Ultrasound Enhanced Anti-tumor Effect of Temozolomide in Glioblastoma Cells and Glioblastoma Mouse Model

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Abstract

Introduction—Glioblastoma is the most aggressive cancer that begins within the brain. In clinic, temozolomide was used as anti-tumor drugs for glioblastoma chemotherapy, but showed limited effect. Therefore, how to improve the effect of temozolomide to glioblastoma is urgently needed.

Methods—The cell viability of T98G cells was detected by cell counting kit-8 (CCK-8) assay. Apoptosis was detected using the Annexin-V-FITC & PI apoptosis kit and assessed by flow cytometry. The expression levels of Bax, B cell lymphoma 2 (Bcl-2), phos-Jun N-terminal kinases (JNK), phos-extracellular signal-regulated kinases (ERK) and phos-p38 were determined by western blot. The effect of ultrasound and temozolomide combination in mice was determined by survival analysis.

Results—Compared with temozolomide treatment alone, ultrasound and temozolomide combination inhibited the cell viability, and promotes apoptosis of human glioblastoma T98G cells. Bax level increased, while Bcl-2 level decreased in combination group. Mechanically, combination treatment promoted apoptosis *via* JNK and p38 pathways. In mouse glioblastoma model, combination treatment improved overall survival.

Conclusions—Ultrasound enhanced anti-tumor effect of temozolomide in glioblastoma cells *via* JNK and p38 pathways.

Keywords—Glioblastoma, Ultrasound, Temozolomide, Apoptosis, JNK and p38 pathways.

INTRODUCTION

Glioblastoma is one of the most common cancers worldwide.¹⁹ In primary brain tumors, glioblastoma is the most aggressive and most frequent type.^{3,28} The

incidence of glioblastoma is about 5–6 cases in 100,000 people every year.^{2,24} Currently, the gold standard treatment for glioblastoma in clinic is maximal resection safely, followed by combination of radiotherapy and chemotherapy with temozolomide.^{17,26} However, the effect of combined therapy was limited and glioblastoma relapsed in almost all patients.⁵ In addition, there is no widely accepted treatment for recurrent glioblastoma.¹⁷ The patients with glioblastoma have poor prognosis with the median overall survival of about 15–17 months.^{2,25} As temozolomide is commonly and widely used for glioblastoma treatment in clinic, how to improve the effect of temozolomide to glioblastoma is urgently needed for carrying a better prognosis.

Sonodynamic therapy is a promising approach for cancer therapy. Ultrasound irradiation administration has great influence on physical and chemical modification of cellular structure.²¹ After ultrasound irradiation, tumor cells prefer to uptake sonosensitizing drugs, which are also activated by ultrasound irradiation.¹² Moreover, sonodynamic therapy is non-invasive treatment, and has the advantage to deeply penetrate into and focus on small area of tumor tissues.¹³ Accumulating evidence has showed that sonodynamic therapy represent for a novel option for cancer treatment. Combination of Chlorin e6 (Ce6) mediated sonodynamic therapy (Ce6-SPDT) and photodynamic therapy markedly inhibited cell viability, promoted intracellular reactive oxygen species (ROS) and apoptosis of breast cancer cell lines (MDA-MB-231, MCF-7 and 4T1). In 4T1 breast cancer xenograft model, Ce6-SPDT significantly inhibited tumor volume, tumor weight and lung metastasis *via* matrix metalloproteinase 9 (MMP-9) and vascular endothelial growth factor (VEGF) signaling pathways.²² In endometrial cancer cell lines, sonodynamic

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therapy leaded to suppressed cell viability, and enhanced ROS production and cell apoptosis. Moreover, sonodynamic therapy regulated the expression levels of apoptosis related proteins (Bax, Fas, Fas-L, Bcl-2 and Survivin).²⁰ All the evidence demonstrated that sonodynamic therapy have promising roles in cancer treatment.

In the present study, we explored the effect of combined ultrasound and temozolomide in glioblastoma and found that ultrasound and temozolomide combination inhibited the cell viability and promotes apoptosis of T98G cells. The apoptosis related proteins Bax level increased, while Bcl-2 level decreased in combination group. Mechanically, combination treatment promoted apoptosis *via* JNK and p38 pathways. In mouse model, combination treatment improved overall survival. Therefore, ultrasound enhanced anti-tumor effect of temozolomide in glioblastoma cells *via* JNK and p38 pathways.

