Flow cytometry measurements of the percentage of positive nuclei for c-myc varied from 1.5–98.5%, the median positivity was 70.2% with greater than 50% positivity being present in 70% cases. There was strong positive correlation between c-myc positivity and favourable outcome as shown in Figure 1. This was significant in the log rank test (p <0.01) and was an independent variable predicting death in the Cox proportional hazards regression model (Table 1). There was no correlation of c-myc with LTD ($r = 0.096$, NS).

#### BCL-2 SCORE

The staining pattern for Bcl-2 showed some variability between tumour cells within the tumour, but was often uniform within large areas with predominantly cytoplasmic staining. A subjective grading system was therefore used to assess the degree of expression. Typical results are shown in Figure 2 for both positive and negatively stained tumours. There was moderate or strong positivity in 70% cases. Bcl-2 positivity (that is, moderate to strong staining) was not a strong prognostic factor in Kaplan–Meier univariate analysis (Fig 3), nor in multivariate analysis (Table 1). There was no staining of normal iris or choroidal melanocytes elsewhere in the eye.

#### P53 EXPRESSION

No nuclear p53 positive tumours were identified by immunohistochemistry despite strong staining of a positive control in all batches stained, and only two tumours showed any evidence of cytoplasmic staining. Further assessment was not performed.

#### COEXPRESSION AND CLINICAL OUTCOME

To determine the joint prognostic significance of c-myc and Bcl-2, the original data from this study and previous studies was entered Table 2. Comparison of c-myc positivity (>50% cells positive on FACS analysis) and Bcl-2 positivity (moderate/strong immunostaining) in uveal melanoma ($n=69$)

<table>
<thead>
<tr>
<th></th>
<th>c-myc+</th>
<th>c-myc−</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2+</td>
<td>34 (49%)</td>
<td>14 (20%)</td>
<td>48 (70%)</td>
</tr>
<tr>
<td>Bcl-2−</td>
<td>15 (22%)</td>
<td>6 (9%)</td>
<td>21 (30%)</td>
</tr>
<tr>
<td>Total</td>
<td>49 (71%)</td>
<td>20 (29%)</td>
<td>69 (100%)</td>
</tr>
</tbody>
</table>

Figure 2 Representative examples of Bcl-2 staining in choroidal melanomas. Section (A) was graded +++ and section (B) was graded negative (original magnification ×400).

Figure 3 Kaplan–Meier graph for Bcl-2 showing no influence on survival ($n=96$, log rank test, NS). (a) High Bcl-2, (b) low Bcl-2.
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However, a similar finding of an adverse relation in skin cancer.26 The finding in this study that high nuclear c-myc−/Bcl-2− tumours, but these differences are not statistically significant and the groups are small (Fig 4).

**Figure 4** Kaplan–Meier graph for grouped c-myc and Bcl-2 showing influence on survival (n = 96, log rank test, df=3, NS). (a) c-myc+/Bcl-2+; (b) c-myc+/Bcl-2−; (c) c-myc−/Bcl-2+; (d) c-myc−/Bcl-2−.

into a Cox proportional hazards model together with the c-myc data alone and then with Bcl-2 forced into the model (Table 1). While Bcl-2 added no significant predictive value to the model, it was interesting to note that those patients with Bcl-2 and c-myc positive tumours accounted for 49% of the cases examined (Table 2). The best survival was seen in c-myc+/Bcl-2+ tumours, followed by c-myc+/ Bcl-2− and c-myc+/Bcl-2+ with the worst in c-myc−/Bcl-2− tumours, but these differences are not statistically significant and the groups are small (Fig 4).

**Discussion**

The finding in this study that high nuclear c-myc positivity was associated with a good prognosis was unexpected, given the recent finding of an adverse relation in skin melanoma.14 15 However, a similar finding of good prognosis associated with high nuclear c-myc levels has been reported in testicular cancer.16 Since c-myc is thought to act as a nuclear transcription factor, high levels of the protein would be expected to correlate with uncontrolled proliferation and faster growth. However, there was no evidence of an association between c-myc and LTD.

