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Original Article

Fas, FasL, and cleaved caspases 8 and 3 in glioblastomas: A tissue microarray-based study☆

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ABSTRACT

This investigation analyzed the immunoexpression of FasL, Fas, cleaved caspase-8, and cleaved caspase-3 in glioblastomas. Formalin-fixed and paraffin-embedded glioblastoma tissues and control brain tissues from 97 patients were analyzed by tissue microarrays and immunohistochemistry. Patients with glioblastomas that were negative or weakly stained (<50% of cells positive) for cleaved caspase-8 had worse cancer-specific overall survival (median = 8.5 months) than did patients with tumors that highly expressed cleaved caspase-8 (median = 11.7 months; $P = 0.0325$), independent of clinical variables. There was no association of other markers with survival, treatment, sex, age, tumor size, and primary site. Among the tumors, there were reasonable to good positive correlations between the expression of FasL and Fas ($r = 0.47$) and between Fas and cleaved caspase-8 ($r = 0.41$), and there were poor positive correlations between Fas and cleaved caspase-3 ($r = 0.26$), FasL and cleaved caspase-8 ($r = 0.22$), and cleaved caspase-8 and -3 ($r = 0.31$). Our results suggest that Fas-Fas-ligand signal transduction could be inhibited, especially at the stage of caspase-8 activation, thereby establishing a major mechanism for evasion of apoptosis by these tumors. The absence or low expression of cleaved caspase-8 in the tumors was a negative prognostic indicator for patient survival.

Introduction

Glioblastoma (GBM) is the most common malignant primary brain cancer, and it has a dismal outcome. Despite advances in diagnosis and treatment, the median survival of patients who suffer from GBM remains approximately 15 months, according to the more recent studies with temozolomide, because of inherent resistance to both chemo- and radiotherapy [8,9]. For decades, surgery and radiotherapy have been the traditional cornerstones of therapy for GBM. Several chemotherapeutic agents, including the nitrosourea derivatives and temozolomide, have also been used with limited success, resulting in median survival times of 12–15 months and long-term remissions in a few temozolomide patients [9,39]. The poor efficacy of these agents is mostly attributed to the highly mutated genome of GBM, which is manifested by the deregulation of many key signaling pathways involving growth, proliferation, survival, and apoptosis [24]. Moreover, O^6-methylguanine-DNA methyltransferase (MGMT), a repair protein that specifically removes promutagenic alkyl groups from the O^6 position of guanine in DNA, protects cells against alkylating drugs, resulting in resistance of GBM to these chemotherapeutic agents [25].

Apoptosis is a basic biological process that promotes survival of the organism at the expense of individual cells. It is widely used by multicellular organisms to remove undesirable cells without injuring neighboring cells or eliciting an inflammatory reaction [32]. Nevertheless, tumor cells can evade apoptosis, and thus perturb

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the balance between apoptosis and cell proliferation [14]. Because cytotoxic drugs and radiation therapy induce tumor cells to die by apoptosis, understanding the mechanisms involved in the extrinsic apoptotic signaling pathway in glioblastomas may identify target molecules for molecular therapies.

The activation of the extrinsic apoptotic pathway following Fas binding has been well characterized [1,40]. Fas ligand (FasL) is a type II membrane protein with an intracellular domain that contains consensus sequences for phosphorylation and an extended proline-rich region that tightly regulates FasL surface expression in the nervous system [41]. Fas (APO-1/CD95) is a 48-kDa type I membrane protein with a cysteine-rich extracellular domain of 155 amino acids. The triggering of Fas by its ligand induces apoptosis in target cells. Although Fas is ubiquitous in human tissues, it is highly expressed in rapidly proliferating cells and injured tissues [29]. The oligomerization of Fas by FasL recruits the adaptor molecule Fas-associated death domain protein (FADD) to the death domain (DD) of the Fas intracellular region [4,7]. Procaspase-8 (FLICE/MACH1/Mch5), in turn, associates with FADD to form the death-inducing signaling complex (DISC), whereby procaspase-8 converts itself to an active cleaved form [4,27]. Next, the cleaved caspase-8 activates the downstream effector, caspase-3 [21]. Previous reports have demonstrated that the extrinsic apoptotic pathway is severely inhibited in high-grade gliomas [2,13,14,16,19,26,33,35,44].

Several findings have indicated that the deregulation of apoptosis is involved in the development of malignant gliomas. The upregulated expression of FasL and downregulated expression of caspase-3 and caspase-8 in malignant glioma cells are involved in gliomagenesis [19,42]. For example, FasL is implicated in glioblastoma growth and invasion through the induction of apoptosis in infiltrating lymphocytes, which facilitate the evasion of the immune system by the tumor [19]. In addition, it has been shown that glioblastomas are resistant to Fas-related apoptosis, showing absent or low levels of caspases-8 and caspase-3 [2,33,38,42].

