

Original Article

GRP78 overexpression as an unfavorable outcome in glioma patients

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Abstract: Aims: In this study, the GRP78 expression and the correlation between GRP78 expression and clinicopathologic data in patients with glioma, including survival, were examined. Methods and results: The mRNA and protein levels of GRP78 were respectively determined by real-time PCR and immunohistochemical analysis in 30 fresh glioma samples and 19 fresh normal brain samples as well as 156 paraffin-embedded glioma samples and 35 normal paraffin-embedded brain samples. The data showed that GRP78 mRNA is markedly upregulated compared with normal brain tissues. Consistent with this data, the GRP78 protein level was also significantly increased in glioma tissues compared with normal brain tissues. We further observed that high GRP78 protein expression was significantly associated with clinical stage ($P = 0.0013$) but did not correlate with age and gender. High, rather than low, GRP78 protein expression was associated with poor overall survival rates ($P = 0.001$). Multivariate analysis indicated that high GRP78 protein expression was an independent prognostic indicator of patient survival ($P = 0.002$). Conclusions: Our findings demonstrate that GRP78 is overexpressed and plays a significant role in disease progression and poor outcome in patients with glioma.

Keywords: GRP78, overexpression, glioma, disease progression, outcome

Introduction

The most common primary (intrinsic) brain tumors are referred to as gliomas graded according to the WHO classification system, which has implications for prognosis and management [1]. The current therapy mode includes maximal safe resection, followed by radiotherapy combined with temozolomide [2]. Generally, a majority of patients succumb to the disease within 2 years of diagnosis [3].

The exact causes of gliomas remain unidentified. Hereditary genetic disorders, such as neurofibromatosis (types 1 and 2) and tuberous sclerosis complex predispose to their development. Several studies on diet and vitamin supplementation suggest that dietary N-nitroso compounds could influence the risks of both childhood and adult brain tumors. Notably, gliomas have been associated with electromagnetic radiation from cell phones [8], although several large studies have found no conclu-

sive evidence. Together, these causes finally induced changes in the expression of many tumor-associated genes [4-10] and led to the transition from normal brain to glioma.

Glucose-regulated protein 78 (GRP78) is found in the endoplasmic reticulum and regulates the unfolded protein response [11]. In previous studies, the role of GRP78 in various cancers has been explored [12-19]. Its increased expression mainly promoted tumor growth, invasion, metastasis, and drug resistance [12-14]. Several studies have also confirmed that the upregulation of GRP78 is significantly associated with an unfavorable prognosis in breast cancer and prostate cancer, among others [15, 16]. Meanwhile, GRP78 has been reported to potentially predict favorable outcomes in breast cancer and thymic carcinoma [17, 18]. Thus, the prognostic value of GRP78 remains controversial in tumors. In glioblastomas, GRP78 has been observed to be upregulated [19]. However, the clinical features and prognostic corre-

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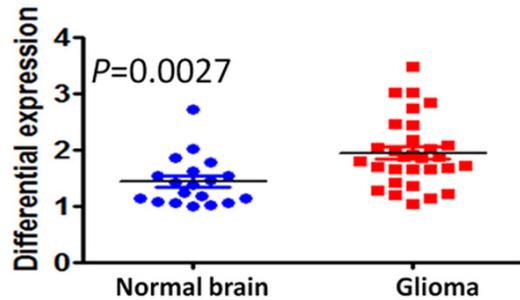


Figure 1. Reduced GRP78 mRNA was shown in glioma tissues compared to normal brain tissues.

lation of patients with GRP78 expression have yet to be investigated in glioma.

In the present study, we evaluated the GRP78 expression in human patient samples and explored the correlation of GRP78 expression with the clinical features and prognosis of glioma. Our data indicated that overexpression of GRP78 is an unfavorable factor promoting the pathogenesis of glioma.

Materials and methods

Sample collection

A total of 30 fresh glioma samples and 19 fresh normal brain samples, as well as 156 paraffin-embedded glioma samples and 35 normal paraffin-embedded brain samples, were obtained from the Nanfang Hospital of Southern Medical University, Guangzhou, China. These samples were obtained from 106 males and 50 females aged 15-78 y (median, 43.8 y). Prior Consent from patients and approval from the Ethics Committee of Nanfang Hospital were obtained prior to the use of these clinical materials for research purposes. All samples had confirmed pathological diagnosis and were classified in accordance with the criteria set by the World Health Organization (WHO).

