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Original Article

Quercetin sensitizes human glioblastoma cells to temozolomide *in vitro* via inhibition of Hsp27

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Aim: Quercetin is an effective Hsp27 inhibitor and has been reported to facilitate tumor cell apoptosis. The aim of this study was to investigate whether quercetin could sensitize human glioblastoma cells to temozolomide (TMZ) *in vitro*.
Methods: Both U251 and U87 human glioblastoma cells were treated with quercetin and/or TMZ for 48 h. Cell viability was detected using the MTT assay. Cell apoptosis was analyzed with caspase-3 activity kits and flow cytometry. Hsp27 expression and phosphorylation were examined using Western blot analysis. RNA interference using Hsp27 siRNA oligos was performed to knock down the gene expression of Hsp27.
Results: TMZ (200 or 400 μmol/L) alone effectively inhibited the viability of U251 and U87 cells. When combined with quercetin (30 μmol/L), TMZ (100 μmol/L) significantly inhibited the cell viability, and the inhibition of TMZ (200 and 400 μmol/L) was enhanced. TMZ or quercetin anole did not affect caspase-3 activity and cell apoptosis, while TMZ combined with quercetin significantly increased caspase-3 activity and induced cell apoptosis. TMZ anole significantly increased Hsp27 phosphorylation in U251 and U87 cells, while quercetin or Hsp27 siRNA oligos combined with TMZ attenuated TMZ-induced Hsp27 phosphorylation and significantly inhibited Hsp27 expression.
Conclusion: Combined treatment with TMZ and quercetin efficiently suppressed human glioblastoma cell survival *in vitro*.

Keywords: glioma; quercetin; temozolomide; chemotherapy; apoptosis; caspase-3; heat shock protein 27; RNA interference

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Introduction

Glioblastoma (GBM) is the most common and the most malignant tumor of the brain. The prognosis for GBM patients remains poor, with a median overall survival of 9.9 to 10.2 months after surgery and an average survival time of only 12 to 15 months[1–3], despite progress in the development of surgical techniques and even with the standard chemotherapy of temozolomide (TMZ) plus radiotherapy. TMZ is an orally delivered alkylating agent that is considered the most efficient drug in GBM therapy. Survival is significantly prolonged in patients with newly diagnosed GBM who are treated with TMZ combined with radiotherapy[4]. However, TMZ treatment usually fails due to the chemoresistance of GBM cells. Therefore, the development of new treatment modalities to attenuate drug resistance and/or to enhance TMZ anti-tumor effects is required.

TMZ treatment results in the formation of O6-methylguanine in DNA and then causes cell-cycle G_2/M phase arrest in glioma cells and ultimately induces cell death. O6-methylguanine DNA methyltransferase (MGMT) expression and MGMT gene promoter methylation are considered to be implicated in chemosensitivity to TMZ[5]. Studies have revealed that MGMT is not the only factor that regulates chemosensitivity to TMZ[6–9]. Hsp27 is a member of the small heat shock protein family and has been found to increase tumorigenicity or induce treatment resistance and apoptosis inhibition[10–12]. It has been reported to be associated with drug resistance of breast cancer, oral cancer and non-small cell lung carcinoma (NSCLC)[11, 13, 14]. Recently, the inhibition of Hsp27 was also reported to enhance the chemosensitivity of glioma to TMZ[15]. Quercetin (3,3′,4′,5,7-pentahydroxyflavone), a natural flavonoid found in various fruits and vegetables, is an efficient Hsp27 inhibitor and has been reported to facilitate apoptosis of tumor cells[14, 16, 17]. In the present study, we try to investigate whether quercetin could sensitize glioblastoma cells to TMZ by Hsp27 inhibition.

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Materials and methods

Agents

Quercetin and temozolomide were purchased from Sigma-Aldrich (St Louis, MO, USA). In all assays, these agents were dissolved in dimethyl sulfoxide (DMSO) and subsequently diluted in serum-supplemented medium immediately before use. The DMSO concentration never exceeded 0.1% (*v*/*v*).