Because the extrinsic apoptotic pathway in treatment-naïve human GBMs has not yet been systematically studied, we aimed to determine the expression of FasL, Fas, cleaved caspase-8, and cleaved caspase-3 in not otherwise specified GBMs using tissue microarrays and immunohistochemistry and to correlate the expression of these molecules with various clinical findings. We also reviewed the molecular basis of Fas-mediated apoptosis in malignant gliomas.

Materials and methods

Tissues

Glioblastoma specimens from 97 patients who had not been previously treated were retrieved from the archives of the Departments of Pathology at São Paulo Federal University ($n = 60$) and Ribeirão Preto Medicine Faculty at São Paulo University ($n = 37$). The tumor specimens were re-examined and confirmed to be glioblastomas according to the criteria of the most recent WHO Classification of Central Nervous System Tumors [22]. All of the patients had undergone surgery during the 15-year period from 1992 through 2006. This study was approved by the Ethics Committees of both institutions (Resolution No. 196 of Brazilian National Health Council).

Tissue microarrays (TMAs)

Histological sections (4 μm) were cut from each tissue block, stained by hematoxylin–eosin, and carefully reviewed by 3 independent pathologists. The areas most representative of each tumor were selected for analysis. Cylindrical cores were removed and used in the construction of tissue microarray (TMA) blocks. Five TMA blocks were constructed using a Beecher tissue array instrument™ (Beecher Instruments, Silver Spring, MD, USA), according to the manufacturer's instructions, in the following stages: (1) Two different areas of the tumor were marked in the original donor block for sampling (necrotic zones and perinecrotic palisading cells were not included in the samples), (2) cylindrical holes were created in the receptor block using the TMA platform. Positions were created in the receptor blocks and were separated by approximately 500 μm such that a matrix of holes for the tissue samples was created, (3) 1-mm diameter cylinders of tissue were extracted from the areas of interest in the donor blocks using a 1-mm-diameter needle (TMArrayer Punch Beecher Instruments™), (4) the cylindrical tissues obtained from the donor blocks were transferred to the holes in the receptor blocks, and (5) finally, the quality of the blocks (representativeness of the tumor samples) was assessed before storage.

Twenty-five control cores obtained from normal brains harvested from 25 autopsied patients (6–12 h postmortem) were included as controls.

Immunohistochemistry

The immunohistochemical procedures were performed on 4-μm-thick sections that were obtained from the TMA blocks and mounted on slides pretreated with 3-minopropyl-triethoxysilane (Sigma). To aid in the adhesion of the slices from the TMA blocks to the silane-treated slides, an adhesive tape system (Instrumedics Inc., Hackensak, NJ, USA) was also used.

Briefly, for immunostaining, the slides were deparaffinized, and rehydrated through a graded ethanol series. For antigen retrieval, slides were placed in a 0.01 M citrate buffer (pH 6.0), heated in a steam bath for 3 min, and allowed to cool at room temperature for 30 min. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 15 min, followed by washing in 0.05 M Tris buffer (pH 9.5). The slides were then subjected to microwave irradiation at 700 W for 7 min. The slides were again placed in phosphate buffered saline (0.01 M PBS [pH 7.4]) and allowed to cool at room temperature for 30 min. All of the immunomarkers that were evaluated were examined on slides that underwent treatment for antigen retrieval. The endogenous biotin was blocked using 0.02 M PBS/0.3% Triton X100 (pH 7.4) and 5% skim milk for 4 h at room temperature. Incubation with anti-FasL rabbit polyclonal antibody (C-178, 1:500; Santa Cruz Biotechnology), anti-Fas rabbit polyclonal antibody (FL-335, 1:200; Santa Cruz Biotechnology), anti-cleaved caspase-8 mouse monoclonal antibody (AP1013, 1:100; Calbiochem), anti-cleaved caspase-3 rabbit polyclonal antibody (AP1027, 1:500; Calbiochem), anti-IDH1 rabbit polyclonal antibody (AP7454a, 1:50; Abgent), or anti-MGMT mouse monoclonal antibody (SPM287, 1:150; Santa Cruz Biotechnology) diluted in PBS with 1.0% bovine serum albumin (Sigma, USA) lasted for 12 h in a moist chamber at 4 °C. The slides were then washed in PBS and incubated with secondary biotinylated antibody followed by streptavidin–biotin-peroxidase (anti-mouse or anti-rabbit Kit LSAB, DAKO) for 30 min each. Finally, to visualize the reactions, the slides were incubated with light-sensitive 3,3′-diaminobenzidine tetrahydrochloride (Sigma) in 0.05 M PBS (pH 7.6) and quickly counterstained with Harris hematoxylin. Coverslips were applied using Entellan (Sigma). A positive reaction was visualized as a brown deposit in the cell that indicated an area where the antigen–antibody reaction had occurred. Negative and positive controls were run simultaneously. Lymphoid tonsil tissue with follicular germinative centers was used as a positive control for FasL, Fas, cleaved caspase-8, and cleaved caspase-3. Placenta and normal colon, which had immunohistochemistry performed separately from the TMAs, were used as positive controls for IDH1

