

TRPC5-induced autophagy promotes the TMZ-resistance of glioma cells via the CAMKK β /AMPK α /mTOR pathway

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Abstract. Temozolomide (TMZ) is the first choice chemotherapy agent against glioblastoma, but the TMZ chemotherapy resistance has restricted the clinical application. Although autophagy is considered an adaptive response for cell survival under the pressure of chemotherapy and associated with chemotherapy resistance, its initiator and the precise molecular mechanism remains unknown. In the present study, it was determined that TMZ increases the transient receptor potential cation channel subfamily C member 5 (TRPC5) protein expression and the basal autophagy level, and the upregulation of autophagy is mediated by TRPC5 in glioma cells. Additionally, knockdown of TRPC5 upregulated the chemotherapy sensitivity *in vitro* and *in vivo*. Furthermore, TRPC5-small interfering RNA and pharmacological inhibition indicated that the Ca²⁺/calmodulin dependent protein kinase β (CaMKK β)/AMP-activated protein kinase α (AMPK α)/mechanistic target of rapamycin kinase (mTOR) pathway mediates cell survival autophagy during TMZ treatment. In addition, TMZ-resistant U87/TMZ cells retained a high basal autophagy level, while silence of TRPC5 expression or inhibition of autophagy reversed TMZ resistance. Thus, the present study revealed that TRPC5, an initiator of autophagy, upregulated TMZ resistance via the CaMKK β /AMPK α /mTOR pathway and this indicated a novel therapeutic site for drug resistance in glioma chemotherapy.

Introduction

Glioma is the most common malignancy type in the central neuronal system, according to prevalence studies conducted in USA in 2010, while temozolomide (TMZ), is the first-choice

chemotherapy agent against glioma (1-3). However, TMZ drug-resistance is a main cause of clinical treatment failure (1). A number of studies demonstrated that the abnormal transport of drugs attributes to resistance (4,5). Furthermore, it was also demonstrated that there are a number of mechanisms contributing to drug resistance, including activation of DNA repair system, impairment of apoptotic signaling and reduction in the drug uptake into cells, but the precise mechanisms remain under investigation (6-8). Therefore, understanding the precise mechanism underlying the drug resistance of glioma cells is critical for developing novel therapeutic strategies to overcome TMZ resistance.

Transient receptor potential cation channel subfamily C member 5 (TRPC5) is a Ca²⁺-permeable channel that is expressed in numerous types of cells and organs, including endothelial and muscle cells, and the lungs and kidneys (9-11), and attributes to a number of neuronal and vascular diseases, including Huntington's disease and infantile hypertrophic pyloric stenosis (12-14). Other studies demonstrated that TRPC5 is involved in cancer chemotherapy. For example, TRPC5 was determined to mediate the Adriamycin resistance in breast carcinoma via P-glycoprotein induction (15). Additionally, it activated autophagy in chemotherapy-resistant breast cells under Adriamycin exposure (16). Therefore, we hypothesized that TRPC5 may be a potential molecular target in glioma chemotherapy treatment.

Macroautophagy (hereafter termed as autophagy) is a catabolic process for the degradation and recycled use of cytosolic excess proteins, and impaired or defective organelles in autolysosomes (17). The hallmark of autophagy is the formation of double- or multi-membrane vesicles in the cytosol, termed autophagosomes. It encapsulates bulk cytoplasm or cytoplasmic organelles, and then fuses with the endocytic compartments, including early and late endosomes, and multivesicular bodies (18). Following maturation, it combines with the compartment of lysosomes to form autolysosomes, where the cargo is degraded by acidic lysosomal hydrolases (19,20). The contents of autolysosomes are digested to recycle the fragment products and generate energy to confer stress tolerance (21,22). In cancer initiation and development, autophagy also serves a controversial role and there is no precise and novel conclusion (23-25). A number of studies demonstrated that autophagy may support cancer survival (26-28); while in contrast, other studies indicated that autophagy is involved in programmed cell

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death (29-31). Different tumor types, stages, genomic contexts and settings may attribute to the different roles of autophagy in cancer. An autophagic response simultaneously triggers the apoptotic cell death induced by a number of anticancer drugs, including Glychionide-A Flavonoid in pancreatic carcinoma and Thioridazine in glioma (32,33). In cancer chemotherapy, the majority of studies prefer autophagy as a protective pathway that postpones or reverses apoptosis (34,35). However, the precise mechanism for protective autophagy induced by chemotherapy and the potential initiating factor remain unknown.

Based on previous research demonstrating that TRPC5 mediates drug resistance via autophagy in other cancer cells (16), we hypothesized TRPC5 as an autophagy initiator during glioma chemotherapy. In the present study, the molecular mechanism of TRPC5 in autophagy and chemotherapy was examined.

Materials and methods

Cell culture. U87 wild-type (U87/WT) cells were obtained from Chinese Academy of Sciences (Shanghai, China), and then cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 culture medium containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA). TMZ-resistant human glioma cells (U87/TMZ) were induced by exposing U87/WT cells to TMZ in high-dose therapy at 400 nM for 6 months. TMZ was reconstituted with dimethyl sulfoxide (DMSO) prior to use, resulting in an effective TMZ concentration of 25 μ M. When the cells were in the logarithmic growth phase, TMZ was combined with DMEM to a final concentration of 400 nM. Subsequently, at every 24-h incubation interval, the medium was discarded and replaced with fresh medium with the identical TMZ concentration. Dead cells were discarded with a wash with PBS after 3 days and the remaining cells were diluted at 2×10^5 cells/ml by DMEM containing 10% FBS and replanted in a 6 cm cell culture dish, and this procedure was repeated for 6 months. Finally, a cell line resistant to 400 nM TMZ (termed U87/TMZ) was derived from U87/WT after 6 months. All cells were incubated at 37°C in 5% CO₂ humidified air.

For the inhibitor experiments, all inhibitors were dissolved in DMSO and control experiments were performed with equal volumes of DMSO. Cells were treated for 6 h at 37°C with Bafilomycin A₁ (BAF₁; 400 nmol/l), mTOR-inhibitor PP242 (400 nmol/l), autophagy activator chloroquine (CQ; 20 μ mol/l), CAMKK inhibitor KN-93 (10 μ mol/l) and AMPK inhibitor dorsomorphin (10 μ mol/l) (all from MedChemExpress; Monmouth Junction, NJ, USA) following the transfection of TRPC5 overexpression plasmid for 18 h, according to the subsequent protocol.

