Research Article

Autophagy contributes to ING4-induced glioma cell death

Aihua Gong, Sisi Ye, Ermeng Xiong, Wenjie Guo, Yan Zhang, Wanxin Peng, Genbao Shao, Jie Jin, Zhijian Zhang, Jicheng Yang, Jing Gao

School of Medicine, Jiangsu University, Zhenjiang 212013, PR China
School of Pharmacy, Jiangsu University, Zhenjiang 212013, PR China
School of Medicine, Suzhou University, PR China

ABSTRACT

Previous studies suggest that ING4, a novel member of ING (inhibitor of growth) family, can inhibit brain tumor growth. However, whether autophagy is involved in ING4-induced cell death still remains unknown. In this study, we found that in addition to apoptosis, autophagy also contributed to cell death induced by ING4. Autophagy levels were elevated following the exposure to Ad-ING4, including enhanced fluorescence intensity of monodansylcadaverine (MDC), a specific in vivo marker for autophagic vacuoles, and increased expression levels of the LC3-II and Beclin-1, whereas the autophagic levels were attenuated following the pretreatment of 3-MA, the inhibitor of autophagy, which significantly decreased the Ad-ING4-induced cell death compared with caspase inhibitor zVAD. Furthermore, ING4 also induced mitochondrial dysfunction, such as mitophagy, collapse of mitochondrial membrane potential and the intracellular ROS, which indicated that mitochondria might be associated with the process of autophagic cell death of glioma cells. Finally, the relationship among Bax, Bcl-2, Beclin-1 and caspase family proteins levels were analyzed in glioma cells U251MG and LN229 infected with Ad-ING4 or Ad-lacZ. It is suggested that both autophagy and apoptosis could contribute to ING4-induced glioma cell death, and mitochondria might play an important role in this process. Our findings reveal novel aspects of the autophagy in glioma cells that underlie the cytotoxic action of ING4, possibly providing new insights in the development of combinatorial therapies for gliomas.

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Introduction

High-grade gliomas (HGGs), which include glioblastoma (GBM) and anaplastic astrocytoma (AA), are the most common intrinsic brain tumors in adults and are nearly uniformly fatal [1]. Despite recent advances in therapy, two-year survival for glioblastoma with optimal therapy is less than 30% [1]. Emerging evidence suggests that the integrated approach to glioma treatment, including surgery, radiation therapy, chemotherapy and even gene-therapy, is a promising therapeutic strategy [1,2]. Preclinical studies have proved that cancer gene therapy can significantly improve the sensitivity of radiotherapy or chemotherapy [3]. Recent studies
Materials and methods

Materials

3-[4,5-dimethyl-2-thiazoly]-2, 5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from A meresco. 5,5′,6′,6′-tetra chloro-1,3,3,3′-tetraethyl benzimidazolylcarbocyanine iodide (JC-1) from Invitrogen. 3-Methyladenine (3-MA) was purchased from Sigma (Shanghai, USA). Benzyloxycarbonyl-Val–Ala–Asp (G7231, zVADfmk, zVAD) was purchased from Promega (Shanghai, USA). Trypsin and EDTA-2 Na were purchased from Gibco. Fetal bovine serum was obtained from Sailing Biological Engineering Materials (Shanghai, China). Antibodies against β-actin were from Abcam (Cambridge, UK), and other antibodies were purchased from Santa Cruz Biotechnology (CA, USA). All the other chemicals were of high purity from commercial sources. The siRNAs specific for human beclin-1 and atg-7 were purchased from Sigma (Shanghai, USA).

Cell culture

Human cancer cell lines U251 and LN229 were obtained from Cancer Cell Repository (Shanghai cell bank) and ATCC (USA). Cells were maintained in DMEM medium (Gibco, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, antibiotics (100 U/ml penicillin and 100 U/ml streptomycin), at 37 °C in a humidified atmosphere of 5% CO2.

