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Immunohistochemical and Prognostic Analysis of Apoptosis and Proliferation in Uveal Melanoma

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Neoplasia can be defined as deregulated tissue homeostasis caused by an imbalance between proliferation and apoptosis. Many genes are involved in the maintenance of tissue homeostasis, eg, the c-myc oncoprotein, which is an important regulator of cell proliferation and Bcl-2 protein, which is involved in the regulation of apoptosis. We studied retrospectively indices of proliferation, such as mitotic count and the Mib-1 index, on 51 uveal melanomas and compared their prognostic significance with established indicators of prognosis such as cell type and tumor size. Along the same line we investigated the expression of the regulating proteins c-myc and Bcl-2. Of all parameters tested, the largest tumor diameter and mitotic count were most strongly associated with tumor-related death (P < 0.001 and P = 0.005, respectively). In addition, cell type, the presence of epithelioid cells, the Mib-1 index, and the percentage of cytoplasmic c-myc-positive cells were significant predictive factors. Multivariate analysis showed that the Mib-1 index, largest tumor diameter, and the percentage of cytoplasmic c-myc-positive cells were independent prognostic parameters. Bcl-2 expression did not correlate with clinical outcome. The Mib-1 index correlated with the presence of epithelioid cells (P < 0.03) and the presence of apoptotic bodies (P < 0.001) and c-myc. A strong inverse relationship was found between (nuclear and cytoplasmic) c-myc and Bcl-2 (P < 0.00004 and P < 0.006, respectively), suggesting that Bcl-2 cooperates with c-myc to immortalize uveal melanoma cells. (Am J Pathol 1995, 147:1097–1104)

The maintenance of homeostasis in normal tissue can be viewed as a tightly regulated balance between cell production and cell death.[1] Neoplasia can arise when tissue homeostasis is deregulated. Most of the knowledge concerning oncogenic events has concentrated on mechanisms of increased cell growth. However, decreased cell death also would result in an expansion of the cell mass.[1] Cells can die either by necrosis (inactively) or by apoptosis (actively). Individual cell disintegration is a constant finding in malignant neoplastic tissue, and these dying or dead cells, morphologically characterized by volume contraction and nuclear condensation, have been called apoptotic.[2] Monoclonal antibodies (MAbs) against proteins involved in the regulation of cell proliferation and death can be used to visualize the dynamics of tissue homeostasis. The *Bcl-2* protein blocks apoptosis and thus prolongs cell survival. In human fetal tissues *Bcl-2* appears to be involved in tissue homeostasis as well as morphogenesis.[3] Only few reports concerning *Bcl-2* expression in nonhematopoietic malignancy have been published,[4–11] including cutaneous melanoma.[12] The c-*myc* protein is involved in the control of cell proliferation but is also a potent inducer of apoptosis.[13] c-*myc* expression is frequently deregulated in neoplasms and is often implicated in their genesis.[14] The c-*myc* gene is located on chromosome 8q24.1; chromosomal abnormalities involving chromosome 8q have been specifically associated with uveal melanoma.[15] It has been found that staining for c-*myc* protein correlates with proliferative index in diploid uveal melanomas, in line with the role of c-*myc* protein progression through the cell cycle.[16] Proliferative indices may provide information independent of other histological and clinical prognostic variables.[17] The MAb Mib-1 recognizes the Ki-67 antigen, which is expressed by proliferating cells and can be used on formaldehyde-fixed paraffin sections.[18]

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The purpose of this study was to determine in uveal melanomas the relationship between the expression of c-*myc* and *Bcl-2* and to investigate how their expression is related to cell proliferation and programmed cell death. Furthermore, the prognostic significance of these parameters in comparison with cell type and tumor size was investigated.

Materials and Methods

Histological Specimens

To correlate immunohistochemical findings with prognosis, a retrospective analysis of 51 formalin-fixed paraffin-embedded uveal melanomas was undertaken. From 1973 to 1987 consecutive cases were entered in the study on the basis of availability of adequate histological material. Follow-up data were obtained by contacting the local ophthalmologist and/or the general practitioner, and these data were reviewed to define tumor-related death or death as a result of other causes.

