

# Sevoflurane: A dual modulator of miR-211-5p and mitochondrial apoptosis in glioma therapy

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**Abstract.** The present study aimed to investigate how sevoflurane (SEV) regulated the apoptosis of glioma cells through the mitochondrial apoptosis pathway. First, an evaluation was performed on the viability, apoptosis, mitochondrial reactive oxygen species levels, mitochondrial membrane potential and apoptosis and autophagy-related protein expression of glioma cells according to experimental groups. Next, the expression of microRNA-211-5p (miR-211-5p), silent information regulator 1 (SIRT1) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway was detected by reverse transcription-quantitative PCR or western blotting. Dual luciferase reporter gene assay confirmed the targeting relationship between miR-211-5p and SIRT1. In addition, SEV suppressed the proliferation and induced the apoptosis in human glioma cell line cells via the mitochondrial apoptosis pathway. In mechanistic analysis, the miR-211-5p level in glioma cells was low, while following SEV treatment, it was increased. Furthermore, SEV regulated SIRT1 by upregulating miR-211-5p expression, thereby blocking the PI3K/AKT signaling pathway activation. Moreover, functional rescue experiments showed that downregulation of SIRT1 or miR-211-5p could reverse the effects of SEV on glioma cells. Collectively, SEV promoted apoptosis in glioma cells by inducing miR-211-5p, which regulated SIRT1/PI3K/AKT pathway, mediating

mitochondria-dependent apoptosis pathway. This finding may open new possibilities for SEV as a potential treatment for glioma in the future.

## Introduction

Glioma, a common primary tumor in the human central nervous system, is distinguished by its high invasiveness and destructive characteristics. At present, the main clinical treatments of glioma include surgical resection, chemotherapy and radiation therapy (1). However, due to the invasive growth pattern of glioma, the presence of non-regenerative nerve tissue and the critical functions involved, glioma is difficult to surgically resect and has a high recurrence rate. In addition, the existence of blood-brain barrier leads to poor efficacy of glioma chemotherapy. Apparently, traditional treatments for glioma are inadequate in achieving a cure, particularly for high-grade glioma with high nausea (2). Gene therapy and molecular targeted therapy, which have become popular in recent years, can be used in combination with traditional treatments to enhance the inhibitory or killing effect on tumour tissue. This approach effectively addresses metastasis and spread while overcoming the drawbacks of traditional treatments, such as incomplete efficacy, ease of metastasis and significant side effects. In addition to accurately killing cancer cells, the combined treatment can also repair and improve the patient's immune system (3,4). Therefore, a thorough investigation of the cellular and molecular mechanisms underlying glioma pathogenesis and prognosis is crucial for identifying therapeutic targets and developing new treatment strategies.

The occurrence of tumors is not only the result of malignant cell proliferation, but also closely related to the apoptosis disorder of tumor cells. The mitochondrial pathway (intracellular apoptosis pathway) plays a crucial role in apoptosis, serving as one of the key signal cascades that control the apoptosis of tumor cells (5). It is noteworthy that the apoptotic signaling cascade of abnormal energy metabolism and mitochondrial dysfunction is often observed in malignant gliomas (6,7). Therefore, mediating the mitochondrial pathway to restart tumor cell apoptosis is an important strategy for the treatment of glioma. Moreover, investigating mitochondrial proteomic abnormalities may provide a new therapeutic target for glioma. Silent information regulators (SIRT1s), which have

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**Abbreviations:** SIRT1, silent Information regulator 1; SEV, sevoflurane; miRNA, microRNA; U251, human glioma cell line; NHA, normal human astrocytes; ECL, enhanced chemiluminescence; SIRT1-WT, SIRT1-wild-type; SIRT1-MUT, mutant vector; RISC, RNA-induced silencing complex

**Key words:** glioma, sevoflurane, microRNA-211-5p, silent information regulator 1, mitochondria-dependent apoptosis, protein kinase pathway, cancer therapy

been discovered recently, play a key role in simultaneously regulating multiple downstream pathways. Silent Information regulators (SIRT1s) can also lead to changes in metabolic and enzyme pathways, or regulate complex mechanisms that result in cell death in gliomas (8). Among SIRT1s family members, SIRT1 is widely regarded as a key epigenetic regulator. It is involved in a number of biological processes, including metabolism, genome stability maintenance, reprogramming, autophagy, aging, mitochondrial apoptosis and tumourgenesis (9). It has been confirmed that SIRT1 is highly expressed in glioma tissues and silencing SIRT1 gene can hinder the occurrence of tumors (10). The present study also noted that SIRT1 can inactivate the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway in a deacetylase-dependent manner (11). The PI3K/AKT pathway, a known negative regulator of glioma (12,13), plays an important role in blocking mitochondrial apoptosis. Consequently, thoroughly examining how SIRT1 modulates the PI3K/AKT pathway to mediate mitochondrial apoptosis in glioma cells could offer a novel targeted approach for glioma therapy.

