

The Unfolded Protein Response Regulator GRP78/BiP as a Novel Target for Increasing Chemosensitivity in Malignant Gliomas

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Abstract

Poor chemosensitivity and the development of chemoresistance remain major obstacles to successful chemotherapy of malignant gliomas. GRP78 is a key regulator of the unfolded protein response (UPR). As a Ca²⁺-binding molecular chaperone in the endoplasmic reticulum (ER), GRP78 maintains ER homeostasis, suppresses stress-induced apoptosis, and controls UPR signaling. We report here that GRP78 is expressed at low levels in normal adult brain, but is significantly elevated in malignant glioma specimens and human malignant glioma cell lines, correlating with their rate of proliferation. Down-regulation of GRP78 by small interfering RNA leads to a slowdown in glioma cell growth. Our studies further reveal that temozolomide, the chemotherapeutic agent of choice for treatment of malignant gliomas, leads to induction of CHOP, a major proapoptotic arm of the UPR. Knockdown of GRP78 in glioblastoma cell lines induces CHOP and activates caspase-7 in temozolomide-treated cells. Colony survival assays further establish that knockdown of GRP78 lowers resistance of glioma cells to temozolomide, and, conversely, overexpression of GRP78 confers higher resistance. Knockdown of GRP78 also sensitizes glioma cells to 5-fluorouracil and CPT-11. Treatment of glioma cells with (–)-epigallocatechin gallate, which targets the ATP-binding domain of GRP78 and blocks its protective function, sensitizes glioma cells to temozolomide. These results identify a novel chemoresistance mechanism in malignant gliomas and show that combination of drugs capable of suppressing GRP78 with conventional agents such as temozolomide might represent a novel approach to eliminate residual tumor cells after surgery and increase the effectiveness of malignant glioma chemotherapy. [Cancer Res 2007;67(20):9809–16]

Introduction

Malignant gliomas are the most commonly diagnosed malignant adult primary brain tumors, including anaplastic astrocytoma (grade 3) and glioblastoma multiforme (GBM; grade 4). Median survival for GBM is ~12 to 15 months. Surgical resection or diagnostic biopsy is usually the first step in therapy, followed by adjuvant

radiation and chemotherapy (1). Despite significant improvements in the surgical treatment of malignant gliomas, a complete resection is impossible; after diffuse brain invasion, cells were often found at great distances from the site of the major tumor (2).

Because a cure remains elusive, it is important to identify new treatment modalities, as well as to modify existing therapies, to possibly change malignant gliomas from a deadly disease into a chronic one. A promising approach to achieving these goals is to enhance glioma chemosensitivity to existing treatment regimens. Temozolomide (Temodar) is currently the standard of care for treatment of newly diagnosed and recurrent malignant gliomas. This well-tolerated drug has been shown to delay tumor progression and prolong patient survival (3). The action of temozolomide is associated with its ability to damage DNA through DNA methylation and subsequent substitution of cytosine by thymine. This leads to the activation of the mismatch repair mechanism, which recognizes a recurring error and triggers apoptosis (4). In patient samples, resistance to temozolomide in GBMs has been linked to expression of O⁶-methylguanine DNA methyltransferase (5).

The unfolded protein response (UPR) consists of a set of adaptive pathways that are triggered by disparate perturbations in normal function of the endoplasmic reticulum (ER) that lead to the production of misfolded proteins. The UPR alleviates ER stress by arresting general translation, up-regulation of chaperones and folding enzymes, and degradation of misfolded proteins; however, in the case of intense or persistent ER stress, the UPR will trigger apoptosis resulting in cell death (6). A major UPR target is the induction of the glucose-regulated protein 78 (GRP78/BiP), which has important roles in protein folding and assembly, targeting misfolded proteins for degradation, ER Ca²⁺ binding, and controlling the activation of transmembrane ER stress sensors (7). Thus, GRP78 represents a prosurvival arm of the UPR. On the other hand, the CCAAT/enhancer binding protein homologous transcription factor (CHOP/GADD153) is one of the critical executioners of the proapoptotic arm of the UPR (8). Induction of CHOP signals that the cells are experiencing ER stress and hence initiating the cell death process.

As malignant gliomas represent tumors that are highly resistant to chemotherapy and elimination of residual tumor cells remains a major challenge, we initiated this study to determine whether GRP78 plays a role in the chemoresistance of such cancers. Here, we provide evidence that malignant glioma cells overexpress GRP78. Our results reveal that knockdown of GRP78 suppresses glioma cell proliferation, induces CHOP, and activates caspase-7 in temozolomide-treated cells. Through colony survival assays, we have established that manipulation of GRP78 levels alters the sensitivity of glioma cells to temozolomide and other chemotherapeutic agents. Further, combination treatment of glioma cells with temozolomide and (–)-epigallocatechin gallate (EGCG), a known

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inhibitor of the protective function of GRP78 (9), sensitizes glioma cells to temozolomide. These results show that GRP78 status is a potential prognostic marker for malignant gliomas and that GRP78 is a novel chemosensitizing target for malignant brain tumors.

Materials and Methods

Cell lines, culture, and drug treatment conditions. U87 cell lines were obtained from the American Tissue Culture Collection. All other malignant glioma cell lines were provided by Frank B. Furnari and Webster K. Cavenee (Ludwig Institute of Cancer Research, La Jolla, CA). The cells were propagated in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 0.1 mg/mL streptomycin at 37°C, and 5% CO₂. Human astrocytes and the astrocyte medium, in which astrocytes were grown, were purchased from ScienCell Research Laboratories. Temozolomide (Temodar) was purchased from Schering Plough and dissolved in DMSO as a 25 mmol/L stock solution. 5-Fluorouracil (5-FU) and irinotecan (CPT-11) were obtained from Sigma-Aldrich.