To test the antibody specificity we used frozen tissue from one of the patients included in this series and a cell line (OMM-1) obtained from metastatic uveal melanoma tissue.[19] A colon carcinoma and a breast carcinoma served as a control for c-*myc*. Paraffin sections were cut at 5 to 6 μm and stained with hematoxylin and eosin (H&E). In these sections we determined the following parameters: largest tumor diameter (LTD) ($\leq$10 mm, 10 to 15 mm, or >15 mm), cell type, mitotic rate, and the presence of apoptotic bodies. The tumors were histologically classified in two groups: (1) according to cell type, using the three categories of the modified Callender classification (spindle cell, mixed cell, and epithelioid cell type)[20] and (2) according to the presence or absence of any epithelioid cells (spindle cell melanoma *versus* a combination of mixed cell type and epithelioid tumors).[21] Mitoses were counted in 15 high power fields (HPF) with a total magnification of $\times$400, using an eyepiece grid. This was repeated three times and the number of mitoses was averaged. Apoptotic bodies were recognized by volume contraction and nuclear condensation of tumor cells.[5] With light microscopy, uncertainties in defining apoptotic bodies remain; therefore we did not use an index for apoptotic bodies[5] but scored for the presence or absence of apoptosis. Areas with tissue necrosis were excluded from the counting.

Immunohistochemistry

Formalin-fixed and paraffin-embedded 5-μm sections were mounted on aminopropyltriethoxysilane (Sigma Chemical Co., St. Louis, MO) coated glass slides and dried overnight at 37°C. After deparaffinizing and rehydrating, the slides were placed in 0.01 mol/L citrate buffer and antigen retrieval was performed by microwave irradiation (Bio-Rad, Richmond, CA) twice 5 minutes each. The slides were preincubated with normal goat serum in a dilution of 1:10 for 15 minutes.

The following specific antibodies were used: (1) The MAb raised against the carboxyl-terminal peptide (9E10; amino acids 408 through 439) of the human c-*myc* protein (Oncogene Science, New York, NY) was used in a dilution of 1:1600 and incubated overnight. The slides were incubated for 30 minutes at room temperature (RT) with biotinylated multilink immunoglobulin (BioGenex, Mainz, Germany) in a dilution of 1:75 in phosphate-buffered saline (PBS) with 5% bovine serum albumin. After washing in PBS/Tween 0.5%, the slides were incubated with the streptavidin-biotin-peroxidase complex (BioGenex) in a dilution of 1:50. The alkaline phosphatase anti-alkaline phosphatase technique was used as the detection system with fast red as chromogen. As specificity control, sections were incubated with antibody preincubated overnight with 10 μg/ml excess of the peptide (Oncogene Science). (2) The MAb Mib-1, reacting with the proliferation-associated antigen Ki-67 (Dianova-Immunotech, Hamburg, Germany) was used in a dilution of 1:200, in an overnight incubation protocol at 4°C. (3) The MAb specific for *Bcl-2* oncoprotein (clone 124) was obtained from Dakopatts (Glostrup, Denmark) and used in a dilution of 1:60. Slides were incubated for 60 minutes at RT.

After incubation with MAbs Mib-1 and *Bcl-2*, the slides were incubated for 30 minutes at RT with biotinylated goat anti-mouse immunoglobulin (Dakopatts) in a dilution of 1:400, in PBS with 2% human serum and normal goat serum. After washing in PBS, the slides were incubated with the streptavidin-biotin-peroxidase complex (Dakopatts) in a dilution of 1:200. The peroxidase was visualized with hydrogen peroxide in *N,N,*-dimethylformamide with 3-amino-9-ethylcarbazole dimethylformamide as chromogenic substrate. As a negative control, specimens were stained following the same incubation protocol without use of the primary MAbs. All sections were counterstained with Mayer's hematoxylin and mounted with glycerin/gelatin. As positive control for c-*myc*, *Bcl-2*, and Mib-1, sections of a breast carci-