Cell culture

U251 and U87 human glioblastoma cell lines were purchased from the ATCC (American Type Culture Collection). All cells were maintained in DMEM (Dulbecco's modified Eagle's medium) containing 10% fetal bovine serum and 1% penicillin/streptomycin (complete medium). Cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

MTT assay

To study the effects of TMZ and quercetin on U251 or U87 cells, the MTT assay was performed. Cells were cultured and then seeded in 96-well plate at a density of 5000 cells/well in 200 μL of culture medium. Cells were separately treated with DMSO (vehicle control), 30 μmol/L quercetin, TMZ (in concentrations of 100, 200, or 400 μmol/L) or various concentrations of TMZ plus 30 μmol/L quercetin for 48 h. Then, the cells were cultured in a humidified incubator containing 5% CO_2. After 48 h, the original culture medium was discarded, 100 μL of culture medium with 5 mg/mL MTT was added to each well and cells were cultured. After 4 h, the supernatant was removed, and DMSO was added to each well. Ten minutes later, the optical density (*OD*) value (absorbance) was measured at 570 nm using an enzyme-linked immunosorbent assay plate reader (Bio-Rad Laboratories, Inc, Berkeley, CA, USA).

Apoptosis analysis by flow cytometry

For apoptosis analysis, cells were plated in 10-cm culture dishes. Cells were separately treated with DMSO (vehicle control), 30 μmol/L quercetin, 200 μmol/L TMZ or 30 μmol/L quercetin plus 200 μmol/L TMZ for 48 h. Cells were subsequently collected by trypsinization, centrifuged (3500 r/min for 5 min) and washed twice with PBS. Cells were then centrifuged, the supernatant was discarded, and the pellet was incubated for 15 min at room temperature with Annexin V-FITC and propidium iodide before analysis with a FACSAria III flow cytometer (BD Biosciences, San Jose, CA, USA).

Western blot analysis

For Western blot analysis, whole cell lysates were separated by SDS-PAGE followed by transfer to an Immobilon-P membrane (Millipore Corporation, Bedford, MA, USA). Membranes were probed with primary antibodies followed by incubation with secondary antibody. Proteins were visualized with chemiluminescence luminol reagents (Beyotime Institute of Biotechnology, Shanghai, China). Antibodies against GAPDH (#2118), Hsp27 (#2402), and phos-Hsp27 (Ser78) (#2405) were purchased from Cell Signaling Technology (Beverly, MA, USA). The densitometry of the immunoblotting bands was performed using the publicly available ImageJ software (National Institutes of Health, USA).

Caspase-3 activity assay

The Caspase-3 Activity Assay kit was used to measure caspase-3 activity in cell samples (Millipore, Kankakee, IL, USA). The level of caspase-3 activity was represented by absorbance of the substrate, Ac-DEVD-pNA, read at 405 nm in a microtiter plate reader. According to the manufacturer's instructions, cell samples were resuspended and incubated in chilled 1× Cell Lysis Buffer for 10 min before being centrifuged for 5 min at 4 °C in a microcentrifuge (10000×*g*). The supernatants were added to a 96-well plate and incubated for 1 h at 37 °C in the working solution containing Ac-DEVD-pNA. The fold-increase in caspase-3 activity was determined by comparing the absorbance from an apoptotic sample with that of an uninduced control after subtracting the background reading from cell lysates and buffers.