and MGMT, respectively. Negative controls consisted of slides that underwent the same procedure, except the incubation with primary antibody was eliminated.

The staining patterns were analyzed according to their distribution and intensity, and the pathologists were blinded to the clinicopathological data of the GBM patients. A numerical scoring system consisting of 2 categories was used to assess FasL, Fas, cleaved caspase-8 and cleaved caspase-3 expression. Category A documented the number of immunoreactive cells (only ones with their respective nuclei inside were counted) as follows: 0 or negative (no immunoreactive cells or <10% immunoreactive cells), 1 (≥10% and <50% immunoreactive cells), or 2 (≥50% immunoreactive cells).

Category B documented the intensity of the immunostaining as follows: 0 or 1 (no immunostaining or weak staining, respectively) or 2 (moderate or strong). The values for categories A and B were summed to provide an "immunoreactivity score", which could range from 0 to 4. Scores of 0, 1, and 2 were considered to be negative to weak immunoreactivity and called "low immunoexpression". Scores of 3 (moderate) and 4 (strong) were considered to be "high immunoexpression".

IDH1 immunostaining was scored in the nuclei and/or cytoplasm, and MGMT were scored in the nuclei of tumor cells as negative (no stain or limited to <10% positive tumor cells) or positive (≥10% tumor cells).

Statistical analysis

The immunohistochemistry scores determined for FasL, Fas, cleaved caspase-8, and cleaved caspase-3 expression of the TMAs and control nervous tissues were compared using the Mann–Whitney test, and correlations in each group were determined using the nonparametric Spearman test.

To construct the survival curves illustrating overall survival between the patient groups with "low expression" (scores 0, 1, and 2) vs. "high expression" (scores 3 and 4) immunohistochemistry scores for FasL, Fas, cleaved casp-8 and -3, IDH1, and MGMT, we used the Kaplan–Meier method. To compare the overall survival curves, we used the log-rank test.

To simultaneously analyze the prognostic effect of the various factors (treatment, age, gender, tumor size, tumor location, and the immunoexpression scores of low and high expression of FasL, Fas, cleaved caspase-8, and -3) on the time of survival, we used a multivariate analysis with the Cox proportional-hazards regression model using a covariate of primary interest and adjustment covariates. All statistical analyses and graph constructions were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software Inc., San Diego, CA, USA), and SAS version 9 for Windows (SAS Institute, Inc.; Cary, NC, USA). The level of significance was 0.05 ($P < 0.05$). Unless specified, data are presented as the mean ± SD or median.

Results

Age and sex distribution

The mean age of patients at diagnosis was 55.5 ± 14 years (range, 18–78 years; median = 56 years), with 64.9% of patients ≥50 years of age, and 35.1% <50 years. The age distribution of patients was as follows: <39 years, 12.4%; 40–49 years, 22.7%; 50–59 years, 23.7%; 60–69 years, 26.8%; and ≥70 years, 14.4%. The female/male ratio was 0.8:1 (Table 1). There were no differences in the survival among the age groups ($P = 0.78$) or the genders ($P = 0.24$) as determined by both univariate and multivariate analyses (Table 3).

Table 1
Clinical data of patients with glioblastomas.

Characteristics		No. (%)
Gender	Male	53 (54.6)
	Female	44 (45.4)
Age	≥50 years	63 (64.9)
	<50 years	34 (35.1)
Tumor size[a]		
Supratentorial	≤5 cm	5 (9.1)
	>5 cm	7 (12.7)
	>5 cm with ventricular invasion or compression	33 (60)
	>5 cm with middle-line crossover or infratentorial invasion	6 (10.9)
Infratentorial	>5 cm	4 (7.3)
Primary site		
	Frontal lobe	25 (25.8)
	Parietal lobe	6 (6.2)
	Temporal lobe	12 (12.4)
	Occipital lobe	1 (1.0)
	Supratentorial sites[b]	46 (47.4)
	Infratentorial	4 (4.1)
	Not available	3 (3.1)
Follow-up[c]		
	Alive at 1 year	29 (38.7)
	Alive at 2 years	8 (10.7)
	Alive at 3 years	3 (4)
	Alive at >3 to 5 years	1 (1.3)
	Lost	10 (10.3)

[a] Tumor sizes available in 55 patients.
[b] Two or more supratentorial sites.
[c] Follow-up of 87 patients (89.7%).