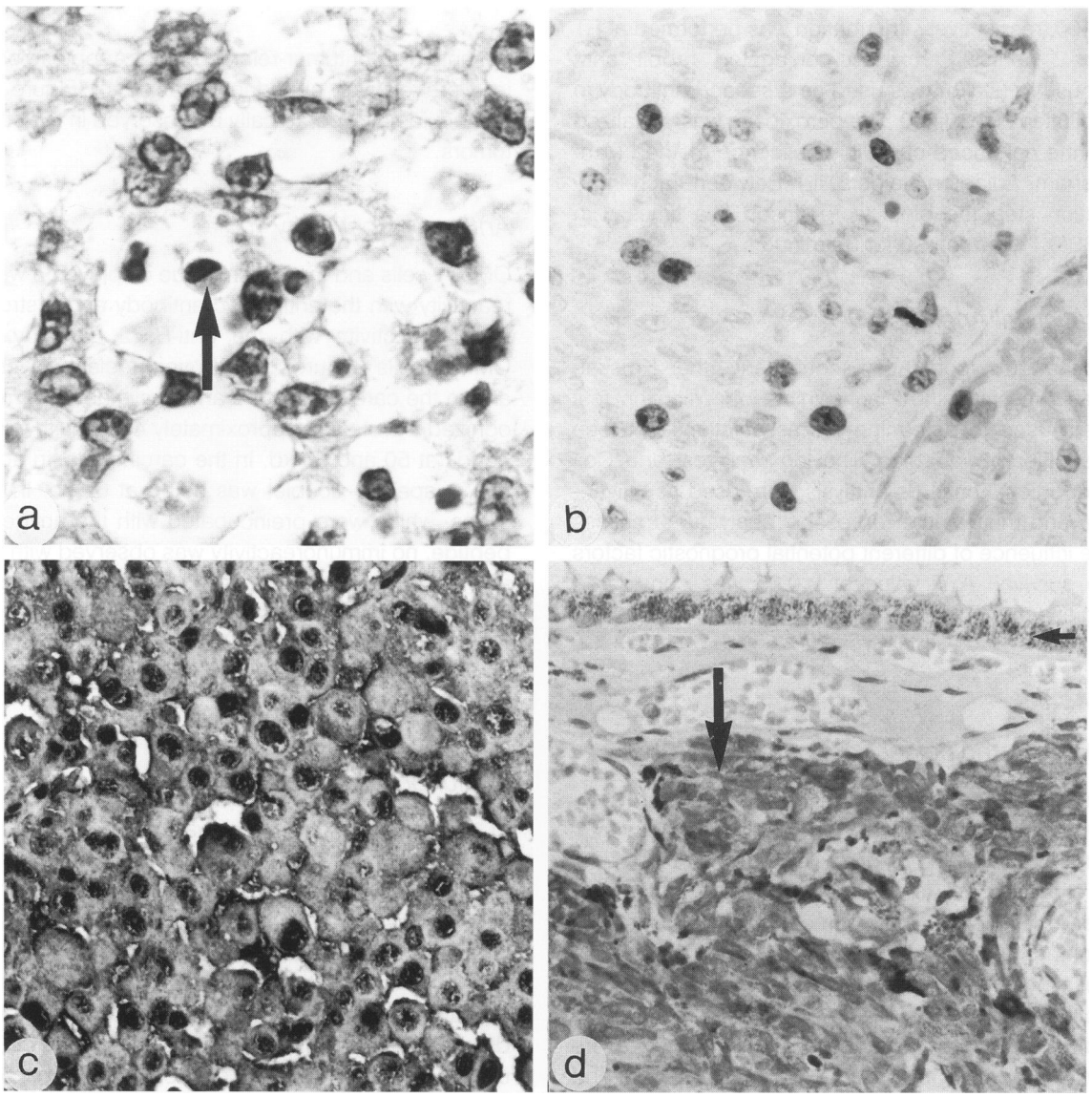
ıoma, normal thymus, and adenocarcinoma of the ›rostate, respectively, were used. In addition, cyto- pin preparations of OMM-1 cells were used as pos- ive control for *Bcl-2* and c-*myc*.