Reagents and antibodies. The following antibodies were used: Anti- β -actin (cat. no. sc-47778; 1:200) from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA); anti-TRPC5 (cat. no. ACC-020; 1:200) from Alomone Labs (Jerusalem, Israel); anti-phospho-CaMKK β (Ser286) (cat. no. 12716; 1:500), anti-CaMKK β (cat. no. 4436; 1:500), anti-phospho-AMPK α (Ser172) (cat. no. 2535; 1:500), anti-AMPK α (cat. no. 2532S; 1:500), anti-phospho-mTOR (Ser2448) (cat. no. 2971; 1:500), anti-mTOR (cat. no. 2972; 1:500) and anti-microtubule

associated protein 1 light chain 3 α (LC3; cat. no. 12741; 1:500) from Cell Signaling Technology, Inc. (Danvers, MA, USA); and AlexaFluor 488-conjugated goat anti-mouse IgG (cat. no. R37120; 1:2,000) and Alexa Fluor 555-conjugated goat anti-rabbit IgG (cat. no. A21428; 1:2,000) from Thermo Fisher Scientific, Inc.

Plasmid and transfection. The pcDNA3.1-TRPC5 plasmid and control plasmid were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The pcDNA3.1-GFP-LC3 plasmid was obtained from Suzhou University (Suzhou, China). TRPC5 and/or LC3 plasmid transfection was conducted by Lipofectamine[®] 3000 Transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. The concentration of plasmids was 0.5 μ g/ml for 6-well plates and 0.4 μ g/ml for 24-well plates for both cell lines. After transfection for 24 h, the subsequent experiments were conducted.

Cell viability assay. U87/WT and U87/TMZ cells were added to 96-well plates (5,000 cells/well) overnight at 37°C, and then treated by 400 nM TMZ for 48 h with or without TRPC5 transfection at 37°C, according to the aforementioned protocol. cell viability was measured by treating cells with MTT (20 μ l; 5 mg/ml; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 4 h. Subsequently, medium was replaced by 150 μ l DMSO in each well prior to measurement at 490 nm with a spectrophotometer.

Intracellular calcium level measurement. U87/WT cells were added to 96-well plates (5,000 cells/well) overnight at 37°C and transfected with TRPC5 plasmid or TRPC5-siRNA for 24 h. Subsequently Fluo-4 (2 mM/l; cat. no. F14201; Thermo Fisher Scientific, Inc.) was added for 30 min at 37°C and measured at 485 nm with a spectrophotometer.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cultured cells with the use of TRIzol[®] reagent (Takara Bio, Inc., Otsu, Japan), following the manufacturer's protocols. cDNA synthesis was performed with a PrimeScript RT Reagent kit (Takara Bio, Inc.). RT-qPCR was performed using the 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) at conditions of 95°C for 2 min, and 40 cycles of 95°C for 10 sec and 60°C for 40 sec. Relative expression of the miRNA was calculated using the comparative C_q method (36). The expression was normalized to GAPDH. The primer sequences used were: TRPC5, forward, 5'-TGAACCTCCCTCTACCTGGCAAC-3', and reverse, 5'-CGAAGAGTGCTTCCGCAATCAGT-3'; LC3, forward, 5'-TACGAGCAGGAGAAAGACGAGG-3', and reverse, 5'-GGCAGAGTARGGTGGGTTGGTG-3'; and GAPDH, forward, 5'-AAGGTCGGA GTCAACGGATTTGGT-3', and reverse, 5'-AGTGATGGCATGGACTGTGGTTCAT-3'.

Western blot analysis. Tissues from mice and cultured cells were lysed in radio immunoprecipitation assay buffer containing protease inhibitor and phosphatase inhibitor (Cell Signaling Technology, Inc.). The protein concentration was quantified with the Bicinchoninic Acid method using

a Protein Assay kit (Beyotime Institute of Biotechnology, Nanjing, China). The samples (15 μ g) were loaded onto 15 (for LC3 detection) or 8% (for other proteins) SDS-PAGE. Subsequently, total protein was transferred to the polyvinylidene fluoride membrane (350 mA for 2 h) and the membrane was blocked at room temperature for 1 h with 5% bovine serum albumin (BSA; Beijing Solarbio Science & Technology Co., Ltd.). Following three washes with PBS for 10 min each, the membranes were immunostained with primary and secondary antibodies at 4°C overnight. The bands were detected by Enhanced Chemiluminescent Western Blotting HRP Substrate (EMD Millipore, Billerica, MA, USA), according to the manufacturer's protocols. The band intensity was analyzed with ImageJ software 1.48 (National Institutes of Health, Bethesda, MD, USA) and normalized to β -actin.

Small-interfering RNA (siRNA) transfection. Human TRPC5 siRNA was synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). Briefly, cells (2x10⁵ cells/well) were seeded in 6-well plates and transfected with 40 nM siRNA (cat. no. 4392420; Thermo Fisher Scientific, Inc.) using Lipofectamine[®] 3000, according to the manufacturer's protocol. The sequences of TRPC5 are: Forward, 5'-CCAAUG GACUGAACCAGCUUUACUU-3', and reverse, 5'-UGUCGU GGAAUGGAUGAUUU-3'. After 24 h, the cells were lysed for further treatments with PCR or western blot analysis.

Immunocytochemistry. U87/WT or U87/TMZ cells were plated at 1x10⁴ cells/ml and co-transfected with GFP-LC3 and TRPC5 or control plasmid, according to the aforementioned protocol. Subsequently, cells were fixed with 4% paraformaldehyde overnight at room temperature, permeabilized with 0.1% Triton X-100 at room temperature for 15 min, and blocked for 1 h at room temperature in 1% BSA. Primary antibodies (TRPC5; cat. no. ACC-020; 1:200; and LC3, cat. no. 12741; 1:500) were added overnight at 4°C and fluorescent secondary antibody were used for 2 h at room temperature. Sections were counterstained with DAPI (Beijing Solarbio Science & Technology Co., Ltd.) for 10 min at room temperature. Images were acquired using a LSM 700 confocal microscope (x400 magnification) and analyzed using Zeiss software 2011 and Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA). A total of 30-50 cells selected randomly from 3 or 4 replicated experiments were quantified.

Intracellular calcium measurement. U87/WT cells were plated at 5x10³ cells/ml and co-transfected with TRPC5 or TRPC5-siRNA for 24 h, according to the aforementioned protocol. Subsequently, cells were incubated with the AM ester for 30 min at 37°C (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. Following this, each well was measured with a spectrophotometer.

Mouse xenograft models. A total of 6 male mice (age, 6-8 weeks; weight, 20-25 g) were obtained from the Central Laboratorial Animal Facility at the Jiangsu Institute of Parasitic Diseases (Wuxi, China). Mice were housed in cages under controlled environmental condition at 25°C with 55-65% humidity, a 12-h light/dark cycle and had free access to food and water. To generate subcutaneous tumors, U87/WT cells

were first transfected with TRPC5-shRNA or control-shRNA lentiviral particles for 48 h, according to the aforementioned protocol, and then 5x10⁶ cells were injected into the nude mice. All mice were housed in air-filtered pathogen-free condition and administered with TMZ (30 mg/m²). Tumor growth was measured after 5 weeks. Tumor volumes were estimated using the formula: Volume (mm³) = [(width)² x length]/2. All experiments involving animals were approved by the Animal Experimentation Ethics Committee of Nanjing Medical University.

