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ORIGINAL

LC3 siRNA-Mediated Impressed Autophagy in GBM Cells Enhances the Efficacy of Temozolomide: Inhibits Proliferation, Clone Formation and Migration of U87-MG Cells

La autofagia impresa mediada por ARNip de LC3 en células GBM mejora la eficacia de la temozolomida: inhibe la proliferación, la formación de clones y la migración de células U87-MG

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Abstract

Objective: Autophagy is a catabolic process for degrading dysfunctional proteins and organelles and closely associated with cancer cell survival under therapeutic, metabolic stress, hypoxia, starvation and lack of growth factors, contributing to resistance to therapies. However, the role of autophagy in Glioblastoma (GBM) is not clearified.

Methods and results: In the present study, we investigated the role of autophagy in highly aggressive and metastatic GBM cells and demonstrated that the knockdown of light chain 3 (LC3) with LC3-siRNAs which is considered as potential markers of autophagic activity, inhibited autophagy and significantly suppressed cell proliferation, colony formation, migration in GBM cells. Also, combination of Temozolamide (TMZ) and LC3-siRNA significantly inhibited autophagic activity, cell proliferation, colony formation, migration in GBM cells. Furthermore, knockdown of LC3 led to inhibition of multiple proto-oncogenic signaling pathways, including cyclin D1, integrin-β1/Src, and PARP1. Our findings suggest for the first time that LC3 are required for cell proliferation, survival, migration and may contribute to tumor growth and progression in by targeting cyclin D1, integrin-β1, Src, and PARP1 oncogenic signaling in GBM cells.

Conclusion: Overall, these results suggesting, LC3-targeted combination treatments may be a potential therapeutic strategy for GBM and enhanced the efficacy of TMZ.

Key words: GBM, U87-MG, LC3-siRNA, Temozolamide, autophagy.

Resumen

Objetivo: La autofagia es un proceso catabólico para degradar proteínas y orgánulos disfuncionales y está estrechamente asociada con la supervivencia de las células cancerosas bajo estrés terapéutico, metabólico, hipoxia, inanición y falta de factores de crecimiento, contribuyendo a la resistencia a las terapias. Sin embargo, el papel de la autofagia en el glioblastoma (GBM) no está claro.

Métodos y resultados: En el presente estudio, investigamos el papel de la autofagia en células de GBM altamente agresivas y metastásicas y demostramos que el knockdown de la cadena ligera 3 (LC3) con LC3-siRNAs que se considera como marcadores potenciales de la actividad autofágica, inhibió la autofagia y suprimió significativamente la proliferación celular, la formación de colonias, la migración en células de GBM. Asimismo, la combinación de temozolamida (TMZ) y LC3-siRNA inhibió significativamente la actividad autofágica, la proliferación celular, la formación de colonias y la migración en células GBM. Además, el knockdown de LC3 condujo a la inhibición de múltiples vías de señalización proto-oncogénicas, incluyendo ciclina D1, integrina-β1/Src, y PARP1. Nuestros hallazgos sugieren por primera vez que LC3 son necesarios para la proliferación celular, la supervivencia, la migración y pueden contribuir al crecimiento tumoral y la progresión en la ciclina D1, integrina-β1, Src, y PARP1 señalización oncogénica en células GBM.

Conclusiones: En general, estos resultados sugieren que los tratamientos combinados dirigidos a LC3 pueden ser una estrategia terapéutica potencial para GBM y mejorar la eficacia de TMZ.

Palabras clave: GBM, U87-MG, LC3-siRNA, Temozolamida, autofagia.

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Introduction

Glioblastoma (GBM) is the most common[1], aggressive tumor characterized by poor prognosis and high recurrence rates, and is the most lethal type of primary brain tumors with a poor prognosis. The heterogeneity, high proliferation rates and aggressive behavior of GBMs make treatment difficult in GBM patients[2,3]. Traditional treatment strategies for GBM patients are based on surgical resection of the tumor, temozolamide-based chemotherapy, radiotherapy, or a combination of these options[4,5]. However, despite conventional chemotherapy and radiotherapy, patient response is still not at the desired level. Therefore, although great advances have been made in the treatment of GBM, the life expectancy of patients is very low[4,6].

Although temozolomide (TMZ) is the first-line therapy used in the chemotherapy of GBM, natural and acquired resistance conferred by multiple mechanisms causes treatment failure[7,8]. To protect against TMZ cytotoxicity, GBM cells are thought to defend themselves against TMZ by inducing autophagy[9,10]. Therefore, it is suggested that autophagy-targeted therapies in GBM will increase the efficacy of conventional therapies[9,11-13].