\ssessment of Results

ɪmmunohistochemical results were evaluated without .ccess to the follow-up data. The Mib-1 score was letermined as the percentage of Mib-1-positive cells elative to the total number of cells per HPF. Cell ıuclei were considered to be positive if there was .ny nuclear staining present, regardless of the intensity and distribution within the nucleus. *Bcl-2* and c-*myc* scores were semiquantitatively determined as percentage of cytoplasmic or positive cells: 0, 1 to 25%, 25 to 50%, 50 to 75%, or 75 to 100%. Nuclear staining of c-*myc* was scored similarly, with an additional score for focal (<5%) staining.

Western Blotting

The specificity of the c-*myc* and *Bcl-2* MAbs for use in immunohistochemistry was determined by Western blotting of a total protein extract from frozen uveal melanoma tissue and OMM-1 cultured cells.



igure 1. a: *Apoptotic body* (arrow) *in uveal melanoma, characterized by nuclear condensation and volume contraction. H&E; magnification, ×880.* · *Immunohistochemistry of a section incubated with Mib-1. Magnification, ×361. Note the speckled nuclear staining pattern.* c: *Immunohisto- hemistry of a section incubated with* c-myc *with strong nuclear staining. Magnification, ×361.* d: *Immunohistochemistry of a section incubated with* cl-*2 with positive staining of uveal melanoma* (long arrow). *The retinal pigment epithelium is indicated by the* short arrow. *Magnification, ×361.*

Frozen tissue from a colon and a breast carcinoma served as control for c-*myc*. The frozen tissue was homogenized in a buffer containing a mix of proteinase inhibitors. OMM-1 cells were harvested with a cell scraper, sonicated, and freeze/thawed. The homogenate was boiled in denaturation buffer (0.1% dithiothreitol, 1% sodium dodecyl sulfate, 10% sucrose, and Tris-HCL) for 5 minutes. The proteins were loaded on a sodium dodecyl sulfate polyacrylamide gel (12%). The gel was blotted overnight (0.2 A, 33 V, 4°C) on Immobilon P (Millipore) and incubated at RT with 2% bovine serum albumin/0.1% Tween-20/PBS and subsequently with 1% goat serum in 0.1% Tween-20/PBS for 20 minutes. The dilutions used for *Bcl-2* and c-*myc* were 1:3000 and 1:100, respectively. Incubation was performed at RT for 2 hours. Peroxidase-conjugated rabbit anti-mouse Ig (Dako) was used as a second antibody in a dilution of 1:10,000. The peroxidase was visualized by the enhanced chemiluminescence method (Amersham, Arlington Heights, IL). Between each incubation step the blots were rinsed five times with PBS/0.1% Tween-20 for 30 minutes.

Statistical Analysis

Spearman's correlation coefficient, the Kruskal-Wallis test and the Mann-Whitney U Wilcoxon test were used to determine the associations between the different variables. The log rank test and Cox proportional hazards analysis were used as univariate and multivariate regression analyses to assess the influence of different potential prognostic factors on survival. A P value of <0.05 was considered significant.

Results

Clinicopathological Parameters

The mean age at diagnosis was 59.8 years. Thirty-two patients were male, nineteen female. Twenty-three patients died of tumor-related death, nine died of other causes, thirteen were still alive, and six were lost to follow-up. The total mean follow-up was 83.9 months. Twenty tumors were classified as spindle cell type, nineteen as mixed cell type, and twelve as epithelioid cell type; in thirty-one of fifty-one tumors, epithelioid cells were present. Five tumors were small (<10 mm), twenty-four were 10 to 15 mm, and twenty-two were large (>15 mm).

The mitotic rate was low (<2 mitoses per 15 HPF) in thirty-nine of fifty-one tumors. In twelve tumors, a mitotic rate of ≥2 per 15 HPF was noted; six of these patients died of tumor-related death. Apoptotic bodies were relatively abundant in one tumor (Figure 1a) and could only sporadically be observed in ten other tumors.

