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Original Investigation

Increased Expression of GRP78 Correlates with Adverse Outcome in Recurrent Glioblastoma Multiforme Patients

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ABSTRACT

AIM: Resistance to chemotherapy is a significant clinical issue in recurrent glioma. Chemotherapy such as temozolomide is used for glioblastoma multiforme (GBM) treatment. However, the medical diagnosis rate of patients with GBM is lower than expected and the recurrence rate is high. Research on targeted therapies based on novel molecular markers for GBM patients is important. The molecular chaperone Glucose-regulated protein 78 kda (GRP78) is overexpressed in various tumors and in the endoplasmic reticulum.

MATERIAL and METHODS: Western blot analysis and real-time PCR were done to detect the expression of GRP78 in GBM tissues.

RESULTS: It was detected that GRP78 is up-expressed in GBM, especially in recurrent GBM. Radiotherapy (RT) plus temozolomide chemotherapy after primary tumor removal in relapsed patients induces C/EBP homologous protein (CHOP).

CONCLUSION: The results mentioned above illustrate an essential function of GRP78 in the pathogenesis of GBM and suggest this receptor as the latest prognostic marker for further GRP78-targeted molecular cancer therapy and potential diagnosis of GMB.

KEYWORDS: Glioma, Temozolomide, Glucose-regulated protein 78 kda, Chemotherapy

ABBREVIATION: GBM: Glioblastoma multiforme, **GRP78:** Glucose regulated protein 78 kda, **RT:** Radiotherapy, **CHOP:** C/EBP homologous protein, **TMZ:** Temozolomide, **Hsp70:** 70 kilodalton heat shock protein, **ER:** Endoplasmic reticulum, **GADPH:** Glyceraldehyde-3-phosphate dehydrogenase, **UPR:** Unfolded protein response.

INTRODUCTION

Temozolomide (TMZ), a chemotherapy drug, is a chemotherapy standard for treatment of newly diagnosed cases and the treatment of recurrent malignant gliomas, and is used to postpone tumor diffusion and prolong the patient's life (2). It can change DNA by DNA methylation and substitute thymine for cytosine. Meanwhile, it can activate mismatch repair mechanisms as well as identify recurring errors and trigger cell apoptosis (15). However, the rate of recurrence is high and the average survival time of the patients with a poor prognosis is less than 2 years (13,24). Studies have illustrated that the expression of O6-methylguanine DNA methyltransferase connects resistance with temozolomide in GBMs (6).

Glucose-regulated protein 78 kda (GRP78) is a vital factor for oncogenesis residing primarily in the endoplasmic reticulum (ER) (8) and is considered as the member of the HSP70 family (5). Its function involves the correct folding and packaging of functional proteins. In the tumor microenvironment, glucose starvation and hypoxia may activate GRP78 overexpression (12). In fact, GRP78 is fully revealed in a variety of tumors and affects their immune regulation, metastasis and growth (3,9,11,25). It also refers to tumor maintenance and progression (3), and influences cell survival and chemotherapy resistance by interference in various apoptotic signaling pathways (4,14,21). Moreover, it is overexpressed in glioblastomas and increases apoptosis resistance and glioma cell growth (6).

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Several previous reports have implicated GRP78 in glioma pathogenesis (16,19,20) but its prognostic value for recurrent GBM has not yet been shown. In addition, malignant gliomas show that the tumors are highly resistant to chemotherapy, which indicates the removal of residual tumor cells is still a problem. The research intends to settle the overexpression of GRP78 in recurrent GBM and explore its chemoresistance to these cancers.

