BCL-2 Family protein expression in initial and recurrent glioblastomas: modulation by radiochemotherapy

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Abstract

Objective—In vitro studies indicate a role of apoptosis regulatory proteins of the BCL-2 family in the resistance of glioblastoma multiforme to irradiation and chemotherapy. To date, no study has compared the expression of these proteins in initial and recurrent tumours. The differences of expression of BCL-2, BCL-X, BAX, and MCL-1 proteins of paired first resection and recurrence glioblastoma specimens were examined.

Methods—Immunohistochemistry was performed in 37 cases of glioblastoma multiforme with paraffin embedded tissue from first resections and their recurrences in three treatment groups (15 radiochemotherapy, 15 irradiation, seven untreated). Ten high power fields were evaluated with an arbitrary score ($<5\%=1$, $5-50\%=2$, $>50\%=3$), and cumulative scores for each antigen calculated.

Results—In the whole group, we found a significant up regulation of antiapoptotic BCL-2 (median cumulative score of 15 in the primary, 19 at recurrence; $p<0.0001$ in the Wilcoxon test), BCLX (median scores 20 and 25, respectively, $p<0.0001$), and MCL-1 (median scores 11 and 14, $p=0.0395$), and a significant down regulation of proapoptotic BAX (median scores 14 and 11, $p<0.0001$). In the subgroups, these trends were also found. No association between protein expression and treatment regimen was found, although significant changes were restricted to the subgroups that received adjuvant chemotherapy. No significant correlation with clinical prognosis was detected with the Kaplan-Meier method.

Conclusions—In the development from initial to recurrent glioblastoma multiforme, the BCL-2 family rheostat shifts towards antiapoptotic adjustment in vivo. Importantly, the changes in BCL-2 family protein expression characterised here were also seen in the subgroup of patients who did not receive adjuvant radiotherapy or chemotherapy, suggesting that the changes of BCL-2 family protein expression result not only from radiochemotherapy but also reflect the natural course of disease.

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Keywords: glioblastoma; apoptosis; BCL-2

Glioblastoma multiforme is highly malignant with a marked resistance to anticancer treatment. Clinical prognostic factors such as tumour size and extent of resection, age, and Karnofsky performance status are relatively well determined.^{1,2} By contrast, no biological marker with impact on clinical prognosis is known to date. For instance, p53 has not been confirmed as a prognostic marker in malignant glioma.³

The efficacy of adjuvant therapy is limited. Regulators of apoptosis such as p53 and BCL-2 family proteins determine the cellular fate of survival or death after DNA damage induced by irradiation or chemotherapy. BCL-2, BCL-xL (a splicing product of BCL-X), MCL-1, and other BCL-2-related proteins are inhibitors of apoptosis, whereas BAX, BCL-xS (another splicing product of BCL-X), and other proteins such as BAD and BAK promote cell death. In vivo, antiapoptotic BCL-xL is by far the most abundant splice variant present in all tissues tested.⁴ BCL-2 family proteins interact by homo and heterodimerisation.⁵ A rheostat between proapoptotic and antiapoptotic proteins may regulate the susceptibility to death promoting stimuli.

Proteins of the BCL-2 family are thought to contribute to the resistance of glioma cells to anticancer therapy by modulating the apoptotic cascade. For instance, BCL-2 gene transfer into human malignant glioma cells blocked CD95 antibody mediated apoptosis and conferred resistance to chemotherapeutic drugs and irradiation.⁶ The activity of BCL-2 family proteins can be altered by irradiation and chemotherapy. In glioma cell lines expressing wild-type p53, a decrease of BCL-2 and increase of BAX concentrations was found after exposure to camptothecin.⁷ To date, no long term effects of radiochemotherapy on BCL-2 family protein expression have been reported. Few data on the clinical impact of BCL-2 in malignant gliomas have been published.⁸⁻¹¹ One study examining the prognostic impact of BCL-X, MCL-1, and BAX in glioblastoma multiforme found a better prognosis in patients with low MCL-1 expression.¹²

Here, we compared the expression of BCL-2 related proteins in initial and recurrent glioblastoma multiforme in vivo, examined a possible influence of multimodal anticancer treatment on protein expression, and considered whether protein expression and progression free survival were correlated.