Infection of U251 cells with Ad-ING4-GFP or ad-GFP

Previous study showed that the optimal MOI for a maximal transgenic expression of U251 cells or LN229 is 200 [5]. The GFP was examined by fluorescence microscopy. PCR detected the expression of ING4 as previously described [5].

Acidic vesicular organelles (AVO) labeled by monodansylcadaverine (MDC)

U251 or LN229 cells (2.4 × 105) were seeded into 24-well plates and cultured for 24, then Ad-ING4-GFP, Ad-GFP, or PBS were added. After 48 h, the cells were incubated with 50 μmol/L MDC at 37 °C for 15 min, then washed with PBS for 3 times, 5 min every time. The cellular fluorescent changes were observed using fluorescence microscope and FACS.

Flow-cytometric analysis of apoptosis

Apoptosis was analyzed by measuring sub-G1 peaks on a flow cytometer after cells were fixed with ethanol and stained with propidium iodide [21].

Silencing of Beclin 1/atg-7 genes by siRNAs

The same number of cells was seeded in each dish (100 mm) on the first day and incubated at 37 °C and 5% CO2. On the second day, cells (with 50–70% confluency) were transfected with 50 nM siRNA (scramble, beclin-1, atg-7). Transfection of siRNA into cells followed the Invitrogen protocols with some modifications. On the third day, cells from each dish were split in six-well plates with the same amount of cells in each well, and were infected...
with Ad-ING4 or Ad-lacZ after cell attachment. 72 h after culture, cells were harvested and analyzed.

**Western blot analysis**

After incubated by Ad-ING4, Ad-lacZ or PBS, both adherent and floating cells were collected, and then western blot analysis was carried out by the method as described previously [19].

**Mitochondrial transmembrane potential (MMP) assay**

Mitochondrial transmembrane potential were determined by a fluorescent dye, JC-1. Cells were seeded in 96-well plates or chamber slides and were treated with Ad-ING4 for 48 hours later. Following JC-1 staining (2.5 μg/ml of JC-1 at 37 °C for 30 min), cells were observed by fluorescence microscope. The fluorescence intensity was detected by fluorescence spectrometry (Spectra MAX GEMINI, Molecular Devices, USA). Set the excitation wavelength at 488–490 nm and the emission wavelengths to 527 nm for green fluorescence and 590–600 nm for red fluorescence. Using the dual fluorescence characteristic of the dye, the changes in the MMP can be most accurately assessed by comparing the ratios of 590–600 nm (red)/527 nm (green) ODs.

**Determination of intracellular reactive oxygen species (ROS)**

ROS was measured with the non-fluorescent probe 2,7-dichlorofluorescin diacetate (DCFH-DA). DCFH-DA can passively diffuse into cells and be deacetylated by esterase to form non-fluorescent 2,7-dichlorofluorescin (DCFH). In the presence of ROS, DCFH reacts with ROS to form the fluorescent product DCF, which is trapped inside the cells. The cells were washed with ice-cold 1 x PBS and incubated with DCFH-DA at 37 °C for 20 min. Then DCF fluorescence intensity was detected by fluorescence spectrometry (Spectra MAX GEMINI, Molecular Devices, USA) (Ex 488/Em 535). Cells were observed by fluorescence microscope. The results were expressed as relative fluorescence intensity per 10^4 cells.

**Transfection of U251 cells with plasmid pdsred1n1 or RFP-LC3**

U251 cells were transfected with 1 μg of pdsred1n1 or RFP-LC3 expressing plasmid in each slide in 24-well plates using lipofectamine as the manufacturer's instructions (Invitrogen). After 4 h, the medium was refreshed and cells were treated with Ad-ING4, the fluorescence of adenovirus or plasmid was viewed under confocal microscope (Carl Zeiss, LSM 700).

**Statistic analysis**

Comparisons were made by one-way analysis of variance (ANOVA) Differences were considered to be significant when \( P < .05 \). All experiments were repeated at least three times.