Tumor size and location

The sizes of the tumors at the first diagnosis were available for 55 patients (Table 1). Most of them (70.9%) were supratentorial tumors >5 cm that had invaded or compressed the ventricular system (60%) or had crossed over the middle line or invaded infratentorial structures (10.9%). The other 21.8% of the supratentorial tumors for which tumor locations had been recorded in the medical records were more circumscribed, measuring >5 cm (12.7%) or ≤5 cm (9.1%). The frontal lobe alone or in association with the involvement of other supratentorial structures was the most affected (49 out of 94 cases (52.1%)). In 4 patients, the tumor was located in infratentorial structures, with the cerebellum, posterior fossa, and pons/medulla serving as the primary sites. Patient survival was not dependent upon tumor size ($P = 0.22$; Table 3) or the primary sites of the tumor according to both univariate and multivariate analyses ($P = 0.08$; Table 3).

Treatment and follow-up

The study patients were diagnosed with GBM and treated before the advent of temozolomide. Gross total resection (defined as the absence of residual tumor on postoperative CT and/or MR imaging) was achieved in 31 cases (31.9%), and incomplete tumor resection was achieved in 66 (68.1%); however, there was no correlation between the type of surgery and overall survival ($P = 0.65$). Seventy-six of the 97 patients (78.3%) received adjuvant radiotherapy (daily fractions of 1.5–2 Gy given 5 days per week for 6 weeks, for a total mean of 60.09 ± 0.54 Gy), and 57 of the 97 patients (58.8%) underwent 6 cycles of adjuvant carmustine chemotherapy. There was no difference in survival between patients treated with surgery, surgery plus radiotherapy, or surgery plus radiotherapy and chemotherapy according to both univariate ($P = 0.15$) and multivariate analyses ($P = 0.16$, Table 3). At the last follow-up, 87 patients were dead of disease (89.7%) and 10 were lost to

Table 2
FasL, Fas, cleaved caspase-8, and cleaved caspase-3 immunoexpression in glioblastomas and normal brain tissues according to the Mann–Whitney test.

Protein	Glioblastoma		Normal brain tissue		*P*
	N	(%)	*N*	(%)	
FasL					
Positive	46	(50.5)	0	(0)	<0.0001
Negative	45	(49.5)	25	(100)	
Fas					
Positive	62	(68.9)	4	(16)	<0.0001
Negative	28	(31.1)	11	(84)	
Cleaved caspase-8					
Positive	43	(45.7)	8	(32)	0.0134
Negative	51	(54.3)	17	(68)	
Cleaved caspase-3					
Positive	32	(35.2)	1	(4)	
Negative	59	(64.8)	24	(96)	0.0011

follow-up (10.3%). Excluding the patients who died during the immediate postoperative period (8 postoperative weeks) and the 4 infratentorial cases, the mean follow-up period was 57.7 ± 53.6 weeks for 76 patients. All of these patients showed residual or recurrent disease during the follow-up period and died from causes related to their neoplasm. The overall 5-year cancer-specific survival rate was 1.3% (Table 1). Only 1 patient was alive after 5 years of follow-up, and that patient died from disease 5.6 years after diagnosis.

Immunohistochemistry

FasL, Fas, and cleaved caspase 8 were positively expressed (≥10% of tumor cells) in the cytoplasm of glioblastoma cells of 46 (50.5%), 62 (68.9%), and 43 patients (45.7%), respectively. Cleaved caspase-3 was positively expressed in the glioblastoma tissues of 32 patients (35.2%) in the following patterns: cytoplasmic positivity was observed in 16 tumors, nuclear positivity in 10, and both cytoplasmic and nuclear positivity in 6 (Table 2 and Fig. 1). In normal brain tissues (control group), the expression of FasL, Fas, and cleaved caspase-8 and cleaved caspase-3 occurred in the cytoplasm of the glial cells of 0 (0%), 4 (16%), 8 (32%), and 1 (4%) control specimens, respectively (Table 2). The expressions of FasL ($P < 0.0001$), Fas ($P < 0.0001$), cleaved caspase-8 ($P = 0.0134$), and cleaved caspase-3 ($P = 0.0011$) were significantly higher in glioblastoma than in normal glial tissues. Interestingly, only GBMs with high or low expression of cleaved caspase-8 were associated with significant differences in the overall survival ($P = 0.0325$), suggesting that low immunoexpression (scores 0, 1, or 2) of cleaved caspase-8 in glioblastomas was indicative of a more locally aggressive tumor and was a prognostic indicator of reduced survival (median survival, 8.5 months; log-rank = 4.57, $P = 0.0325$, and hazard ratio [95% confidence interval] = 1.64 [1.04–2.74]) (Table 3 and Fig. 2). In addition, cleaved caspase-8 was determined to be an independent prognostic factor according to a multivariate analysis ($P = 0.03$, Table 3).