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Table 1 Patient data and cumulative scores

Patient	Age	Sex	Radiation Gy	Chemoth No courses	BCL-2		BCL-X		BAX		MCL-1		PFS Months
					Prim	Rec	Prim	Rec	Prim	Rec	Prim	Rec	
1	60	F	60	5 AC/Ara-C	15	29	12	19	11	11	11	10	11
2	53	M	60	2 AC/VM 26	12	13	15	20	21	11	13	11	12
3	43	M	60	5 AC/Ara-C	21	23	18	26	11	10	15	16	27
4	40	M	60	3 AC	14	18	24	28	12	10	10	10	8
5	43	M	60	2 CC	21	25	30	30	16	14	16	20	11
6	27	M	60	2 AC/VM 26	12	11	11	28	10	10	10	10	3
7	57	M	60	3 CC	11	17	25	17	16	11	10	10	6
8	51	M	50.4	2 AC/VM 26	17	18	22	24	16	10	10	13	4
9	54	M	60	5 AC	14	19	15	24	12	11	10	10	30
10	55	M	60	2 AC/VM 26	14	21	12	28	10	13	10	13	13
11	38	M	60	6 AC	14	11	12	15	10	10	10	10	46
12	34	M	60	7 AC/VM 26	10	20	12	25	10	11	10	13	16
13	52	F	60	1 AC	13	19	24	25	18	12	11	11	19
14	47	M	60	2 AC/Ara-C	15	20	26	29	15	11	12	10	12
15	53	M	60	4 AC	13	20	20	20	15	11	10	17	9
16	52	M	60	No	15	16	23	22	14	14	10	10	12
17	60	F	54	No	10	15	14	17	13	12	10	11	12
18	57	M	60	No	14	20	23	30	14	12	13	17	6
19	56	M	60	No	13	18	20	27	10	12	10	12	36
20	38	M	55	No	15	14	19	30	11	19	10	19	7
21	50	M	60	No	22	20	21	30	19	14	11	13	12
22	46	M	60	No	17	28	21	30	14	10	10	11	7
23	66	F	60	No	15	16	16	18	13	12	10	10	11
24	46	M	54	No	16	26	30	30	14	16	11	15	18
25	59	M	60	No	24	25	26	26	14	10	10	10	4
26	56	F	38	No	10	16	11	21	11	11	10	11	2
27	48	M	60	No	19	14	25	20	16	12	13	10	7
28	49	M	60	No	22	26	13	17	10	10	10	13	3
29	67	M	60	No	13	17	16	17	14	10	10	13	16
30	45	F	60	No	11	14	14	15	11	11	11	11	20
31	51	M	No	No	13	10	18	11	11	12	10	10	7
32	16	M	No	No	15	25	27	26	21	17	22	11	1
33	79	F	No	No	15	27	17	23	11	13	11	11	6
34	61	F	No	No	15	11	22	29	15	12	14	15	1
35	54	M	No	No	12	17	20	30	12	12	11	14	3
36	49	M	No	No	17	22	30	30	18	21	17	13	8
37	61	F	No	No	20	29	22	29	15	10	11	11	8

AC=ACNU, CC=CCNU; PFS=progression free survival; prim=primary; rec=recurrent.

Materials and methods

PATIENT DATA

We collected consecutive cases with paraffin embedded tissue from initial and recurrent glioblastoma multiforme in the same patient. Starting from 1985, 37 cases were eligible for examination by immunohistochemistry. Patient data are shown in table 1.

All specimens of the primary tumours were obtained before radiotherapy or chemotherapy. Tissue samples were divided into three treatment groups: 15 patients were treated with radiotherapy with an average dose of 57.4 Gy. Another 15 patients received adjuvant chemotherapy in addition to radiotherapy (mean 59.4 Gy). Chemotherapy regimens were not standardised, but always contained a nitrosourea (ACNU or CCNU) as a single agent or combined with either Ara-C or VM 26 (table 1). Seven patients received not more than one or two chemotherapy courses, due to haematological complications or early recurrence. Seven other patients refused any specific post-operative treatment (table 2).

Table 2 Patient data in treatment groups

	Whole group	Radiochemotherapy	Radiotherapy	Untreated
Patients	37	15	15	7
Mean age (SEM) (y)	51 (1.87)	47 (2.4)	53 (2.09)	53 (7.23)
Female	12	4	5	3
Male	25	11	10	4
Radiation (mean dose)	30 patients	57.4 Gy	59.4 Gy	No
Chemotherapy	15 patients	ACNU/CCNU ± Ara-C/VM26	No	No