The only previous study concerned with c-myc and clinical outcome in uveal melanoma was reported by Mooy et al.19 In their study immunohistochemistry was used to detect c-myc, Bcl-2, and Ki-67 antigen. The striking feature of their study was the prevalence of cytoplasmic (80%) rather than nuclear (33%) expression of c-myc. Although expression in these two cellular compartments was correlated, only cytoplasmic accumulation of c-myc was found to be a significant adverse prognostic factor. This represents a much lower nuclear positivity than we report in this study using flow cytometry and is also in contrast with our finding that overexpression of c-myc was associated with favourable outcome. This difference could be attributed to the different techniques and antibodies used. It is of interest that the 9E10 monoclonal antibody used in the study by Mooy et al.19 predominately recognised a 40 kDa protein in the uveal melanoma cells rather than the expected 64–67 kDa protein. It is known that this antibody may recognise breakdown products of c-myc, and that other antibodies have similar problems.

However, the reasons underlying the different prognostic significance in nuclear c-myc expression between skin and uveal melanoma are likely to be complex. Possible technical explanations for the paradox could include post-translational modification of the c-myc protein in poor prognosis tumours leading to lower antibody affinity and a false indication of low c-myc within the cells. Since we have studied nuclear c-myc expression, we cannot exclude the possibility that cytoplasmic c-myc expression was high in some tumours with low nuclear levels reflecting greater cell cycle deregulation. Nevertheless, c-myc is reported to act mainly as a transcriptional activator28 and a mechanism by which cytoplasmic expression might influence prognosis is not apparent.

In the case of uveal melanoma, our study and others19 22 show that most tumours express Bcl-2 but presence of the proto-oncogene is without influence on clinical outcome. This is in contrast with cutaneous melanoma in which Bcl-2 expression decreases with tumour progression29 30 and has been shown to be an adverse prognostic marker in metastatic melanoma.31 p53 is not overexpressed in our series and appears to be wild type in most uveal melanomas, in contrast with early indications.32 33 Autocrine secretion of survival factors by uveal melanoma cells may assist their apparent slow growth and resistance to apoptosis.34 We have not assessed the influence of other genes in the apoptosis pathway and it is possible that Bcl-xS, Bcl-xL, BAK, BAD, or BAX might have an effect which would influence the possible biological role of the observed high c-myc expression. Activation of c-myc has been shown to increase the susceptibility to apoptosis in cells subjected to stress, such as adverse microenvironmental conditions, through a p53 dependent mechanism.20 22 23

Unlike cutaneous melanoma, the prognosis of uveal melanoma depends solely on the development of haematogenous metastasis and the treatment for metastatic disease has, until recently, been completely ineffective.35 The clues to the biological role of high c-myc levels in uveal melanoma may lie in consideration of the different selective pressures and stresses imposed on metastasising cells by the microenvironment in the blood and in the tissues where they subsequently lodge. In this scenario, cells with high nuclear c-myc which were susceptible to apoptosis via an intact p53 pathway would be less capable of metastasis. Thus, tumours with an intact p53 pathway and high c-myc would be expected to have a better prognosis. In cutaneous melanoma, p53 mutation is a late event36 37 and may predispose to metastasis. The tumours have a higher mitotic and apoptotic rate than uveal melanoma,38 but their p53 mutation triggers apoptosis during metastasis less likely. High c-myc in such cases would result in faster growth and metastasis, resulting in a poor prognosis.
These findings have some implications for treatment, since most cytotoxic drugs are thought to induce apoptosis by mechanisms which would be influenced in many cell types by c-myc, p53, and Bcl-2 status.\textsuperscript{34-38} Uveal melanoma is remarkably chemoresistant,\textsuperscript{39} but can be made sensitive to alkylating agents ex vivo by the addition of inhibitors of DNA repair.\textsuperscript{40-42} It will be of interest to see whether the c-myc status of the tumour influences this response.

In summary, nuclear c-myc expression is associated with a good prognosis in uveal melanoma. There is differential expression of Bcl-2, but no evidence that it has prognostic significance. p53 is not expressed in most or even all tumours. These results contrast with skin melanoma and provide further evidence of the difference in the biology and behaviour of these tumours, despite their common melanocytic derivation.

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