Immunoblots and antibodies. Fifty micrograms of total cell lysate prepared in radioimmunoprecipitation assay buffer were processed for Western blot analysis as described (10). The antibodies against GRP78, CHOP, actin (Santa Cruz Biotechnology, Inc.), and caspase-7 (BD PharMingen) were used according to the manufacturer's recommendations. The secondary antibodies were coupled to horseradish peroxidase, and were detected by chemiluminescence using the SuperSignal West substrate (Pierce). Each immunoblot was done at least twice to confirm the results. The blots were quantified using ImageJ software (11).

Immunohistochemistry. Malignant glioma tissues were obtained from glioblastoma patients undergoing primary resection or biopsy of their tumor. No exposure to radiation or chemotherapy had been experienced by this group of patients. The peritumoral tissue was obtained from patients

undergoing a partial or full lobectomy for their GBM. Consent for use of tissue specimens was obtained from the University of Southern California Institutional Review Board. Immunohistochemical analysis of GRP78 expression in tumor tissues and cell lines was done with anti-GRP78 antibody (Santa Cruz Biotechnology) diluted 1:100 in 2% goat blocking serum as described (12). Specificity of the antibody was tested as previously described (12).

Transfections. For knockdown of GRP78, the glioblastoma cells were transfected with small interfering RNA (siRNA) against GRP78: sense 5'-GG-AGCGCAUUGAUACUAGATT-3' and antisense 5'-UCUAGUAUCAAUGCG-CUCCTT-3' (13). The siRNA targeted against green fluorescent protein (GFP) purchased from Qiagen served as negative control. A final concentration of 80 nmol/L was used for both siRNAs. Transfections were done in six-well plates following Qiagen's instructions. Ten microliters of each siRNA were used with 5 µL of Lipofectamine 2000 per well.

Lentivirus infection. Lentiviruses expressing siRNA against GRP78 as described above or full-length human GRP78 were constructed as described (14). All lentiviral constructs expressed GFP. For infection, 10⁴ cells were plated in six-well plates and infected with the lentivirus at titers of 5 × 10⁶ units/mL. Infected cells were monitored for GFP under the fluorescence microscope.

Colony formation assays. Twenty-four hours after transfection with siRNA, the cells were seeded in six-well plates at 200 cells per well (when higher concentrations of temozolomide were used, appropriately increased numbers of cells were seeded). The next day, the medium was changed to include 20 to 160 µmol/L temozolomide. After a change of fresh medium 48 h later, the cells were allowed to form colonies for 14 days in the absence of drug. The colonies were stained with 1% methylene blue (in methanol) for 4 h and then counted. The experiments were done in three or six replicates and each experiment was done at least twice. Statistical analysis was done using Student's *t* test, two-tailed distribution, assuming equal variance for the samples.

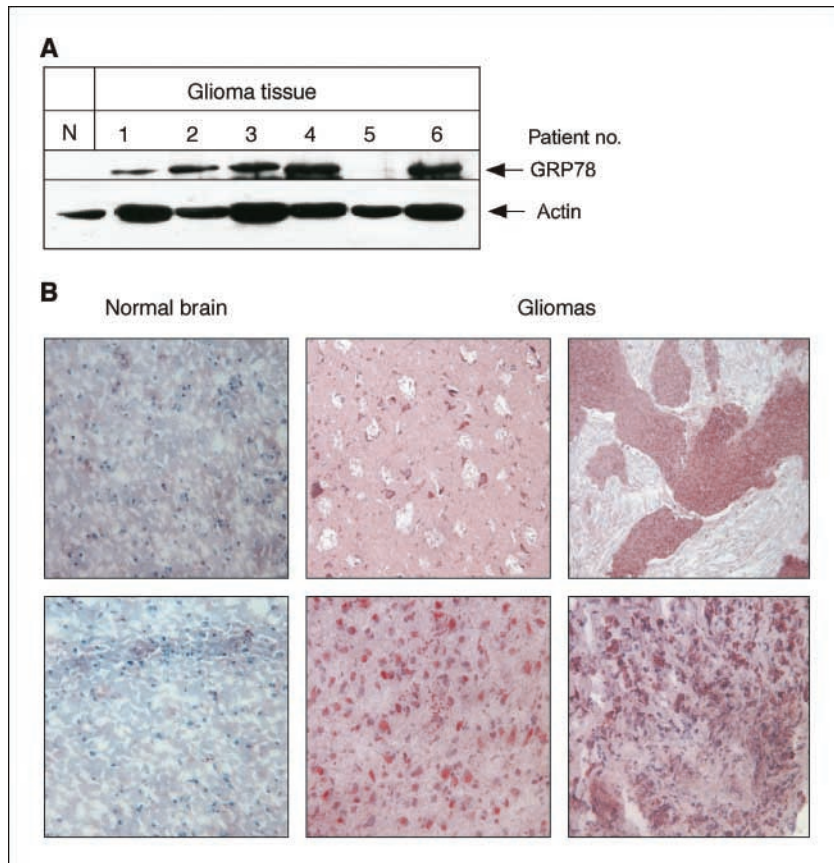


Figure 1. Elevation of GRP78 level in human glioma tissues. *A*, the human glioma tumors were ground in liquid nitrogen, lysed, and tested for the level of GRP78 protein by Western blot. For comparison, a lysate of the peritumoral brain (N) was also used. Actin levels were assessed as a loading control. *B*, immunohistochemical staining of GRP78 in human glioma tissues and normal brain. Various glioma tissues were cut in the cryostat, after which they were stained with anti-GRP78 antibody. Red, GRP78 staining; blue, hematoxylin was used as background staining.