MATERIALS AND METHODS

Cell Line

The human glioblastoma cell line T98G was purchased from American Type Culture Collection (ATCC). T98G cells were cultured in ATCC-formulated Eagle's Minimum Essential Medium. The following components were added into the base medium to make complete growth medium: 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 g/mL streptomycin. The cells were cultured in humidified 5% (v/v) CO₂ at 37 °C incubator.

CCK-8 Assay

The cell viability of T98G cells was detected by CCK-8 kit. Briefly, 1×10^4 cells were seeded into 96 well plate, and cultured in incubator of 5% (v/v) CO₂ at 37 °C. Forty-eight hours later, total of 10 μ L CCK-8 was added into the wells, followed by 4 h incubation at 37 °C. Then, Microplate Reader (Model 680, BIO-RAD, USA) was used to measure the absorbance at wavelength of 450 nm. The experiments were performed three times.

Ultrasound (US) Treatment

Ultrasonic therapy instruments (Ultrasonic Therapy Model CSL, Shanghai, No. 822 Factory, China) was used to generate ultrasound. The instrument consists of a ceramic cylindrical transducer combined with a function generator. The resonance frequency of piezoelectric element (round ceramic plate 20 mm in diam-

eter and 1 mm in thickness) is 2 MHz. The irradiance was detected by the radiometer system. In this study, total of 6 light doses were used to treat T98G cells: US1: 1 MHz, 2 W/cm²; US2: 1 MHz, 10 W/cm²; US3: 2 MHz, 2 W/cm²; US4: 2 MHz, 10 W/cm²; US5: 4 MHz, 2 W/cm²; US6: 4 MHz, 10 W/cm². After treated with 100 μ M temozolomide for 30 min, the cells were treated with continues ultrasound for 15 min.

Cell Apoptosis Analysis

Total of 2×10^6 T98G cells were plated into 6 cm dish and treated with 100 μ M temozolomide or combination of temozolomide and Ultrasounds. Then, the cells were harvested by centrifugation at 1500 r/min for 5 min. The Annexin V/PI kit was used to detect cell apoptosis according to manufacturer's instructions,

Flow Cytometry Analysis

The T98G cells were harvested and washed twice using PBS. Then, the cells passed through 40- μ m nylon mesh to get the single cell suspension. 1×10^6 cells were used for staining according to the standard procedure. Then, flow cytometry analysis was performed using FACS Calibur (BD Biosciences, San Diego, CA).

Western Blot

The T98G cells were harvested after TZM or TZM + US treatment, followed by washing with cold PBS. Then, the cells were lysated by RIPA buffer on ice. The amounts of proteins were detected electrophoresed into SDS-PAGE gel. Then, western blot was performed as previously described.¹⁵ The antibodies were purchased from Cell Signaling Technology: Bax, Bcl-2, phos-JNK, phos-ERK, phos-p38, JNK, ERK, GAPDH. Signals were determined by GeneTools software. GAPDH was used as control.

Xenograft Model

To detect the role of temozolomide and ultrasound in overall survival of mice, 1×10^5 T98G cells were slowly injected into the pons of athymic mice.¹⁶ Mice were divided into three groups: T98G, T98G + TZM and T98G + TZM + US. The treatment initiated 7 days after tumor cell implantation. The temozolomide was given by intraperitoneal (*i.p.*) injection with the amount of 25 mg/kg daily for 2 weeks. The mice that were treated with US3 (2 MHz, 2 W/cm²) for 30 min after the injection of temozolomide. Ultrasonic therapy instruments (Ultrasonic Therapy Model CSL, Shanghai, No. 822 Factory, China) were used. This

study was approved by the ethics committee of Yantai Yuhuangding Hospital.

Statistics

All data were presented as mean $\pm$ SD. The significances between groups were detected with one-way ANOVA followed by Tukey's post-test. The overall survival was determined using log-rank (Mantel-Cox) test. *, ** and *** represents $p < 0.05$, 0.01 and 0.001 respectively.