In the glioblastomas, there were reasonable to good positive correlations between the expressions of FasL vs. Fas ($r = 0.47$, $P < 0.0001$) and between Fas vs. cleaved caspase-8 ($r = 0.41$, $P < 0.0001$) and poor positive correlations between Fas vs. cleaved caspase-3 ($r = 0.26$, $P = 0.014$), FasL vs. cleaved caspase-8 ($r = 0.22$, $P = 0.0388$), and cleaved caspase-8 and -3 ($r = 0.31$, $P = 0.0026$). No correlations were found among FasL, Fas, and cleaved caspase-8 and cleaved caspase-3 in normal nervous tissue.

Both IDH1 and MGMT were negatively expressed in all 97 GBMs despite the positive controls used for immunohistochemistry.

Table 3
Multivariate analysis of the Cox proportional-hazards regression model for survival in patients with GBM.

Variable	HR	95% CI	*P*
Age	1.18	0.38–3.67	0.78
Gender	2.07	0.61–7.01	0.24
Tumor size	4.37	0.42–45.89	0.22
Primary site	4.83	0.83–28.14	0.08
Treatment			
Surgery	1		
Surg. + Radiotherapy	1		0.16
Surg. + Rad. + Chemo	1.76	0.80–3.89	
FasL			
High expression	1		0.85
Low expression	1.13	0.33–3.93	
Fas			
High expression	1		0.11
Low expression	0.37	0.11–1.27	
Cleaved caspase-8			
High expression	1		0.03
Low expression	11.5	1.25–106.95	
Cleaved caspase-3			
High expression	1		0.08
Low expression	2.50	0.90–6.93	

Discussion

Deregulation of the normal mechanism for programmed cell death plays an important role in the pathogenesis and progression of gliomas [14,16,20,33]. Although evidence has accumulated that gene mutations [22], microRNAs [11,36,47], growth factors [17,18,37], RNA-binding proteins [45], DNA-binding transcription factors [23], Ca^{2+} binding proteins [31], signal transduction proteins [5,31], and DNA methylation [15] have critical roles in regulating cell apoptosis, the significance of the extrinsic apoptotic signaling pathway for glioblastomas remains unclear [19,26]. In this study, we used TMA technology and immunohistochemistry to assess the expression of proteins involved in the extrinsic pathway. We looked at FasL, Fas, cleaved caspase-8, and cleaved caspase-3 in treatment-naïve human glioblastomas and normal glial cells from control brains and examined these immunohistochemistry findings in the context of the clinicopathological data of the study patients.

Death receptors of the tumor necrosis factor (TNF) family, including TNFR1, Fas (CD95/Apo-1), DR4/DR5, Apo-3 (DR3), and their respective cognate ligands TNF-α, FasL (CD95L/Apo-1L), TNF-related apoptosis-inducing ligand (TRAIL/Apo-2L), and Apo-3L can induce the extrinsic apoptotic pathway in the cytoplasm of tumor and normal glial cells [1]. Molecular assays of the Fas signaling pathway using yeast and eukaryotic cells have shown that after the binding of FasL to the Fas receptor, Fas binds directly to the adapter protein FADD (Mort1) and leads to apoptotic signal transduction. In turn, FADD interacts with caspase-8 through its death effector domain (DED), leading to DISC assembly and caspase-8 oligomerization, which drives its own activation in the cytoplasm through self-cleavage. Subsequently, cleaved caspase-8 molecules in the DISC activate downstream effector caspases, leading to the cleavage of caspase-3 and apoptosis [4,7,21,27].

We demonstrated that malignant glial cells of glioblastomas express Fas and FasL, an inducer of immunocyte cell death via the Fas-mediated pathway of apoptosis. Because activated leukocytes express abundant cell-surface Fas, the expression of FasL potentially enables glioblastomas to counterattack and kill Fas-sensitive, antitumor immune effector cells [19]. We observed the augmented expression of FasL in 50.5% of glioblastomas, in contrast to the absence of its expression in normal glial tissue.