**Fig. 1** – ING4 induces glioma cell death. (A) U251 cells were infected with Ad-GFP, Ad-ING4-GFP respectively, and examined under by fluorescence microscopy (100 x ). (B) RT-PCR analyzed the expression of ING4 in U251 cells. Total RNA was obtained from U251 cells infected with Ad-GFP, Ad-ING4-GFP. The first strand cDNA was synthesized from RNA using reverse transcriptase, and PCRs were conducted using primer sets for ING4 and the housekeep gene β-actin used as control. (C) U251 or LN229 cells were exposed to Ad-ING4-GFP, Ad-lacZ or PBS. Cell death was determined by typan blue staining using FACS in indicated time points. Bar is 50 μm.
Results

**Ad-ING4 induces glioma cell death**

To evaluate the infection efficiency of glioma cells to Adenovirus, U251MG or LN229 cells were infected with Ad-GFP, Ad-ING4-GFP, Ad-ING and Ad-lacZ at MOI of 200 respectively, and examined by fluorescence microscopy. As shown in Fig. 1A, more than 95% GFP expression was found in U251 cells transfected with Ad-GFP and Ad-ING4-GFP. To assess ING4 expression, the total RNA extracted from infected U251MG cells was subjected to RT-PCR. As shown in Fig. 1B, a significant amount of ING4 expression was found in Ad-ING4-GFP, but not in Ad-GFP and the control U251 cells, the data from LN229 unshown.

To evaluate the cytotoxic effect of the ING4 on cell death, U251 or LN229 cells were cultured in presence or absence of Ad-ING4 or Ad-lacZ. The tumor cell growth was examined routinely at different time points (24, 48, 72 h) by FACS using typan blue staining. Ad-ING4 significantly induced U251 and LN229 cell death in a time-dependent way, compared to the control (PBS treated as control group) and Ad-lacZ. (Fig. 1C), indicating that

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**Fig. 2** – ING4 induces autophagy in glioma cells. (A) LC3 and (B) Beclin-1 expression was detected by western blotting in U251MG and LN229 infected with Ad-ING4 or Ad-lacZ or PBS for 72 h. β-actin was used as a loading control. (C) U251 cells were transfected using pcDNA3.1 LC3-REP, and after 24 h, were treated with PBS, Ad-lacZ, Ad-ING4. Cells were observed under the fluorescence microscope. (D) Cells were treated with Ad-ING4, Ad-lacZ at MOI of 200 or PBS for 72 h. After treatment, the cells were stained with MDC fluorescent dye. MDC staining was analyzed by flow cytometry, and observed under the fluorescence microscope (E). Bar is 50 μm.
transgenic ING4 expression efficiently resulted in glioma cell death in vitro.

**Ad-ING4 induces glioma cell autophagy**

To explore the potential mechanism of Ad-ING4 induced-glioma cell death, we analyzed the autophagy levels in U251MG or LN229 cells treated with Ad-ING4, Ad-lacZ and PBS for 72 h. It is usually considered that the conversion of soluble LC3-I to lipid bound LC3-II is associated with the formation of autophagosomes, and tracking the conversion of LC3-I to LC3-II is indicative of autophagic activity [21]. Thus, we first detected the expression level of LC3-I (18 kDa) and LC3-II (16 kDa) by western blotting. We found that the conversion of LC3-I to LC3-II was significantly increased in Ad-ING4 group, while there was hardly conversion in the other two groups (Fig. 2A). Besides the increased conversion of LC3-I to LC3-II, another autophagy marker Beclin-1 was also markedly increased by Ad-ING4 (Fig. 2B) in both U251MG and LN229 cells. To directly view the change of autophagy levels induced by Ad-ING4, cells were transfected with RFP/RFP-LC3 plasmid. The fluorescence of RFP-LC3 was viewed under a fluorescent microscope. RFP-LC3-labeled vacuoles (autophagosomes) were obviously increased in Ad-ING4 group relative to control and Ad-lacZ group (Fig. 2C).

