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Allicin induces apoptosis through activation of both intrinsic and extrinsic pathways in glioma cells

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Abstract. Allicin is an extract purified from *Allium sativum* (garlic), and previous research has indicated that Allicin has an inhibitory effect on many kinds of tumor cells. The aim of the present study was to explore the anticancer activity of Allicin on human glioma cells and investigate the underlying mechanism. MTT and colony-formation assays were performed to detect glioma cell proliferation, and explore the effect of Allicin at various doses and time-points. The apoptosis of glioma cells was measured by fluorescence microscopy with Hoechst 33258 staining, and then flow cytometry was used to analyzed changes in glioma cell apoptosis. Reverse transcription-quantitative polymerase chain reaction and western blot analysis were used to detect the effect of Allicin on the expression levels of Fas/Fas ligand (FasL), caspase-3, B-cell lymphoma 2 and Bcl-2-associated X protein. Allicin suppressed the proliferation and colony formation ability of U251 cells in a dose- and time-dependent manner. A cytotoxic effect of Allicin was observed in glioma cells in a dose-dependent manner. Changes in nuclear morphology were observed in U251 cells with Hoechst 33258 staining. The activity of caspases were significantly elevated and Fas/FasL expression levels were increased following treatment with Allicin, at both the mRNA and protein level. These results demonstrated that Allicin suppresses proliferation and induces glioma cell apoptosis *in vitro*. Both intrinsic mitochondrial and extrinsic Fas/FasL-mediated pathways react in glioma cell after treating with Allicin, which then activate major apoptotic cascades. These results implicate Allicin as a novel antitumor agent in treating glioma.

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Introduction

Glioblastoma (GBM) is the most aggressive subset of primary brain tumor in adults, and is responsible for ~50% of all cranial tumors. GBMs are highly infiltrative which results in difficulty for them to be resected completely (1-3). Comprehensive therapy including radiotherapy and chemotherapy is the main approach used for treatment; however, the overall survival of glioma patients is only 12-14 months post-diagnosis (4).

Allicin (Fig. 1A) is extracted from freshly crushed garlic (*Allium sativum*). The anti-bacterial and antiinflammatory effects of Allicin have been indicated, and previous studies have demonstrated the antitumor capacity which may inhibit tumor growth and induce apoptosis (5-7). Allicin has been demonstrated to have an inhibitory effect on different kinds of tumors including lung cancer, colorectal carcinoma, stomach cancer and liver cancer (8,9). Previous studies have further explored the effect of Allicin as an anticancer drug. However, the molecular mechanism underlying the apoptosis effect of Allicin in glioma remains to be clarified.

Activation of apoptosis signaling pathways may be involved in the treatment of malignant tumor. Two main apoptosis-associated signaling pathways have been addressed: The extrinsic death receptor pathway and the intrinsic mitochondrion pathway (10). The activation of Fas binding to its Fas ligand (FasL) could initiate the extrinsic pathway of apoptosis and serve a key role involved in death signaling in many cancer types (11). Both signaling pathways contain mitochondrial membrane and B-cell lymphoma 2 (Bcl-2) family proteins. Fas-associated protein with death domain (FADD) together with caspase-8 leads to autoproteolysis, and results in enzymatic activation of caspase-8 which in turn activates caspase-3, -6 and -7, resulting in the hydrolysis of cytosolic and substrates.

However, the roles of Allicin in human glioma remain largely unclear. The present study aimed to investigate the apoptotic activity of Allicin in human glioma cell lines, and explore the underlying mechanism. These results provide

evidence that Allicin could inhibit proliferation and induce glioma cell apoptosis *in vitro*.

Materials and methods

Cell line and culture. The U251 human glioma cell line was obtained from the Chinese Academy of Sciences Cell Bank. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Hyclone; GE Healthcare Life Sciences, Chicago, IL, USA), supplemented with 10% (FBS) at 37˚C with 5% CO_2. Allicin (purity ≥90%) was purchased from Shanghai Harvest Pharmaceutical Co., Ltd. (Shanghai, China).

Cytotoxicity assay. Glioma cells were cultured at 5,000-8,000 cells/well in 96-well plates overnight. Cells were treated with 15, 30, 60 or 90 μg/ml of Allicin for 20 h. Subsequently, 5 mg/ml MTT (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added for additional 4 h at 37˚C. Optical density (OD) was assessed by using a TECAN microplate reader at 490 nm.