MATERIALS and METHODS

Sample and Clinical Information

28 specimens of 23 patients with histologically-confirmed GBM, WHO grade IV (18 primary GBMs and 10 recurrent GBMs, involving 5 pairs of the same patients) and 5 normal brain specimens were from the neurosurgery department, Wuhan General Hospital of Guangzhou Military (Wuhan, China) from 2008 to 2015. The recurrent patients were treated with TMZ (Schering Plough, Shanghai, China) chemotherapy plus radiotherapy (RT) after primary tumor removal. All human tissue specimens have been approved by the ethics committee of Hubei Polytechnic Institute on December 3, 2015 and have been performed according to the ethical standards set forth in Declaration of Helsinki (1964). The patient study detailed in our manuscript was conducted with informed consent and followed guidelines for all human subject experimental studies. In the study, informed consent was obtained prior to all persons' inclusion.

Immunohistochemical Staining and Evaluation

To detect the GRP78 expression in GBMs and normal brain specimens, anti-GRP78 antibody in mice (Santa Cruz, CA, USA) was used for immunohistochemical analysis as the primary antibody and the horseradish peroxidase (HRP)-conjugated rabbit anti- mouse polyclonal antibody was used as the secondary antibody (Santa Cruz, CA, USA).

Real-Time PCR

On the basis of the manufacturer's instructions, RNAs were extracted from tumor tissues and normal tissues with TRIzol (Invitrogen, Carlsbad, USA). Real-time PCR was completed by an iQ5 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The following primers were used:

GRP78 forward: 5'-GACCCTTACTCGGGCCAAATT-3';
GRP78 reverse: 5'-GTAGAGCGGAACAGGTCCATGT-3';
GAPDH forward: 5'-GGTCACCAGGGCTGCTTTTA-3';
GAPDH reverse: 5'-GAGGGATCTCGCTCCTGGA-3'.

GAPDH was chosen as the reference gene.

Western Blot Analysis

We detected the expression of CHOP and GRP78 in GBMs and normal brain specimens by western blot analysis. Mouse anti-CHOP or GRP78 antibody (Santa Cruz, CA, USA) was used as primary antibody, and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse polyclonal antibody (Santa Cruz, CA, USA) was used as secondary antibody.

Statistical Analysis

Statistical significance of the data was examined by ANOVA via SPSS 10.0 statistical software. The significance level was $p<0.05$.

Table I: Clinical Characteristics for all Patients with Primary and Recurrent Gliomas

Patient number	Age(years)	Gender	Tumor location
Primary 1	46	M	Rt. temporal
Recurrence 1	47		
Primary 2	65	M	Lt. temporal
Recurrence 2	66		
Primary 3	56	M	Rt. parietal
Recurrence 3	57		
Primary 4	49	F	Lt. temporal
Recurrence 4	49		
Primary 5	51	F	Lt. frontal
Recurrence 5	52		
Primary 6	55	F	Rt. frontal
Recurrence 6	47		
Primary 7	38	M	Rt. temporal
Recurrence 7	62		
Primary 8	40	M	Rt. frontal
Recurrence 8	55		
Primary 9	35	F	Rt. parietal
Recurrence 9	50		
Primary 10	47	M	Lt. frontal
Recurrence 10	38		
Primary 11	57	M	Rt. parietal
Primary 12	44	M	Rt. frontal
Primary 13	39	F	Lt. frontal
Primary 14	68	M	Rt. frontal
Primary 15	58	F	Lt. parietal
Primary 16	59	F	Lt. temporal
Primary 17	65	M	Rt. temporal
Primary 18	48	M	Rt. temporal

Note: *Clinical characteristics for all 23 patients with primary and recurrent gliomas. 5 pairs of specimens (primary and recurrence specimens) of patient No. 1 to 5 were from the same patients, 5 pairs of specimens (primary and recurrence specimens) of patient No. 6 to 10 were from different 10 patients. Each pair of patient No. 6 to 10 has the same gender and tumor location, but not the same patient.*

RESULTS

28 specimens of 23 patients with histologically-confirmed GBM, WHO grade IV (18 primary GBMs and 10 recurrent GBMs, including 5 pairs of the same patients) brain specimens were studied. As shown in Table I, the diagnostic age was between 35 and 68. There was no significant difference in age distribution based on gender.