Autophagy is also characterized by the formation of acidic vesicular organelles (AVOs) (autophagosomes and autolysosomes) [21]. Thus, we subsequently quantitatively detected the AVOs using FACS through staining of MDC. The result indicated that the percentage of MDC-positive cells induced by Ad-ING4 was 53.19%, more twice or three times than the other two groups (Fig. 2D). We further observed AVO formation under a fluorescence microscope. There were significantly higher fluorescent density and more MDC-labeled particles in U251 cells in Ad-ING4 group, compared with control and Ad-lacZ group (Fig. 2E). The above results confirmed that ING4 can significantly induce autophagy levels in glioma cells. However, we were interested to know the roles of ING4-induced autophagy in glioma cell death.

**Autophagy contributes to Ad-ING4 induced-glioma cell death**

Recent evidence indicates that autophagy is a double-edged sword, cytoprotective and cytotoxic autophagy [12,14]. To clarify the effects of ING4-induced autophagy in glioma cells, we used the inhibitor of autophagy 3-MA to treat glioma cells, and found 3MA could significantly decrease the percentage from 53.19% to 18.1% in Ad-ING4 group (Figs. 2D, 3A). The relative quantitative result was shown in Fig. 3B. Subsequently, we focused on a question whether 3-MA could rescue ING4-induced cell death. Thus, we analyzed the cell death in infected glioma cells treated with or without 3 MA. Actually, 3-MA dramatically decreased ING4-induced cell death in time-dependent manner (24, 48, 72 h) in both U251MG and LN229. However, 3-MA only rescued ING4-cell death by 35% in 72 h, which is different from control group.

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**Fig. 3** – ING4 induces autophagic cell death in glioma cells. (A) U251 cells were treatment with 3-MA(5 mM) and infected with Ad-ING4, Ad-lacZ or PBS for 72 h, MDC staining analyzed by flow cytometry. Relative quantification of MDC positive cells was shown in (B). (C) U251 cells were treatment with 3-MA (5 mM), and infected with Ad-ING4, Ad-lacZ or PBS in indicated time points, and then the cell death was analyzed by flow cytometry using Typan blue staining. (D) U251 cells were treated as above decribed, and cell death was analyzed as above.
and Ad-lacZ group (Fig. 3C and D). As previous studies suggest that ING4 can to different extent induce cell apoptosis, we speculated that cell apoptosis might in part contribute to ING4-induced cell death.

**Beclin-1 and ATG-7 knockdown rescue glioma cell death induced by ING4**

To understand the relationship between autophagy and apoptosis in ING4-induced glioma cell death, we investigated whether there was the synergistic or additive effect between autophagy and apoptosis on cell death. First, we used 3-MA or/and zVAD (caspase inhibitor) to treat U251 and LN229 cells, cell death was analyzed by FACS. The result indicated that both 3-MA and zVAD could to different extent inhibited the cell death in U251MG and LN229 cells, whereas combination of 3-MA and zVAD almost absolutely inhibited cell death induced by ING4 (Fig. 4A), indicating that there had additive contribution between apoptosis to cell death. To further confirm the above result, we subsequently detected the effect of 3-MA on apoptosis levels by analyzing subG1 using FACS. In spite of 3-MA treatment, the percentage of subG1 still maintained 20–25% in both U251 MG and LN229 cells infected with Ad-ING4, 5-FU was used as a positive control. The result further proved that autophagy had no significant effect on ING4-induced cell apoptosis (Fig. 4B), which suggested that ING4 could induced parallel pathway of cell death in glioma cells.