Statistical analysis. Each experiment was independently repeated at least 3 times. Two-tailed Student's test for two groups or one-way analysis of variance with post hoc test least significant difference test for more than two groups were performed using SPSS software 16.0 (SPSS, Inc., Chicago, IL, USA). Values are presented as the means \pm standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

Results

Chemotherapy upregulates the expression of TRPC5 and autophagy level in glioma cells. Firstly, to confirm the effect of TMZ to U87/WT cells, U87/WT cells were exposed to 400 nM TMZ for 48 h, and then the cell viability was assessed by MTT, which demonstrated that it was significantly reduced, compared with the control (Fig. S1A). Furthermore, the transcriptional level of TRPC5 was assessed by RT-qPCR to determine the influence of TMZ on TRPC5, and the results indicated that TRPC5 mRNA expression was also significantly increased following exposure to TMZ, compared with the control (Fig. S1B). Additionally, TRPC5-siRNA was applied to confirm the effect of TMZ on TRPC5, and the result indicated that the mRNA expression of TRPC5 was also decreased (Fig. S1C).

Subsequently whether TMZ increases TRPC5 protein expression level and basal autophagy level in glioma cells was investigated by analyzing TRPC5 and LC3 protein expression. LC3 is a reliable marker of autophagy, and LC3-II is associated with the amount of autophagosomes (16). While, the TRPC5 and LC3 expression was significantly increased, compared with the control (Fig. 1A). To further determine the autophagic flux, the LC3 expression in U87/WT cells exposed to TMZ combined with the lysosomal protease inhibitor BAF₁, a proton pump inhibitor that raises lysosomal pH and blocks the activity of acid hydrolases to restrict its proteolytic degradation and autophagosome-lysosome fusion (37), was assessed. It was determined that BAF₁ significantly increased the LC3-II expression level, indicating that increased LC3-II levels were attributed to promotion of autophagy, rather than disruption of autophagic degradation (Fig. 1B). Subsequently, the autophagy level in TMZ-resistant glioma cells (U87/TMZ), generated by high-dose concentrations of TMZ over 6 months, was detected. As depicted, U87/TMZ cells had enhanced LC3-II levels, compared with U87/WT cells. Additionally, BAF₁ increased the LC3-II expression in U87/TMZ cells (Fig. 1C). These results indicated that U87/TMZ cells have increased basal levels of autophagy.