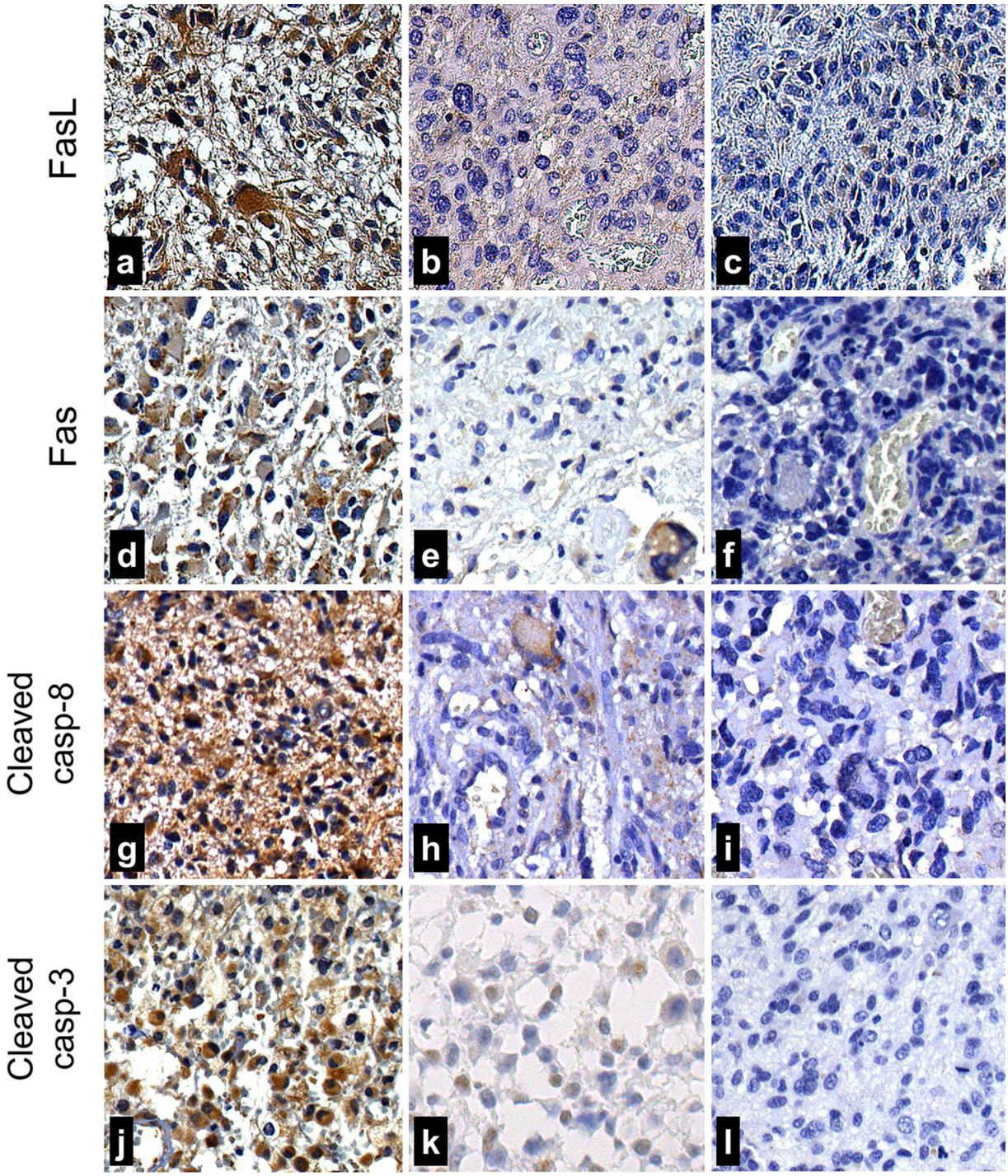

Fig. 1. Immunoexpression of FasL, Fas, cleaved caspase-8 and cleaved caspase-3 in glioblastomas. (A–C) Cytoplasmic expression of FasL is observed in tumor cells; (A) shows a tumor with high FasL expression (score of 3); (B) (score of 1) and (C) (score of 1) show tumors with low FasL expression. (D–F) Cytoplasmic expression of Fas is observed in tumor cells; (D) shows a tumor with high Fas expression (score 3); and E (score 1) and F (score 0) show tumors with low-to-negative Fas expression. (G–I) Cytoplasmic expression of cleaved caspase-8 is observed in tumor cells; (G) shows a tumor with high cleaved caspase-8 expression (score 4); and H (score 1) and I (score 0) show tumors with low and negative cleaved caspase-8 expression, respectively. (J–L) Expression of cleaved caspase-3 in cytoplasm and/or in nuclei is observed in tumor cells. (J) shows high expression (score 4) of cleaved caspase-3 in tumor cells, whereas K (score 2) and L (score 0) show tumor cells with low and negative expressions, respectively (original magnifications: ×200).