RNA extraction and real-time PCR

RNA was extracted from glioma tissues and brain tissues by using Trizol (Takara, Shiga, Japan). RNA was transcribed into cDNA and amplified with specific sense: 5'-GTGCAGCAGGACATCAAGTT-3', antisense primer: 5'-AGCAATAGTTCCAGCGTCTT-3'. The GAPDH gene was used as an internal control using the sense primer 5'-CGGAGTCAACGGATTTGGTCGTAT-3' and the antisense primer 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'. Assays were conducted

in accordance with the manufacturer's instructions (Takara, Shiga, Japan). Cycling conditions were set as follows: 95°C for 10 min to activate the DNA polymerase, followed by 45 cycles at 95°C for 15 s, 56°C for 15 s, and 72°C for 12 s. PCR reactions for each gene were repeated 3 times. Independent experiments were conducted in triplicate.

Immunohistochemistry

In accordance with standard protocols, paraffin sections (3 μm) of lung adenocarcinoma were deparaffinized in 100% xylene and rehydrated in descending ethanol series (100%, 90%, 80%, and 70% ethanol). Heat-induced antigen retrieval was performed in 10 mM citrate buffer for 2 min at 100°C. A peroxidase blocking reagent containing 3% hydrogen peroxide and serum to block endogenous peroxidase activity and non-specific antigen was followed by incubation with a mouse anti-human GRP78 polyclonal antibody at a concentration of 1:100 (Santa Cruz Biotechnology, Inc., CA, USA) at 4°C overnight. The sections were visualized with 3,3'-diaminobenzidine (DAB) and counterstained with hematoxylin, mounted in a neutral gum and analyzed using a bright-field microscope.

Evaluation of staining

The stained tissue sections were reviewed separately by 2 pathologists blinded to the clinical parameters. The tissue sections were then evaluated for presence of cytoplasm staining.

The score was evaluated according to the sum of cytoplasm staining intensity and the percentage of positive staining areas of cells. The staining intensity was scored as follows: negative expression, 1; weak expression, 2; positive expression, 3; and strong expression, 4. The percentage of positive staining areas of cells was defined in the scale of 0-3 (0: <10%, 1: 10%-25%, 2: 26%-75%, and 3: >76%). For statistical analysis, final staining scores of 0-5 and 6-7 in the cytoplasm were considered as low- and high-expression, respectively.

Statistical analyses

All statistical analyses were conducted using SPSS 20.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5 (<http://www.graphpad.com/company/>). Two-tailed Student's t-test was used for comparison between groups. A Chi-

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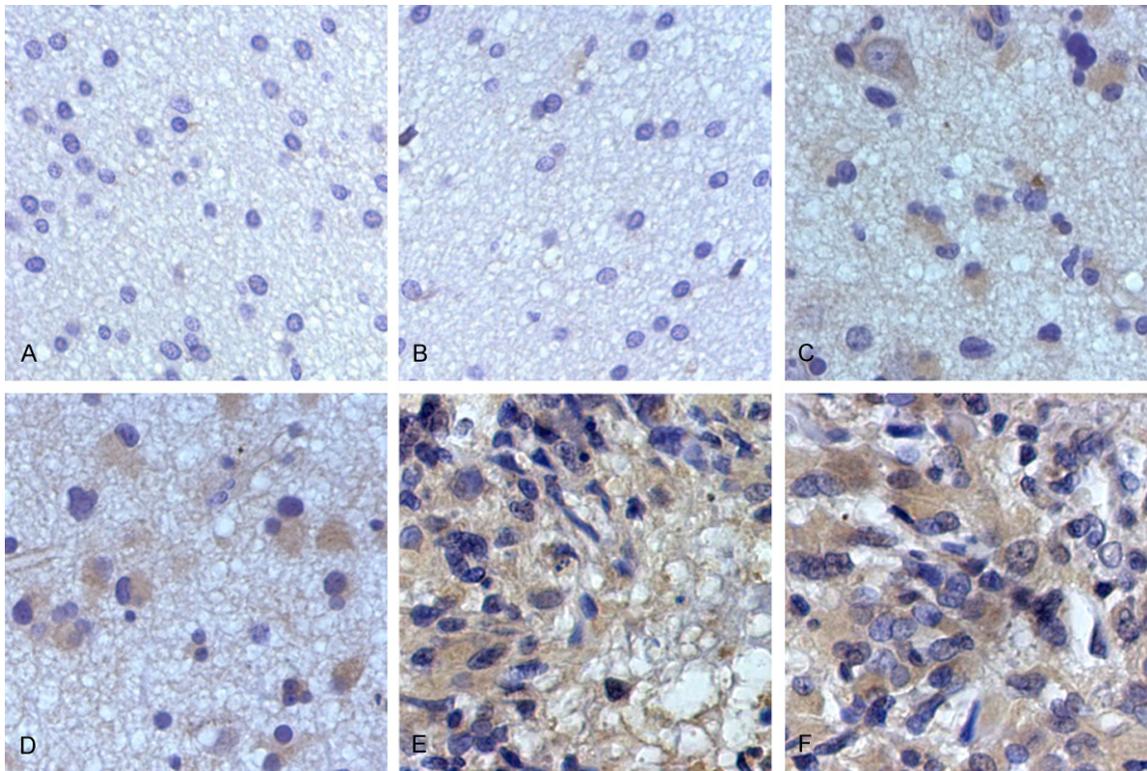