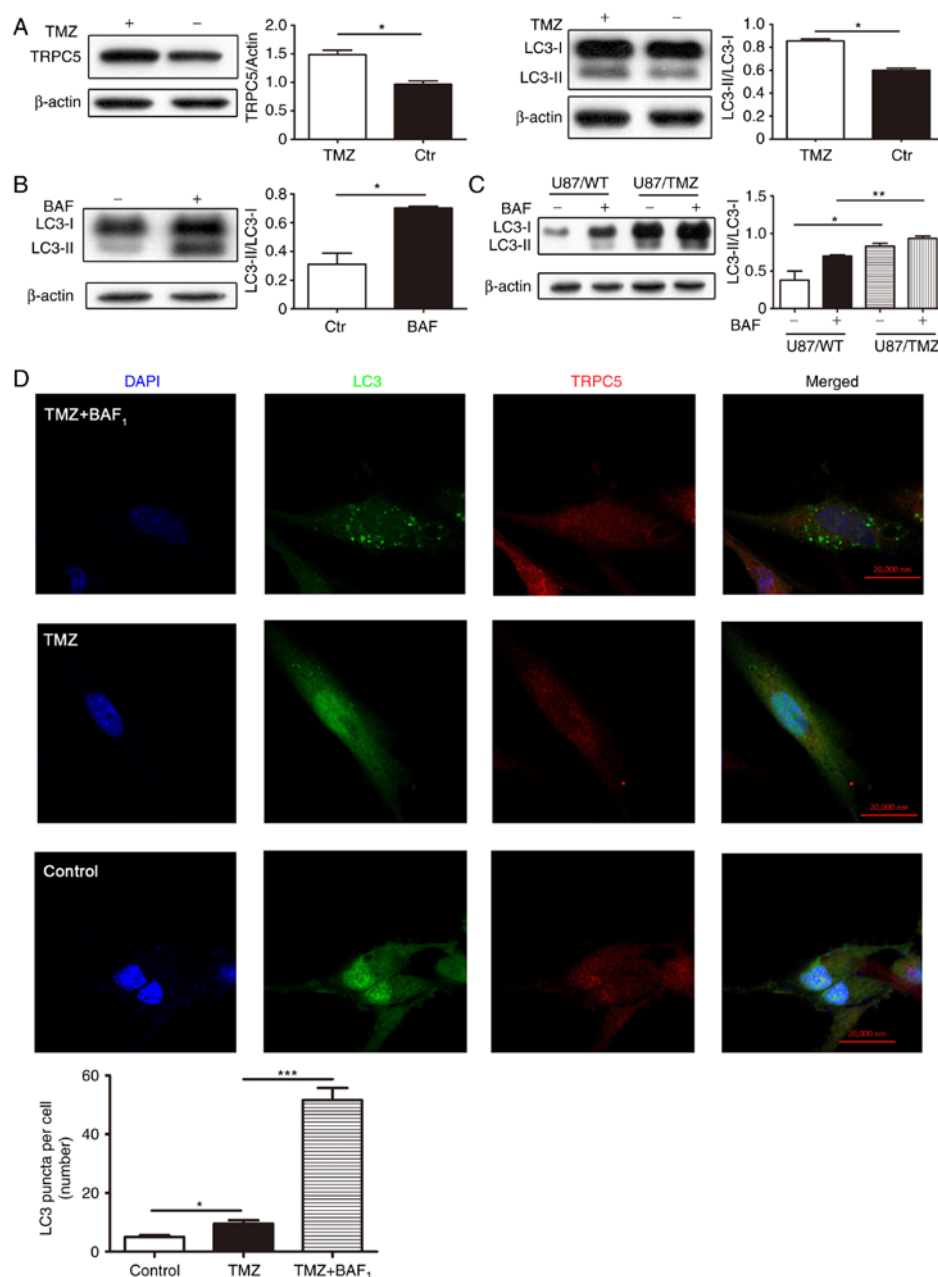


Figure 1. Chemotherapy upregulates TRPC5 protein expression and autophagy level in glioma U87/WT cells. (A) Glioma U87/WT cells were treated with 400 nM TMZ for 48 h, and the protein expression of TRPC5 and LC3 were analyzed by western blot analysis. It indicated that TMZ enhances TRPC5 and LC3-II expression in U87/WT cells. Values are presented as the means \pm SEM of 4-6 experiments. (B) Glioma U87/WT cells were treated with 400 nM TMZ for 48 h and the lysosomal protease inhibitor 400 μ M BAF₁ for 6 h, and the LC3 expression was analyzed by western blot analysis. Representative western blotting indicated that BAF₁ accelerates TMZ-induced LC3-II accumulation. Values are presented as the means \pm SEM of 4-6 experiments. (C) Glioma U87/TMZ cells were treated with 400 nM TMZ for 48 h and the lysosomal protease inhibitor 400 μ M BAF₁ for 6 h, and the LC3 expression was analyzed by western blot analysis. Values are presented as the means \pm SEM of 4-6 experiments. (D) Representative immunofluorescence images of the accumulation of autophagosomes in U87/WT cells, and the number of LC3 dots was calculated in 40-50 cells. Scale bar, 20 μ m. SEM, standard error of the mean; BAF₁, Bafilomycin A₁; WT, wild-type; TRPC5, transient receptor potential cation channel subfamily C member 5; TMZ, temozolomide; Ctr, control; LC3, microtubule associated protein 1 light chain 3 α . *P<0.05, **P<0.01, ***P<0.001.

To further determine the effect of TMZ on the increase of the expression level of LC3-II, the basal level of autophagy was visualized by using a LC3-GFP plasmid to immunostain autophagosomes. Upon autophagy, LC3-II is localized on autophagosomes and LC3 puncta is used as a marker for autophagosomes. U87/WT cells were transfected with GFP-LC3 plasmid for 24 h, and treated with or without TMZ for 24 h. As depicted, compared with the number of LC3 dots contained in the control group, a significantly increased number

of LC3 dots were detected in cells treated with TMZ. This indicated that TMZ treatment accelerates the formation of LC3B and autophagosomes (Fig. 1D). Additionally, the present results demonstrated that TRPC5 expression and basal autophagy level in glioma cells are upregulated during TMZ exposure.

TRPC5 initiates TMZ-induced autophagy in glioma cells. To confirm whether TRPC5 initiates autophagy under exposure to TMZ, the LC3-II protein level and LC3 dots formation in

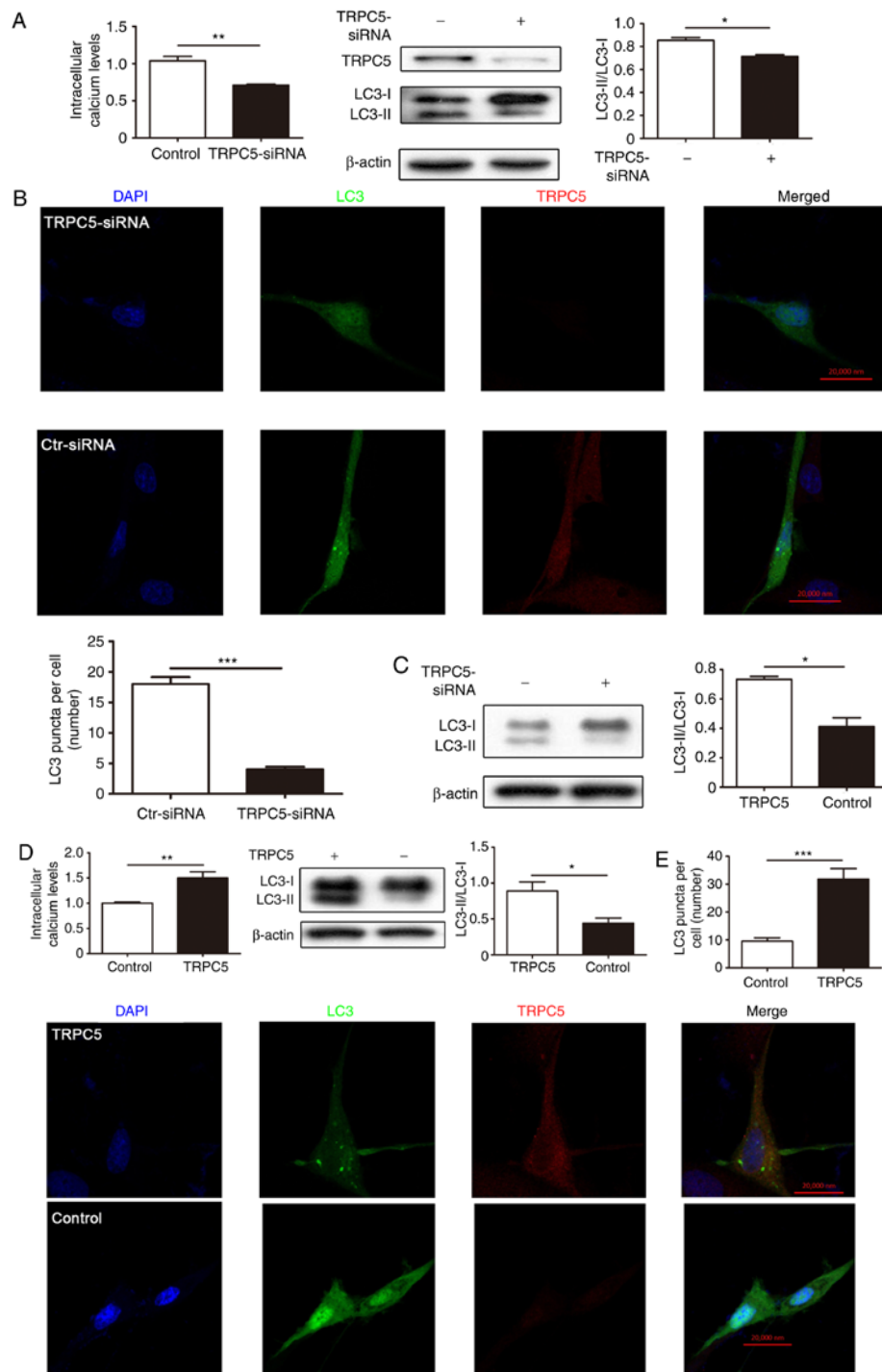


Figure 2. TRPC5 regulates autophagy induced by chemotherapy in glioma cells. (A) U87/WT cells were transfected with TRPC5-siRNA or scramble-siRNA for 24 h, and then exposed to TMZ for 48 h. The intracellular calcium levels were first measured and the intracellular calcium levels were decreased following TRPC5-siRNA transfection. The protein expression of TRPC5 and LC3 were analyzed by western blot analysis. Representative images indicated that TRPC5-siRNA decreased TRPC5 and LC3-II protein expression. (B) Representative immunofluorescence images demonstrated that TRPC5-siRNA decreased the mean number of LC3 dots per cell in U87/WT cells. (C) U87/TMZ cells were transfected with TRPC5-siRNA or scramble-siRNA for 24 h, and then exposed to TMZ for 48 h. The knockdown of TRPC5 restricted the accumulation of LC3-II in U87/TMZ cells. (D) The intracellular calcium levels were measured and the intracellular calcium levels were increased following TRPC5-plasmid transfection. Representative western blot and densitometric analyses normalized to β-actin demonstrated the effect of TRPC5 overexpression on the accumulation of LC3-II in indicated cells. (E) Representative immunofluorescence images of the accumulation of autophagosomes in indicated cells transfected by TRPC5 plasmid, and the mean number of LC3 dots was calculated in 40-50 cells. Scale bar, 20 μm. Values are presented as the mean ± standard error of the mean of 3-6 experiments. WT, wild-type; TRPC5, transient receptor potential cation channel subfamily C member 5; TMZ, temozolomide; LC3, microtubule associated protein 1 light chain 3 α; siRNA, small interfering RNA. *P<0.05, **P<0.01, ***P<0.001.