Autophagy is consistently used by both normal and cancer cells and is an evolutionarily conserved catabolic process involving lysosome-dependent degradation of defective cytoplasmic materials and organelles[14-16]. In normal cells; autophagy can play a role as a tumor suppressor mechanism for eliminating toxic materials, damaged organelles, misfolded proteins, and reducing oxidative stress and protecting cells from genetic damage[17]. And also, autophagy may act as a survival pathway under conditions such as starvation, hypoxia and therapy-induced stress in tumor cells[15]. Because of autophagy in cancer cells, it is suggested that suppression of autophagy will increase the anti-cancer therapeutic effect[18-20].

Autophagy is a highly complicated process regulated by expression of at least 15 genes and consists of several well-coordinated phases, including initiation, nucleation, elongation and fusion with lysosome. In these phases several autophagy-related (Atg) genes/proteins including microtubule-associated light chain 3 (LC3, homolog of yeast Atg8 gene) play an important role and are often considered as potential markers of autophagic activity[14,16]. LC3 exists in two forms, LC3-I and LC3-II (a LC3- phospholipid conjugate). LC3-I is localized in the cytoplasm under normal conditions. When autophagy is induced by various stresses (such as starvation, hypoxia and growth factor deprivation), a cytosolic form of LC3 (LC3-I) is converted to LC3-II, by conjugation of a lipid molecule called phosphatidyl ethanolamine (PE) for incorporation into membrane of autophagosomes. Therefore, LC3-II is a marker of autophagy[14,15,21]. We also believe that LC3 can be used as a therapeutic target in cancer. However, data on LC3 in glial tumors are scarce and further research is needed on this subject.

In the present study, we investigated the role of LC3 in GBM cells, and demonstrated that LC3 gene is involved in promotion of cell proliferation, colony formation and migration in highly metastatic GBM cells. In addition, we found that LC3 expression is in involved in expression of cyclin D1, integrin-β1 and PARP proteins as well as the activity of Src, all of which are well known as mediators of the cell cycle, cell survival, and cell migration. Further more we demonstrated that LC3-siRNA and TMZ combination significantly supressed GBM cell prolifereation, colony formation and migration more and LC3 specific siRNAs enhanced effect of TMZ in GBM cells. Overall, our findings suggesting for the first time that LC3 may contribute to GBM cell growth, survival and progression, and also combination of LC3-siRNA and TMZ may be a good alternative therapeutic strategy in GBM therapy.

Materials and methods

Cell lines, culture conditions, and reagents

Human U87-MG (cat# HTB-14) cell was purchased from the American Type Culture Collection (Manassas, VA, USA). U87-MG cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO). Temozolamide (TMZ) (CAS: 85622-93-1 was purchased from Sigma-Aldrich (St. Louis, MO) and was dissolved in Dimethyl sulfoxide (DMSO) (Sigma Aldrich, D2650) to prepare stock solution. The stock solution of TMZ was diluted within FBS free medium and applied to U87-MG cells at different concentrations for 72h.

Transfection with siRNA

Two different small interfering RNAs (LC3#1 siRNA, SASI_Hs02 00356118_AS, 50nM, Sigma-Aldrich LC3#2 siRNA, SASI_Hs01 00212378_AS, 50nM, Sigma-Aldrich) targeting LC3 gene and non-silencing control siRNA (Cat# WD00909801) were purchased from Sigma-Aldrich. Exponentially growing U87-MG GBM cells were plated 24h before transfection and transfected with two different LC3 siRNAs or control siRNA at a final concentration of 50 nM for 72h, using HiPerFect Transfection Reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol. Non-silencing control siRNA-transfected cells were used as negative controls[22-25]. After treatment, the cells were harvested and processed for further analysis.