Cell proliferation assay and colony formation assay. Cell viability was measured by MTT assay. Cells were seeded at $5x10^3$ cells/well into a 96-well plate and incubated with Allicin at various concentrations (15, 30, 60 and 90 μg/ml) for different periods (24, 48 and 72 h). The OD was assessed by using a TECAN microplate reader at 490 nm. For the colony formation assay, cells were cultured in a 6 cm dish ($0.5x10^3$ cells/well) and incubated at 37˚C. Following this, different concentrations of Allicin were added for 24 h. The medium was then removed and cells were cultured for another 12 days. The colonies formed were fixed with 10% formalin for 10 min. Giemsa staining was used to stain the samples obtained for 30 min in room temperature and the number of colonies (>50 cells) was counted by using upright light microscope (Leica DM2000; Leica Microsystems, GmbH, Wetzlar, Germany).

Western blot analysis. U251 cells were treated with different concentrations Allicin (30 and 60 μg/ml) for 48 h, and then washed with ice-cold PBS. The cell lysates were centrifuged at 6,037 x g for 15 min at 4˚C and the total protein was extracted using radioimmunoprecipitation buffer (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with protease inhibitors, then subsequently separated via 4-10% Tris glycin/SDS-PAGE. A bicinchoninic acid protein assay kit was used to determine the concentration of protein. The total quantity of protein loaded onto each lane was 40 μg; separated proteins were electrotransferred to ECL nitrocellulose membranes (IPFL00010; EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk (Wuhan Boster Biological Technology, Ltd., Wuhan, China) at room temperature for 1 h and incubated separately with mouse anti-human Fas (1:1,000 dilution, cat. no. 8023S; Cell Signaling Technology, Inc., Danvers, MA, USA), mouse anti-human FasL (1:1,000 dilution, cat. no. 4273; Cell Signaling Technology, Inc.), mouse anti-human Bcl-2 (1:500 dilution, cat. no. sc-509; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit anti-human Bcl-2-associated X protein (Bax; 1:500 dilution, cat. no. sc-526; Santa Cruz Biotechnology, Inc.), rabbit anti-human caspase-3 (1:1,000 dilution, cat. no. 9665; Cell Signaling Technology, Inc.) and mouse anti-human β-actin (1:500 dilution, cat. no. 376421; Santa Cruz Biotechnology, Inc.) at 4˚C overnight, and then incubated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution, cat. no. ab150077; Abcam, Cambridge, MA, USA) for 1 h. The protein levels were visualized using an enhanced chemiluminescence kit (BeyoECL Plus, cat. no. P0018; Beyotime Institute of Biotechnology, Haimen, China) and determined by a Gel Doc 2000 imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total U251 cell RNA treated with different concentration of Allicin (0, 30 or 60 μg/ml) were isolated using TRIzol reagent (Thermo Fisher Scientific, Inc.). PCR was performed with ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The thermocycling condition were as follows: 95˚C for 30 sec, then followed by 40 cycles at 95˚C for 5 sec, 60˚C for 30 sec, and 72˚C for 15 sec. The PCR primers were as follows: Fas sense, 5'-TTCTGCCATAAGCCCT GTC-3' and antisense, 5'-TTGGTGTTGCTGGTGAGT-3' (amplification fragment 320 bp); FasL sense, 5'-TTCAGCTCT TCCACCTACAG-3' and antisense, 5'-ACATTCTCGGTG CCTGTAAC-3' (amplification fragment 599 bp); caspase-3 sense, 5'-GACAGACAGTGGAAGCGACTGGAT-3' and antisense, 5'-GCATGGCACAAAGCGACTGGAT-3'; Bcl-2 sense, 5'-CGCCCTGTGGATGACTGAGTA-3' and antisense, 5'-GGGCCGTACAGTTCCACAAAG-3'; Bax sense, 5'-CCCTTTTGCTTCAGGGTTTCATCCA-3' and antisense, 5'-CTTGAGACACTCGCTCAGCTTCTTG-3'; U6 sense, 5'-TGCGGGTGCTCGCTTCGGCAGC-3' and antisense, 5'-CCAGTGCAGGGTCCGAGGT-3'. Fas/FasL, caspase-3, Bcl-2 and Bax mRNA expression levels were determined using a SYBR PrimeScript RT-PCR kit (Takara, Bio, Inc., Otsu, Japan) and normalized to U6 mRNA. The relative expression levels were analyzed using the $2^{-\Delta\Delta Cq}$ method (12).