To prove if GRP78 is up-regulated in recurrent GBM tissues, primary and recurrent GBM specimens from patients were examined in three ways: immunohistochemistry, real-time PCR, and western blot analysis. Recurrent patients underwent TMZ chemotherapy plus RT after primary tumor removal. Compared to specimens from normal brains, the immunohistochemical staining displayed that the expression of GRP78 in GBM specimens increased overall (Figure 1A-F). Furthermore, a strong expression of GRP78 mRNA was found in recurrent GBM specimens on the basis of real-time PCR analysis. A significant difference in GRP78 expression was found between the primary and recurrent GBM specimens (Figure 2). In GBM specimens, a higher level of GRP78 mRNA was detected and a strong level of GRP78 mRNA emerged in recurrent GBM specimens while normal brain specimens showed lower expression of this mRNA. Apparently, the increase of GRP78 level was correlated with tumor recurrence.

Temozolomide, an anti-glioma drug, is used widely. Its effect on GRP78 and CHOP verified whether it would affect ER stress or unfolded protein response (UPR) levels. As can be seen from Figure 3, temozolomide significantly stimulated induction of CHOP which is similar to GRP78.

DISCUSSION

As a member of the HSP70 family, GRP78 induces oxidative stress, hypoxia and glucose starvation under certain stress conditions, which is characteristic of the tumor microenvironment (12). In fact, GRP78 is up-expressed in a series of cancer tissues, including gastric tumors (25), colon (23), breast (11), lung (22), prostate (18), and gliomas (6); however, its expression and function in recurrent GBM have not yet been expressed.

