Immunohistochemical and Prognostic Analysis of Apoptosis and Proliferation in Uveal Melanoma

Cornelia M. Mooy,*† Gregorius P. M. Luyten,†
Paulus T. V. M. de Jong,† Theo M. Luider,†
Theo Stijnen,‡ Frieda van de Ham,*
Cornelus C. J. van Vroonhoven,* and
Fred T. Bosman*

From the Departments of Pathology,* Ophthalmology,† and
Biostatistics,‡ Erasmus University, Rotterdam,
The Netherlands

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, e.g., 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 non-hematopoietic 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 MAAb 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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Address reprint requests to Dr. C. M. Mooy, Department of Pathology, PO Box 1738, 3000 DR Rotterdam, The Netherlands.
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. 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) (<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) 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).

Mitoses were counted in 15 high power fields (HPF) with a total magnification of ×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. With light microscopy, uncertainties in defining apoptotic bodies remain; therefore we did not use an index for apoptotic bodies 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 multi-link 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 MAb-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-
noma, normal thymus, and adenocarcinoma of the prostate, respectively, were used. In addition, cyto-
spin preparations of OMM-1 cells were used as posi-
tive control for Bcl-2 and c-myc.

Assessment of Results

Immunohistochemical results were evaluated without access to the follow-up data. The Mib-1 score was determined as the percentage of Mib-1-positive cells relative to the total number of cells per HPF. Cell nuclei were considered to be positive if there was any nuclear staining present, regardless of the inten-
sity 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.

Figure 1. a. Apoptotic body (arrow) in uveal melanoma, characterized by nuclear condensation and volume contraction. H&E, magnification ×880. b. Immunohistochemistry of a section incubated with Mib-1. Magnification, ×361. Note the speckled nuclear staining pattern. c. Immunohistochemistry of a section incubated with c-myc with strong nuclear staining. Magnification, ×361. d. Immunohistochemistry of a section incubated with Bcl-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

**Western blotting/c-myc and bcl-2**

![Western blot analysis from cell line OMM-1 with c-myc (lane 1) and Bcl-2 (lane 2).](image)

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.

**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) andveal 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.

**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, non-tumor 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-
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>
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<th>Examined parameters</th>
<th>Two-sided P value</th>
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<tbody>
<tr>
<td>c-myc n</td>
<td>c-myc c</td>
</tr>
<tr>
<td>c-myc n</td>
<td>Bcl-2</td>
</tr>
<tr>
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<td>Bcl-2</td>
</tr>
<tr>
<td>c-myc c</td>
<td>Mib-1 score</td>
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<tr>
<td>Presence of epithelioid cells</td>
<td>Mib-1 score</td>
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<td>Apoptosis</td>
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<tr>
<td>Mitotic rate</td>
<td>Mib-1 score</td>
</tr>
<tr>
<td>Mitotic rate</td>
<td>Cell type</td>
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n, nuclear staining of c-myc; c, cytoplasmic staining of c-myc.
*Inverse relationship.
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 immuno-
reactivity is somewhat elusive. It has been shown by immunoprecipitation and immunoblotting that the
clon e9E10 antibody reacts with a 67-kd c-myc pro-
tein and with its cleavage products. The results of
blocking experiments, with the antigenic peptide,
support the specificity of the observed immunoreac-
tivity. In immunoblot of uveal melanoma, however,
we found immunoreactivity at 40 kd. In the carcino-
mas, a 40-kd band as well as a 67-kd doublet were
noted. The 40-kd c-myc immunoreactivity were
detected in the uveal melanoma as well as in the carci-
nomas 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.

We found c-myc-like immunoreactivity in both nu-
clei and cytoplasm of neoplastic cells. In uveal melano-
am, Roys et al reported a higher percentage of
c-myc-positive lesions than we found in our series.
However, all of their melanomas were of the mixed
epithelial cell type and classified as large tu-
mors (>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 phosphopro-
teins. It has been suggested that newly synthe-
sized myc protein is retained in polyribosomes. Pu-
tatively, 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.
The unusual pattern of intracellular accumulation of

Table 2. Log rank test

<table>
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<th>Univariate regression analysis</th>
<th>P value</th>
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<tr>
<td>Cell type</td>
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<tr>
<td>Presence of epithelioid cells</td>
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<tr>
<td>LTD</td>
<td>&lt;0.001</td>
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<tr>
<td>Mitotic rate</td>
<td>0.005</td>
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<tr>
<td>Mib-1 score</td>
<td>0.045</td>
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<tr>
<td>Cytoplasmic staining c-myc</td>
<td>0.05</td>
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c-myc protein might be caused by abnormal post-
translational modification or altered nuclear import
of c-myc protein. This might be caused by deregula-
tion of cell proliferation but might also be respon-
sible 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 furd-
thermore demonstrated that c-myc is a predictive
indicator of clinical outcome. In agreement with the
findings on PC-10 (proliferating cell nuclear anti-
gen) staining but in contrast to earlier findings with
Ki-67-defined proliferative activity, 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 mela-
nomas. Its expression was not related to patient
survival. A strong inverse relationship was found be-
tween 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,
but c-myc is also a potent inducer of apoptosis when
expressed in the absence of serum or growth fac-
tors. It has been shown that Bcl-2 prevents c-
myc-induced apoptosis, which provides a mecha-
nism whereby cells can express c-myc without
undergoing apoptosis. 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 out-
come.

Although apoptosis was not found to be a promi-
nent 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
could 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 impor-
tant findings in this study are that in uveal melanoma
c-myc and Bcl-2 are expressed in inversely correlat-
ing 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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References


