Analysis of Bcl-2 protein expression in choroidal melanomas

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Background: Bcl-2 protooncogene alterations are involved in tumorigenesis and may have prognostic ramifications.

Aims: To investigate normal ocular structures and choroidal melanoma for: (1) Bcl-2 protein expression (semiquantitative staining values: SI, staining intensity; PP, percentage of positive cells; and IRS, immunoreactivity score) and (2) any associations between the staining values and clinicopathological features in these lesions.

Materials/Methods: Bcl-2 protein expression was analysed in 24 choroidal melanomas using immunoperoxidase staining methods.

Results: Bcl-2 protein expression was seen in corneal epithelium, lens epithelium, the ciliary body, and retinal cells. In these structures, the mean (SEM) values were: 1.1 (0.1), 1.6 (0.3), 1.1 (0.1), and 2.3 (0.3), respectively, for SI; 1.6 (0.2), 1.7 (0.1), 1.7 (0.2), and 1.7 (0.2) for PP, respectively; and 1.9 (0.4), 2.7 (0.5), 1.9 (0.1), and 4.0 (0.8), respectively, for IRS. Based on Bcl-2 immunoreactivity, the lesions were divided into two groups. The first group comprised 12 tumours with Bcl-2 expression. Bcl-2 expression was significantly higher in this group compared with normal ocular structures (1.5 (0.1) v 2.8 (0.2), 1.7 (0.1) v 3.5 (0.1), and 2.6 (0.3) v 9.3 (0.9) for mean (SEM) SI, PP, and IRS scores, respectively; $p = 0.00$). The second group comprised 12 tumours lacking Bcl-2 protein expression. There was no significant correlation between Bcl-2 protein expression and most of the clinicopathological features of these lesions.

Conclusions: Bcl-2 protein expression is altered in choroidal melanomas.

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Uveal melanoma is the most common primary intra-ocular malignancy in adults and can cause both death and blindness. It originates from the melanocytes of the uveal tract (iris, ciliary body, and choroid).¹ The growth of these melanocytes is regulated by a delicate balance between molecules controlling cell survival (such as Bcl-2) and cell death (such as p53). This regulation is perturbed during melanomagenesis, with an increase in the volume of the cell mass as a result of enhanced cellular proliferation.^{2–3} Bcl-2 is an antiapoptotic membrane associated molecule that resides in the nuclear envelope and mitochondria. Bcl-2 exerts its antiapoptotic functions by modulating the mitochondrial release of cytochrome c and the interaction of apoptosis activating factors with caspase 9 and Bax (Bcl-2 associated X protein).^{4–5}

“This study investigates the hypothesis that Bcl-2 protein expression is altered in choroidal melanomas, and that these alterations impact upon the clinicopathological features of these lesions”

Although some studies have reported Bcl-2 protein expression in choroidal melanomas, semiquantitative analysis of this protein in these lesions and in the normal ocular structures, in addition to its association with clinicopathological features, is still lacking.^{6–9} This study investigates the hypothesis that Bcl-2 protein expression is altered in choroidal melanomas, and that these alterations impact upon the clinicopathological features of these lesions. To examine these issues, 24 choroidal melanomas were investigated using immunoperoxidase staining methods and mouse monoclonal antibodies.

MATERIALS AND METHODS

Tissue specimens

The formalin fixed, paraffin wax embedded tissues of 24 enucleated eyes with choroidal melanoma were stained with haematoxylin and eosin for routine histological examination and immunohistochemical stains (Bcl-2), at the department of pathology, Assuit University Hospital, Assuit, Egypt. None of the patients studied had associated conditions such as diabetes or glaucoma.

Immunohistochemical analysis

Immunostaining was carried out as described previously.^{10–11} Briefly, sections mounted on glass slides were dewaxed and rehydrated through graded alcohols to water. Endogenous peroxidase activity was blocked with 0.6% H₂O₂. Sections were then immersed in the retrieval solution (10mM sodium citrate buffer, pH 6.0) and subjected to heat induced antigen retrieval for 20 minutes. The slides, in plastic Coplin jars containing retrieval solution, were microwaved in a microwave set at high (~750 W) for four cycles of five minutes duration each. Non-specific protein binding was blocked by a 10 minute exposure to 10% normal goat serum. Sections were then incubated with mouse monoclonal antibody for 30 minutes at 37°C (clone 124, IgG1κ; Dako, Glostrup, Denmark). A catalysed signal amplification system (K1500; Dako) was used according to the manufacturer's instructions. Sections were then treated with peroxidase labelled strept-avidin for 30 minutes at 37°C and incubated with 14-diaminobenzidine for five minutes. They were counterstained with haematoxylin, dehydrated in alcohol, cleared in xylene,

Abbreviations: IRS, immunoreactivity score; PP, percentage of positive cells; SI, staining intensity

Figure 1 Bcl-2 protein expression in (A) the positive control (lymph node), (B) lens epithelium, (C) ciliary body, (D) retinal cells, (E) corneal epithelium, and (F) choroidal melanoma. Staining intensity was scored as 1 for weak (C), 2 for medium (B and E), and 3 for intense (A, D, and F) staining.

and cover slipped. The slides were independently evaluated by two observers (Drs Al-Sabae and Hussein).