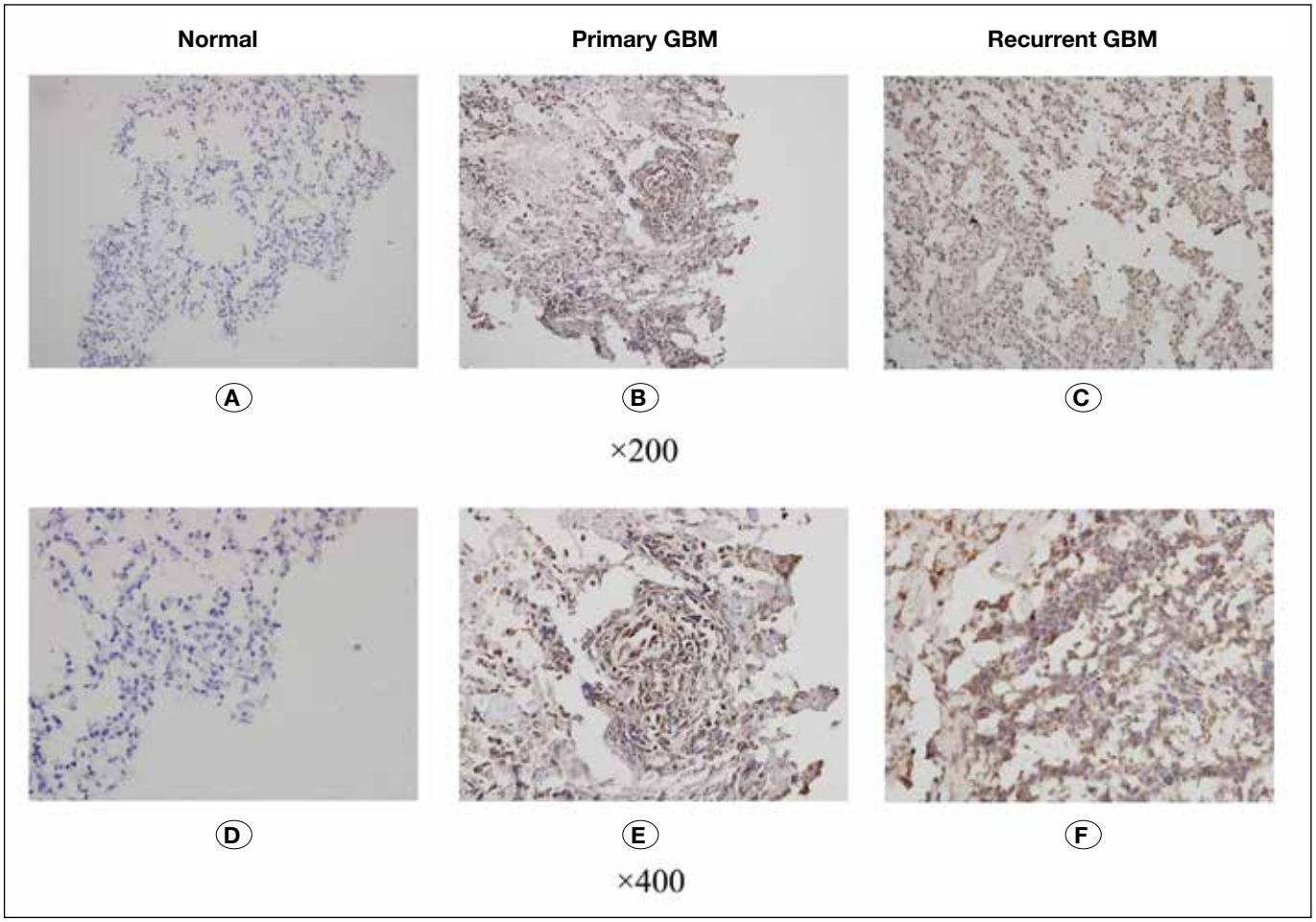

Figure 1: Immunohistochemical staining of GRP78 in recurrent GBM specimens, primary specimens and normal brain specimens. Immunohistochemical staining of GRP78 in the specimen demonstrated a series of GRP78 expression from weakly focused positive (normal specimens) to higher diffusely positive (primary GBM specimens) to strongly diffuse positive (recurrent GBM specimens); original magnification, x200 (**A, B** and **C**) and x400 (**D, E** and **F**).