Western blot analysis

For western blot analysis, cells were seeded in T-25 culture U87-MG cells were transfected with siRNAs (50 nM) for 72h. Then, the cells were collected, washed twice in ice-cold phosphate-buffered saline (PBS) and lysed in a lysis buffer at 4°C. The protein concentrations were measured with a protein assay kit (DC kit; Bio-

Rad, Hercules, CA). A total of 40 µg of protein from each sample was separated by Sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis with a 4-20% gradient and transferred to polyvinylidenedifluoride membranes. The membranes were blocked with a blocking buffer (0.1 Triton X-100 with 5% dry milk in Tris-buffered saline–Tween 20 (TBS-T) for 60min. After being washed with TBS-T, the membranes were probed with the following primary antibodies: LC3 (2775S, Cell Signalling) Cyclin-D (60186-1-IG, Proteintech,Integrin-β (12594-1-IG, Proteintech), pSrc (2101S, Cell Signalling), Src (11097-1-AP, Proteintech),and β-actin (60008-1-IG, Proteintech). After being washed with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit (Bio-Rad, #170-6515) or anti-mouse secondary antibody ((Biorad, #1706515). β-actin was used as a loading control. All antibodies were diluted in TBS-T containing 5% dry milk. Chemiluminescence detection was performed with Clarity Western ECL Substrate (Biorad) and the blots were visualized with a ChemiDoc MP Imaging System (Biorad) and quantified with a densitometer using the ChemiDoc MP Imager application program (Biorad)[23-26].

Cell viability and proliferation assays

Cell viability and proliferation were measured by MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega, Madison, WI) as described previously [24-26]. U87-MG cells were seeded in 96-well plates (1.20 x10^3 cells/well) and treated with increasing doses of TMZ (10, 20, 40, 80, 100, 200, 400, 500 and 600 µM). Also, U87-MG cells were seeded in 96-well plates (1.20 x10^3 cells/well) and transfected with siRNAs (50 nM) for 72h. Furthermore, U87-MG cells were seeded in 96-well plates (1.20 x10^3 cells/well) and treated with TMZ (20 and 40 µM) and at the same time transfected with siRNAs (50 nM) for 72h. Following treatments, a solution containing MTS and phenazine methosulfate (20:1 v/v) was added to the cells. After 3h of incubation at 37°C, the number of viable growing cells was estimated by measuring absorption at 490 nM using Elisa Reader based on generation of formazan by the cells[23-26].

Detection of Acidic Vesicular Organelles (AVOs)

Autophagosomes were detected by formation of acidic vesicular organelles (AVOs). AVOs were acridine orange staining was performed[27]. Briefly, cells were seeded in 6-well plates (1x10^5cells/2 ml medium). U87-MG cells were treated with TMZ (20 and 40 µM) for 72h. Also, U87-MG cells were seeded in 6-well plates (1x10^5cells/2 ml medium) and transfected with siRNAs (50 nM) for 72h. Furthermore, cells were seeded in 6-well plates (1x10^5cells/2 ml medium) and treated with TMZ (20 and 40 µM) and at the same time transfected with siRNAs (50 nM) for 72h. Following treatments, the cells were stained with 1 µg/ml acridine orange for 15min AVO staining was examined using a fluorescence microscope (Nicon Eclipse Ti)[24,28].

Colony formation and Clonogenic assays

U87-MG cells were seeded in 6-well plates (1.5 × 10^3 cells/ well) and treated with TMZ (20 and 40 µM) and incubated at 37°C for 2 weeks to form colonies. Also, cells were seeded in 6-well plates (1.5 x 10^3 cells/well) transfected with a non-silencing control siRNA or LC3 siRNAs (50 nM) and grown for 2 weeks. Furthermore, cells were seeded in 6-well plates (1.5 x 10^3 cells/well) and treated with TMZ (20 and 40 µM) and at the same time transfected with siRNAs (50 nM) and grown for 2 weeks. Then, the cells were washed with PBS and stained with crystal violet, and visible colonies were counted[23-26,28].

Cell migration and motility assay

U87-MG cells were seeded in 6-well plates (5 x 10^5 cells/ well) and treated with TMZ (20 and 40 µM) for 72h. Also, cells were seeded in six-well plates (5 x 10^5 cells/well) and transfected with the control siRNA or two different LC3 siRNAs (50 nM) for 72h. Furthermore, U87-MG cells were seeded in 6-well plates (5 x 10^5 cells/well)) and treated with 20 and 40 µM TMZ at the same time transfected with siRNAs for 72h. Following treatments each cell monolayer was carefully scratched using a 20-µl sterile tip and cellular debris was removed by washing with medium, which was then replaced with fresh medium. Cells in the scratched area were imaged at start and 24th h using light microscopy, and the distance traveled by cells at the leading edge of the wound was measured at each time point. The results were expressed as average distance between the edges of the gap[23,24].

Statistical analysis

All experiments were conducted at least in triplicate, and the results were summarized as means with Standard Deviations (SD). Statistical significance was determined using the Student-t test. P- values less than 0.05 were considered statistically significant.