Detection of caspase activity. A caspase activity kit (Beyotime Institute of Biotechnology) was used to detected the activity of caspase-3, -8 and -9. Cells ($1x10^6$ cells/well) were added to Allicin (0, 30 and 60 μg/ml) for 24 h. Data was obtained by measuring the enzyme labeling meter with the wavelength of 490 nm via a microplate reader (Infinite F50 Tecan Group, Ltd., Männedorf, Switzerland). The U251 cells were isolated with caspase assay buffer (Nanjing Kaiji Materials, Co., Ltd., Nanjing, China) and then supernatant was collected and centrifuged at 7,546 x g at 4˚C for 10 min. The relative activity of caspases was measuring by an enzyme-labeling meter with a microplate reader.

Hoechst 33258 staining. Cells were treated with Allicin for 24 h. The media was then removed and cells were fixed with 4% formaldehyde. Cells were then stained with 200 μM Hoechst 33258 for 10 min at room temperature, and the slides were examined under a fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Cell apoptosis assay. U251 cells were seeded onto a 6-well plate at $5x10^5$ cells/well for 24 h and treated with different amounts of Allicin (0, 30 and 60 μg/ml) for 48 h. The samples were collected at a concentration of $1x10^6$ cells/ml with 4˚C PBS and

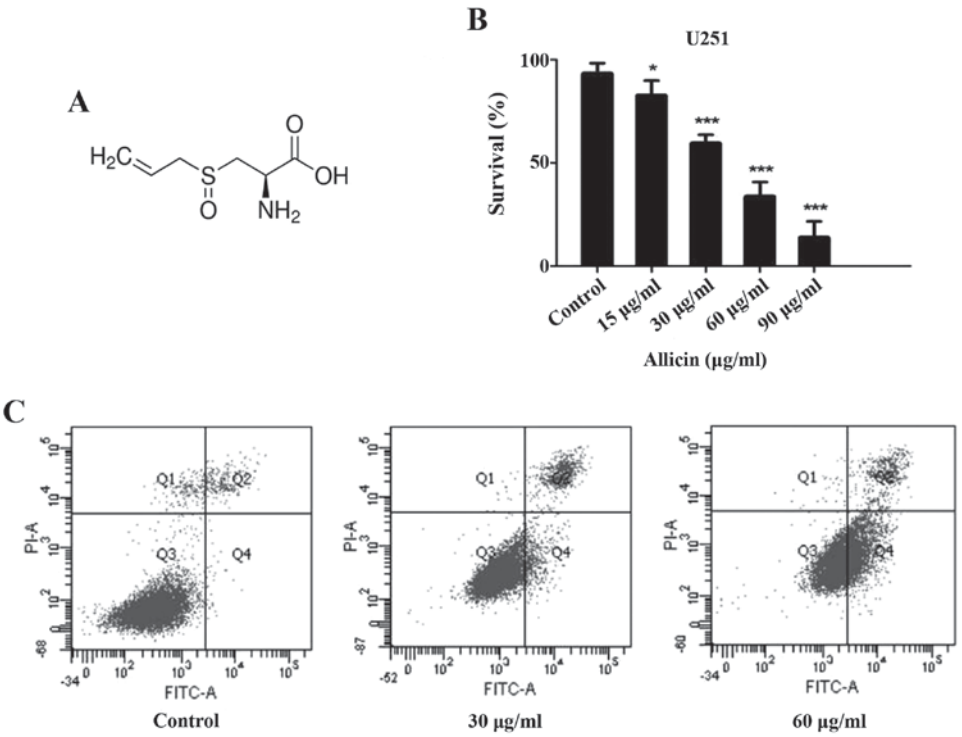

Figure 1. (A) Chemical structure of Allicin. (B) Allicin had a cytotoxic effect on human U251 glioma cells. MTT assay was used to detect cell death. (C) Annexin V-FITC/PI staining was used to examine U251 cell apoptosis by flow cytometry. Data are presented as the mean ± standard error of three independent experiments. *P<0.05 and ***P<0.001 vs. control. FITC, fluorescein isothiocyanate; PI, propidium iodide.