In recent years, the influence of anesthetics on the prognosis of surgical cancer treatment has received extensive attention. Sevoflurane (SEV) is a commonly used inhalation anesthetic in clinics. In addition to its good anesthetic effect, SEV is found to have anti-cancer effects in a variety of cancers, including glioma (14-16). Specifically, SEV limits the progression of glioma by regulating the balance between proliferation and apoptosis of glioma cells (17). In other diseases, SEV has been found to modulate tumor progression through the mitochondrial apoptosis pathway (18). However, few studies have investigated the effect of SEV-mediated mitochondrial apoptosis pathway on apoptosis of glioma cells. Furthermore, a previous study has shown that SEV inhibits the development of glioma by regulating the circular RNA\_0079593 (circ\_0079593)/microRNA-633 (miR-633)/rho associated coiled-coil containing protein kinase 1 axis (19). Additionally, SEV also suppresses the malignant phenotype of glioma by regulating the miR-146b-5p/nuclear factor I/B axis (20). Further research indicates that SEV impedes glioma progression by modulating the circ\_0037655/miR-130a-5p/ribophorin II axis and the circ\_0000215/miR-1200/natural killer cell cytotoxicity receptor 3 ligand 1 axis (21). However, the precise anti-cancer molecular mechanisms of SEV in glioma require further elucidation. The important regulatory functions of miRNA in tumors have been comprehensively studied. A previous study showed that SEV can promote neuronal apoptosis by inducing miR-211-5p expression (22). As a mature sequence of miR-211, miR-211-5p is identified to be under-expressed in gliomas and involved in the regulation of apoptosis pathways in glioma cells (23). Based on this, it was hypothesized that SEV might play an anticancer role in glioma by inducing miR-211-5p expression. Importantly, the online bioinformatics analysis databases StarBase, PITA and miRmap predicted that miR-211-5p and SIRT1 have targeted binding sites and miR-211-5p may target SIRT1 to regulate the PI3K/AKT pathway and mediate the role of SEV in glioma progression.

Based on previous studies, the present study hypothesised that SEV promotes apoptosis in glioma cells through the induction of miR-211-5p, which in turn regulates the SIRT1/

PI3K/AKT pathway and mediates mitochondria-dependent apoptosis. The present study identified the role and potential molecular regulatory mechanisms of SEV in mitochondrial apoptosis of glioma cells. Although the present study only examined short-term effects, it may also open up new possibilities for SEV as a potential treatment for glioma in the future.

## Materials and methods

**Cellular model.** Human glioma cell line (U251, IM-H421, IMMOCELL) and normal human astrocyte (NHA, IMP-H223, IMMOCELL) were obtained from the American Type Culture Collection and cultured according to the corresponding instructions. All cell lines were confirmed by short tandem repeat analysis and mycoplasma contamination detection.

**Experimental grouping and administration.** For SEV exposure, the U251 cells were seeded onto a plate and cultured overnight at 37°C, then the cell plate was placed in a closed glass chamber connected to the anesthesia machine. SEV was fed into the chamber using an anesthetic carburetor and the concentration of SEV was continuously monitored by a gas monitor. Treated with SEV at different concentrations (0, 2, 4 and 6%), U251 cells were randomly divided into Control group, 2% SEV group, 4% SEV group and 6% SEV group. Upon treatment with the optimal concentration of SEV at different times (0, 4, 6 and 8 h), U251 cells were randomly divided into the Control group, the 4 h group, the 6 h group and the 8 h group. According to cell viability and apoptosis results, 6% sevoflurane (100 nM) and an induction time of 8 h were selected as the optimal conditions for subsequent experiments. miR-211-5p mimic (100 nM), miR-211-5p inhibitor (in-miR-211-5p; 100 nM), SIRT1-targeted small interfering (si)RNA (50 nM), SIRT1 overexpression plasmid (1 µg) and their negative controls (NC; miR-NC, in-NC, si-NC and pcDNA 3.1) were used at a concentration of 50 nM for siRNAs and 1 µg for plasmids, obtained from Guangzhou Ruibo Co., Ltd. When the cells adhered to the wall and the cell confluence reached 80%, the aforementioned plasmids and RNA were transfected into U251 cells using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the experimental groups. The transfection process was carried out at 37°C for 4 h. After transfection, the cells were incubated in culture medium for 24 h and then subjected to subsequent experiments. The negative controls used in the experiments included scrambled or non-targeting sequences for siRNA (si-NC); controls for miRNA mimics and inhibitors (miR-NC, in-NC); and an empty vector for overexpression plasmids (Tables I and II).