Positive and negative controls

Specimens used as positive controls consisted of lymph nodes with reactive hyperplasia.¹² Additional sections, run in parallel but with omission of the primary antibody, served as negative controls.^{10–12}

Semiquantitation of Bcl-2

The average weighted score was evaluated by multiplying the percentage of positive cells (PP%) and the staining intensity (SI). First, the PP% was scored as 0 for < 5%, 1 for 5–25%, 2 for 25–50%, 3 for 50–75%, and 4 for > 75%. Second, the SI was scored as 1 for weak, 2 for medium, and 3 for intense staining, as described previously.^{12–13}

Evaluation of Bcl-2 staining

The expression of Bcl-2 protein was identified as diffuse golden yellow cytoplasmic staining (sites of the mitochondria). Bcl-2 protein expression was considered negative only when more than 95% of the lesional cells were negative.¹²

Table 1 Bcl-2 immunostaining values in normal ocular structures and choroidal melanomas

Structure	SI	PP	IRS
Corneal epithelium	1.1 (0.1)	1.6 (0.2)	1.9 (0.4)
Lens epithelium	1.6 (0.3)	1.7 (0.1)	2.7 (0.5)
Ciliary body	1.1 (0.1)	1.7 (0.2)	1.9 (0.1)
Retina	2.3 (0.3)	1.7 (0.2)	4.0 (0.8)
Sclera	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Optic nerve	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Choroidal melanoma	2.8 (0.2)	3.5 (0.1)	9.3 (0.9)

Values are mean (SEM).

IRS, immunoreactivity score; PP, percentage of positive cells; SI, staining intensity.

Statistical analysis

Statistical analysis was done using ANOVA (for comparison among mean values), the Student's *t* test, and Pearson's correlation coefficient test (Statistix for Windows, Analytical Software Program). The results were presented as mean (SEM) and *p* values < 0.05 were considered significant.¹²

RESULTS

The positive and negative controls were consistently immunoreactive and non-immunoreactive, respectively, indicating the validity of the results.

Bcl-2 expression in normal ocular structures

Bcl-2 protein immunostaining was seen in the corneal epithelium, endothelium, lens epithelium, several layers of the retinal cells, and the ciliary body. The following was noted in these structures: (1) Bcl-2 immunoreactivity appeared as diffuse golden yellow cytoplasmic staining; (2) the nuclei were completely lacking Bcl-2 immunoreactivity; (3) the staining intensity was mild to moderate (cornea, lens, and ciliary body) or strong (retina); and (4) the staining was homogeneous rather than heterogeneous (fig 1). The mean (SEM) staining scores in the corneal epithelium, lens epithelium, ciliary body, and retinal cells were: 1.1 (0.1), 1.6 (0.3), 1.1 (0.1), and 2.3 (0.3), respectively, for SI; 1.6 (0.2), 1.7 (0.1), 1.7 (0.2), and 1.7 (0.2), respectively, for PP; and 1.9 (0.4), 2.7 (0.5), 1.9 (0.1), and 4.0 (0.8), respectively, for IRS. These values were highest in retinal cells (tables 1 and 2). In the cornea, expression was seen in the basal layer of the stratified squamous epithelium and the endothelium. In the retina, expression was pronounced in the inner nuclear and outer plexiform layers. When these values were summed together, the SI, PP, and IRS values were 1.5 (0.1), 1.7 (0.1), and 2.6 (0.3). Table 1 shows a summary of these results. In contrast, Bcl-2 protein expression was absent in the optic nerve and the sclera.