As 3-MA is a non-specific autophagy suppressor that inhibits PI3K-III (autophagy inducer) and PI3K-I (autophagy suppressor) [21], the autophagy regulators Beclin-1 or ATG-7 siRNA was used to treat glioma cells to confirm that autophagy contributes to cell death. To further confirm the above result, we subsequently detected the effect of 3-MA on apoptosis levels by analyzing subG1 using FACS. In spite of 3-MA treatment, the percentage of subG1 still maintained 20–25% in both U251 MG and LN229 cells infected with Ad-ING4, 5-FU was used as a positive control. The result further proved that autophagy had no significant effect on ING4-induced cell apoptosis (Fig. 4B), which suggested that ING4 could induced parallel pathway of cell death in glioma cells.

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**Fig. 4** – Beclin-1 or ATG-7 knockdown rescue ING4 induces autophagic cell death in glioma cells. (A) U251 or LN229 cells were treated with 3-MA (5 mM) and/or zVAD (10 mM) or PBS, and infected with Ad-ING4 for 72 h, and then the cell death was analyzed by flow cytometry using Trypan blue staining. (B) U251 or LN229 cells were treated with 3-MA (5 mM) or PBS infected with Ad-ING4 or Ad-lacZ for 72 h, and then the cell subG1 was analyzed by flow cytometry using PI staining. (C) Cells were transfected with Beclin-1 siRNA or Scramble or PBS, and then infected with Ad-ING4, after treatment for 72 h, cell lysis was subjected to WB with Beclin-1 antibody. (D) Cells were transfected with ATG-7 siRNA or Scramble or PBS, and then infected with Ad-ING4, after treatment for 72 h, cell lysis was subjected to WB with ATG-7 antibody. Mitochondrial dysfunction induced by Ad-ING4. (E) Cells were transfected with Beclin-1 or ATG-7 siRNA or Scramble, and then infected with Ad-ING4, after treatment for 72 h, MDC staining analyzed by flow cytometry. Relative quantification of MDC positive cells was shown. (F) Cells were treated as above, and cell death was analyzed by flow cytometry using Trypan blue staining. *P<0.01 vs. control group.
death induced by ING4. The efficiency of RNA interfering was determined using WB. We found that Beclin-1 or ATG-7 protein levels were significantly downregulated compared with control and scramble in both glioma cells infected with Ad-ING4 (Fig. 4C and D). The autophagy levels were further analyzed by detecting AVOs using FACS, and significantly inhibited by Beclin-1 or ATG-7 siRNA in glioma cells infected with Ad-ING4 (Fig. 4E). Finally, the cell death was examined in above cells, and the result was consistent with that of 3-MA inhibition (Fig. 4F). The above data indicated that autophagy contributed to ING4-induced glioma cell death, which is a parallel cell death pathway to apoptosis in above process described.

**Ad-ING4 induces mitochondrial dysfunction**

Mitochondria have an essential role in autophagosome biogenesis. ROS of mitochondrial origin plays a regulatory role for as signaling molecules in autophagy under different circumstances, leading to either survival or cell death [19]. To examine whether ING4 affected the mitochondria function during the autophagy process, we detected the changes of intracellular ROS under fluorescence microscope. ROS was increased in U251 cells after treatment with Ad-ING4 for three days, as shown in Fig. 5A. And then DCF fluorescence intensity was detected by fluorescence spectrometry. It was found that ROS formation increased significantly as detected by higher fluorescence intensity compared with control group in both glioma cells (p < 0.01) (Fig. 5B).

Next, we detected the potential of mitochondrial membrane using JC-1 staining. The decreased red fluorescence intensity and increased green fluorescence intensity of JC-1 was observed by fluorescence microscope (Fig. 5C), and the relative ratio red to green of fluorescence intensity was detected by fluorescence spectrometry, and found that the ratio markedly was decreased in Ad-ING4 by 25%-35%, compared with that of control and

![Image](image-url)