U87/WT cells following TRPC5-siRNA transfection were detected. Knockdown of TRPC5 significantly decreased

the intracellular calcium level and the LC3-II expression in U87/WT cells under exposure to TMZ, compared with the

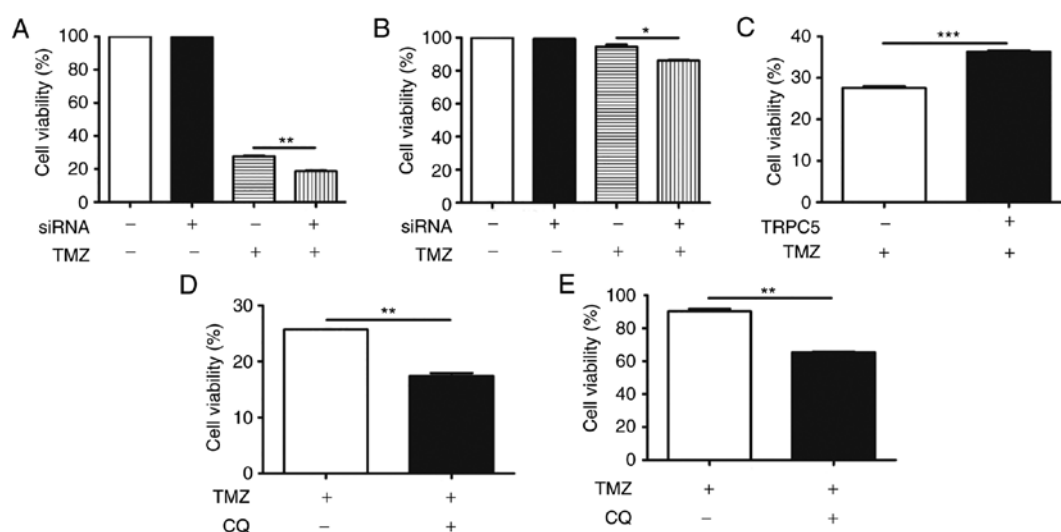


Figure 3. TRPC5 knockdown or inhibition of autophagy enhances the glioma cell sensitivity to chemotherapy. (A) U87/WT cells were transfected with TRPC5-siRNA or scramble-siRNA for 24 h, and treated with 400 nM TMZ for 48 h. U87/WT cells exhibited enhanced cell sensitivity to TMZ following TRPC5 knockdown. (B) U87/TMZ cells were transfected with TRPC5-siRNA or scramble-siRNA for 24 h, and treated with 400 nM TMZ for 48 h. U87/TMZ cells exhibited enhanced cell sensitivity to TMZ following TRPC5 knockdown. (C) TRPC5 overexpression increases cell viability in U87/WT cells exposed to TMZ. (D) U87/WT cells were treated with 10 μmol/l CQ and U87/WT cell viability decreased during TMZ exposure. (E) U87/TMZ cells were treated with 10 μmol/l CQ and U87/TMZ cell viability decreased during TMZ exposure. Values are presented as the mean ± standard error of 4-6 experiments. CQ, chloroquine; WT, wild-type; TRPC5, transient receptor potential cation channel subfamily C member 5; TMZ, temozolomide; siRNA, small interfering RNA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

controls (Fig. 2A). The LC3 dots number per cell was significantly decreased following TRPC5-knockdown and TMZ exposure, compared with the control (Fig. 2B). Furthermore, TRPC5-siRNA also significantly decreased the LC3-II protein level in U87/TMZ cells. (Fig. 2C). To further confirm autophagy induced by TRPC5, U87/WT cells were transfected with TRPC5 plasmid and it was determined that TRPC5 overexpression significantly upregulated intracellular calcium level, LC3-II expression and LC3 dots formation, compared with the control (Fig. 2D and E). Furthermore, the LC3 mRNA level was also determined following the overexpression or silencing of TRPC5 and there was no significant difference (data not shown). Collectively, the present data indicated that chemotherapy may induce autophagy and TRPC5 initiated TMZ-induced autophagy in glioma cells.

TRPC5 silencing or autophagy blockage enhances glioma cell chemotherapy sensitivity to TMZ. TRPC5-siRNA was transfected into U87/WT cells to determine whether autophagy induced by TRPC5 is involved in cell survival under exposure to TMZ. The results demonstrated that cell viability reduced to 28% in 400 nmol/l TMZ, compared with untreated cells (100%). Additionally, downregulation of TRPC5 caused TMZ to decrease proliferation of U87/WT cells to 18%, relative to the siRNA control (Fig. 3A). Furthermore, the effect of TRPC5 knockdown in drug-resistant U87/TMZ cells was measured. It also demonstrated a significant proliferation reduction, compared with TMZ exposure alone (Fig. 3B). This indicates that knockdown of TRPC5 sensitizes U87/WT cells to TMZ-induced damage. The TRPC5-plasmid was also transfected into U87/WT cells and it became significantly more resistant to TMZ-induced injury, compared with the control (Fig. 3C). Additionally, CQ, an inhibitor of autophagy by inhibiting lysosomal acidification (37), significantly

reduced cell viability with TMZ in U87/WT cells, compared with the control (Fig. 3D). CQ also significantly restricted the proliferation of drug-resistant U87/TMZ cells, compared with TMZ alone. (Fig. 3E) These results indicated that TRPC5 knockdown or autophagy inhibition increases the chemotherapy sensitivity of glioma cells.