Results

TMZ inhibits MDA-MB-231 Cell Proliferation, Colony Formation and Migration

We first treated cells with TMZ in increasing concentration (10, 20, 40, 80, 100, 200, 400, 500 and 600 µM) for 72h. The MTS analysis revealed that TMZ treatment reduced cell proliferation of U87-MG cells compared to untreated (NT) and DMSO-treated control cells (**Figure 1**).

We found that 20 µM and 40 µM concentrations of TMZ treatment led to about 20% and 25% reduction in cell viability, respectively, in U87-MG cells compared to NT and DMSO-treated cells.

We then investigated the effects of TMZ on colony formation of U87-MG cells. TMZ treatment 20, 40 µM) led to a significant reduction in the number of colonies in TMZ cells compared DMSO-treated cells, and 20 µM

and 40 µM concentrations of TMZ treatment led to about 30% and 40% reduction in cell viability, respectively, of U87-MG cells compared to NT cells and DMSO-treated cells (**Figure 2**). However, TMZ treatment at doses starting at 400 µM and 600 µM led to almost complete elimination viable cells.

We also investigated the effects of TMZ on motility and migration of U87-MG cells using wound healing or scratch assay. After 72h of treatment with TMZ (20, 40 and 80 µM), a single scratch wound was created in wells and the cells were monitored for 24h. While the control cells treated with DMSO, the wounded areas completely closed by migrating into the open areas, TMZ treated cells had a larger open areas with much less number of cells at 24h time point. In treated cells with TMZ in increasing concentrations (20, 40 and 80 µM), the width of the wound was found to be between mean 120-150 µm compared to NT and DMSO-treated cells (**Figure 3**).

TMZ inhibits autophagic activity in U87-MG cells

GBM cells represent highly aggressive and metastatic phenotype of brain tumors that are known to be relatively resistant to chemotherapeutics[29,30]. The development of resistance often limits the therapeutic benefit of temozolomide, particularly in GBM. A number of resistance mechanisms have been proposed including the development of cytoprotective autophagy. Therefore, we first investigated the effect of TMZ on autophagic activity.

Autophagy is characterized by the formation of acidic vesicular organelles (AVOs), which represent formation of autophagolysosomes. AVOs were detected by acridine orange (AO) staining, which stain nuclear and cytoplasm with bright green color, while it stains AVOs with bright red color[24,25,28]. After treatment with TMZ, (20 and 40 µM) U87-MG cells were examined by inverted fluorescence microscope (Nikon Eclipse Ti-E) using green filter. FITC, excitation wavelength: 465-495 nm; dichroic mirror (DM): 505 nm) and red filter (Tx Red, excitation wavelength: 540-580 nm; dichroic mirror (DM): 595 nm). The percentage of cells with redfluorescence stains (indicating AVOs) was calculated. We found that that TMZ treatment led to about only 30% reduction of autophagosome formation in U87-MG cells compared with control cells (DMSO-treated cells) by AO staining (**Figure 4**). Therefore, we thought that continued autophagic activity may reduces the therapeutic effects of TMZ against GBM cells.

LC3 siRNA inhibits autophagic activity and suppresses cell proliferation, colony formation and migration in U87-MG cells

LC3 considered important mediators of autophagy[24,31,32]. Therefore, to clarify the function of LC3 and autophagic process in cells, we knocked down LC3 in U87-MG cells and investigated the effects on cell proliferation, clonogenicity and migration compared to the control cells transfected with control siRNA. We first demonstrated that siRNA mediated knockdown of LC3 using two different siRNAs (72h). The siRNAs targeted LC3 mRNA efficiently reduced expression of LC3-I and LC3-II proteins (**Figure 5**). These results showed that the LC3 siRNAs can effectively knockdown LC3 expression in the U87-MG. Also, we found that LC3#2 siRNA supppressed expression of LC3 more than LC3#1 siRNA in cells.

Next, to investigate the effect of LC3 silencing in inhibition of autophagy we also evaluated autophagy induction in U87-MG cells. The cells were seeded in a six-well plates and transfected with LC3, or control siRNA for 72 hours. and stained with AO to detected AVOs by fluorescent microscopy. Knockdown of LC3 efficiently suppressed autophagy as evidenced by reduced AVO formation compared to control siRNA transfected cells (**Figure 4**).

Then, we tested the effect of LC3 knockdown on cell proliferation, viability and colony formation or clonogenicity. To this end, U87-MG were transfected by LC3 siRNAs and 72h later cell viability was detected by MTS assay. Cell viability was significantly reduced after LC3 knocked down in U87-MG cells as examined by MTS assay (**Figure 1**).