RNA interference

The siRNA oligos of Hsp27 (sc-29350) and negative control siRNA oligos were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). The Hsp27 siRNA oligos consisted of three target-specific siRNAs and were designed and applied as a pool to knock down gene expression of Hsp27. The target sequences were listed below: sc-29350A: Sense: GAGUGGUCGCAGUGGUUAGtt; Antisense: 5′-CUAACCACUGCGACCACUCtt-3′. sc-29350B: Sense: 5′-GACGAGCUGACGGUCAAGAtt-3′; Antisense: 5′-UCUUGACCGUCAGCUCGUCtt-3′. sc-29350C: Sense: 5′-CCACGCAGUCCAACGAGAUtt-3′; Antisense: 5′-AUCUCGUUGGACUGCGUGGtt-3′. Lipofectamine RNAiMAX transfection reagent (Invitrogen, USA) was used for siRNA transfection following the manufacturer's protocol. For a 6-well plate, the final volume of siRNA (10 μmol/L) used per well was 2.5 μL (25 pmol), and the final volume of Lipofectamine RNAiMAX used per well was 7.5 μL. For a 96-well plate, the final volume of siRNA (10 μmol/L) used per well was 0.1 μL (1 pmol), and the final volume of Lipofectamine RNAiMAX used per well was 0.3 μL.

Statistical analysis

Three experiments were performed for each assay. All data were analyzed using the Student-Newman-Keul's test, and are expressed as the mean±standard deviation (SD). $P<0.05$ was considered significant.

Results

Quercetin enhances TMZ induced cell growth inhibition

U251 and U87 cells were separately treated with DMSO (vehicle control), 30 μmol/L quercetin, TMZ (at concentrations of 100, 200, or 400 μmol/L) or various concentrations of TMZ plus 30 μmol/L quercetin for 48 h. Cell viability was detected using the MTT assay. As shown in Figure 1, cell growth was inhibited in a concentration-dependent manner when the TMZ concentration reached 200 μmol/L ($P<0.05$), while 100 μmol/L

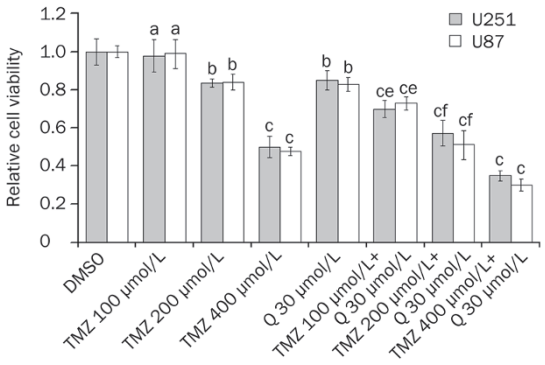

Figure 1. Quercetin (Q) enhances TMZ-induced cell growth inhibition. The U251 and U87 cells were treated separately. The optical density (*OD*) value (absorbance) was measured, and the relative cell viability was calculated. The cell growth was inhibited in a concentration-dependent manner when the TMZ concentration reached 200 μmol/L ($P<0.05$); 100 μmol/L TMZ caused no significant growth inhibition ($P>0.05$). When combined with 30 μmol/L quercetin, TMZ can induce cell growth inhibition at a concentration of 100 μmol/L ($P<0.05$) and more intense inhibition at 200 μmol/L ($P<0.01$). $^{a}P>0.05$, $^{b}P<0.05$, $^{c}P<0.01$ *vs* DMSO. $^{e}P<0.05$, $^{f}P<0.01$ *vs* corresponding single TMZ treatment group.

TMZ caused no significant growth inhibition ($P>0.05$). When combined with 30 μmol/L quercetin, TMZ can induce significant cell growth inhibition at a concentration of 100 μmol/L ($P<0.01$) and more intense inhibition at 200 μmol/L ($P<0.01$). This result indicated that quercetin can enhance TMZ-induced cell growth inhibition in U251 and U87 cells.