RESULTS

Ultrasound Inhibited T98G Cell Viability in Combination of Temozolomide

To explore whether Ultrasound (US) treatment will increase the anti-tumor effect of temozolomide (TZM), we performed 15 min ultrasound treatment to T98G cells at different light doses: US1: 1 MHz, 2 W/cm²; US2: 1 MHz, 10 W/cm²; US3: 2 MHz, 2 W/cm²; US4: 2 MHz, 10 W/cm²; US5: 4 MHz, 2 W/cm²; US6: 4 MHz, 10 W/cm². The cell viability was detected by CCK-8 assay. As shown in Fig. 1, TZM alone inhibited cell viability as compared without TZM. TZM + US3 and TZM + US6 significantly inhibited cell viability of T98G cells as compared without TZM treatment, while some other light doses of US have no great influence on cell viability. As US6 treatment will lead to excessive heat (The temperatures of the medium are as follows: US1: 37.12 ± 0.12 ; US2: 37.53 ± 0.09 ; US3: 37.34 ± 0.02 ; US4: 37.34 ± 0.02 ; US5: 37.52 ± 0.22 ; US6: 38.16 ± 0.27 ; US6: 40.13 ± 0.31 °C), which may have influence on T98G cells, we chose US3 in the following experiments. These data suggested that US treatment promoted the anti-tumor effect of TZM.

37.52 $\pm$ 0.22; US5: 38.16 $\pm$ 0.27; US6: 40.13 $\pm$ 0.31 °C), which may have influence on T98G cells, we chose US3 in the following experiments. These data suggested that US treatment promoted the anti-tumor effect of TZM.

TZM Combined with Ultrasound Promoted Apoptosis Induced by Temozolomide in T98G Cells

We next detected how US treatment promotes the anti-tumor effect of TZM. As an anti-tumor drug, TZM treatment induced apoptosis of T98G cells. US or TZM alone significantly increased the percentage of cell apoptosis. Moreover, combination of TZM and US further obviously increased the percentage of apoptosis compared with TZM treatment alone (Figs. 2a and 2b). These data suggest that US treatment promoted TZM induced apoptosis.

TZM Combined with Ultrasound Promoted Apoptosis via the Mitochondrial Pathway

Next, we detected how US treatment promoted apoptosis. After US, TZM or TZM + US treatment, we harvested T98G cells and detected the expression levels of Bax and Bcl-2. As shown in Fig. 3a, US alone treatment has little effect on Bax and Bcl-2 expression. After TZM treatment, Bax expression level in T98G cells increased, while Bcl-2 expression level decreased. The combination of TZM and US treatment further significantly increased Bax expression level and decreased Bcl-2 expression level as compared with TZM treatment alone. The statistics of the relative protein level showed that Bax relative level significantly increased (Fig. 3b), while Bcl-2 relative level significantly increased after combination treatment (Fig. 3c). US alone treatment has little effect on Caspase3 and Caspase9 expression, while TZM treatment increased Caspase3 and Caspase9 levels. Moreover, Caspase3 and Caspase9 levels significantly increased after combination treatment (Figs. 3a, 3d and 3e). These data demonstrated that US promoted apoptosis via the mitochondrial pathway.

FIGURE 1. Ultrasound inhibited T98G cells in the combination of temozolomide. CCK-8 assay showing the cell viability of the T98G cells with the different treatments as indicated. The different condition of ultrasounds was indicated in the right. After treated with 100 μ M TZM for 30 min, the cells were treated with continues ultrasound for 15 min. Data were represented as mean $\pm$ SD; $n = 3$ independent experiments. *** $p < 0.001$. TZM temozolomide, US ultrasound.

Ultrasound Promoted Apoptosis via JNK and p38 Pathway

We next detected how the signals would be transduced into the cells after treatment. The T98G cells were harvested after treated with US, TZM or TZM + US. As shown in Figs. 4a and 4b, the protein levels of phos-JNK, phos-ERK and phos-p38 showed no great difference after US treatment, while significantly increased after TZM treatment as compared non-treatment. The combination treatment of TZM

FIGURE 2. Ultrasound promoted apoptosis induced by temozolomide in T98G cells. (a) The apoptosis of the T98G cells with different treatment as indicated was determined by using the Annexin-V-FITC & PI Apoptosis Kit and assessed by flow cytometry. $n = 5$ independent experiments and this panel presented one of these repeats. (b) Statistic of percentage of the apoptosis cells performed in panel (a). Data showed the Annexin-V positive cells. Data were represented as mean $\pm$ SD; $n = 5$ independent experiments. ** $p < 0.01$, *** $p < 0.001$. TZM temozolomide, US ultrasound.

and US significantly promoted phospho-JNK and phospho-p38 levels when compared with TZM treatment alone. However, the expression level of phospho-ERK showed no great difference after combination treatment. In addition, the statistics of the relative protein level showed that phospho-JNK and phospho-p38 increased after combination treatment, while no great difference of phospho-ERK after combination treatment. These data demonstrated that US promoted apoptosis *via* JNK and p38 pathway.