In addition, we observed a significant difference in Fas expression between glioblastomas (68.9%) and normal glial tissue (16%) and reasonable to good positive correlations between both FasL and Fas and Fas and cleaved caspase-8 in glioblastomas. Taken together, our findings suggest that neoplastically transformed glial cells increase the expression of FasL, Fas, and cleaved caspase-8, indicating the initiation of the extrinsic apoptotic pathway. Molecular studies have demonstrated the high expression of Fas and FasL in malignant glioma cells, and these findings support the conclusion that the FasL-Fas-dependent apoptotic mechanism is intact and functional [14,33].

When the expression of cleaved caspase-8 and cleaved caspase-3 proteins was analyzed, we found a significant expression of cleaved caspase-8 in 45.7% of the glioblastomas and 32% of the

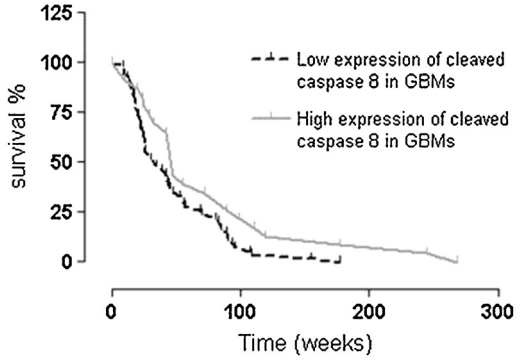

Fig. 2. Survival curves according to low (scores 0–2) and high (scores 3 and 4) immunoexpression of cleaved caspase-8 in glioblastomas. Patients with tumors that expressed low levels of the protein showed lower median survival (8.5 months) compared to patients with tumors that expressed high levels of the protein (median survival = 11.7 months) (Log-rank = 4.57, $P = 0.0325$, and hazard ratio [95% confidence interval] = 1.64 [1.04–2.74]).

normal glial tissues. Cleaved caspase-3 was expressed in 35.2% of the glioblastomas and in only 4% of the normal glial tissues. In addition, we found that the low level of expression of cleaved caspase-8 in glioblastomas was associated with a median survival of 8.5 months, which represents a significant decrease in overall survival compared to patients with glioblastomas expressing high levels of cleaved caspase-8 (median survival of 11.7 months). This effect on survival was independent of treatment, gender, age, tumor size, and tumor location. Using a quantitative immunoblotting method, Ashley et al. [2] also found that the caspase-8 protein levels in *ex vivo* malignant gliomas varied substantially. Taken together, our findings suggest that high- or low-levels of expression of cleaved caspase-8 and cleaved caspase-3 are independent of clinicopathological features and are likely implicated in tumor progression.

We observed poor correlations between Fas and cleaved caspase-3, between FasL and cleaved caspase-8, and between cleaved caspase-8 and cleaved caspase-3 in the tumors. These results suggest that Fas-induced apoptosis is activated by the extrinsic pathway but is inhibited downstream. In fact, the Fas-mediated apoptotic pathway can be inhibited in glioblastomas at several stages by RIP (receptor-interacting protein) [3], by c-FLIP (cellular Fas-associated death domain-like interleukin-1beta-converting enzyme-inhibitory protein) [13], by PEA-15/PED (phosphoprotein enriched astrocytes-15 kDa/phosphoprotein enriched in diabetes) [14,37], by Bcl-2 [10,12,42] or by the cytokine response modifier A (CrmA) [28]. In addition, the activation of caspase-3 by caspase-9 can be blocked by the high expression of inhibitor of apoptosis proteins (IAPs) in glioblastomas [28,35,44].

According to the literature, after FasL is bound to the Fas receptor, apoptotic signal transduction via the extrinsic pathway results in the interaction of FADD with caspase-8 through their DED; however, in our study, we found poor correlations of cleaved caspase-8 expression with FasL, Fas, and cleaved caspase-3 levels. This finding might be explained by the high expression in glioblastomas of c-FLIP and PED/PEA-15, which are protein inhibitors of caspase-8 activation and contain DED domains and can modify DISCs in the non-raft fractions of the plasma membrane [3,13,43]. In fact, Bellail et al. [3] showed that RIP, c-FLIP, and PED/PEA-15 can modify the DR5-mediated DISC in TRAIL-sensitive and resistant glioblastoma cells, leading to the inhibition of caspase-8 cleavage and NF-κB activation.