Quercetin sensitizes glioblastoma cells to TMZ by Hsp27 inhibition

Quercetin is a widely distributed bioflavonoid and is known to have antitumor effects. It has been reported to effectively reduce lung tumor growth by Hsp27 inhibition when combined with traditional chemotherapy for drug-resistant lung cancer[13]. In the present study, to investigate whether quercetin sensitizes glioblastoma cells to TMZ by Hsp27 inhibition, knockdown experiments to silence the expression of Hsp27 using specific siRNA were performed. As shown in Figures 2A and 2B, treatment with 200 μmol/L TMZ increased the phosphorylation level of Hsp27. Quercetin and siRNA against Hsp27 both decreased Hsp27 protein expression and blocked the phosphorylation of Hsp27. After pretreatment with quercetin or siRNA against Hsp27, TMZ no longer increased the Hsp27 phosphorylation levels; Hsp27 expression and phosphorylation were both inhibited. The results of the cell viability assay (Figure 2C) showed that with either Hsp27 knockdown or quercetin treatment, TMZ induced more intense cell growth inhibition ($P<0.01$). This result indicates that quercetin treatment can sensitize glioblastoma cells to TMZ by Hsp27 inhibition.

Quercetin facilitates TMZ to induce apoptosis

U251 and U87 cells were separately treated with DMSO (vehicle control), 30 μmol/L quercetin, 200 μmol/L TMZ, or 30 μmol/L quercetin plus 200 μmol/L TMZ for 48 h. Cell apoptosis was evaluated with flow cytometry using annexin V-FITC conjugates and propidium iodide double staining. As shown in Figure 3, in DMSO (vehicle control)-treated cells, the apoptosis proportions (Lower Right plus Upper Right section) were 4.8%±1.3% for U251 and 5.7%±1.1% for U87 (Figures 3A and 3E). Cells treated only with 200 μmol/L TMZ for 48 h exhibited no significant cell apoptosis (5.5%±1.2% for U251 and 5.0%±1.2% for U87) (Figures 3B and 3F). Cells treated with 30 μmol/L quercetin also exhibited no significant apoptosis, with apoptosis proportions of 6.3%±1.2% for U251 and 6.9±1.0% for U87 (Figures 3C and 3G). For cells treated with 30 μmol/L quercetin plus 200 μmol/L TMZ, the apoptosis proportions were significantly increased, with 15.2%±1.3% for U251 ($P<0.01$) and 17.1%±2.1% for U87 ($P<0.01$) (Figures 3D and 3H). The data from the caspase-3 activity assay also showed that the activity of caspase-3 significantly increased ($P<0.01$) in cells treated with TMZ plus quercetin, compared with cells treated only with TMZ or quercetin (Figure 4).

Discussion

MGMT was usually considered the major factor resulting in chemoresistance to TMZ[18, 19]. However, in the present study, we detected that after TMZ treatment, the phosphorylation level of Hsp27 was significantly elevated in U251 and U87 glioblastoma cell lines without cell growth inhibition. It seems that the upregulation of Hsp27 phosphorylation is a result of TMZ treatment or may be a cause of resistance. Interestingly, MGMT expression was undetectable in both the U251 and U87 cell lines[5], although these cells were not sensitive to TMZ until its concentration rose to 200 μmol/L (Figure 1). Thus, we suggest that Hsp27 may also confer chemoresistance in U251 and U87 cells.

Hsp27 is considered to contribute to the malignant properties of cancer cells, including resistance to treatment and the inhibition of apoptosis[20]. Increasing evidence of the role of Hsp27 in drug resistance in a variety of cancers has been reported[21, 22]. Hsp27 is phosphorylated at Ser78, Ser82, and Ser15 by MAPKAPK-2 as a result of the activation of the p38 MAPK pathway. The phosphorylation of Hsp27 causes a change in its tertiary structure, which shifts from large homotypic multimers to dimers and monomers. Elevation of Hsp27 phosphorylation was shown in different metastatic cancer cells and has been indicated to correlate with the metastatic potential of cancer cells[13–15]. Lung cancer stemlike cells have been reported to resist cell death induced by superoxide and cytotoxic agents, cisplatin and gemcitabine, and the resistance to apoptosis involves the activation of Hsp27[13]. In glioma, Hsp27 has been reported to participate in secreted protein acidic and rich in cysteine (SPARC)-induced cell survival. The authors demonstrated that suppressing Hsp27 decreases tumor cell survival in gliomas and that the inhibition of Hsp27 is more effective than is TMZ treatment alone. They conclude that the inhibition of Hsp27 may be an effective therapeutic approach to inhibit SPARC-induced glioma cell invasion and