We next examined the effects of LC3 siRNAs on cell colony formation in U87-MG cells by using a clonogenic assay. Knocdown of LC3 in U87-MG cells resulted in a marked reduction of colony formation (**Figure 2**).

Then, to assess whether autophagy is involved in cell motility migration of GBM cells, we performed an in vitro scratch or wound healing assay. GBM cells were seeded in a six-well plates and transfected with LC3 siRNAs. 72h after transfections, a single scratch wound was created in the well, and the cells were monitored for 24h. While some cell migration was observed in the wounded (sctracted) areas in control cells (control siRNA transfected) and the wounded areas were completely closed by migration of cells (24h), in LC3 knocked down cells, indicating that cells with reduced LC3 expression were unable to migrate (**Figure 3**). These data demonstrated that autophagy is essential for survival, proliferation, colony formation and migration of GBM cells.

Combination of LC3 siRNA and TMZ significantly suppresses autophagic activity and inhibits cell proliferation, colony formation and migration in U87-MG cells

Glioblastoma multiforme (GBM) remains one of the most challenging solid cancers to treat due to its highly aggressive and drug resistant nature[29]. Therefore, to test whether suppressed LC3 increases the sensitivity of cells to TMZ, we investigated the effect of the combination of TMZ and siRNA on cell viability, clonogenicity and migration. To this end, U87-MG cells were seeded in 96-well plates (1.20 x10^3 cells/well) and treated with 20 and 40 µM TMZ and at the same time transfected with siRNAs (50 nM) for 72h later cell viability was detected by

MTS assay. The MTS analysis revealed that LC3 siRNA and TMZ combination treatment significantly reduced cell proliferation of U87-MG cells compared to LC3-siRNA transfected and TMZ treated cells (**Figure 1**).

We next examined the effects of LC3-siRNA and TMZ combination treatment on cell colony formation in U87-MG cells by using a clonogenic assay, LC3 siRNA and TMZ combination treatment in U87-MG cells resulted in a marked reduction of colony formation (**Figure 2**).

Even, LC3 siRNA and TMZ combination treatment led to almost complete elimination of colony formation. Then, to identify the effects of LC3 siRNA and TMZ combination treatment on cell motility and migration, U87-MG cells were treated with 20, 40 and 80 μM TMZ and at the same time transfected with siRNAs (50 nM) for 72h. In LC3-siRNA and TMZ combination treatment in U87-MG cells were unable to migrate (**Figure 3**).

Figure 1. LC3-siRNA and TMZ combination treatment inhibits cell proliferation in U87-MG cells. Cells were treated with increasing concentration of TMZ, and cell proliferation was evaluated after 72 h by MTS assay (a). U87-MG cells were transfected with indicated siRNAs and proliferation/ cell viability was detected by an MTS assay. LC3-siRNA-mediated knockdown of autophagy gene inhibits U87-MG cell proliferation/viability (b). Cells were treated with LC3-siRNA and TMZ combination, and cell proliferation was evaluated after 72 h by MTS assay. Combination of LC3-siRNA and TMZ significantly inhibit U87-MG cell proliferation (c). The data are presented as means with standard deviations. (ns: non-significant, *p <0.05, **p <0.01, ****p <0.0001).