**Fig. 5** – Ad-ING4 induces mitochondrial dysfunction. (A) ROS was measured with the non-fluorescent probe DCFH-DA after treatment with Ad-ING4, Ad-lacZ or PBS for 72 h. The DCF in cells was observed under fluorescence microscope. (B) The relative levels of DCF fluorescence were quantified by fluorescence spectrometry. Values are the mean ± SD (n = 3) of fluorescence intensity for 10⁴ cells (FAU: fluorescence arbitrary units). ** P < 0.01 vs. control group. (C) ING4 induced MMP collapse. Cells were treated with Ad-ING4, Ad-lacZ or PBS for 48 h. After treatment, the cells were stained with JC-1 fluorescent dye and observed under the fluorescence microscope. (D) The relative levels of red and green fluorescence were quantified by fluorescence spectrometry. The relative ratio of red to green was shown. Values were the mean ± SD (n = 3) of fluorescence intensity for 10⁴ cells. ** P < 0.01 vs. Control group. (E) Decrease in number of mitochondrial induced by Ad-ING4. U251 cells were transfected with pdsred1-n1 (mitochondria marker). Photos were taken by confocal Fluorescence microscope 48 h after Ad-ING4-GFP infection. (F) Relative quantitation of mitochondrial number was shown. Values were the mean ± SD (n = 3) of fluorescence intensity for 10⁴ cells. ** P < 0.01 vs. Control group. Bar is 50 μm.
Ad-ING4 by 100–90% (Fig. 5D). The result indicated that Ad-ING4 resulted in depolarization of mitochondrial membrane in glioma cells.

Mitophagy is an autophagic process that degrades mitochondria, and can prevent or slow the accumulation of malfunctioning mitochondria, which are thought in turn to underlie central aspects of the aging process in eukaryotic organisms [19]. In order to know whether mitochondria plays an important role during the above process, we transfected U251 with pdsred1mito (red fluorescence protein, RFP) to label mitochondria, and then Ad-ING4-GFP was added 4 h after transfection. As shown in Fig. 5E, less RFP particles were found in U251 cells treated with Ad-ING4-GFP. Relative quantitative result was shown in Fig. 5F. Ad-ING4 significantly led to decrease in mitochondria volume. It is implied that Ad-ING4 might induce mitophagy and result in increase in autophagy levels in glioma cells.

**Ad-ING4 induces autophagy and apoptosis via the mitochondria-mediated pathway**

Since Bcl-2 family members play the vital role in the regulation of the mitochondrial apoptotic and autophagy pathway [22,23], the levels of Bax/Bcl-2 and Beclin-1 were detected by Western blot in Fig. 6A, after treatment with Ad-ING4, the levels of Bax were increased in time-dependent manner and the decreased trend of Bcl-2 expression was observed. On the contrary, the levels of Beclin-1 were increased in time-dependent manner, which was consistent with the profile of ING4-induced glioma cell death above described (Fig. 6A and B). The above result also further confirmed that both autophagy and apoptosis contributed to ING4-induced cell death in glioma cells. Interestingly, it is noted that Bcl-2 was a little upregulated at 48 h after infection of glioma cells with Ad-ING4, suggesting that ING4 overexpression probably induced stress response of survival signal and could not affect autophagy levels, but further secondarily improved autophagy levels.

Additionally, we investigated the involvement of apoptosis protein caspase-8, caspase-9 and caspase-3 in ING4-induced cell death. As shown in Fig. 5B, the active forms of caspase-3, caspase-8 and caspase-9 were to lower extent increased during Ad-ING4 treatment. These results indicated that ING4 induced lower levels of apoptosis of glioma cells, which also contributed to cell death along with autophagy. The data further confirmed that Ad-ING4 induced cell death via the mitochondria-mediated pathway, consistent with above described.