Combination of Temozolomide and Ultrasound Improved Survival in Mice Model

At last, we determined whether combination of TZM and US could improve survival. T98G cells were slowly injected into the pons to induce glioblastoma. TZM treatment increased the survival of mice as

compared with non-treatment. In addition, the combination of TZM and US significantly improved survival when compared with TZM treatment alone (Fig. 5). These data demonstrated that combination treatment using TZM and US improve survival in glioblastoma survival.

DISCUSSION

Glioblastoma is an aggressive type of cancer that originates from brain or spinal cord, and leads to 3–4% of all cancer related deaths every year in US.²³ The treatment for glioblastoma includes surgery, radiation therapy, chemotherapy and targeted drug therapy.¹ However, either single treatment or combined treatment showed no totally inhibition to glioblastoma development, which finally relapses in almost all patient.⁹ In clinic, temozolomide is commonly and

FIGURE 3. Ultrasound promoted apoptosis *via* the mitochondrial pathway. (a) Western bolt showing the protein expression level of Bax, Bcl-2, cleaved caspase-3 and cleaved caspase-9 in the treatment with temozolomide in the presence or absence of ultrasound. $n = 3$ independent experiments and this panel presented one of these repeats. (b) Statistic of the relative protein level of Bax in panel (a). (c) Statistic of the relative protein level of Bcl-2 in panel (a). (d) Statistic of the relative protein level of cleaved caspase-3 in panel (a). (e) Statistic of the relative protein level of cleaved caspase-9 in panel (a). Data were represented as mean $\pm$ SD; $n = 3$ independent experiments. $\#p > 0.05$, $**p < 0.01$, $***p < 0.001$. TZM temozolomide, US ultrasound.

widely used as a chemotherapy drug for glioblastoma treatment.⁶ Therefore, it is urgently needed to explore the novel way to improve the effect of temozolomide for glioblastoma treatment. As ultrasound was used to overcome blood–brain barrier to promote drug or gene delivery, we explored whether combined treatment of ultrasound and temozolomide will have improved effect for glioblastoma treatment. We found that combined treatment inhibited cell viability and promotes apoptosis of T98G cells. The expression levels of Bax

increased, while Bcl-2 decreased in combination group. In addition, combination treatment promoted T98G apoptosis *via* JNK and p38 pathways. In mouse model, combination treatment improved overall survival. Therefore, this study provided a novel way to improve the effect of temozolomide for glioblastoma therapy.

The blood–brain barrier is a highly selective anatomical and biochemical barrier that separate blood from brain and extracellular fluid in the central nervous system.^{7,27} Blood–brain barrier allows water,

FIGURE 4. Ultrasound promoted apoptosis *via* JNK and p38 pathway. (a) Western bolt showing the protein expression level of JNK, phos-JNK, ERK, phos-ERK, p38 and phos-p38 in the treatment with temozolomide in the presence or absence of ultrasound. $n = 3$ independent experiments and this panel presented one of these repeats. (b) Statistic of the relative protein level of phos-JNK, phos-ERK and phos-p38 in panel (a). Data were represented as mean $\pm$ SD; $n = 3$ independent experiments. ** $p < 0.01$, *** $p < 0.001$ and # $p > 0.05$. TzM temozolomide, US ultrasound.