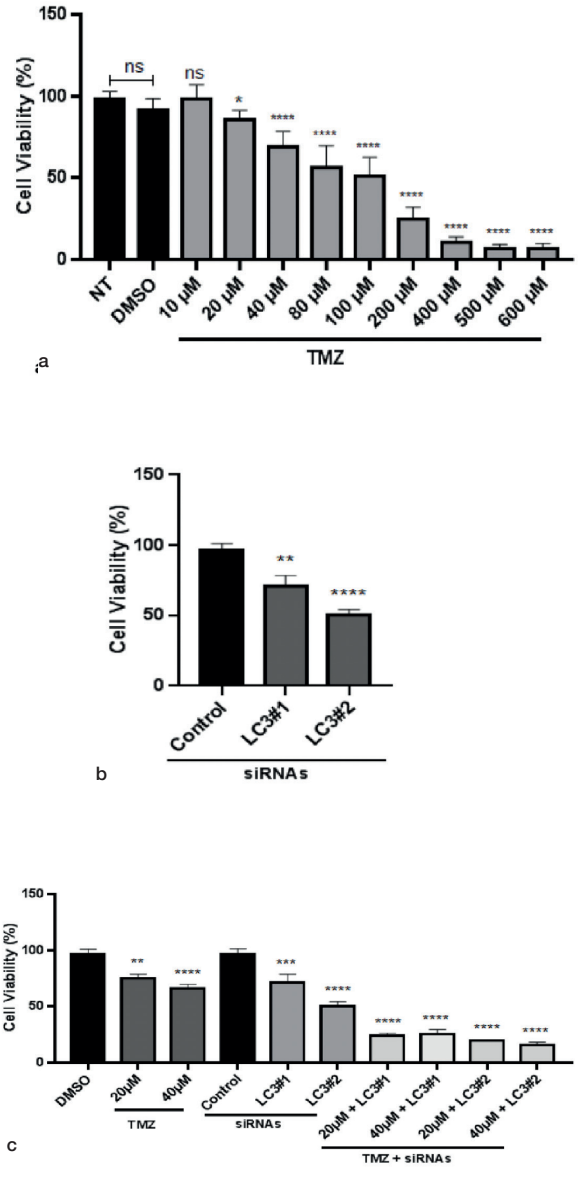


Figure 2. LC3-siRNA and TMZ combination treatment inhibits colony formation in U87-MG cells. Cells were treated TMZ and evaluated for colony formation by crystal violet staining at the end of the 14 days in U-87MG cells (a). U87-MG cells were transfected with two different LC3-siRNAs or control siRNA and evaluated for colony formation by crystal violet staining at the end of the 14 days (b). Cells were treated with LC3-siRNA and TMZ combination, and was evaluated for colony formation by crystal violet staining at the end of the 14 days (c). Colony areas measured densitometrically and image J at the at the end of the 14 days . The data are presented as means with standard deviations (d) (ns: non-significant, ***p <0.0001).

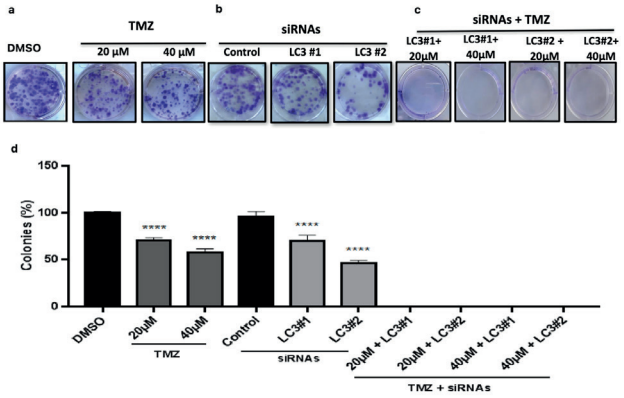
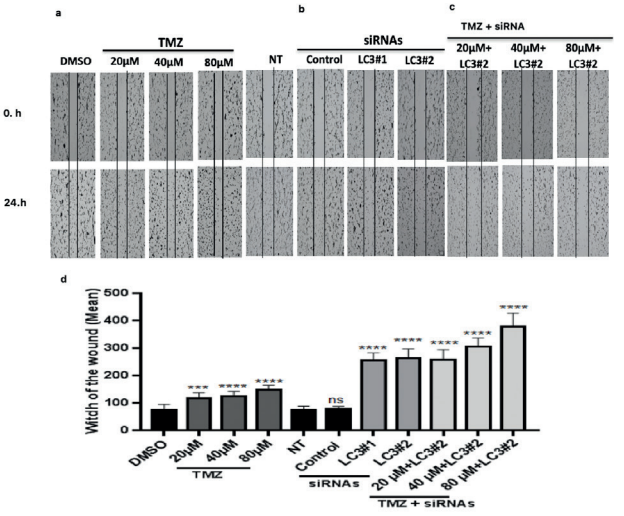


Figure 3. LC3-siRNA and TMZ combination treatment inhibits migration in U87-MG cells. Cells were treated with TMZ and evaluated after 72 h for migration and visualized by light microscopy. (a). Cells were transfected with two different LC3 siRNAs. The cell migration was visualized by light microscopy (b). Cells were treated with LC3-siRNA and TMZ combination, and cell migration was visualized by light microscopy (c). Cell migration was measured by a scratch wound healing assay. A single scratch was made in the center of the confluent cell monolayer. Images were taken immediately (0 h), and after 24 h of scratching the cultures. The bar graph shows the percentages of the migrating cells to scratched/wounded area, and the data are presented as means with standard deviations (d) (ns: non-significant, ***p <0.0001).