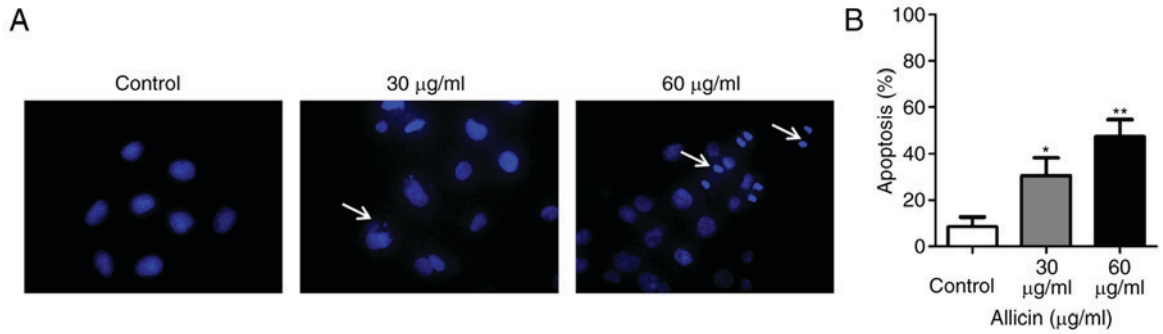

Figure 2. Apoptosis of glioma cells. (A) Representative fluorescent microscope images and (B) quantification of Hoechst-stained cell apoptosis after treatment with Allicin for 48 h (magnification, x400). *P<0.05 and **P<0.01 vs. control. White arrows indicate apoptotic cells.

suspended in 400 μl binding buffer. Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI; BD Pharmingen; BD Biosciences, Franklin Lakes, NJ, USA) were added into the labeled tube and cells were incubated for 20 min. The samples were examined by using flow cytometry (BD Biosciences; Clontech, Palo Alto, CA, USA).

Statistical analysis. All results were summarized from three independent experiments. Results are expressed as the mean ± standard error. Comparisons of each treatment data with control were carried out for statistical difference by the paired t-test. One way analysis of variance with followed by a Turkey's post-hoc test was used to analyze statistical differences between groups by using the statistical software SPSS 17.0 (SPSS, Inc., Chicago, IL, USA); graphs were produced using by GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Cytotoxic effects of Allicin on U251 glioma cells. The cytotoxic effect of Allicin (P<0.05; Fig. 1B) on U251 cells was measured by MTT assay. The results demonstrated that Allicin decreased cell survival in a dose-dependent manner, indicating its cytotoxicity. The median IC_{50} was 41.97 μg/ml; therefore, 30 and 60 μg/ml doses of Allicin were chosen for the present study.

Allicin induces apoptosis of glioma cells. In the present study, Annexin V/PI staining was used to demonstrate the apoptosis-inducing effect of Allicin. The results demonstrated