CaMKKβ/AMPKα/mTOR pathway activated by TRPC5 mediates autophagy under chemotherapy. To confirm the potential mechanism of TRPC5 activation in autophagy, the autophagy-associated kinases downstream of TRPC5 were investigated. Previous studies reported that overexpression of TRPC5 activated CaMKKβ in breast cells (16). After exposing U87/WT cells to TMZ, the phospho-CaMKKβ level was determined to be significantly increased, compared with the control, which is a downstream kinase of TRPC5. Phosphorylation of CaMKKβ may activate AMPKα, therefore, AMPKα activity was also detected and it was demonstrated to be significantly increased in the U87/WT cell line under TMZ exposure, compared with the control (Fig. 4A). Previous research indicated that phospho-AMPKα may inhibit the expression of mTOR to activate autophagy (38). The present data demonstrated that phospho-mTOR is significantly downregulated under exposure to TMZ, compared with the control (Fig. 4A). All of these results indicated that the CaMKKβ/AMPKα/mTOR pathway contributed to activation of autophagy when exposed to TMZ. To further investigate the function of TRPC5, whether the CaMKKβ/AMPKα/mTOR pathway was activated was investigated, and TRPC5-siRNA was used and the key proteins of this pathway were assessed. It was determined that knockdown of TRPC5 significantly downregulated the phospho-CaMKKβ and phospho-AMPKα levels, and upregulated the phospho-mTOR levels under TMZ exposure, compared with the controls (Fig. 4B). Thus, TRPC5 may be attributed to

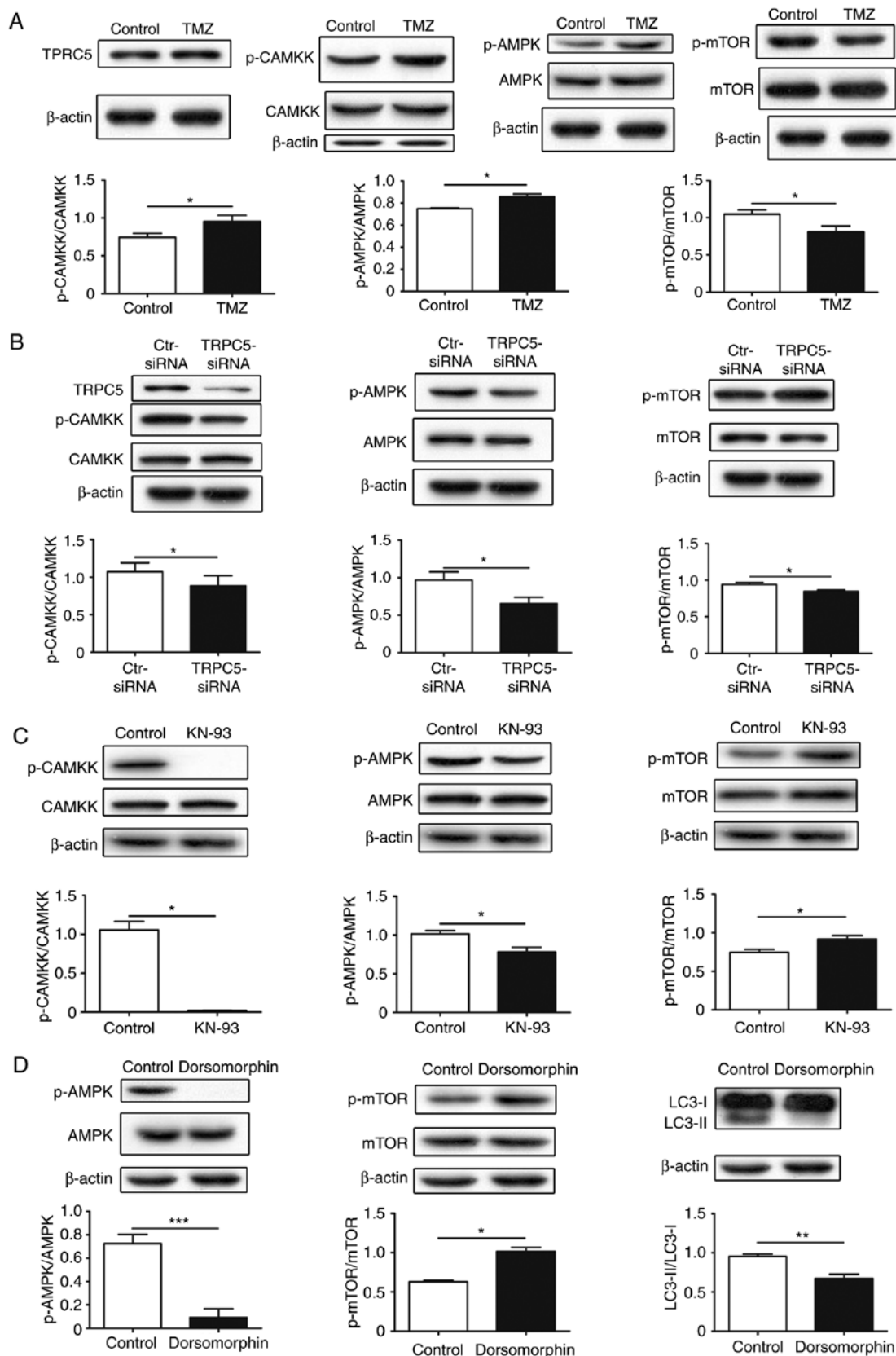


Figure 4. TRPC5 initiates autophagy via the CaMKK β /AMPK α /mTOR pathway during chemotherapy. (A) U87/WT cells were exposed to TMZ for 48 h and the protein levels of the CaMKK β /AMPK α /mTOR pathway were analyzed by western blot analysis. p-CaMKK β and p-AMPK α levels were increased, while p-mTOR/mTOR levels decreased, compared with the control group. (B) U87/WT cells were treated with TRPC5-siRNA and the protein levels of the CaMKK β /AMPK α /mTOR pathway were analyzed in U87/WT cells exposed to TMZ. The p-CaMKK β and p-AMPK α levels were decreased, while p-mTOR levels were increased, compared with the control group. (C) CaMKK β inhibited by KN-93 decreased p-CaMKK β and p-AMPK α levels, and increased p-mTOR levels in U87/WT cells treated with TMZ. (D) AMPK α silencing by dorsomorphin decreased p-AMPK α levels, increased p-mTOR levels and downregulated LC3-II levels in U87/WT cells exposed to TMZ. Values are presented as the mean \pm standard error of the mean of 3-6 experiments. CaMKK β , Ca²⁺/calmodulin dependent protein kinase β ; AMPK α , AMP-activated protein kinase α ; p-, phospho-; mTOR, mechanistic target of rapamycin kinase; TRPC5, transient receptor potential cation channel subfamily C member 5; TMZ, temozolomide; siRNA, small interfering RNA; Ctr, control. *P<0.05, **P<0.01, ***P<0.001.