To investigate the effect of combination of LC3-siRNA and TMZ in inhibition of autophagy we also evaluated autophagy induction in U87-MG cells. The cells were seeded in a six-well plates and transfected with LC3-siRNA treated with 20 and 40 μM TMZ for 72 hours were stained with AO to detected AVOs by fluorescent microscopy. LC3-siRNA and TMZ combination efficiently suppressed autophagy as evidenced by reduced AVO formation (**Figure 4**).

These results showed that LC3-siRNA by suppressing autophagy and increased sensitivity and efficiency against TMZ in U87 cells.

Figure 4. LC3-siRNA and TMZ combination treatment decreases autophagy in U87-MG cells. Formation of acidic vesicular organelles (AVO) were detected in U87 cells following TMZtreatments by AO staining and fluorescent microscopy (a). Formation of AVO in transfected cells with indicated siRNAs were demonstrated by AO staining and fluorescent microscopy (b). Cells were treated with LC3-siRNA and TMZ combination, and formation of AVO was visualized by AO staining and fluorescent microscopy (c). The data are presented as means with standard deviations (d) (ns: non-significant, ****p <0.0001).

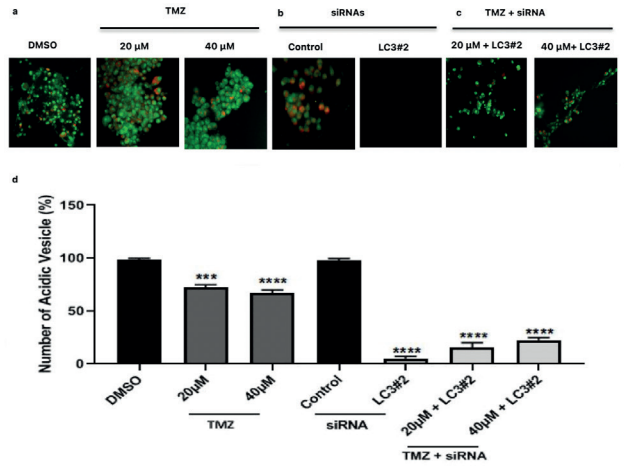
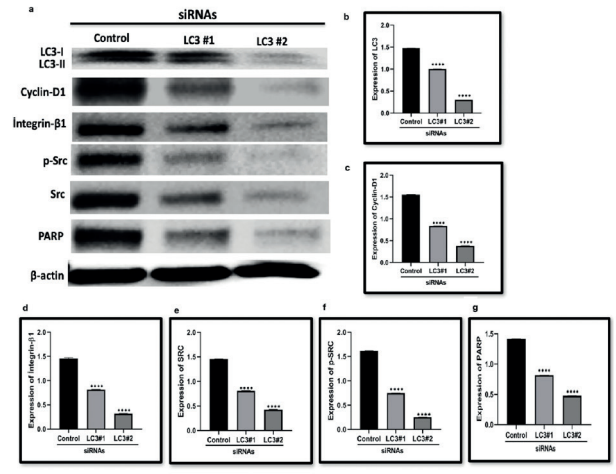


Figure 5. Down-regulation of autopgahgy by LC3 siRNAs inhibits expression of mediators of the cell cycle, cell survival, and cell migration. Cells were transfected with 50 nM LC3 siRNA or control siRNA. Protein extracts were isolated 72 h after transfection.β1-Actin was used as a loading control. Knockdown of LC3 by siRNA decreased expression levels of Cyclin D, Integrin-β1 and p-Src, Src and PARP. α/β-Tubulin was used as a loading control. The data are presented as means with standard deviations (d) (ns: non-significant, ****p <0.0001).



Knockdown of LC3 inhibits mediators of the cell cycle, survival, and cell migration/invasion in U87-MG cells

To elucidate the molecular mechanims by which autophagy inhibition reduces cell survival, migration and invasion we investigated related signaling pathways after knocdown of autophagy-regulating gene (LC3). We found that inhibition of autophagy markedly reduced the expressions of cyclin D1, which promotes the cell cycle by inducing G1 phase, Integrin-β1/p-Src, both of which are the most important mediators of cell survival, migration and invasion[23-25], PARP, which is involved in DNA repair and cell death, and plays an important role in GBM (**Figure 5**). Overall, our findings suggest that LC3 may provide protumorigenic effects by inducing imporant signaling pathways in U87-MG cells.

Discussion

Genomic studies on tumour cells from GBM patients have shown that mutations in multiple signalling pathways have an effect on regulating autophagy (33). Furthermore, in GBM cell cultures, autophagy has been observed to be frequently activated as a stress response upon treatment with therapeutic agents[34]. Cellular and xenograft experiments suggest that autophagy plays an important role in GBM[12,35]. Mouse studies have also shown that autophagy is necessary for GBM growth[36].