Figure 2. GRP78 protein expression in glioma (original magnification: $\times 400$). A, B. Negative expression of GRP78 protein in brain tissues. C, D. Low expression of GRP78 protein in glioma tissues of I-II stage. E, F. High expression of GRP78 protein in glioma tissues of III stage.

Table 1. Differential protein expression of GRP78 in glioma and brain tissues

	N	GRP78 protein expression		P value
		High	Low	
Glioma	156	88	68	P=0.006
Brain tissues	35	11	25	

square test was used to analyze the correlation between the GRP78 expression and the clinicopathologic parameters of glioma. The association between the GRP78 expression and survival was examined by Kaplan-Meier analysis with the log-rank test. A P value < 0.05 was considered statistically significant.

Results

GRP78 expression is increased in glioma

To assess the role of GRP78 in glioma, we performed real-time PCR to measure the expression of GRP78 mRNA transcripts in 30 freshly collected glioma tissues and 19 freshly collected normal brain tissues. Compared with normal

brain tissues, glioma tissues exhibited higher expression of GRP78 mRNA ($P = 0.0027$) (**Figure 1**).

Immunohistochemistry of GRP78 in glioma tissues

We examined the expression level of GRP78 protein in 156 archived paraffinembedded glioma samples and 35 paraffin-embedded brain samples by immunohistochemical staining. Specific GRP78 protein staining was detected in the cytoplasm of brain and tumor tissues (**Figure 2A-F**). Moreover, GRP78 expression was significantly increased in the glioma tissues (88/156, 56.4%) relative to that in the brain tissues (11/35, 31.4%) (**Table 1**).

Correlation between GRP78 expression and clinicopathologic parameters in patients with glioma

The correlations between GRP78 expression and clinicopathologic parameters in patients with glioma are summarized. As shown in **Table 2**, no significant relationship was observed

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Table 2. The correlation of GRP78 protein expression with clinical features of glioma

Clinical parameter	n	The expression of GRP78		P value
		High expression (n)	Low expression (n)	
Age (year)				
<50	74	44	30	0.519
≥50	82	44	38	
Gender				
Female	50	30	20	0.605
Male	106	58	48	
Clinical stage				
I-II	60	26	34	0.013
III	96	62	34	

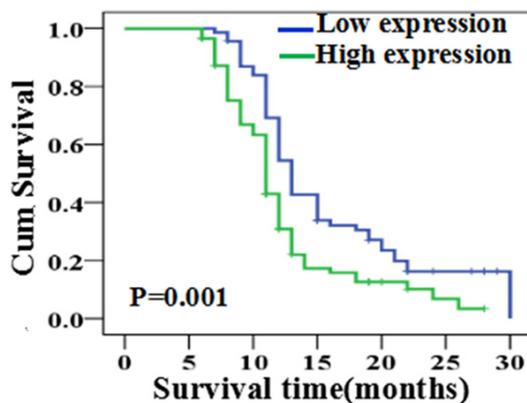


Figure 3. High expression of GRP78 protein as an unfavorable factor reduces the overall survival time of glioma patients.

between GRP78 protein expression and age and gender in 156 patients with glioma. However, high GRP78 protein expression was associated with the clinical stage of the disease ($P = 0.0013$).