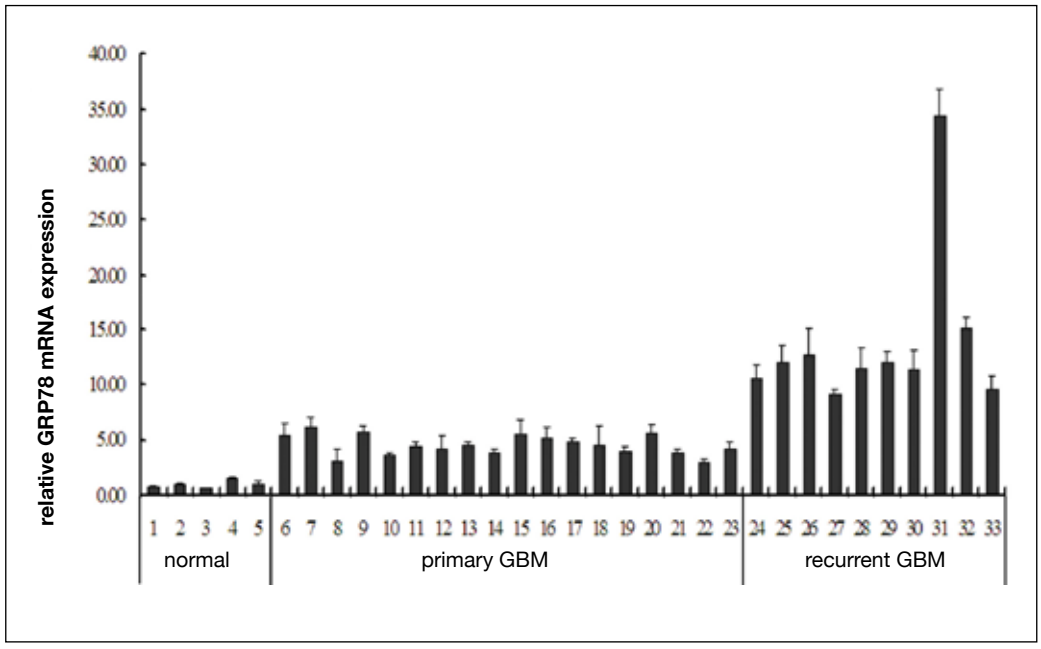

Figure 2: Real-time PCR analysis of GRP78 expression in recurrent GBM specimens, primary specimens and normal brain specimens. A collection of normal (1-5), primary GBM (6-23) and recurrent GBM tissues (24-33) was analyzed for GRP78 expression with real-time PCR. The consequences normalized to the level of GAPDH mRNA and demonstrated correlation with reference samples. Data were exhibited as the mean ± SD of three independent experiments, normal versus primary GBM, $p<0.05$; recurrent GBM versus primary GBM, $p<0.05$.