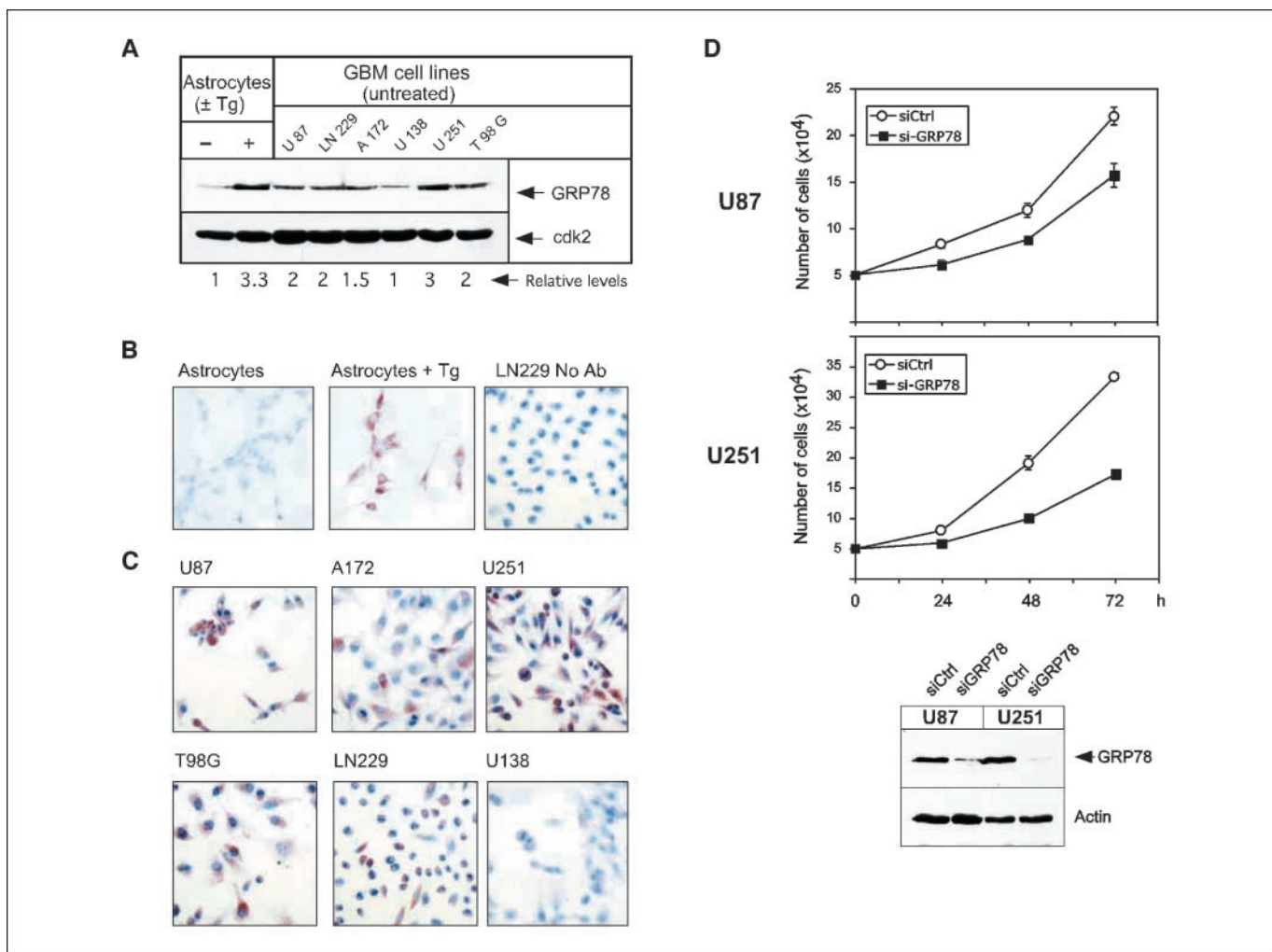


Figure 2. GRP78 expression level in glioma cell lines. *A*, various glioblastoma cell lines were grown under standard conditions for 24 h, after which they were lysed and their GRP78 protein levels were determined by Western blot. For comparison, untreated human astrocytes and astrocytes treated with 1 μmol/L thapsigargin (Tg) were run in parallel. cdk2 levels were assessed as a loading control. Numbers below the blot, relative levels of GRP78 protein relative to the untreated astrocytes control set at 1. *B*, experimental controls for immunohistochemical staining of GRP78 in glioma cells. Human astrocytes were grown under standard conditions or in the presence of 1 μmol/L thapsigargin on glass coverslips, then stained with anti-GRP78 antibody. LN229 cell line stained without the GRP78 primary antibody was also shown. *C*, various glioma cell lines were grown under standard conditions for 24 h on glass coverslips, then stained using anti-GRP78 antibody. *D*, down-regulation of GRP78 causes reduced growth of U87 and U251 cells. U251 and U87 glioma cells were transfected with siRNA against GRP78 (*si-GRP78*) or the GFP (*siCtrl*). Twenty-four hours posttransfection, 5 × 10⁴ cells per well were plated in a six-well plate. Twenty-four, 48, and 72 h after the plating, the cells were counted. Down-regulation of GRP78 was confirmed by Western blot (*bottom*).

Results

Elevation of GRP78 levels in human glioma tissue specimens.