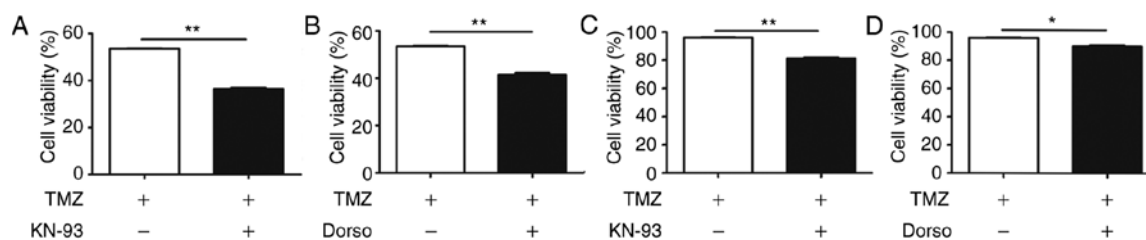


Figure 5. Downregulation of the Ca^{2+} /calmodulin dependent protein kinase β /AMP-activated protein kinase α /mechanistic target of rapamycin kinase pathway enhances the glioma cell sensitivity to chemotherapy. (A) KN-93 enhanced the TMZ-induced cell death in U87/wild-type cells. MTT assays were used to assess the cell viability. (B) Dorso enhanced the TMZ-induced cell death in U87/wild-type cells. MTT assays were used to assess the cell viability. (C) KN-93 enhanced the TMZ-induced cell death in U87/TMZ cells. MTT assays were used to assess the cell viability. (D) Dorso enhanced the TMZ-induced cell death in U87/TMZ cells. MTT assays were used to assess the cell viability. Values are presented as the mean \pm standard error of the mean of 3 experiments. TMZ, temozolomide; Dorso, dorsomorphin. * $P < 0.05$, ** $P < 0.01$.

the initiation of autophagy via the CaMKK β /AMPK α /mTOR pathway. Subsequently, CaMKK β was significantly inhibited using KN-93, an inhibitor of CaMK (39), in U87/WT cells, and AMPK α activity was significantly inhibited and phospho-mTOR expression was significantly enhanced on exposure to TMZ (Fig. 4C). Furthermore, AMPK α was significantly inhibited by dorsomorphin, an inhibitor of AMPK (40), and it also significantly upregulated the phospho-mTOR levels and significantly attenuated the LC3-II levels (Fig. 4D). Additionally, inhibition of the CaMKK β /AMPK α /mTOR pathway also significantly increased the TMZ sensitivity of U87/TMZ cells (Fig. 5A-D). The present data indicated that TRPC5 upregulated the CaMKK β /AMPK α /mTOR pathway to activate cytoprotective autophagy during TMZ exposure.

Downregulation of TRPC5 to suppress autophagy increases TMZ sensitivity *in vivo*. To determine whether inhibition of autophagy induced by TRPC5 also upregulates sensitivity to TMZ *in vivo*, nude mice were injected subcutaneously with U87/WT cells previously treated with TRPC5 short hairpin (sh) RNA or control shRNA lentiviral particles. The tumor size of TRPC5 shRNA-treated cancer cells was significantly reduced, compared with cells transfected with control shRNA following TMZ exposure (Fig. 6A). Additionally, it was determined that tumors treated with TRPC5 shRNA exhibited reduced autophagy following TMZ exposure. Furthermore, the LC3 level was significantly upregulated in TRPC5-shRNA treated U87/WT xenografts, compared with in control-shRNA treated U87/WT xenografts (Fig. 6B).

Discussion

Numerous patients with glioma acquire resistance to the first-choice drug TMZ and chemotherapy resistance is the major cause for recurrence and mortality. Autophagy induced by chemotherapy is considered as a novel participant in drug resistance in cancer cells (16), but the precise mechanism and initiator of autophagy remains unknown. In the present study, it was determined that TRPC5-activated autophagy serves as a novel participant in the occurrence and development of TMZ resistance in glioma cells. Blockage of TRPC5 or autophagy accelerated glioma cell death under exposure to TMZ. The present results also indicated significant inhibition of autophagy and xenografts treated with TRPC5 shRNA in response to TMZ *in vivo*.

A number of mechanisms, including abnormal ex-transport of drug, inhibition of cell death pathways and activation of the DNA repair system, are considered to contribute to chemotherapy resistance (15,41,42). In the present research, TMZ exposure enhanced TRPC5 protein expression in U87/WT cells. Furthermore, silencing TRPC5 expression enhances drug sensitivity and restricts resistance in glioma cells exposed to TMZ. Similar results were also determined in U87/TMZ cells. Collectively, it was demonstrated that TRPC5 acts as a positive regulator against TMZ-induced cell death in glioma cells. Chemotherapy destroys cancer cells by resulting in cell death via a number of mechanisms, including apoptosis and damage to DNA duplication (42,43); however, autophagy is considered to accelerate the degradation of and recycle damaged or excess components to maintain survival (19,44,45). Previous research demonstrated that a number of types of TRP channels, including TRP mucolipin 1 (TRPML1), TRPML3, TRPV1, TRPC1 and TRPM7, are involved in the regulation of autophagy via different mechanisms (46). TRPML1 impairs lysosomal pH, and accumulates autophagosomes, abnormal mitochondria, p62 and ubiquitin proteins to regulate autophagy (47-50). TRPV1 activates autophagy through the reactive oxygen species-associated AMPK and autophagy related 4C cysteine peptidase pathway (51). TRPC1 serves as a key regulator in hypoxia and nutrient depletion dependent autophagy (52), while TRPM7 regulates basal autophagy (53). Autophagy is also exhibited in a number of cancer cells, including glioma and lung cells, to maintain cell survival under chemotherapy. In gastric cancer cells, TRPM2 downregulation inhibited the c-Jun N-terminal kinase signal pathway, accumulated the damaged mitochondria and upregulated the chemosensitivity to paclitaxel and doxorubicin (54). In the present study, increased autophagy occurs under chemotherapy in glioma cells. In line with these results, TMZ exposure was determined to increase the LC3-II protein expression and accelerate LC3 dots formation in glioma cells. U87/TMZ cells acquired an increased basic autophagy level, compared with U87/WT cells. The combination of CQ and TMZ facilitates the cell death of sensitive or drug-resistant glioma cells, compared with TMZ alone, indicating that autophagy may be a main mechanism of cell survival. Subsequently, the association between TRPC5 and autophagy was determined. The present results indicated that overexpression of TRPC5 significantly upregulates LC3-II levels and accelerates LC3 dots formation in glioma cells under exposure to TMZ. Knockdown of TRPC5 reduced LC3-II expression and facilitated cell death