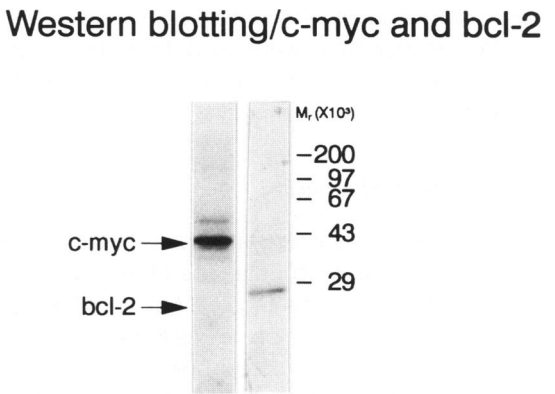


Figure 2. *Western blot analysis from cell line OMM-1 with* c-myc (lane 1) *and* Bcl-2 (lane 2).

Antibody Specificity

OMM-1 cells and the frozen tissue displayed a weak reactivity with the anti-c-*myc* antibody and a strong immunoreactivity with the anti-*Bcl-2* antibody. In OMM-1 cells (Figure 2) and uveal melanoma cells and in the carcinomas, the anti-c-*myc* antibody recognized a protein of approximately 40 kd, with minor bands at 50 and 36 kd. In the carcinomas, in addition, a specific doublet was noted at 67 kd. In the slides, which were preincubated with the antigenic peptide, no immunoreactivity was observed with the anti-c-*myc* antibody. The anti-*Bcl-2* antibody bound a protein in OMM-1 cells with an apparent molecular mass of 25 kd (Figure 2), which is in agreement with the described molecular mass of *Bcl-2* in other tumors.[4]

Immunohistochemistry

In 15 tumors the Mib-1 score was >1.8% (Figure 1b); eight patients in this group died of tumor-related death. In 1 tumor, c-*myc* staining could not be reliably assessed. In 16 of 50 tumors (33%), both nuclear and cytoplasmic staining was noted (Figure 1c); in 40 of 50 tumors (80%) cytoplasmic staining, regardless of nuclear staining, was observed. The distribution of the different scores is reflected in Figure 3. As internal positive control in the same sections, nontumor ocular tissue staining was noted in the photoreceptor inner segments of the retina.

In 49 melanomas, cytoplasmic *Bcl-2* staining was found (Figure 1d), and 2 were negative. The distribu-