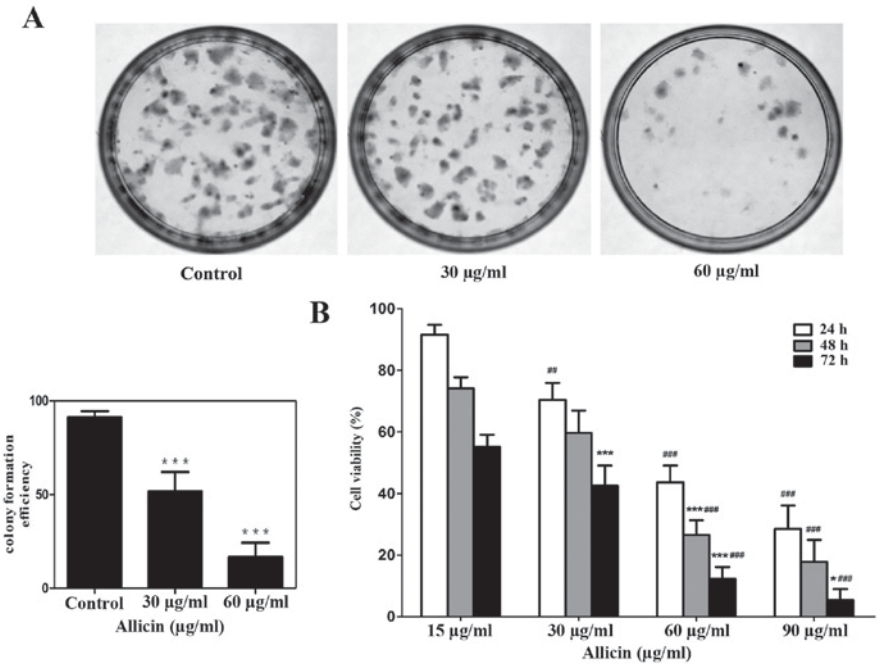

Figure 3. Allicin suppresses the proliferation of glioma cells. (A) Treatment with Allicin suppressed colony formation compared with the control group. ***P<0.001. (B) Cell viability was detected by MTT assay. The percentage of cell viability is presented. Dose- and time-dependent inhibition of cell growth could be observed after 96 h. Data are expressed as the mean ± standard error. *P<0.05 and ***P<0.001 vs. 24 h; ##P<0.01 and ###P<0.001 vs. 15 μg/ml Allicin group.

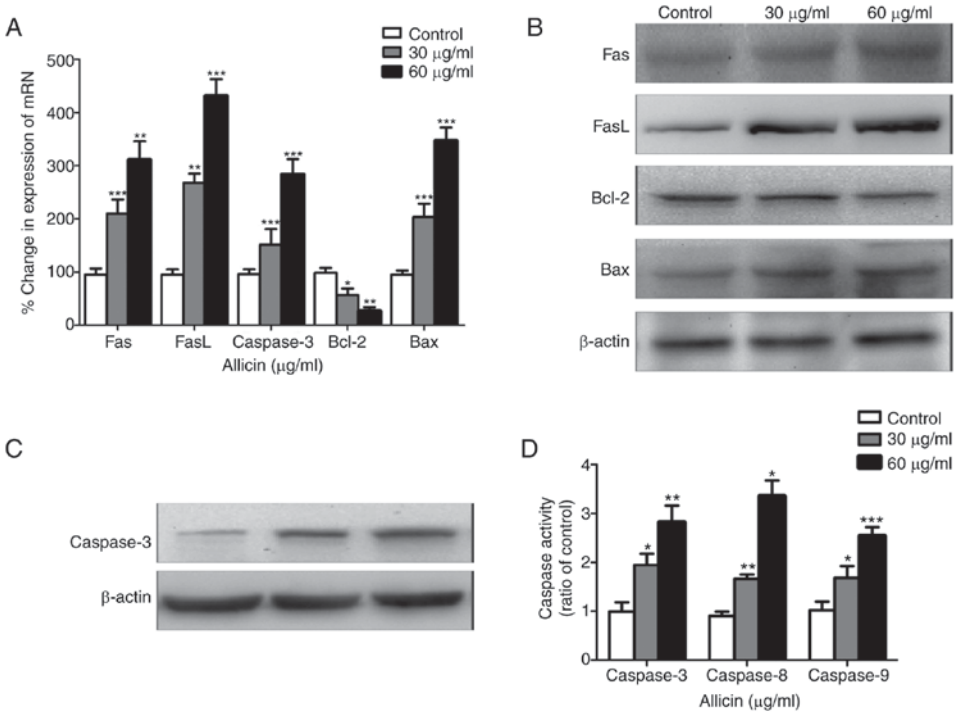

Figure 4. Allicin increases apoptosis in glioma cells. (A) mRNA and (B) protein expression levels of apoptosis-associated factors. (C) Caspase-3 protein expression levels. (D) The activity of caspase-3, -8 and -9 were detected by using colorimetric assay. Data are presented as the mean ± standard error. *P<0.05, **P<0.01 and ***P<0.001 vs. control. FasL, Fas ligand; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

that the rate of apoptosis increased (30 μg/ml for 9.1±3.2% and 60 μg/ml for 51.4±3.8%) at higher Allicin concentrations compared with control cells (3.3±1.5%). Flow cytometry assay demonstrated that the rate of apoptosis increased in response to treatment with Allicin in a dose-dependent manner (P<0.05; Fig. 1C).

U251 cells were treated with Allicin for 24 h, then stained with Hoechst 33258. The morphological changes of apoptosis cells (such as condensation of chromatin and nuclear fragmentation) was detected under a fluorescence microscope (Fig. 2A). The level of apoptosis was increased with the dose of Allicin in U251 cells (Fig. 2B). These results

demonstrated that Allicin induced apoptosis in a dose-dependent manner.