Tumor specimens from patients undergoing craniotomy before adjunctive therapy, including radiation and chemotherapy, were examined using both Western blot analysis and immunohistochemistry to determine whether GRP78 is up-regulated in human glioma tissue. The Western blot results show that GRP78 level was generally elevated with varying degrees in the glioma tissues (Fig. 1A). For example, compared with normal brain tissue where GRP78 was below detection limit, specimens 4 and 6 showed high GRP78 level, specimens 2 and 3 showed intermediate level, specimen 1 was at low level, and specimen 5 was at very low level. GRP78 overexpression was confirmed by immunohistochemical staining of tissue sections from human glioma specimens that were different from those used in the Western blots. All four sections were positive for GRP78 staining (Fig. 1B). In agreement with the

immunoblot result, peritumoral brain tissue showed very little GRP78 staining (Fig. 1B). These results reveal that GRP78 is highly elevated in this sample of human malignant gliomas.

GRP78 expression level correlates with proliferation rates and is required for glioma cell growth.

A panel of malignant glioma cell lines was tested for GRP78 expression in Western blot analysis to address whether human glioma cells constitutively express this protein and at what level. The results show that compared with human astrocytes grown under normal culture conditions, five of the six glioma cell lines constitutively expressed higher level of GRP78, with the apparent exception of the U138 cell line (Fig. 2A). The level of GRP78 overexpression ranged from 2- to 3-fold, with the upper range comparable with the level of GRP78 induction observed in human astrocytes treated with thapsigargin, an inhibitor of the ER Ca²⁺-ATPase, and an established ER stress inducer. Interestingly, the level of GRP78 overexpression in glioma cell lines seems to correlate with their rate of proliferation. For

example, highly replicating lines (U251, LN229, T98G, U87, and A172) with relatively shorter doubling times (19, 22, 22, 24, and 37.5 h, respectively) showed higher level of GRP78 (3-, 2-, 2-, 2-, and 1.5-fold increase) compared with the slower replicating U138 cells, which have a doubling time of >80 h, for which the GRP78 level was similar to the baseline control. Doubling times of U251, T98G, A172, and U87 cells had previously been reported and were confirmed in our laboratory (15–17). Doubling times of U138 and LN229 cells were obtained in our laboratory.

To confirm the immunoblot results, immunohistochemical staining was done. Human astrocytes grown under normal conditions and treated with thapsigargin were used as negative and positive controls, respectively (Fig. 2B). As shown in Fig. 2C, the majority (>90%) of U251 cells stained positively for GRP78; the majority of T98G, LN229, and U-87 cells (>70%) also stained positively for GRP78. A172 cells showed intermediate staining, with ~25% of the cells stained. By contrast, U138 cell staining was comparable with the negative control with only an occasional positive cell, consistent with the immunoblot results. The cellular staining pattern of GRP78 was perinuclear, within the cytosol, demonstrating a staining pattern consistent with ER localization.

GRP78 was down-regulated using specific GRP78 siRNA (siGRP78) to determine whether it was required for glioma cell growth. Two glioma cell lines (U87 and U251) were examined (Fig. 2D). Twenty-four hours posttransfection, equal numbers of cells transfected with siGRP78 or an siRNA directed against GFP (siCtrl) were plated in six-well plates. Subsequently, one-well per group was trypsinized and counted each day for 3 days and the numbers of cells were plotted against time. Down-regulation of GRP78 resulted in a slower growth rate for both cell lines, with the effect being more severe in U251 than in U87 cells, correlating with near-complete versus partial knockdown of GRP78 in U251 and U87 cells, respectively (Fig. 2D). Taken together, these data show

that the majority of malignant glioma cell lines express elevated levels of GRP78 protein, with the fastest proliferating cells exhibiting the highest level of overexpression. In addition, down-regulation of GRP78 protein levels results in a significant slowing down of the glioma cell growth.

Temozolomide treatment of glioma cells induces CHOP/GADD153 and GRP78. Temozolomide is widely used as an anti-glioma drug. The effect of temozolomide on GRP78 (i.e., the protective arm of the UPR) as well as on CHOP (i.e., the proapoptotic component of the UPR) were examined to determine whether this drug would impinge on ER stress or UPR levels. First, U87 cells were treated with a range (30–500 $\mu\text{mol/L}$) of temozolomide concentrations for 72 h, and then subjected to Western blot analysis. As shown in Fig. 3A, CHOP induction was dramatically stimulated by temozolomide in a dosage-dependent manner, whereas a moderate induction of GRP78 was detected in parallel. We then tested whether this observation is applicable to other glioma cells and to determine the kinetics of CHOP and GRP78 induction. U251 cells were incubated with 250 $\mu\text{mol/L}$ of temozolomide from 4 to 72 h. The results showed that temozolomide could induce GRP78 and CHOP in the U251 cells; the induction became evident after 36 h, and intensified and persisted at least through 72 h of treatment (Fig. 3B). Thus, the induction of the representative UPR markers GRP78 and CHOP indicates that temozolomide is a novel inducer of the ER stress response.