Cells in logarithmic growth stages were selected for experimental intervention and grouping. They were divided into 10 groups: the Control group (normal control group), the SEV group (cells treated with SEV), the SEV + in-NC group (cells treated with SEV + transfection inhibitor negative control), the SEV + in-miR-211-5p group (cells treated with SEV + transfection miR-211-5p inhibitor), the SEV + pcDNA 3.1 group (cells were treated with SEV + transfection of blank overexpressed plasmid), the SEV + pcDNA-SIRT1 group (cells treated with SEV + transfection of SIRT1 targeted overexpressed plasmid), the si-NC group (transfection of si-NC),

Table I. miR-211-5p transfection sequences.

| Gene                 | Primer sequences (5'-3')                                                          |
|----------------------|-----------------------------------------------------------------------------------|
| miR-211-5p-mimic     | Forward: 5'-UUCCCUUUGUCAUCCUUCGCCT-3'<br>Reverse: 5'-GCGAAGGAUGACAAAGGGAANN-3'    |
| mimic-NC             | Forward: 5'-UCACAACCUCUAGAAAGAGUAGA-3'<br>Reverse: 5'-UCUACUCUUUCUAGGAGGUUGUGA-3' |
| miR-211-5p-inhibitor | AGGCGAAGGAUGACAAAGGG                                                              |
| inhibitor-NC         | UCUACUCUUUCUAGGAGGUUGUGA                                                          |

miR, microRNA; NC, negative control.

Table II. SIRT1 transfection sequences.

| Gene          | Primer sequences (5'-3')                                                                                                                                           |
|---------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| SIRT1 siRNA-1 | Forward: 5'-TTAAAAGTGGTTTTTTGTGTTTTCAAGAGAAACACAAAAAACCACTTTTAA-3'<br>Reverse: 5'-CACAAAAAACCACTTTTAAATAAGTTCTCTATTTAAAAGTGGTTTTTTGTG-3'                           |
| SIRT1 siRNA-2 | Forward: 5'-ATTTAAAAGTGGTTTTTTGTGTTCAAGAGACACAAAAAACCACTTTTAAAT-3'<br>Reverse: 5'-CAAAAAAACCACTTTTAAATGGAAGTTCTCTCCATTTAAAAGTGGTTTTTTG-3'                          |
| SIRT1 siRNA-3 | Forward: 5'-AAACATAAATGTTTAGTCCGTTTCAAGAGAACGGACTAAACATTTATGTTT-3'<br>Reverse: 5'-GGACTAAACATTTATGTTTCAAAGTTCTCTTGAAACATAAATGTTTAGTCC-3'                           |
| si-NC         | Forward: 5'-CACCGTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCCGGAGAA<br>TTTTTTG-3'<br>Reverse: 5'-GATCCAAAAAATTCTCCGAACGTGTCACGTTCTCTTGAAACGTGACACGTTCCG<br>GAGAAC-3' |

SIRT1, silent information regulator 1; si, small interfering; NC, negative control.

the si-SIRT1 group (transfection of SIRT1-targeted siRNA), the si-SIRT1 + in-NC group (negative control transfected with SIRT1-targeted siRNA + inhibitors) and the si-SIRT1 + in-miR-211-5p (transfection with SIRT1-targeted siRNA + miR-211-5p inhibitors).

**Reverse transcription-quantitative (RT-q) PCR.** The RNA extraction was performed using the RNA extraction buffer (Vazyme Biotech Co., Ltd.) with a cell density of  $1 \times 10^6$  cells/well. The concentration and purity of RNA was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. HiScript III 1st Strand cDNA Synthesis Kit (Vazyme Biotech Co., Ltd.) was used for RT according to the manufacturer's protocol. qPCR was conducted using Taq Pro Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd.) under the following conditions: 95°C for 5 min (pre-denaturation), followed by 40 cycles at 95°C for 10 sec (denaturation) and 60°C for 1 min (annealing/extension). U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as internal controls for miR-211-5p and messenger RNA (mRNA) normalization, respectively. The primer sequences are listed in Table III. The relative transcript levels were analyzed by the  $2^{-\Delta\Delta C_q}$  method (24).