The present study showed that suppression of LC3, the main element of autophagy mechanism, by two different siRNAs significantly inhibited cell proliferation, viability and migration. Thus, it was shown that autophagy inhibition via LC3 inhibits cell proliferation, clone formation and migration in GBM cells.

In addition, the present study showed that suppression of LC3 expression in GBM cells via two different LC3-siRNAs suppressed CyclinD1/Integrin β1/Src signalling expression, which is involved in cell proliferation, viability, and migration, and PARP expression, which plays an important role in DNA repair and cell death.

As previously reported, TMZ is an alkylating agent used in the first line chemotherapy of GBM[7]. However, although TMZ is the first-line chemotherapy for glioma patients, natural and acquired resistance provided by multiple mechanisms leads to treatment failure. Studies have shown that TMZ induces autophagy[10]. It has also been shown that pharmacological inhibition of autophagy at different stages has different results on TMZ-induced autophagy. However, in the present study, TMZ (20 and 30 μM) was found to suppress autophagy by 30% in U87-MG cells. Accordingly, it was found that cell viability decreased by approximately 20-25% in cells treated with 20 and 30 μM TMZ. For all these reasons, inhibition of the autophagic process is thought to significantly affect the antitumour effect of TMZ[10].

In the light of all these, it was thought that suppression of LC3 by two different LC3-siRNAs, which are markers of autophagy, and LC3-siRNA-mediated suppressed autophagy would increase the anticarcinogenic activity of TMZ in U87-MG cells. LC3-siRNA and TMZ were co-administered simultaneously. The combination of LC3-siRNA and TMZ significantly decreased cell viability, clonogenic formation and migration compared to TMZ-treated cells. This result indicated that LC3-siRNA-mediated suppressed autophagy may increase the sensitivity of U87-MG cells to TMZ.

In addition, our study demonstrated for the first time that when the expression of LC3 was suppressed by two different LC3 siRNAs, the expression of PARP protein was also significantly suppressed. Poly (ADP-ribose) polymerase 1 (PARP) is a protein involved in DNA repair and cell death and is generally highly expressed in cancers[37]. Our results showed that autophagy promoted cell survival by preventing apoptotic cell death in GBM cells. Our study also showed for the first time that suppression of LC3 expression in GBM cells through two different LC3-siRNAs suppresses CyclinD1/Integrin β1/Src[22,23] expression, which is involved in cell proliferation, viability, adhesion and migration, and leads to inhibition of Src-phosphorylation.

GBMs are aggressive brain tumours characterised by poor prognosis and high recurrence rates and are the most lethal type of primary brain tumours with poor prognosis. The heterogeneity, high proliferation rates and aggressive behaviour of GBMs make the treatment of GBM patients difficult. Therefore, although great advances have been made in the treatment of GBM, the survival of patients is still very low[4,6].

Conventional treatment strategies for GBM patients are based on tumour resection (neurosurgery), TMZ-based chemotherapy, radiotherapy or a combination of these options[4]. However, patients respond poorly to conventional chemotherapy and radiation.

In particular, temozolomide (TMZ) is used as an effective chemotherapeutic in the treatment of GBM[38,39]. However, GBM cells become resistant to TMZ-induced cytotoxicity and this resistance limits the efficacy of TMZ[9,40]. To protect against TMZ cytotoxicity, GBM cells are thought to defend themselves against TMZ by inducing autophagy[9,10]. Therefore, autophagy-targeted therapies in GBM are suggested to improve the efficacy of conventional therapies[9,11,13].

Conclusion

Our results showed that LC3, and thus autophagy, promotes important oncogenic biological processes such as GBM cell survival, proliferation, migration and resistance to apoptosis and that LC3-siRNA-mediated suppression of autophagy significantly blocks these mechanisms and that autophagy may be a critical factor in the progression of GBM and that LC3, and thus autophagy, represents a potential therapeutic target in the treatment of GBM. LC3-targeted combination therapies may be a potential therapeutic strategy for GBM and may enhance the efficacy of TMZ. Therefore, our results suggest that the combination of LC3-siRNA and TMZ may be a novel therapeutic strategy for the treatment of GBM. Further research is needed for this, and it is suggested that these results obtained in vitro should be transferred to in vivo environment and their effects in the living body should be revealed.

Conflict of interest

The authors declare that they have no competing interests.

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