Interestingly, the suppression of GRP78 expression with the use of siRNA resulted in a substantial increase in CHOP levels in U251 cells, even in the absence of any drug treatment (Fig. 3C). This result was consistently obtained in U87 and LN229 cells as well (data not shown). Therefore, GRP78 suppresses CHOP induction. GRP78 is known to interact with procaspase-7, suppressing its activation (9, 18, 19). U251 cells infected with lentivirus expressing

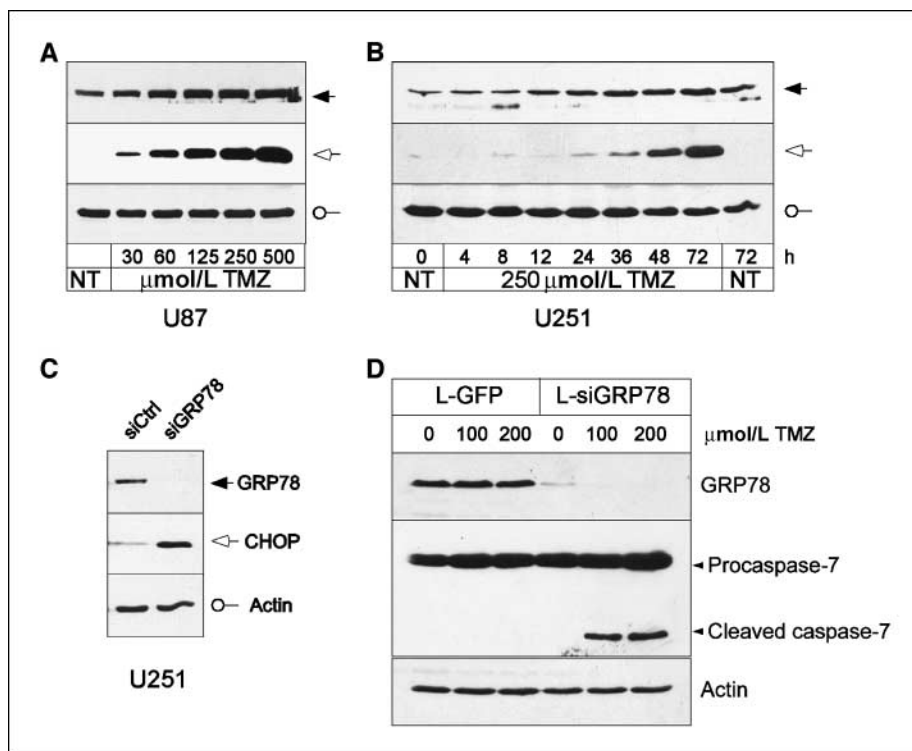
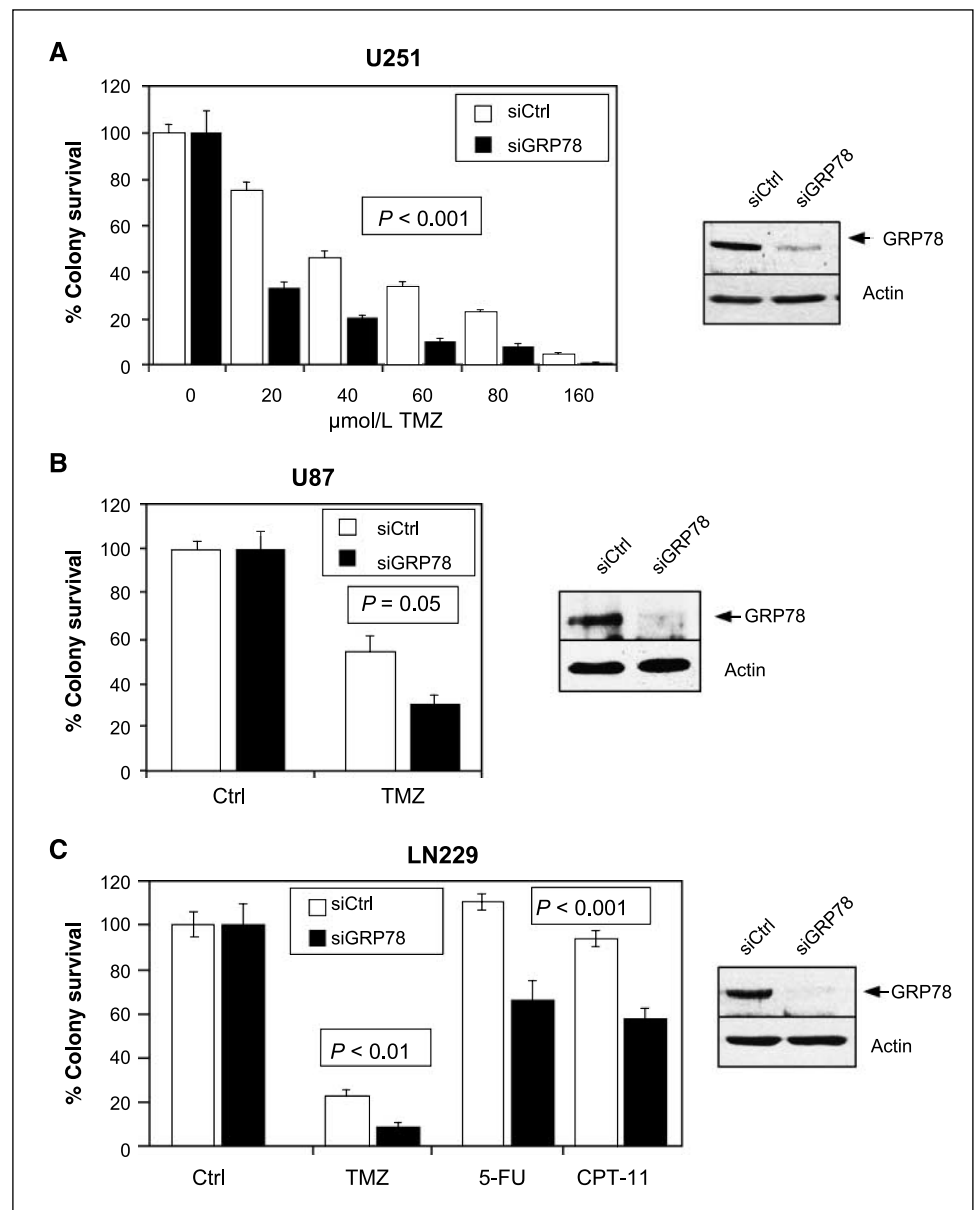