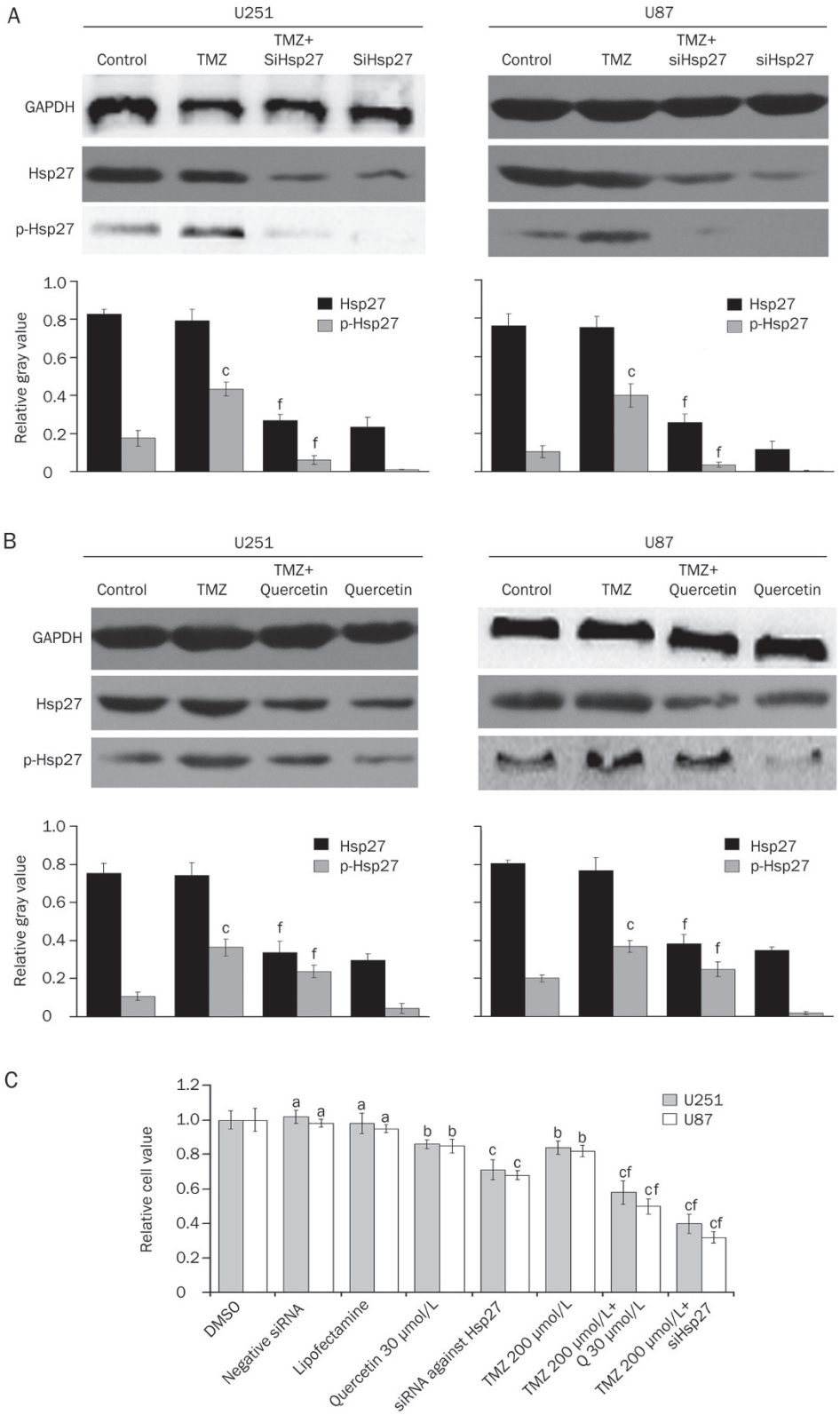

Figure 2. Quercetin sensitizes glioblastoma cells to TMZ by Hsp27 inhibition. (A) U251 and U87 cells were separately treated with negative control siRNA oligos, 200 μmol/L TMZ, 200 μmol/L TMZ plus siRNA against Hsp27 or only siRNA against Hsp27 for 48 h; (B) U251 and U87 cells were treated with DMSO (vehicle control), 200 μmol/L TMZ, 200 μmol/L TMZ plus 30 μmol/L quercetin or only 30 μmol/L quercetin. GAPDH served as a loading control. The relative gray values were measured using ImageJ software. (C) U251 and U87 cells were separately treated with DMSO (vehicle control), control siRNA oligos, Lipofectamine, 30 μmol/L quercetin, siRNA against Hsp27, 200 μmol/L TMZ, 200 μmol/L TMZ plus 30 μmol/L quercetin, or 200 μmol/L TMZ plus siRNA against Hsp27. Cell viability was detected using the MTT assay. Q, quercetin; siHsp27, siRNA against Hsp27. [a]$P>0.05$, [b]$P<0.05$, [c]$P<0.01$ *vs* DMSO. [f]$P<0.01$ *vs* TMZ 200 μmol/L.