IMMUNOHISTOCHEMISTRY

Paraffin embedded samples were cut, deparaffinised, and rehydrated. The 3 µm sections were immersed in 0.01 M citrate buffer and irradiated for 5×5 minutes in a microwave oven set at 750 W. Endogenous peroxidase was blocked with 1% H₂O₂ in methanol. Non-specific protein binding domains were blocked by application of standard porcine serum (Seromed®, Biochrom, Berlin, Germany) for 15 minutes. Monoclonal antibodies to BCL-2 (Clone 124, monoclonal, Dakopatts, Glostrup, Denmark) and polyclonal antibodies to BCL-X (BCL-xL and BCL-xS), BAX and MCL-1 (BCL-X L19, BAX P19, MCL-1 S 19; Santa Cruz, Santa Cruz, CA, USA) were diluted in Tris buffered saline (TBS) and applied as primary antibodies for 1 hour at a concentration of 1:100 (BCL-2) or 1:1000 (BCL-X, BAX, MCL-1). Biotinylated secondary antibodies, either monoclonal to mouse IgG (Dakopatts) or polyclonal to antirabbit IgG (Dakopatts) were both diluted 1:400 in TBS and incubated for 30 minutes to the respective primary antibodies. Streptavidin-biotin horseradish peroxidase complex (Dakopatts) was diluted 1:200 in TBS+albumin and incubated for 30 minutes. Labelled antigens were visualised by application of diaminobenzidine for 10 minutes. Negative controls without primary antibody were done for each probe. All sections were counterstained with haematoxylin.

Figure 1 (A) In the primary tumour only single cells are immunolabelled with BCL-2. (B) The recurrent tumour of the same patient, by contrast, is characterised by more than 50% positive cells (magnification $\times 400$, scale bar = 50 μm).

STATISTICAL ANALYSIS

For evaluation of the protein expression, 10 high power fields were assessed with the following score: score 1 = 0–5% stained cells, score 2 = 5–50% stained cells, score 3 = >50% stained cells.

For each protein and each patient, cumulative scores of the 10 high power fields were calculated in primary and recurrent tumour. Cumulative score 10 was classified negative,¹³ scores 11–14 as weak expression, 15–20 as intermediate, and scores >20 were classified as strong expression.

To assess changes in protein expression, the cumulative scores were compared for each protein with the Wilcoxon test for paired probes in all initial and recurrent tumours and in the three treatment groups. In the subgroups, we adjusted the significance level of the p values with the Bonferroni method for multiple significance testing and presumed p values <0.017 (three subgroups: $\alpha = 0.05/3 = 0.0166$) to be significant at the 0.05 level. The differences between the median cumulative scores in primary and recurrent tumour in the three treatment groups (untreated, radiotherapy, radiochemotherapy) were compared for each protein with the Kruskal-Wallis test to allow a descriptive analysis of the influence of different treatment modalities.

To estimate the influence of protein expression on the clinical prognosis, progression free survival was determined as the time between first and second resection in each case. Median progression free survival was calculated with the Kaplan-Meier method and confirmed with the logrank test in pooled subgroups (for instance, negative and weak expression compared with intermediate and strong expression of BCL-2 (fig 1).

Results

STAINING PATTERNS

All antibodies showed mainly cytoplasmic staining, but with BCL-2 and BCL-X, nuclear staining was also seen. Antibodies to BCL-X and to BCL-2 stained strongly. Staining was predominantly homogeneous, but with BAX, a granular pattern also occurred.

PROTEIN EXPRESSION

We found BCL-2 positivity in a high percentage of initial (34/37 (92%)) and recurrent (36/37 (97%)) tumours. The percentage of protein expressing cells was up regulated at recurrence. This difference was statistically significant in the whole series and in the subgroup with postoperative therapy (also after adjustment with the Bonferroni method in the subgroups, see statistical analysis). Staining in neoplastic gemistocytes accounted predominantly for this rise in protein expression in the recurrent tumours, as could be seen morphologically as well as in the statistical analysis (positive correlation between the rise in BCL-2 expression and content of gemistocytes between first and second resection, $p = 0.0022$).

Table 3 Protein expression: median cumulative scores, quartiles (25% and 75% percentiles), and p values

	BCL-2		BCL-X		BAX		MCL-1	
	Initial	Recurrence	Initial	Recurrence	Initial	Recurrence	Initial	Recurrence
Whole group (n=37)	15	19	20	25	14	11	10	11
Quartiles	13–16	16–22	15–23	23–29	11–15	10–12	10–11	10–13
p Value	< 0.0001		< 0.0001		< 0.0001		0.0395	
Radiochemoth (n=15)	14	19	18	25	12	11	10	11
Quartiles	12.5–15	17.5–20.5	12–24	22–28	10.5–16	10–11	10–11.5	10–13
p Value	0.0012		0.0031		0.0122		0.0977	
Radiotherapy (n=15)	15	17	20	22	14	12	10	11
Quartiles	13–18	15.5–22.5	15–23	17.5–30	11–14	10.5–13	10–11	10.5–13
p Value	0.0125		0.081		0.2402		0.0186	
Untreated (n=7)	15	22	22	29	15	12	11.00	11.00
Quartiles	14–16	14–26	19–24.5	24.5–29.5	11.5–16.5	12–15	11–15.5	11–13.5
p Value	0.0781		0.3125		0.4375		0.6250	

According to the Bonferroni method, only p values <0.017 are presumed to be significant at the 0.05 level in the subgroups.