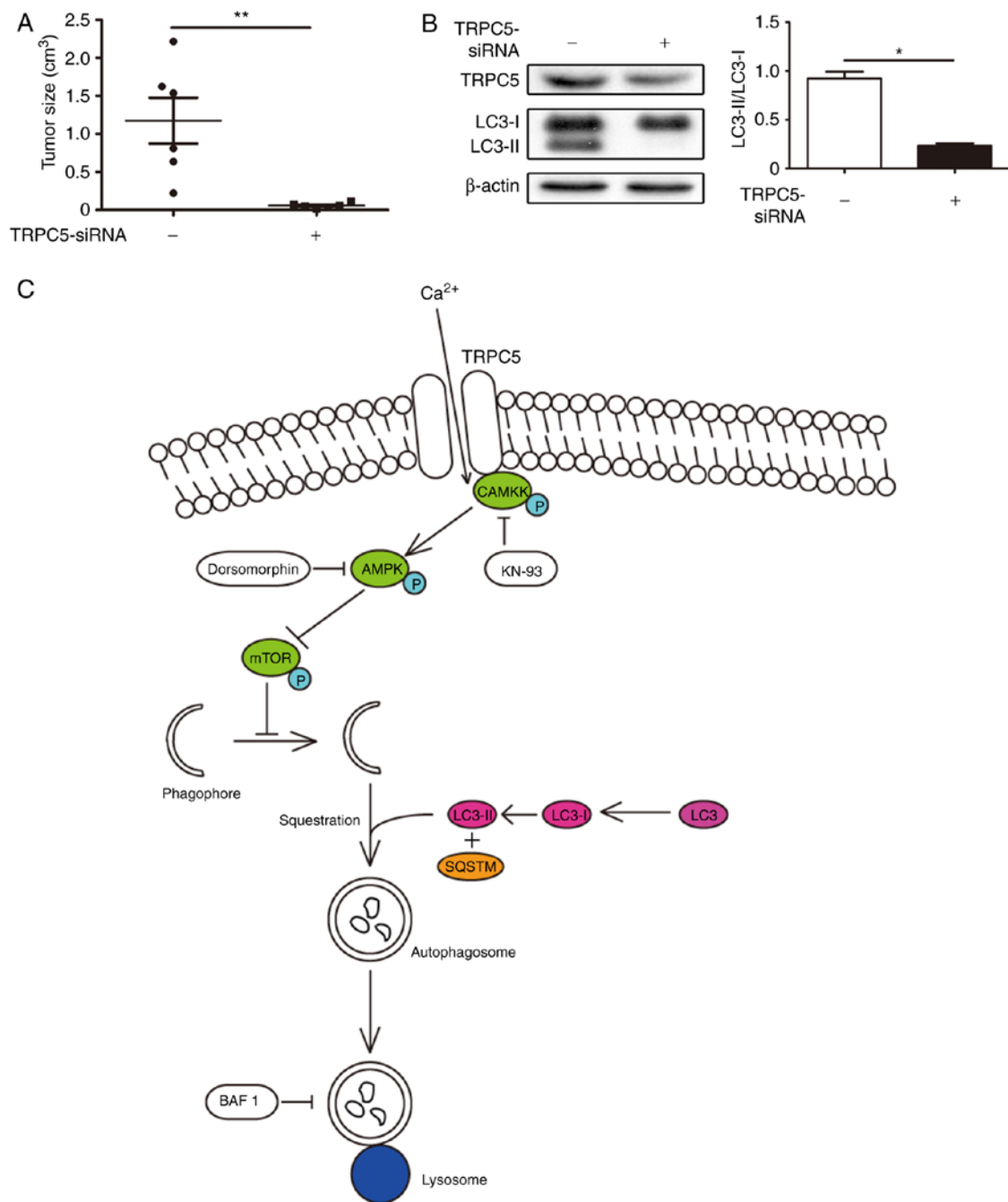


Figure 6. Inhibition of autophagy by TRPC5 knockdown enhances sensitivity to TMZ *in vivo*. (A) Nude mice were inoculated with U87/WT cells pre-transfected with TRPC5 or control shRNA lentivirus and administered with TMZ (30 mg/m²) after 5 weeks (n=3 in each group). The tumor size was measured after 5 weeks. (B) TRPC5 and LC3-II protein expression were measured by western blot analysis. Values are presented as the mean ± standard error of the mean. (C) Signal pathway involved in TRPC5-activated autophagy in glioma cells under exposure to TMZ. TMZ increases TRPC5 expression and activates phospho-CaMKKβ, and then activates phospho-AMPKα. AMPKα activation negatively regulates phospho-mTOR, resulting in upregulated basic autophagy level. TRPC5 induces autophagy during chemotherapy and accelerates glioma cell survival. Arrows represent upregulation events, blunt arrows represent downregulation events. TRPC5, transient receptor potential cation channel subfamily C member 5; siRNA, small interfering RNA; LC3, microtubule associated protein 1 light chain 3 α; CaMKKβ, Ca²⁺/calmodulin dependent protein kinase; AMPK, AMP-activated protein kinase; P, phosphate; mTOR, mechanistic target of rapamycin kinase; BAF1, Bafilomycin A1; SQSTM1, sequestosome; TMZ, temozolomide. *P<0.05, **P<0.01.

of glioma cells in response to TMZ. Furthermore, U87/WT cells treated with TRPC5 shRNA lentiviral particles acquired decreased autophagy level and restricted tumor size with TMZ chemotherapy. In conclusion, TRPC5 mediates glioma cell survival to TMZ via autophagy activation.

Autophagy is negatively regulated by mTOR via regulating the binding of the ULK1-ATG13-FIP200 complex (55). Therefore, the function of mTOR may depend on a number

of upstream components. AMPK, the upstream molecule of mTOR, functions as a positive regulator in the activation of autophagy (56,57). AMPKα is phosphorylated and activated by CaMKKβ (58). The activation of Ca²⁺ influx via TRPC5 in response to numerous physiological stimuli to activate CaMKKβ has been determined (59). Thus, to confirm whether autophagy activation by TRPC5 exposed to chemotherapy via the CaMKKβ/AMPKα/mTOR pathway, the effects of

TRPC5-siRNA and pharmacological agents on this pathway were examined. Downregulation of TRPC5 inhibited the phosphorylated activity of CaMKK β and AMPK α , and increased the phosphorylated activity of mTOR under TMZ exposure. Furthermore, KN-93 to silence CaMKK β , and dorsomorphin to silence AMPK α , restricted the autophagy activation and accelerated cell death under exposure to TMZ. In line with previous research (16), the present results indicated that TRPC5-induced autophagy is mTOR-dependent in glioma cells mediating chemotherapy. Therefore, the present data indicated that the CaMKK β /AMPK α /mTOR pathway is involved in TRPC5-induced autophagy in chemotherapy.

In conclusion, the present results indicated that TRPC5-mediated autophagy facilitated the cell viability of glioma cells via the CaMKK β /AMPK α /mTOR pathway under exposure to TMZ (Fig. 6C). TRPC5 expression has a positive correlation with autophagy *in vivo* prior to and following TMZ chemotherapy. This research confirmed TRPC5 to be an initiator of autophagy, and revealed a novel mechanism for drug resistance in chemotherapy for glioma.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

XJL and YZ conceived and designed the experiments. YZ, JW, SZ and MC performed the experiments. YZ, XDZ and ZLM analyzed the data. YZ and XJL wrote the paper. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All animals were kept in a pathogen-free environment and fed *ad libitum*. The procedures for care and use of animals were approved by the Ethics Committee of the Affiliated

No. 2 Hospital of Nanjing Medical University (Wuxi, China) and all applicable institutional and governmental regulations concerning the ethical use of animals were followed.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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