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

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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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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.² 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 9.³⁻⁶ Little is known about the molecular pathogenesis of uveal melanoma. However, *p53* abnormalities do not appear to be of major importance,⁷ despite early indications to the contrary⁸ and variable immunohistochemical results.⁹ Recent studies¹⁰ have implicated *p16* in at least some uveal melanomas, although mutations have not yet been found.¹¹

Overexpression of the *c-myc* oncogene is known to influence outcome in a number of tumours,^{12,13} including skin melanoma in which FACS analysis of nuclear *c-myc* correlates with prognosis.^{14,15} *c-myc* is expressed in uveal melanoma.¹⁶ Multiplication of chromosome 8 is common in uveal melanoma^{3,5,11,17} and has been associated with poor prognosis.¹⁸ In addition, chromosome 8 multiplication has been found to correlate with growth in uveal melanoma.¹⁸ *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.¹⁹ 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.^{22,23} 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.

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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.¹ Local treatment

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.²⁴ 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), added at 1 in 300 dilution in TBS and incubated for 45 minutes at room temperature. After washing, the sections were incubated with a tertiary streptavidin-alkaline phosphatase reagent (K0391, Dako). The sections were again washed in TBS and incubated in Vector Red (Vector Laboratories, Peterborough) for 15 minutes, washed, and lightly counterstained with Mayer's haematoxylin for 25 seconds. Sections were viewed by direct microscopy and the positivity of the melanoma cells assessed qualitatively. Positive and negative (no primary antibody) controls were included with each batch of sections. The slides stained for Bcl-2 were graded by a single pathologist (IAC) without reference to previous data as negative (0), weakly positive (+), moderately positive (++), or strongly positive (+++) based on staining intensity rather than the number of cells stained.

FLOW CYTOMETRY

Flow cytometry for c-myc was performed using a modified version of the method originally described by Watson *et al.*^{25, 26} 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 10⁶ 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 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

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.

Table 1 Cox regression model for *c-myc* in uveal melanoma, taking into account other prognostic factors of known importance. There were 71 cases in total, 10 with missing values, 0 valid cases with non-positive times, and 3 censored cases before the earliest event in a stratum. Thus, 13 cases were dropped and 58 were available for the analysis. $\text{Exp}(\beta)$ is equivalent to relative risk, for which confidence intervals are given. The test statistic (Wald), degrees of freedom (df), and significance (p value) are also shown

Variable	$\text{Exp}(\beta)$	95% CI for $\text{Exp}(\beta)$		Wald	df	p Value
		Lower	Upper			
<i>c-myc</i>	0.977	0.960	0.993	7.7724	1	0.005
LTD	1.036	0.936	1.146	.4603	1	0.496
Vessel count	1.034	1.013	1.055	9.9281	1	0.002
CB involvement	0.137	0.044	0.423	11.9271	1	0.001
Age	0.026	0.998	1.053	3.4073	1	0.065
<i>Bcl-2</i>	0.658	0.210	2.064	.5148	1	0.473
Extraocular ext	151391	0.000	NA	.0003	1	0.986

previously recorded for these patients.²⁴ 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.

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

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*.

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,^{24–27} 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 ($n=69$)

	<i>c-myc</i> +	<i>c-myc</i> –	Total
<i>Bcl-2</i> +	34 (49%)	14 (20%)	48 (70%)
<i>Bcl-2</i> –	15 (22%)	6 (9%)	21 (30%)
Total	49 (71%)	20 (29%)	69 (100%)

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.²⁶ 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 predominantly recognised a 40 kDa protein in the uveal melanoma cells rather than the expected 64–67 kDa pro-

tein. 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 others^{19 22 23} show that most tumours express *Bcl-2* but presence of the proto-oncogene is without influence on clinical outcome. This is again in contrast with cutaneous melanoma in which *Bcl-2* expression decreases with tumour progression^{29 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,⁷ in contrast with early indications.⁸ Autocrine secretion of survival factors by uveal melanoma cells may assist their apparent slow growth and resistance to apoptosis.³² 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 33 34}

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 event^{36 37} and may predispose to metastasis. The tumours have a higher mitotic and apoptotic rate than uveal melanoma,³² 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.

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.^{34, 38} Uveal melanoma is remarkably chemoresistant,³⁵ but can be made sensitive to alkylating agents *ex vivo* by the addition of inhibitors of DNA repair.³⁹ 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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