High GRP78 expression is associated with poor overall survival

To evaluate the prognostic value of *GRP78 protein* expression in glioma tissues, we used Kaplan-Meier analysis with the log-rank test. The association between *GRP78 protein* expression and patient survival was analyzed. *GRP78 protein* expression was shown to be negatively associated with the overall survival time of patients with glioma. Patients with high *GRP78 protein* expression had a worse prognosis than patients with low *GRP78 protein* expression (**Figure 3**; $P = 0.001$).

High GRP78 expression is an independent prognostic factor for patients with glioma

We used the univariate and multivariate Cox proportional hazards model to analyze the significance of various variables in survival to investigate the potential high expression of *GRP78 protein* is an independent prognostic factor. Both univariate and multivariate analyses suggested that *GRP78 protein* expression was significantly

associated with patient survival ($P = 0.002$ and $P = 0.002$). High *GRP78* expression is an independent prognostic marker for patients with glioma (**Table 3**).

Discussion

GRP78 encodes glucose-regulated protein 78 and is a member of the heat shock protein 70 (HSP70) family. It is localized in the lumen of the endoplasmic reticulum and is responsible for protein folding and denaturing to maintain cellular integrity. GRP78 is highly conserved among eukaryotes, including mammals. In addition, it is widely expressed among all tissue types in humans [20]. Many reports demonstrating that abnormal GRP78 expression correlates with the pathogenesis of some diseases, including autoimmune disease, type 2 diabetes, Alzheimer's disease, and tumors, among others [12-17, 21-23].

Glioma is a type of tumor that predominantly occurs in the brain. In the current study, we first observed that GRP78 mRNA and protein are overexpressed in glioma tissues, compared with the normal brain tissue. This finding was similar to that found in the research on glioblastomas by Lee [24] and other studies, including that by Du et al. on esophageal squamous cell carcinoma [25], Su et al. in hepatocellular carcinoma [26], and Xing et al. in colon cancer [27]. These results preliminarily demonstrated that GRP78 may function as a tumor-promoted role and participates in the pathogenesis of glioma. However, the correlation of the GRP78 protein expression with the clinical features of glioma was still determined.

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Table 3. Univariate analysis and Multivariate analysis of GRP78 protein expression in glioma

Parameter	Univariate analysis			Multivariate analysis		
	P	HR	95% CI	P	HR	95% CI
Age (year)						
≥50 vs <50	0.007	0.615	0.431-0.876	0.011	0.629	0.439-0.901
Clinical stage						
I-II vs III	0.001	2.000	1.319-3.033	0.002	1.963	1.287-2.995
Gender						
Female vs Male	0.442	0.864	0.595-1.255			
GRP78 expression						
Low vs High	0.002	1.743	1.221-2.490	0.002	1.747	1.220-2.503

In previous studies, overexpression of GRP78 was positively correlated with the clinical progression of some tumors [25-27]. In the current study, overexpression of GRP78 was found to be positively associated with the progression of glioma. This result was analogous to the previous findings [25-27] and consistently suggested that overexpression of GRP78 can play an unfavorable role in glioma pathogenesis. However, the correlation between GRP78 expression and the survival of patients with glioma has never been reported.

In recent years, overexpression of GRP78 in tumors was shown to be of favorable or unfavorable prognostic significance depending on the type of tumor [15-18]. More evidence indicated that overexpression of GRP78 in cancer cells was not a favorable prognosis factor in breast cancer and prostate cancer [15, 16]. By contrast, two recent studies demonstrated that high GRP78 expression could be used as a valuable predictor of favorable outcomes in patients with advanced thymic carcinoma and breast cancer [17, 18]. Therefore, the roles of GRP78 in tumors remain complex.

In the present study, we proved that GRP78 protein expression in glioma was inversely correlated with the overall survival of patients. The patients with higher GRP78 expression had shorter survival times. Multivariate analyses showed that increased GRP78 expression was a significant predictor of poor prognosis in patients with glioma. These results were analogous to those reported by Shimizu et al. [28] in which high GRP78 expression was found to be an independent prognostic factor for predicting poor survival against malignant melanoma. Further, high GRP78 expression was also iden-

tified as an independent prognostic factor in tongue cancer and pancreatic cancer [29, 30].

Conclusion

Our study indicated that GRP78 expression was significantly increased and correlated with the malignant status of glioma. Our data also showed that increased GRP78 was an unfavorable prognostic factor for glioma.

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Disclosure of conflict of interest

None.

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