Figure 3. Temozolomide induces CHOP and GRP78 in glioblastoma cell lines. A, U87 cells were grown in the presence of increasing doses of temozolomide as indicated, harvested, and analyzed by Western blot with antibodies against GRP78 (black arrows), CHOP/GADD153 (white arrows), and actin (white circle). NT, no treatment. B, U251 cells were grown for the indicated number of hours in the presence of 250 $\mu\text{mol/L}$ temozolomide, harvested, and analyzed by Western blot with antibodies against GRP78, CHOP/GADD153, and actin. Untreated 72 h control is also shown. C, U251 cells were transfected with siRNA against GRP78 or the GFP. Forty-eight hours posttransfection, the cells were harvested and analyzed by Western blot with antibodies against GRP78, CHOP, and actin. D, GRP78 knockdown results in caspase-7 cleavage in temozolomide-treated glioma cells. U251 cells were infected with lentivirus expressing GRP78 siRNA (L-siGRP78) or GFP alone (L-GFP). The cells were treated with temozolomide for 72 h and analyzed by Western blot with antibodies to caspase-7, GRP78, and actin.

Figure 4. Knockdown of GRP78 sensitizes glioma cells to temozolomide. **A**, U251 cells were transfected with siRNA against GRP78 or GFP. Twenty-four hours posttransfection, 200 cells per well in control, and increasing number of cells in temozolomide treatment groups (200, 400, 600, 800, and 2,000 cells per well for 20, 40, 60, 80, and 160 $\mu\text{mol/L}$ temozolomide, respectively), were seeded in a six-well plate. Twenty-four hours later, the cells were treated with the indicated concentration of temozolomide. The percent of colony survival was plotted against the temozolomide dosage. **Columns**, mean value of six experiments; **bars**, SE. P values for all temozolomide concentrations are indicated. In parallel, untreated cells transfected with siGRP78 and siCtrl were harvested and the protein lysates were analyzed by Western blot with antibodies against GRP78 and actin. **B**, U87 cells were treated with 20 $\mu\text{mol/L}$ temozolomide and subjected to colony survival assay and Western blot analysis as in **A**. **Columns**, mean value of three and six experiments; **bars**, SE. The P value is indicated. The result of colony survival is presented with an accompanying Western blot on the right. **C**, LN229 cells were treated with 20 $\mu\text{mol/L}$ temozolomide, 2 $\mu\text{mol/L}$ 5-FU, or 200 nmol/L CPT-11 and subjected to colony survival assay and Western blot analysis as in **A**. **Columns**, mean value of six experiments; **bars**, SE. $P < 0.01$ for temozolomide treatment; $P < 0.001$ for 5-FU and CPT-11 treatments. The result of colony survival is presented with an accompanying Western blot on the right.



siRNA against GRP78 (L-siGRP78) or the GFP alone (L-GFP) were treated with temozolomide. Whole cellular lysates were analyzed by Western blot with antibodies to GRP78 and caspase-7. The cells that detached and were floating in the medium were included in this analysis. We observed significant caspase-7 cleavage in temozolomide-treated U251 cells in which GRP78 level was down-regulated by siRNA (Fig. 3D). These results imply that elevated level of GRP78 in gliomas may contribute to increased resistance to cell death of these tumor cells.

Knockdown of GRP78 or treatment with EGCG sensitizes gliomas to temozolomide. GRP78 knockdown experiments were done to test directly whether gliomas depend on GRP78 for protection against temozolomide-mediated cell death. U251, U87, and LN229 glioma cells were transfected with siRNA targeted against human GRP78 (siGRP78) or control siRNA (siCtrl) targeted at GFP. Colony survival assays were done using a range of temozolomide concentrations to evaluate the drug sensitivity of the transfected cells (Fig. 4A–C). Western blot assays verified that

siRNA against GRP78 was highly effective in suppressing GRP78 expression in all three cell lines (Fig. 4A–C). The results showed that suppression of GRP78 expression caused a statistically significant increase in drug sensitivity in all three glioma cell lines tested, indicating a critical role of GRP78 as a protector against the cytotoxic effects of temozolomide. To determine whether GRP78 protection extends to drugs other than temozolomide, the same cells were treated with 5-FU or CPT-11. Down-regulation of GRP78 resulted in increased sensitivity of LN229 cell to both drugs (Fig. 4C). Treatment of cells with these drugs did not result in induction of ER stress markers (data not shown).