Our results suggest that these proteins mediate the early stages of the extrinsic apoptotic pathway in glioblastomas. FasL binds to Fas and subsequently binds to FADD, transmitting the signal to activate the extrinsic pathway. At this stage, in glioblastomas, cleaved caspase-8 may be inhibited, and consequently apoptosis of these cells may also be inhibited. One could argue that the signal strengths detected by immunohistochemistry in our study, mainly for cleaved caspases-8 and cleaved caspase-3, did not correlate with the apoptotic morphology in the GBMs. Two fundamental explanations for these results could be postulated. First, perinecrotic palisading cells, where apoptotic figures are more often observed, were not included in the analyzed samples. Second, there is evidence that the molecular modification of the death receptor-mediated DISC by RIP, c-FLIP and PED/PEA-15 may control caspase-8 cleavage and the initiation of apoptosis in glioblastoma cells [3].

In contrast to other studies [6,30], we did not observe any significant differences in the survival of our patient cohort's patient survival between older and younger groups (<50 years vs. ≥50) or between the three different treatment regimens, even when the data were adjusted for the other variables studied. These divergent results may be due to the differences in the age ranges of the cohorts. For example, Ohgaki et al. [30] studied 715 GBM patients in the following age ranges: 6.9% were <39 years, 12.5% between 40 and 49 years, 21.1% between 50 and 59, 29.9% between 60 and 69, 22.1% between 70 and 79, and 7.6% >80 years. In addition, we analyzed a smaller sample of patients ($n = 97$) compared to the Ohgaki et al.'s cohort ($n = 715$). It is important to highlight that the age distribution of the population-based study of Ohgaki et al. [30] showed greater frequency of younger and older patients (40.5% <50 years and 29.7% ≥70) compared to our series (35.1% <50 years and 14.4% ≥70). This difference in the survival outcomes and responses to treatment could be attributed to the different age distributions presented in both studies. Similarly, Burger and Green [6] studied 71 patients with GBM, 35 (49%) of whom were younger than 45 years and 36 were older than 65 years (51%); however, in our series, 35.1% of the patients were ≤49 years, and 41.2% were ≥60 years.

Unfortunately, we did not obtain any conclusive labeling for MGMT (instead, the controls were positive), though we used a robust antibody (SPM287). In fact, the small tissue cores (1.0 mm) and the well-known MGMT immunolabeling heterogeneity may have been limiting factors in our analysis, underscoring some of the difficulties in using immunohistochemistry to assess MGMT expression in formalin-fixed paraffin-embedded GBM tissues, as previously reported in other studies [34,46]. Similarly, the immunohistochemistry for IDH1 was negative in all GBM tissue cores (with positive controls). However, it is important to note that the majority (if not all) of our GBM cases were primary GBMs that did not contain the IDH1 mutation. Although we used a general IDH1-antibody instead of the well-established antibody for the dominant mutant variant of the enzyme (IDH1-R132H), we do not believe that it impacted our results because no IDH1-immunopositive cells could be found in the TMAs. Furthermore, the staining of such small areas with the mutation-specific antibody may be problematic.

In conclusion, 50.5% of the glioblastomas expressed variable levels of FasL, 68.9% expressed Fas, 45.7% expressed cleaved caspase-8, and 35.2% expressed cleaved caspase-3. Moreover, glioblastoma tumors should contain a functional mechanism for the extrinsic apoptotic pathway. Our findings suggest that Fas–Fas-ligand downstream signal transduction could be inhibited, especially at the stage of caspase-8 activation, thereby establishing a major mechanism for the evasion of apoptosis by these tumors. Furthermore, our findings highlight the study of Ho et al. [16], who showed that FasL and Fas delivery by a glioma-specific and cell cycle-dependent HSV-1 amplicon virus enhances apoptosis in high-grade gliomas, and may be useful as an adjuvant therapy to complement the current therapeutic regimens for human gliomas. In addition, the low immunoexpression of cleaved caspase-8 (0 to <50% of faintly positive tumor cells) in glioblastomas was an independent prognosticator of slightly decreased disease-specific survival, compared

with tumors that expressed higher levels of cleaved caspase-8. Further studies examining molecular targets in the extrinsic pathway of apoptosis are needed and may reveal promising treatment strategies for glioblastomas.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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