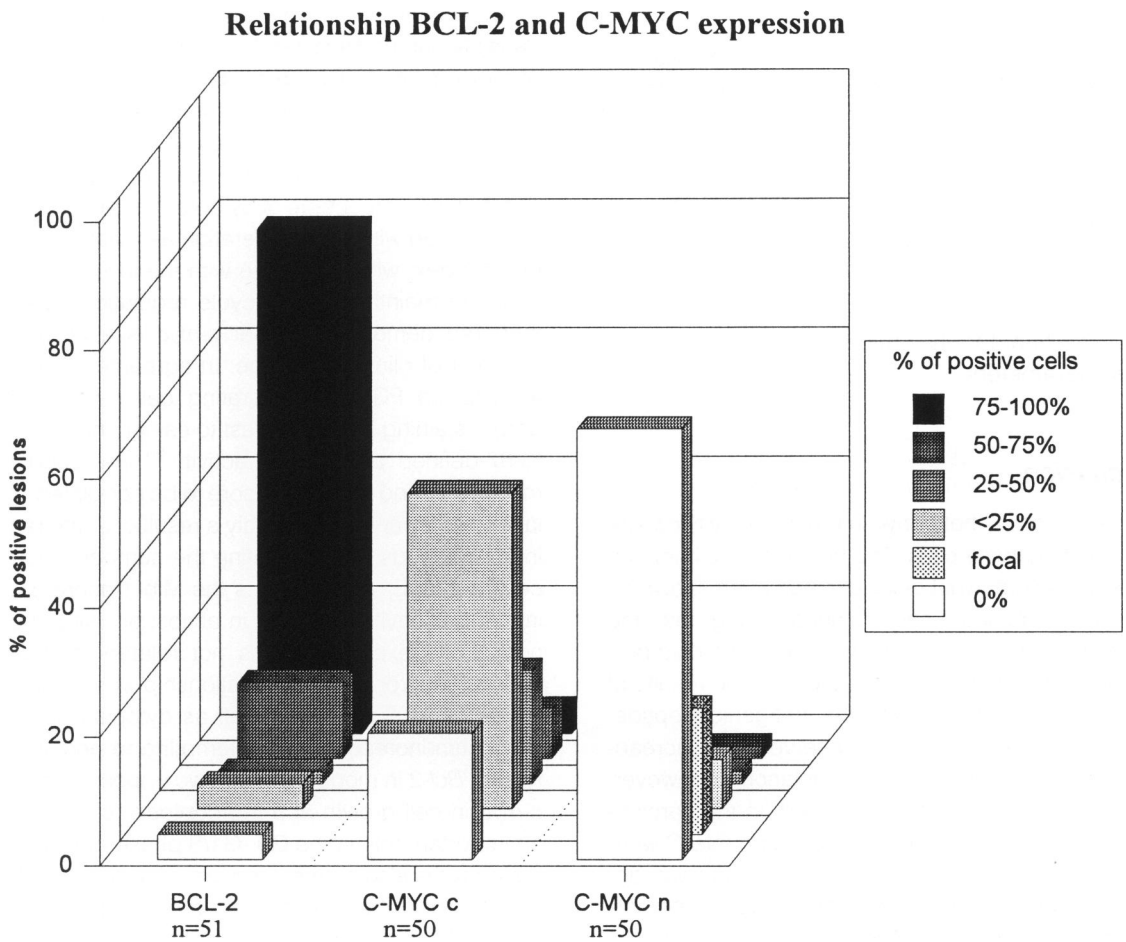


Figure 3. *Percentage of nuclear and cytoplasmic* c-myc-*positive cells and* Bcl-*2-positive cells, expressed as percentage of total number of lesions. c, cytoplasmic staining; n, nuclear staining.*

tion of the scores is reflected in Figure 3. In nontumor ocular tissue, staining of *Bcl-2* was noted as an internal positive control in normal choroidal melanocytes, the retinal pigment epithelium, the nonpigmented epithelium of the ciliary body, tumor-infiltrating lymphocytes, the Müller cells, the plexiform layers of the retina, and the glial cells of the optic nerve. In 1 tumor with abundant apoptotic bodies, *Bcl-2* expression was low, whereas c-*myc* (nuclear and cytoplasmic) expression and the proliferative activity were high (19 mitoses per 15 HPF; Mib-1 score, 2.68%).

Statistical Analysis

Significant associations between different variables were found with Spearman's rank correlation test, which are summarized in Table 1. A strong inverse relationship was found between nuclear as well as cytoplasmic c-*myc*-positive cells and *Bcl-2* expression, which is reflected in Figure 3. The log rank test revealed a significant correlation of survival with cell type, the presence of epithelioid cells, LTD, mitotic rate, the Mib-1 score, and the percentage of c-*myc* cytoplasmic positive cells (Table 2) but not with *Bcl-2* staining. In the multivariate analysis with the Cox proportional hazard model, the correlation of LTD, the Mib-1 index and percentage of c-*myc*-positive cells with survival remained significant af-

Table 1. *Spearman correlation coefficient*

Examined parameters		Two-sided *P* value
c-*myc* n	c-*myc* c	0.0001
c-*myc* n	*Bcl-2*	−0.00004*
c-*myc* c	*Bcl-2*	−0.006*
c-*myc* c	Mib-1 score	0.02
Presence of epithelioid cells	Mib-1 score	0.04
Apoptosis	Mib-1 score	0.001
Mitotic rate	Mib-1 score	0.003
Mitotic rate	Cell type	0.05

n, nuclear staining of c-*myc*; c, cytoplasmic staining of c-*myc*.
*Inverse relationship.

Table 2. *Log rank test*

Univariate regression analysis	*P* value log rank test
Cell type	0.05
Presence of epithelioid cells	0.05
LTD	<0.001
Mitotic rate	0.005
Mib-1 score	0.045
Cytoplasmic staining c-*myc*	0.05

ter correcting for the influence of other investigated parameters.