Allicin inhibits proliferation in U251 glioma cells. Colony formation was used to confirm the proliferation ability by treating with Allicin in U251 glioma cells, which indicated that the inhibition ability of Allicin was irreversible (Fig. 3A). MTT assay was used to detect the effect of Allicin on the growth of glioma cells. Allicin was demonstrated inhibit the viability of glioma cell in a dose- and time-dependent manner (Fig. 3B; $P<0.05$).

Allicin increases Fas and FasL expression in glioma cells. Fas (CD95/APO-1) induces apoptosis by binding to a member of the tumor necrosis factor receptor (TNFR) family (FasL), which is a cell surface receptor protein (13). RT-qPCR was used to measure the expression of Fas and FasL mRNA. The data demonstrated that after treating with different amounts of Alllicin, Fas, FasL and Bax mRNA expression levels were significantly increased, while Bcl-2 expression levels were decreased, in a dose-dependent manner ($P<0.05$, Fig. 4A). These results were reflected at the protein level ($P<0.05$; Fig. 4B and C). These results demonstrated that Allicin induces apoptosis a dose-dependent manner.

Allicin increases the activity of caspases. After treating cells with various amounts of Allicin for 24 h, caspase-3, -8 and -9 enzyme activities were upregulated ($P<0.01$; Fig. 4D). Therefore, apoptosis signaling pathways which were activated by caspases may be involved in the antitumor effects of Allicin.

Discussion

GBM is the most common and aggressive type of brain cancer, has an unfavorable prognosis and treatment still remains great challenge. The majority of chemotherapy options are not recommended because of various severe side effects, and the median survival time of the patients is extremely poor (14).

Garlic is the bulb of Allium plants, and Allicin ($C_6H_{10}S_3$) is the main flavor compound. A previous study reported that Allicin supresses the growth of certain cancers, and may possess anticancer activity *in vitro* and *in vivo* (15). Allicin may induce cell apoptosis, cell cycle arrest and inhibit proliferation, but the underlying mechanisms remain unknown. A previous study demonstrated that Allicin can mediate the apoptosis of cancer cells by activating caspase-3, -8 and -9 (16). Allicin could activate autophagy of human liver cancer cells and induce cell death through apoptosis (7,17).

Apoptosis involves a series of biochemical processes of cell death controlled by several signaling pathways, such as the caspase and mitochondrial pathways. There are two main apoptotic pathways involved: The receptor-mediated pathway and the mitochondrial pathway, which is known as extrinsic and intrinsic. Allicin induces apoptosis by activating the extrinsic and intrinsic apoptosis pathways in gastric cancer cells (18).

The extrinsic pathway is initiated by stimulating the ligand of the death receptors on the cell surface, such as TNFR, Fas and FasL. Fas is a transmembrane protein binding to FasL, then trimerization and recruitment of FADD proteins occurs by activating caspase-8 and -10 (19). Caspase-8 regulates the expression of pro-caspase-3, -6, or -7. Caspase-3 serves an important role in cell death. In the present study, following Allicin treatment, the level of Fas and FasL increased in glioma cells, which has also been demonstrated in gastric cancer cells (20).

This leads to the activation of Bax, which belong to the Bcl-2 family, which leads to the release of cytochrome *c* (21). It has been reported that mitochondria are involved in inducing the intrinsic pathway, which is initiated via the release of signaling factors from the mitochondria. Cytochrome *c* is the first to be released into the cytosol, and interacts with pro-caspase-9, which could regulate caspase-3 or -7 (22).

The Bcl-2 family serves a pivotal a role in the intrinsic apoptosis pathway, which could maintain cell viability by inhibiting the capacity of Bax and impeding the release of cytochrome *c* (23). The results of the present study demonstrated that Allicin may inhibit the proliferation of U251 cells *in vitro* by suppressing Bcl-2 and inducing the expression of Bax.

In conclusion, the results of the present study indicated that Allicin can effectively inhibit proliferation and induce apoptosis in U251 glioma cells *in vitro*. It revealed that Allicin treatment increases the activation of both intrinsic and extrinsic apoptosis signaling pathways in U251 cells. Future studies should evaluate Allicin as a novel antitumor agent in treating glioma.

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