FIGURE 5. Combination of temozolomide and ultrasound improved survival in mice model. Survival curve of the mice injected into the pons with T98G cells (1×10^5). The temozolomide was given by intraperitoneal (*i.p.*) injection with the amount of 25 mg/kg daily for 2 weeks. Ultrasound was performed 30 min after the injection of temozolomide. Each group has 20 mice.

some molecular to pass through passive diffusion. In addition, some amino acids and glucose that essential for neural function also pass through blood–brain barrier *via* selective transport.¹⁸ Because of the blood–brain barrier, the potential harmful substances could not enter into the systemic circulation by the way of active transport.⁸ Blood–brain barrier prevents substances diffusion from the bloodstream into brain,

meanwhile some drugs were significantly limited to accumulation in brain, which inhibits the treatment efficacy of drugs.⁴ Therefore, on clinic, how to deliver the drugs through blood–brain barrier or how to increase efficacy of drugs remains a big problem.

Ultrasound has the ability to go through blood–brain barrier noninvasively to promote drug or gene delivery into brain noninvasively, and the pulsed focused ultrasound was used to open blood–brain barrier to promote drug or gene delivery into brain.¹¹ Temozolomide treatment profoundly prevented brain metastasis of triple negative breast cancer in an *O*(6)-methylguanine-DNA methyltransferase-dependent manner.¹⁴ Encapsulation of temozolomide with “dual-targeting” nanomedicine (scL-TMZ) markedly enhanced killing efficacy of various non- glioblastoma multiforme tumor cells through targeting cancer stem cells. Administration of scL-TMZ significantly improved overall survival of mice bearing intracranial glioblastoma multiforme.¹⁰ In the present study, we provided evidence that ultrasound treatment increased the percentage of T98G cell apoptosis induced by temozolomide. Moreover, ultrasound treatment improved overall survival in glioblastoma mouse model. These data demonstrated that ultrasound improved

the effect of temozolomide, providing theoretical basis for combination of ultrasound and temozolomide in glioblastoma treatment clinically.

However, it is important to note that ultrasound treatment with high doses will lead to excessive heat, which may have influence on brain cells. So, we chose US3 instead of US6 in our experiments. This is consistent with what has reported, during which MRI-guided pulsed focused ultrasound combined with microbubbles led to disruption of blood–brain barrier. The combination treatment results in the elevated levels of HSP70, IL-1, IL-18 and TNF α , which indicated damage-associated inflammatory response. In addition, histological evaluation demonstrated pulsed focused ultrasound combined with microbubbles induced ischemia or mild traumatic brain injury.¹¹

When detected the effect of ultrasound in glioblastoma mouse model, we used the same doses of ultrasound with that *in vitro*, which may not be the most suitable condition *in vivo*. How to optimize the condition for *in vivo* study should be explored in future. In the present study, only T98G cell line was used, while some other glioblastoma cell lines such as A172, U87, LN229, U373 and LN428 should be used to further detect the effect of temozolomide to glioblastoma. The combined treatment using ultrasound and temozolomide significantly improved the overall survival when compared with temozolomide alone in glioblastoma mouse model, indicating the novel effect of ultrasound in glioblastoma. However, the effect of ultrasound in glioblastoma is only determined in one glioblastoma model. Some other glioblastoma models should be used to confirm the effect of ultrasound in glioblastoma. In addition, the combined treatment improved overall survival, but not completely cured mice with glioblastoma. So, more effective therapy methods should be explored. More importantly, the experiments were only performed in mouse model, which could not totally reflect situations glioblastoma patients. Therefore, the effect of combined treatment should be detected in glioblastoma patients.

Taken together, our study demonstrated that ultrasound treatment inhibited cell viability and promotes cell apoptosis of T98G cells. After combination treatment, apoptosis related proteins Bax level increased, while Bcl-2 level decreased as compared with TMZ alone treatment. In glioblastoma mouse model, ultrasound treatment improved overall survival. Mechanically, ultrasound promoted apoptosis of T98G cells *via* JNK and p38 pathways. These data provided theoretical basis for combination of ultrasound and temozolomide in glioblastoma patient treatment.

CONCLUSIONS

Ultrasound enhanced anti-tumor effect of temozolomide in glioblastoma cells *via* JNK and p38 pathways. These data provided theoretical basis for combination of ultrasound and temozolomide in glioblastoma patient treatment.

CONFLICT OF INTEREST

Jie Xue, Yuanyuan Wu, and Na Liu declare that declare that they have no conflicts of interest.

ETHICAL STANDARDS

No human studies were carried out by the authors for this article. All animal studies were carried out in accordance with the institutional guidelines and approved by the ethics committee of Yantai Yuhuangding Hospital.

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