As a direct test that GRP78 overexpression protects glioma cells against temozolomide-induced cell death, U251 cells were infected with a lentivirus expressing human GRP78 (L-GRP78) or a control vector expressing GFP alone (L-GFP). Colony survival assays were done using a range of temozolomide concentrations to evaluate the drug sensitivity of the infected cells (Fig. 5). Western blot assay verified that infection with L-GRP78 increased the level of GRP78

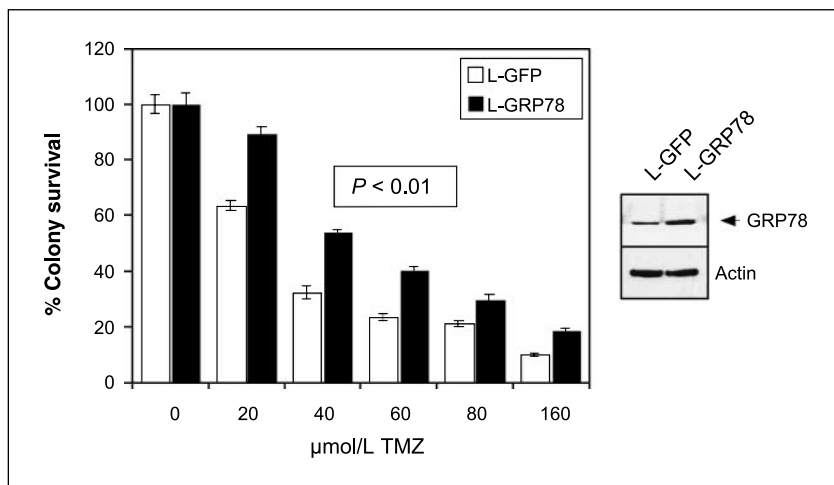


Figure 5. GRP78 protects glioma cells from temozolomide. U251 cells were infected with lentivirus expressing human GRP78 (*L-GRP78*) or GFP alone (*L-GFP*). Five days after infection, 200 cells per well in control, and increasing number of cells in temozolomide treatment groups (200, 400, 600, 800, and 2,000 cells per well for 20, 40, 60, 80, and 160 μmol/L temozolomide, respectively), were seeded in a six-well plate. Twenty-four hours later, the cells were treated with the indicated concentration of temozolomide. The percent of colony survival was plotted against the temozolomide dosage. *Columns*, mean value of six experiments; *bars*, SE. The *P* value for all concentrations of the drug is indicated. *Right*, in parallel, untreated cells infected with *L-GRP78* and *L-GFP* were harvested and the protein lysates were analyzed by Western blot with antibodies against GRP78 and actin.

protein by ~2-fold. The results showed that elevation of GRP78 level caused a statistically significant protection from temozolomide-induced cell death in U251 cells.

Our results predict that down-regulation of GRP78 by compounds directed against GRP78 expression or activity could lead to increased chemosensitivity of glioma cells to temozolomide. One such compound is EGCG, which directly interacts with the ATP-binding domain of intracellular GRP78, blocking its interaction with procaspase-7 and suppressing the protective function of GRP78 (9). In colony survival assays, concomitant treatment of U251 cells with temozolomide and EGCG led to increases in temozolomide chemosensitivity (Fig. 6A and B). Importantly, EGCG at the effective concentrations did not cause cell death in U251 cells by itself. The combination of EGCG and temozolomide, however, caused significantly more cell death than temozolomide alone. These results imply that combination of drugs capable of GRP78 suppression could sensitize glioma cells to temozolomide.

Discussion

Gliomas, and GBM in particular, are among the most chemoresistant tumors (20). In this study, we have made several new observations, which may have important prognostic and therapeutic applications. In our survey of several malignant glioma cell lines, GRP78 overexpression was generally observed and this overexpression was independent of their p53 or PTEN status. Our results showed that glioma cell lines with rapid proliferation rates had the highest levels of GRP78 expression. In addition, down-regulation of GRP78 led to a significant decrease in glioma cell growth. Varying levels of elevated GRP78 expression were also detected in patient biopsies before adjuvant treatment. Taken together, our results raise the possibility that the level of GRP78 in patient biopsies could be used as a predictive factor of aggressiveness of the glioblastoma, independent of their p53 and PTEN status. Our findings predict that tumors with low GRP78 expression represent slow growing, less aggressive tumors, whereas

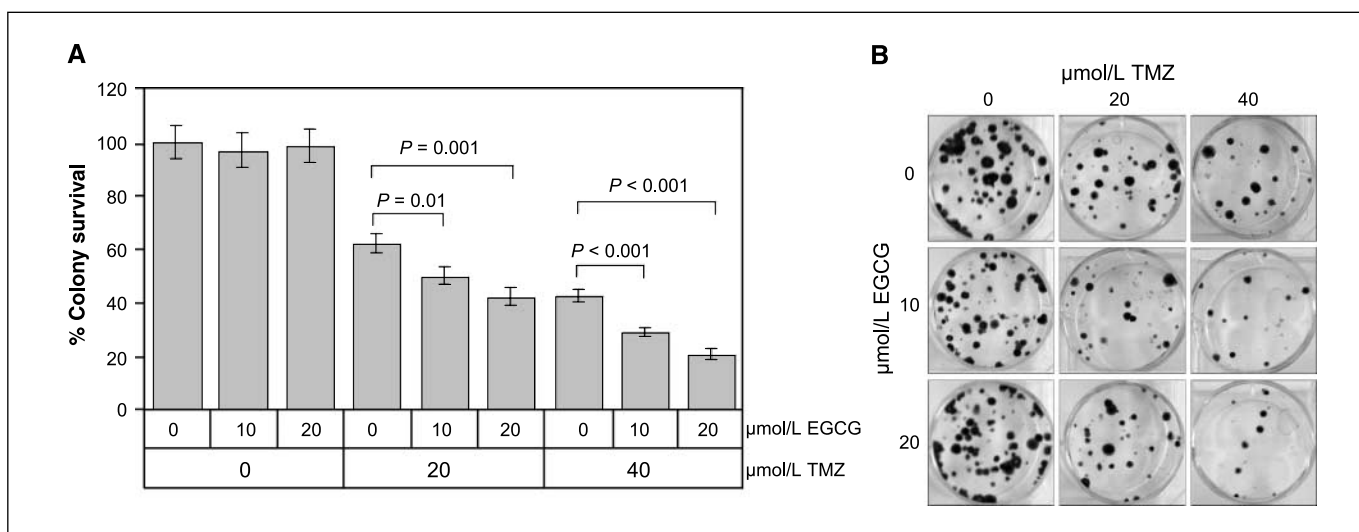


Figure 6. Treatment with EGCG sensitizes U251 glioma cells to temozolomide. *A*, 200 U251 glioma cells per well were seeded in six-well plates and treated with temozolomide in the presence or absence of the indicated concentration of EGCG for 48 h, after which the medium was changed and the cells were allowed to spawn colonies for 14 d. The colonies were stained with methylene blue, counted, and percentage colony survival was plotted. *P* values are indicated. *B*, pictures of the colonies from all treatment groups.