Bcl-2 expression in choroidal melanoma

Bcl-2 positivity was seen in 12 cases of choroidal melanoma. Bcl-2 expression was significantly higher in these lesions

Table 2 The clinicopathological features and Bcl-2 immunoreactivity scores of the choroidal melanomas

Clinicopathological features						Invasion of the adjacent structures							Bcl-2 IRS	
Case	Age	Sex	Site	Type	MF	LCN	EEC	TV	EOE	Sclera	CB	ON	Normal	Tumour
1	53	M	L	MCM	0	-	-	-	-	-	-	-	3	12
2	54	M	L	MCM	2	-	+	-	-	-	-	-	1.7	8
3	64	M	L	MCM	3	-	+	-	-	-	-	-	3.1	8
4	46	M	L	MCM	2	-	-	-	-	-	-	-	2	12
5	50	M	L	MCM	0	-	-	-	-	-	-	-	2.3	9
6	64	F	R	ECM	2	-	+	+	-	-	-	-	3.1	12
7	64	F	R	MCM	0	-	+	-	-	-	-	-	1.7	9
8	56	M	R	MCM	2	-	-	-	-	-	-	-	1.7	9
9	57	F	L	MCM	0	-	-	+	-	-	-	-	2	8
10	62	M	R	MCM	0	-	-	-	-	-	-	-	1.7	6
11	60	M	R	ECM	0	-	-	-	-	-	-	-	2	8
12	50	M	R	ECM	0	-	-	-	-	-	-	-	1.7	8
13	97	M	R	MCM	0	+	-	-	-	-	-	-	1.7	0.0
14	69	M	R	MCM	0	-	+	-	-	+	-	-	3.1	0.0
15	72	M	R	MCM	0	-	-	-	-	-	-	-	1.7	0.0
16	73	F	L	MCM	0	-	-	-	-	-	-	-	2	0.0
17	52	M	R	ECM	0	-	-	-	-	-	+	-	2	0.0
18	67	M	L	MCM	0	-	-	-	-	-	-	-	1.7	0.0
19	71	F	L	MCM	2	-	-	+	-	-	-	-	2.3	0.0
20	34	M	L	JPM	0	-	+	+	-	-	-	-	1.7	0.0
21	66	M	L	MCM	0	-	-	-	-	-	-	+	3	0.0
22	65	F	L	MCM	0	-	-	-	-	-	-	-	3	0.0
23	66	M	L	MCM	0	-	-	-	-	-	-	-	2	0.0
24	71	M	R	MCM	0	-	-	-	-	-	-	-	1.7	0.0

Cases 1–12 and 13–24 are tumours with and without Bcl-2 protein expression, respectively.

CB, ciliary body; ECM, epithelioid cell type; EEC, extension through the emissary canal; EOE, extraocular extension; IRS, immunoreactivity score; JPM, juxtapapillary melanoma; L, left; LCN, long ciliary nerve; MCM, mixed cell type; MF, mitotic figures (number of mitotic figures/40 high power fields); ON, optic nerve; R, right; TV, tumour cells in vascular lakes; -, absent; +, present.

The Bcl-2 reactivity in normal tissues is the average reactivity (summed together) in corneal epithelium, endothelium, lens epithelium, several layers of the retinal cells, and the ciliary body.

compared with the normal ocular structures (1.5 (0.1) ν 2.8 (0.2), 1.7 (0.1) ν 3.5 (0.1), and 2.6 (0.3) ν 9.3 (0.9) for SI, PP, and IRS, respectively; $p = 0.00$; tables 1–3; fig 1).

Correlation between Bcl-2 expression and clinicopathological features of choroidal melanoma

The age of the patients ranged from 34 to 97 years. The lesions were stratified into two groups of 12 comprising tumours with and without Bcl-2 expression. Table 3 shows a comparison of the clinicopathological data between these two groups. There were no significant differences for most of the clinicopathological variables between the two groups, but invasion of the long ciliary nerve, optic nerve, and ciliary

body, in addition to extraocular extension were found only in tumours lacking Bcl-2 protein expression ($p = 0.00$). There were no differences in expression according to the patient's age or factors other than melanoma.

DISCUSSION

Currently, our understanding of the Bcl-2 protein expression pattern in normal ocular structures and choroidal melanomas is incomplete. In addition, the relation between Bcl-2 protein expression and the clinicopathological features of these lesions is still unknown. This investigation was carried out to gain insight into these issues. The following three observations were made: (1) Bcl-2 protein expression is seen

Table 3 Clinicopathological characteristics of choroidal melanomas with and without Bcl-2 expression