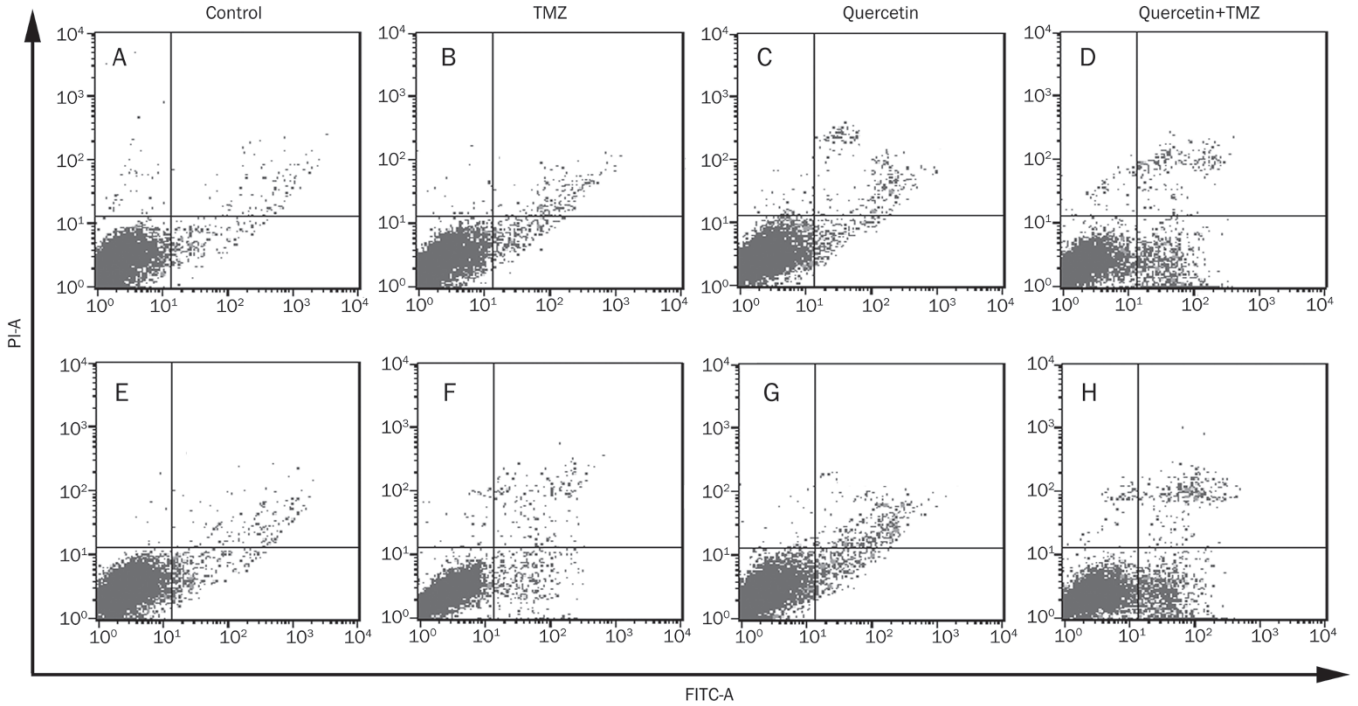

Figure 3. Quercetin facilitates TMZ's induction of apoptosis. U251 and U87 cells were separately treated with DMSO (vehicle control), 30 μmol/L quercetin, 200 μmol/L TMZ, or 30 μmol/L quercetin plus 200 μmol/L TMZ for 48 h. Cell apoptosis was evaluated with flow cytometry using Annexin V-FITC conjugates and propidium iodide double staining. For the vehicle control of U251 or U87 cells, the apoptosis proportions (Low Right plus Right Upper section) were 4.8%±1.3% (A) and 5.7%±1.1% (E). The apoptosis in 200 μmol/L TMZ treated U251 or U87 cells was 5.5%±1.2% (B) or 5.0%±1.2% (F) (P>0.05). The proportion of apoptosis in 30 μmol/L quercetin treated U251 or U87 cells was 6.3%±1.2% (C) or 6.9%±1.0% (G) (P>0.05). For cells pretreated with 30 μmol/L quercetin plus 200 μmol/L TMZ, the proportion of apoptosis was significantly increased by 15.2%±1.3% in U251 (D) or 17.1%±2.1% in U87 (H) (P<0.01).