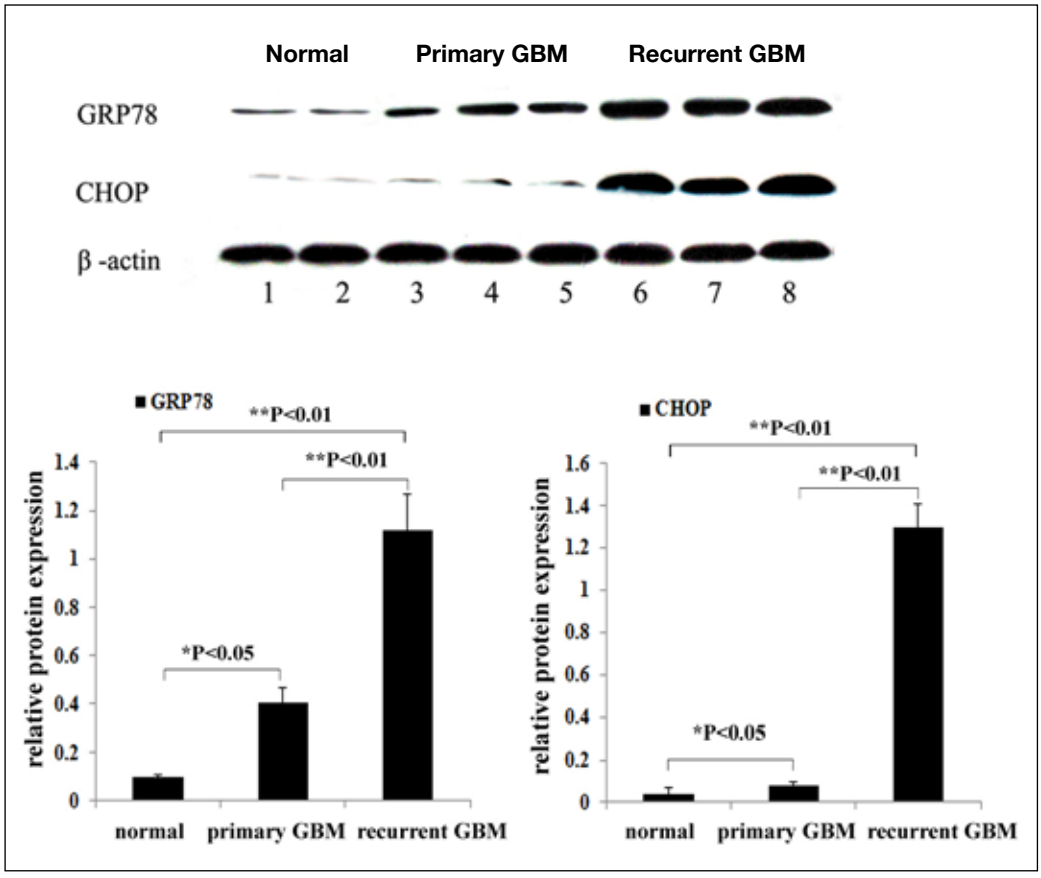

Figure 3: Overexpression of GRP78 and CHOP in recurrent GBM specimens was analyzed by Western blot. Western blot analysis was done to detect the expressions of CHOP and Grp78 in normal brain (1 and 2), primary GBM (3, 4 and 5) and recurrent GBM (6, 7 and 8) specimens. β –actin was the standardization of the average signal intensity. Figures were exhibited as the mean ± SD of three independent experiments, recurrent GBM versus primary GBM, $**p < 0.01$; normal versus primary GBM, $*p<0.05$; normal versus recurrent GBM $**p < 0.01$.

In this study, we have made an entirely new observation with important prognostic and therapeutic applications. Compared to primary GBMs or normal brain specimens, GRP78 was highly expressed in recurrent GBMs on the basis of the survey of malignant glioma specimens.

The key clinical problem in recurrent glioma is its resistance to chemo-RT. Although an alkylator such as temozolomide is used for GBM treatment, the ability to prolong the patient's life is limited, in recurrent GBM in particular. Thus, the increased expression of GRP78 in recurrent GBM may partially explain a protection mechanism against cytotoxic drugs (taking temozolomide as an example) which may apply to prognosis and treatment.

How the ER chaperone GRP78 protects gliomas from temozolomide (DNA damaging agents) has become an important issue. There are some mechanisms that may be used to explain this. UPR is composed of serial adaptive pathways triggered by disparate perturbations in the normal function of the ER, producing misfolded proteins (19). It relieves ER stress through multiple pathways involving disintegration of misfolded proteins, puckering enzymes, strengthening the supervision of chaperones, and general translation capture. However, in a state of persistent ER stress, it triggers cell apoptosis and eventually death. (7). It targets mainly the induction of GRP78, which acts as a significant character in protein folding and assembly as well as targeted misfolded protein disorganization (10). Hence, GRP78 stands for a pro-survival arm of UPR. Moreover, the CCAAT/enhancer binding protein homologous transcription factor (CHOP/GADD153) (19) is one of the key performers of UPR pro-apoptotic molecules (17). When ER stress was activated, GRP78 upregulated and dissociated from the transmembrane protein, and then initiated the transcription of CHOP nuclear expression. Two typical markers of endoplasmic reticulum stress are GRP78 and CHOP.

Our experiments suggested increased levels of GRP78 and CHOP in GBM specimens, especially in recurrent GBM specimens treated with TMZ, indicating that the endoplasmic reticulum stress response pathway may be activated. On one hand, ER stress may be triggered by temozolomide indirectly. On the other hand, ER could be a direct target site for temozolomide (19). Currently, studies (1) report that temozolomide makes pro-apoptotic markers overexpressed in tumor cells and increases the intracellular $Ca2^{+}$ level. It is reported that elevated $Ca2^{+}$ levels activate ER stress responses and induce the expression of GRP78. The function of GRP78 is to be a Ca^{2+} binding protein to prevent Ca^{2+} outflow into the cytosol in ER. Under such circumstances, GRP78 can offer buffers for increased temozolomide-mediated cytoplasmic Ca^{2+} levels so as to suppress apoptosis. Besides, ER stress brings about misfolding of endoplasmic reticulum proteins. Grp78, as an important molecular partner of the ER family, can resist ER and inhibit apoptosis (19).

Overall, we believe GRP78 is overexpressed in GBM, particularly in recurrent GBM. The expression of GRP78 is not only related to the enhancement of glioma cell proliferation, but also to the sensitivity of apoptosis induced by chemotherapeutics (such as temozolomide). These researches confirm that GRP78 may be a therapeutic target for gliomas as well as a potential prognostic marker.

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