Discussion

Our results show that c-*myc* immunoreactivity occurs in uveal melanoma cells. The nature of this immunoreactivity is somewhat elusive. It has been shown by immunoprecipitation and immunoblotting that the clone 9E10 antibody reacts with a 67-kd c-*myc* protein and with its cleavage products.[22] The results of blocking experiments, with the antigenic peptide, support the specificity of the observed immunoreactivity. In immunoblots of uveal melanoma, however, we found immunoreactivity at 40 kd. In the carcinomas, a 40-kd band as well as a 67-kd doublet[23] were noted. The 40-kd c-*myc* immunoreactivity we detected in the uveal melanoma as well as in the carcinomas may be a cleavage product. As an alternative explanation, cross-reactive proteins should be considered. Two proteins of 32 kd and 58 kd have been detected in extracts of human cells, which appeared to be antigenically related to the synthetic peptides against which the c-*myc* antibody was raised.[24]

We found c-*myc*-like immunoreactivity in both nuclei and cytoplasm of neoplastic cells. In uveal melanoma, Royds et al[16] reported a higher percentage of c-*myc*-positive lesions than we found in our series. However, all of their melanomas were of the mixed and epithelioid cell type and classified as large tumors (>15 mm), whereas our series was composed of 39% spindle cell melanomas and 57% small and medium sized tumors. Our finding of cytoplasmic c-*myc* immunoreactivity was unexpected, because the c-*myc* gene encodes two nuclear phosphoproteins.[14,23] It has been suggested that newly synthesized *myc* protein is retained in polyribosomes. Putatively, upon activation of this system c-*myc* migrates to the nucleus, where it has been shown to bind both to specific and relatively nonspecific DNA sequences, perhaps influencing DNA replication.[25] The unusual pattern of intracellular accumulation of c-*myc* protein might be caused by abnormal post-translational modification[26] or altered nuclear import of c-*myc* protein. This might be caused by deregulation of cell proliferation but might also be responsible for abnormalities in cell cycle regulation.

We have demonstrated that c-*myc* expression in terms of the percentage of cytoplasmic positive cells is correlated with cell proliferation as reflected in the Mib-1 index, which is in line with the involvement of c-*myc* in maintaining cell cycle regulation. We furthermore demonstrated that c-*myc* is a predictive indicator of clinical outcome. In agreement with the findings on PC-10 (proliferating cell nuclear antigen)[27] staining but in contrast to earlier findings with Ki-67-defined proliferative activity,[28] we found the mitotic rate and the Mib-1 score to be correlated. The latter discrepancy is probably a result of a difference in the methods used to define the number of tumor cells per HPF, which affects the Mib-1 score. *Bcl-2* immunoreactivity occurred in almost all uveal melanomas. Its expression was not related to patient survival. A strong inverse relationship was found between c-*myc* (nuclear as well as cytoplasmic) and *Bcl-2* immunoreactivity. It is tempting to postulate a role for *Bcl-2* in modulating the two opposing roles of c-*myc* in cell growth and in apoptosis. c-*myc* plays an important role in the G0–G1/S phase transition,[29] but c-*myc* is also a potent inducer of apoptosis when expressed in the absence of serum or growth factors.[13,30] It has been shown that *Bcl-2* prevents c-*myc*-induced apoptotis, which provides a mechanism whereby cells can express c-*myc* without undergoing apoptosis.[31–35] *Bcl-2* may also modulate the role of c-*myc* in cell cycle progression. In the presence of growth factors, *Bcl-2*-negative cells may proliferate rapidly. In the absence of growth factors, *Bcl-2* may prevent apoptosis as induced by c-*myc*, which also leads to expansion of cell growth. This would imply that, both in the presence and absence of *Bcl-2* expression, tumor growth (either because of increased proliferation or blocked apoptosis) can occur, which would provide an explanation as to why c-*myc* but not *Bcl-2* expression predicts clinical outcome.

Although apoptosis was not found to be a prominent feature in uveal melanoma, we found the Mib-1 index and apoptosis but not the expression of c-*myc* and apoptosis to be correlated. This discrepancy may also point toward a modifying role of *Bcl-2* on c-*myc* function. The role of growth factors in uveal melanoma needs to be elucidated. The most important findings in this study are that in uveal melanoma c-*myc* and *Bcl-2* are expressed in inversely correlating patterns. The expression of c-*myc* is, in addition

to LTD and the Mib-1 index, a useful independent prognostic parameter for ciliary body and choroidal melanomas. *Bcl-2* expression did not appear to be of prognostic significance.

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