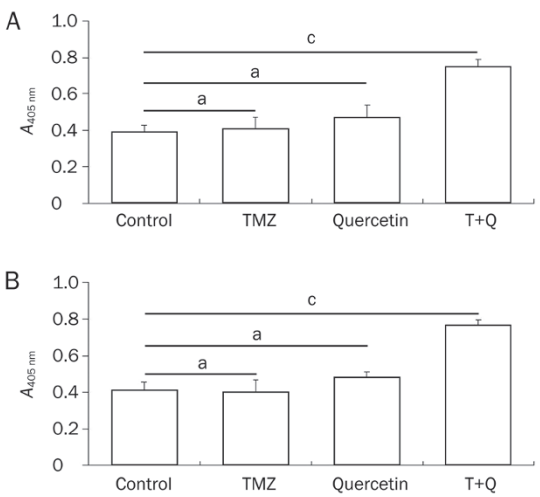

Figure 4. TMZ plus quercetin increases caspase-3 activity. U251 (A) or U87 (B) cells were treated with DMSO (vehicle control), 200 μmol/L TMZ, 30 μmol/L quercetin or 30 μmol/L quercetin plus 200 μmol/L TMZ for 48 h. A caspase-3 activity assay kit was used. Absorbance read at 405 nm represented the level of caspase-3 activity ($A_{405\ nm}$). No significant change of caspase-3 activity was observed in cells only treated with 200 μmol/L TMZ or 30 μmol/L quercetin (P>0.05). Caspase-3 activity was significantly increased in cells treated with 30 μmol/L quercetin plus 200 μmol/L TMZ (P<0.01). T, TMZ; Q, quercetin. [a]P>0.05, [b]P<0.05, [c]P<0.01.

survival[15]. In our study, a natural flavonoid and efficient Hsp27 inhibitor, quercetin, was used to investigate the role of Hsp27 in TMZ treated U251 and U87 cells and whether the inhibition of Hsp27 could sensitize cells to TMZ.