**Discussion**

ING4, as a novel member of the ING family, has a potential effect on tumor inhibition via multiple pathways. Recent studies have demonstrated that Adenovirus-mediated ING4 suppresses different tumor cell growth via induction of cell-cycle alteration, apoptosis, and inhibition of tumor angiogenesis [4–7]. ING4 is also involved in regulating brain tumor growth and angiogenesis. Expression of ING4 is significantly reduced in gliomas as compared with normal human brain tissue, and the extent of reduction correlates with the progression from lower to higher grades of tumors [4,24]. Therefore, ING4 is characterized as a candidate tumor suppressor gene in gliomas. Indeed, it is reported that ING4 can induce higher inhibition of cancer cell growth by 70–80% and lower apoptosis levels by 25–30%. However, the mechanism is unknown in above process. In current study, we actually found that ING4 induced glioma cell death, consistent with previous studies [4–7]. Interestingly, in addition to apoptosis, we also found that autophagy could contribute to ING4-induced glioma cell death, which might be a mechanism to explain the inhibition effect of ING4 on tumor cell growth. Although autophagy can promote the survival of cancer cells in some cases [11,12], autophagy can also potentially lead to cell death [24]. Our finding at the first time provides a new pathway of cell death induced by tumor suppressor gene, which might be a new tumor therapeutic approach.

Although the precise mechanism of autophagy remains to be elucidated, Mitochondria plays an important role in autophagy during programmed cell death and disease [19,21,25,26], including the mitochondrial permeability transition [27], generating of ROS [19]. The reactive oxygen species (ROS) that originated from mitochondrial oxidative stress seems to play a central role [20,25]. In this study, we found that ING4 stimulated production of ROS.
and mitochondrial dysfunction, which indicates that these oxygen radicals are in part toxic and causes loss of mitochondrial viability. In addition, it has been speculated that dysfunction of autophagy may result in abnormal mitochondrial function and oxidative or nitrative stress [28,29]. Nevertheless, it is an interesting topic on cross-talk among autophagy, mitochondria and oxidative stress. In this study, we find that mitochondria are involved in the autophagic cell death induced by Ad-ING4.

In some circumstances, there are multiple connections between apoptotic and autophagic processes that can jointly determine the fate of tumor cells [30,31]. In this study, we found that the long-term treatment of ING4 could induce both autophagy and apoptosis parallel pathways of cell death in glioma cells. Recently, studies confirmed that Bcl-2 plays an important role not only in apoptosis, but also in autophagy [31–35]. Beclin-1 has an N-terminal BH3 domain and is considered to fall into this third subgroup of the Bcl-2 family [33,34], and can interact with Bcl-2/Bcl-xL to inhibit the formation of autophagosome. Compared to these studies, we also found that ING4 could downregulate Bcl-2 and upregulate Bax protein expression, and simultaneously upregulate the Beclin-1 expression, which therefore decreases the probabilities of interaction Beclin-1 and Bcl-2/Bcl-xL and promote apoptosis and autophagy. Accordingly, we proposed that the ING4 induced cell death induced might mainly result from Bcl-2 down-regulation and Beclin-1 up-regulation via ING4 nuclear signal, which mechanism still remains unknown. In addition, caspase and autophagic activities may share complementary roles during the regulation of different types of cell death [36]. First, caspase family cysteine proteases, like ‘molecular switches’, play the essential roles in the induction and execution of apoptosis. When apoptosis occurs, a series caspase would be activated. On the other hand, it is reported that caspase 8 and caspase 10 played a crucial role in apoptotic pathways of cell death in glioma cells. Recently, it has been speculated that dysfunction of autophagy and mitochondria, which indicates that these oxygen radicals are in part toxic and causes loss of mitochondrial viability. In addition, it has been speculated that dysfunction of autophagy may result in abnormal mitochondrial function and oxidative or nitrative stress [28,29]. Nevertheless, it is an interesting topic on cross-talk among autophagy, mitochondria and oxidative stress. In this study, we find that mitochondria are involved in the autophagic cell death induced by Ad-ING4.

In conclusion, this study clearly indicates that Ad-ING4-induced cell death including autophagy and apoptosis might associate with caspase-family activation.

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