Figure 2 Median cumulative scores of protein expression in the whole group.

Figure 3 (A) Immunostaining with BAX in the primary tumour shows several positive cells; (B) all cells are negative in the recurrent tumour.

In the group without adjuvant therapy, the same trend towards up regulation of BCL-2 was found but this did not achieve significance (table 3, figs 1 and 2).

BCL-X was expressed in all (100%) examined initial and recurrent tumours. As with BCL-2, expression was up regulated in all subgroups at recurrence. The differences were significant in the whole series and in the radiochemotherapy group, and the same trend was present in irradiated and untreated patients.

Expression of BAX was found in 31/37 initial (84%) and 27/37 recurrent (73%) tumours. The percentage of protein expressing cells was down-regulated in all subgroups at recurrence, with a significant difference in the whole series and in the radiochemotherapy group (figs 2 and 3) and with a similar trend in irradiated and untreated patients.

Only 17/37 initial tumours (46%) and 11/37 relapsed tumours (30%) were positive for MCL-1. The up-regulation was less pronounced compared with the other proteins and the difference achieved significance in the whole series but not in the subgroups.

Comparing the treatment groups, no significant difference in the up-regulation or down-regulation of any protein was found between primary and recurrent tumour. The statistical analysis, however, shows that significant changes in protein expression occurred in the radiochemotherapy groups for BCL-2, BCL-X, and BAX and in the radiotherapy group for BCL-2, but not in the other treatment groups (table 3).

CORRELATION WITH PROGRESSION FREE SURVIVAL
Median progression free survival was 9 months in the whole group, 12 months in patients with radiochemotherapy, 11 months with radiotherapy alone, and 6 months in untreated

Figure 4 Progression free survival in patients with low and high BCL-2 expression.

patients. In patients with initially negative or weak expression of BCL-2, median progression free survival was 12 months, compared with 8 months with intermediate and strong expression of BCL-2. This difference did not achieve significance ($p=0.0507$, fig 4). Also with all other examined proteins, no significant correlation between protein expression and progression free survival was found (data not shown).

Discussion

In the present study, we compared the expression of BCL-2 family proteins in initial and recurrent glioblastoma multiforme to assess whether in vitro findings on the contribution of these proteins to the resistance to irradiation and chemotherapy can be endorsed by in vivo data.

The frequency of BCL-2, BCL-X, and BAX expression in our primary tumours corresponds with other series.⁸⁻¹⁷ Compared with the other proteins, MCL-1 was expressed less often in our series (48%) and our previous study (65%),¹⁷ whereas other authors had 100% positivity.¹⁸ In the recurrent tumours, we found a significant up-regulation of the antiapoptotic proteins BCL-2, BCL-X, and MCL-1 and a significant down-regulation of proapoptotic BAX. Short term effects of chemotherapy on BCL-2 concentrations in vitro have been reported, with divergent results.¹⁸⁻²³ No reports exist on BCL-2 family protein expression in recurrent, untreated human cancer and no data have been reported on their expression in human glioblastoma multiforme before and after adjuvant therapy. In non-CNS tumours, no change in BCL-2 expression was found in 29 patients with breast cancer after chemotherapy and in 27 patients with colorectal carcinoma after radiotherapy.^{23, 24} Tumour type specificity may account for the difference between our results and those in other solid tumours. Our findings of a correlation between the up regulation of BCL-2 and the content of gemistocytes is in line with a previous report on predominant expression of BCL-2 in gemistocytes.¹⁶ The authors suggest that this may result from an accumulation of gemistocytes that escape apoptosis by BCL-2 overexpression, a hypothesis which is supported by our data.

In accordance with other series,⁸⁻¹² we found no significant association between clinical

prognosis and expression of BCL-2, BCL-X, or BAX. Also with MCL-1, there was no correlation with prognosis.

Our in vivo findings indicate a shift of the BCL-2 rheostat towards resistance to apoptosis in the recurrent glioblastomas. This may be achieved by a positive selection of cells with enhanced antiapoptotic capacity. One possible mechanism of selection is anticancer treatment, consisting of irradiation and chemotherapy. We found, however, the same tendency to higher expression of antiapoptotic BCL-2 related proteins and a reduction on BAX expression in never treated patients (table 1), indicating that the development of a more apoptosis resistant phenotype cannot only be a consequence of adjuvant radiochemotherapy but may reflect a natural process of selection. These findings are in line with two other features of glioblastoma: (1) most of these tumours show only a moderate response to adjuvant therapy, favouring primary resistance rather than acquired resistance through selection; (2) tumour hypoxia is a prominent feature of glioblastoma and would be predicted to select for the changes characterised here.²⁵⁻²⁷

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