Quercetin is widely distributed in various plants and is known as an antitumor agent. Wei et al. reported that quercetin displayed antitumor activity by triggering apoptosis *in vitro*[23]. It has been reported to inhibit Hsp27 expression and has suppressed both tumor growth and the expression of stemness genes, including Oct4, Nanog, and Sox2 in lung cancer[13]. Another study demonstrated that quercetin suppresses Hsp27 phosphorylation, leading to the promotion of apoptosis in drug-resistant spheres of oral cancer cells[14]. The combination of quercetin and TMZ has been reported to treat the human astrocytoma cell line MOGGCCM (WHO grade III). The results indicate that quercetin acts in synergy with TMZ and that when both drugs are used in combination, rather than in separate pharmacological application, they more effectively induce programmed cell death[24]. However, glioblastoma cells (WHO grade IV) are usually TMZ-insensitive and multidrug resistant; thus, investigating the effects of quercetin and TMZ on glioblastoma cell lines is more meaningful.

In our study, cell growth inhibition by quercetin and TMZ was first analyzed, and it was found that for the U251 and U87 cell lines, TMZ inhibits cell growth until its concentra-

tion reaches 200 μmol/L. However, with a pretreatment of 30 μmol/L quercetin, TMZ treatment can induce significant cell growth inhibition at a concentration of 100 μmol/L and more intense inhibition at 200 μmol/L. To further investigate the mechanism by which quercetin enhances TMZ-induced cell growth inhibition, we performed knockdown experiments to silence the expression of Hsp27 using specific siRNAs and measured Hsp27 expression and its phosphorylation level by Western blot. Our results showed that treatment with 200 μmol/L TMZ increased the phosphorylation level of Hsp27; either quercetin or siRNA against Hsp27 can decrease Hsp27 protein expression and block phosphorylation of Hsp27 as well. Quercetin can block TMZ-induced Hsp27 phosphorylation. Our study detected Hsp27 phosporylation levels after TMZ treatment in U251 and U87 cells for the first time, although others have reported TMZ treatment increasing Hsp27 phosporylation levels in the HF2303 glioma cell line[15]. The increased phosphorylation levels of Hsp27 may indicate TMZ resistance.

In the next step, we used the cell viability assay to confirm the function of Hsp27. As we expected, the results showed that with either knockdown of Hsp27 or treatment of quercetin, the two cell lines were significantly sensitized to TMZ treatment with more intense growth inhibition (Figure 2B). Then, the results of the cell apoptosis analysis by flow cytometry and the caspase-3 activity assay showed that quercetin sensitized U251 and U87 cells to TMZ-induced apoptosis by increasing caspase-3 activity. The increased caspase-3 activity suggests apoptosis via the internal (mitochondrial) pathway, indicating that apoptosis induction in U251 and U87 cells after treatment with quercetin plus TMZ was mediated by the mitochondrial pathway. Cell apoptosis induction is very important in tumor chemotherapy, and our research demonstrated that for the U251 and U87 cell lines, treatment with quercetin can enhance TMZ efficacy in cell growth inhibition and cell apoptosis induction.

In conclusion, our data demonstrated that the U251 and U87 human glioblastoma cell lines are insensitive to TMZ. When combined with quercetin, TMZ effectively decreased the survival of U251 and U87 cells, increased caspase-3 activity and significantly induced cell apoptosis. Quercetin sensitized glioblastoma cells to TMZ by inhibiting Hsp27. The combination of quercetin and TMZ may be a potential strategy for the treatment of GBM.

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Author contribution

Dong-ping SANG and Qing LAN designed the research; Dong-ping SANG and Ru-jun LI performed the research; Dong-ping SANG and Ru-jun LI analyzed the data; Dong-ping SANG wrote the paper; and Qing LAN revised the paper.

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