those with high GRP78 are rapidly proliferating, aggressive tumors. Furthermore, elevated GRP78 expression in highly proliferating tumors may provide malignant gliomas with a possible protective mechanism against cytotoxic drugs such as temozolomide. Cytotoxic drugs that induce DNA mismatch work best in rapidly proliferating cells. Rapidly proliferating malignant glioma cells are expected to be more chemosensitive to temozolomide but they may also be protected from temozolomide by higher GRP78 levels.

An important question arises as to how GRP78, an ER molecular chaperone, protects gliomas against temozolomide, a DNA-damaging agent. Our studies suggest that several novel mechanisms may be involved. Evidence is emerging that some therapeutic agents recognized as working through DNA damage can also elicit cytosolic stress independent of the nucleus. For example, cisplatin can induce apoptosis in the absence of DNA damage, at least in part through activation of ER stress as evidenced by GRP78 induction and activation of the ER-associated caspase-12 (21). Our discovery that temozolomide is an inducer of both GRP78 and CHOP suggests that it also causes ER stress. The ER could be a direct target site of temozolomide or temozolomide may indirectly elicit ER stress acting through other cellular pathways. Recently, Das et al. (22) reported that temozolomide treatment at 100 $\mu\text{mol/L}$ for 6 h leads to increased intracellular Ca^{2+} levels and higher expression of proapoptotic markers. Elevated cytoplasmic Ca^{2+} levels have been reported to activate the ER stress response, as exemplified by ATF6 cleavage and GRP78 induction (23). GRP78 is known to function as a Ca^{2+} -binding protein in the ER, thereby preventing Ca^{2+} efflux into the cytosol. In such capacity, GRP78 may provide a buffer against temozolomide-induced increases in cytosolic Ca^{2+} levels, thereby suppressing apoptosis. In addition, ER stress causes protein misfolding in the ER. GRP78, in its capacity as a major ER molecular chaperone, offers protection against ER stress and suppresses apoptosis.

The mechanism of cell death and resistance to temozolomide is just emerging and is likely to be complex, involving apoptotic as well as nonapoptotic pathways (5, 22, 24). In the glioma cell lines, temozolomide induces CHOP, which as a transcription factor, sensitizes cells to ER stress-induced apoptosis through down-regulation of BCL-2 and activation of GADD34 and ER01 α , an ER oxidase (25, 26), and this may represent a new antitumoral mechanism of temozolomide. Our studies reveal that CHOP is constitutively suppressed by GRP78 in glioblastoma. Further, temozolomide treatment leads to activation of caspase-7 when GRP78 level is down-regulated in glioma cells. In view that GRP78 also protects glioma cells against drugs such as 5-FU and CPT-11, which do not induce ER stress, pathways independent of ER stress are likely to be affected by GRP78. These may include its role in cell proliferation and other yet unknown mechanisms (27–30).

Because GRP78 provides protection to tumor cells and endows them with increased chemoresistance, down-regulation of GRP78, therefore, may become an important adjunct in future treatments of malignant gliomas. Our results with siGRP78 show that lowering GRP78 levels significantly increases chemosensitivity to temozolomide. Recent developments of small molecules that can specifically block GRP78 expression and/or its activity may make this approach clinically feasible in the near future (9, 30–34). Recently, Ermakova et al. (9) showed that EGCG, a major component of green tea, can bind to intracellular GRP78, inhibit its protective function, and increase chemosensitivity to etoposide in breast and bladder carcinoma cell lines. In our study, we showed that treatment with EGCG had similar effects as down-regulation of GRP78 by siRNA such that cells treated with the combination of EGCG and temozolomide exhibited more cell death than cells treated with temozolomide alone. EGCG alone did not cause any cell death in glioma cells. EGCG is a water-soluble molecule that may potentially be formulated for i.v. infusions. As a small molecule, it should cross the blood-brain barrier readily. Further *in vivo* studies, including toxicity studies on systemic EGCG administration, are pending. Thus, in our study, we present a target for increasing glioma cell sensitivity to temozolomide (GRP78), and a clinically feasible way to affect this target (treatment with EGCG) in gliomas. Other agents, such as ABT 828, recombinant human plasminogen Kringle 5, has been recently used in phase I/II studies in various carcinomas. Preclinical studies showed that GRP78 is the receptor for Kringle 5, which induces apoptosis of fibrosarcoma and growth-stimulated endothelial cells (18). Its mechanism of action has been hypothesized to be secondary to binding of surface GRP78, internalization of ABT 828 into the cytosol, and blocking of GRP78 binding to the proapoptotic molecule procaspase-7. Combining these and other drugs that can suppress GRP78 with conventional agents, such as temozolomide, may present a novel approach to the elimination of residual tumor cells after surgery, and thereby increase the effectiveness of malignant glioma chemotherapy.

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