Variable	Choroidal melanoma		p Value
	Bcl-2 positive	Bcl-2 negative	
Total	12/24 (50%)	12/24 (50%)	1.00
Mean age (SEM)	56.6 (10.8)	66.9 (14.0)	0.2
Site			
Left eye	6/12 (50%)	7/12 (58%)	0.3
Right eye	6/12 (50%)	5/12 (42%)	0.2
Cell type			
Mixed cell type	9/12 (75%)	10/12 (83%)	0.2
Epithelioid cell type	3/12 (25%)	2/12 (16%)	0.2
Spindle cell type	0/12 (0.0%)	3/12 (25%)	0.0
Juxtapapillary melanoma	0/12 (0.0%)	2/12 (16%)	0.0
Mitotic figures (/10 HPF)	0.9 (0.1)	0.3 (0.1%)	0.2
Invasion of long ciliary nerve	0/12 (0.0%)	1/12 (8.0%)	0.00
Extension through emissary canal	4/12 (33%)	2/12 (16%)	0.00
Tumour cells in vascular lakes	2/12 (16%)	2/12 (16%)	1.0
Extraocular extension	0/12 (0.0%)	0/12 (0.0%)	1.0
Invasion of the sclera	0/12 (0.0%)	1/12 (8.0%)	1.0
Ciliary body involvement	0/12 (0.0%)	1/12 (8.0%)	0.0
Invasion of the optic nerve	0/12 (0.0%)	1/12 (8.0%)	0.00

HPF, high power fields.

Take home messages

- Bcl-2 protein expression is altered in choroidal melanomas: in one group expression was significantly higher than in normal tissue, but in another group Bcl-2 expression was absent
- The higher expression of Bcl-2 seen in some choroidal melanomas suggests that abrogation of apoptosis may be a potential active process in the development of these lesions
- There was no significant correlation between Bcl-2 protein expression and most of the clinicopathological features of these lesions

in some normal ocular structures, (2) Bcl-2 protein expression is high in choroidal melanomas, and (3) there is no correlation between Bcl-2 protein expression and most of the clinicopathological features of choroidal melanoma.

The presence of Bcl-2 reactivity in some normal ocular structures not only agrees with similar findings in rats, but also highlights the cytoprotective effects of this protein in the eye.^{14–16} In this regard, Bcl-2 can protect bovine corneal endothelial cells against apoptosis induced by staurosporin (a broad spectrum kinase inhibitor).¹⁷ These findings also suggest that (1) these Bcl-2 positive cells may have a prolonged survival; (2) apoptosis rarely occurs in normal ocular structures; and (3) Bcl-2 plays a role in regulating apoptosis in the normal eye. Of note, Bcl-2 protein expression in mouse and human corneal endothelium is stimulated by a protein factor present in aqueous humour.^{18, 19}

“The presence of Bcl-2 protein expression in choroidal melanoma suggests that it may be involved in the development of these lesions, perhaps by delaying or preventing p53 induced apoptosis in the melanomatous cells”

In keeping with previous studies, half of the choroidal melanomas in this study had high Bcl-2 expression,^{6–8} perhaps because of low p53 expression, which can antagonise Bcl-2¹² by the induction of several proteins. In addition, this high expression might help to explain the slow growth and resistance of these lesions to apoptosis. It is well known that overexpression of Bcl-2 provides protection against various apoptotic stimuli, including growth factor deprivation, oncogenes such as c-myc, tumour suppressor genes such as p53, radiation, and chemotherapeutic drugs.^{4, 5, 17}

The presence of Bcl-2 protein expression in choroidal melanoma suggests that it may be involved in the development of these lesions, perhaps by delaying or preventing p53 induced apoptosis in the melanomatous cells. Therefore, it would favour the persistence and propagation of DNA mutations by its cytoprotective effects. The discrepancy regarding the frequency of Bcl-2 protein expression between this study (50%) and previous ones (100%) can be explained by: (1) the use of different methodology, including antibody selection, antibody specificity, the assessment of Bcl-2 positivity, and antigen retrieval techniques; (2) cell cycle dependent variations in protein expression; and (3) the genetic heterogeneity of melanoma.^{6–8, 10}

Recently, bcl-2 overexpression has been reported to be associated with an unfavourable outcome in cutaneous malignant melanomas.^{20–22} In the present study, the lack of a significant correlation between Bcl-2 protein expression and clinicopathological features (except invasion) in

choroidal melanomas not only contrasts with findings in cutaneous melanoma, but also suggests the following: (1) Bcl-2 protein expression has no prognostic value in choroidal melanomas because its expression does not influence clinical outcome; (2) the malignant progression of choroidal melanomas may involve pathways other than Bcl-2, such as the p53 pathway^{2, 23}; and (3) single prognostic markers may have limited value.

In conclusion, this study reports the semiquantitative expression of the Bcl-2 protein in choroidal melanomas and in normal ocular structures. The higher expression of Bcl-2 seen in some choroidal melanomas suggests that abrogation of apoptosis may be a potential active process in these lesions. This finding supports a possible relation between Bcl-2 expression and the development of these lesions. Moreover, this finding indicates that the development of choroidal melanomas involves genomic lesions that inhibit apoptotic control.

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