**Western blotting.** Protein was extracted from cell precipitates using a radioimmunoprecipitation assay lysis buffer containing

protease inhibitors (Beyotime Institute of Biotechnology). Protein concentration was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. A total of 30  $\mu$ g protein was loaded per lane and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (NCM Biotech) on 10% gels. The separated proteins were then transferred onto a polyvinylidene fluoride membrane. The membrane was blocked with 5% skimmed milk at room temperature for 2 h and then incubated with primary antibodies at 4°C overnight. Subsequently, the membrane was incubated with secondary antibodies (1:10,000; cat. no. bs-0295G-HRP; BIOSS) at room temperature for 2 h. Protein bands were visualized using enhanced chemiluminescence (NCM Biotech) and semi-quantified using ImageJ software (version 1.53; National Institutes of Health). GAPDH was used as a loading control. Primary antibodies were: B-cell lymphoma-2 (Bcl-2)-associated X protein (Bax) (cat. no. 50599-2-Ig; 1:8,000; Wuhan Sanying Biotechnology); cytochrome c (cat. no. 10993-1-AP; 1:4,000; Wuhan Sanying Biotechnology); cleaved-caspase-3 (cat. no. ab32042; 1:2,000, Abcam); caspase-3 (cat. no. ab184787; 1:2,000, Abcam); caspase-9 (cat. no. ab184786; 1:2,000, Abcam); caspase-9 (cleaved Asp330) antibody (cat. no. PA5-105272; 1:1,000; Invitrogen; Thermo Fisher Scientific, Inc.); Bcl2 antibody (cat. no. 12789-1-AP; 1:9,000; Wuhan Sanying Biotechnology); microtubule-associated protein light chain 3 (LC3)

Table III. Primer sequences.

| Gene           | Primer sequences (5'-3')                                                         |
|----------------|----------------------------------------------------------------------------------|
| hsa-miR-211-5p | Forward: 5'-CGCGTTCCTTTGTCATCCT-3'<br>Reverse: 5'-AGTGCAGGGTCCGAGGTATT-3'        |
| SIRT1          | Forward: 5'-TAGCCTTGTTCAGATAAGGAAGGA-3'<br>Reverse: 5'-ACAGCTTCACAGTCAACTTTGT-3' |
| U6             | Forward: 5'-CGACAAGACGATCCGGGTAAA-3'<br>Reverse: 5'-GGTTGAGGAGTGGGTCCAAG-3'      |
| GAPDH          | Forward: 5'-ACAACTTTGGTATCGTGGAAGG-3'<br>Reverse: 5'-GCCATCACGCCACAGTTTC-3'      |

miR, microRNA; SIRT1, silent information regulator 1.

(cat. no. 14600-1-AP; 1:2,500; Wuhan Sanying Biotechnology); P62 (cat. no. 18420-1-AP; 1:10,000; Wuhan Sanying Biotechnology); SIRT1 (cat. no. 13161-1-AP; 1:3,000; Wuhan Sanying Biotechnology); PI3K antibody (cat. no. 20584-1-AP; 1:300; Wuhan Sanying Biotechnology); phosphorylated (p-)PI3K p85/p55 (Tyr458, Tyr199) (cat. no. PA5-17387; 1:1,000; Invitrogen; Thermo Fisher Scientific, Inc.); AKT (cat. no. 10176-2-AP; 1:1,000; Wuhan Sanying Biotechnology); and p-AKT (Ser473) (cat. no. ab81283; 1:2,000; Abcam).

**MTT assay.** The cells in logarithmic growth stage were seeded into a 96-well plate with 2,000 cells per well and 100  $\mu$ l cell suspension was added to each well. Thereafter, MTT solution (5 mg/ml) was added to each well and incubated in the incubator at 37°C for 4 h. The supernatant was then removed and 150  $\mu$ l of dimethyl sulfoxide was added to each well. The plate was oscillated at room temperature for 10 min to fully dissolve the crystals. The optical density value at a wavelength of 570 nm was measured using a microplate reader.

**Flow cytometry.** The cells were seeded into a 6-well plate ( $5 \times 10^5$  cells/well) and administered or transfected as described aforementioned. After 48 h, staining was carried out in the dark at 25°C with 5  $\mu$ l Annexin V-FITC and 10  $\mu$ l propidium iodide for 5 min. The apoptotic cells were analyzed using a flow cytometer (BD FACSCanto II; BD Biosciences) and the data were processed with FlowJo software (version 10.8.1; BD Biosciences). The apoptotic rate was calculated as the percentage of early apoptotic cells plus late apoptotic cells.

**Mitochondrial membrane potential (MMP) determination.** Referring to previous studies, cells ( $2 \times 10^5$  cells/well) were seeded into a 6-well microplate, cultured at 37°C for 24 h and treated with SEV. After 48 h, the staining was performed with JC-1 (Beyotime Institute of Biotechnology) at 37°C and 5% CO<sub>2</sub> for 30 min. Fluorescence intensity was monitored with an EVOS M5000 fluorescence microscope (Thermo Fisher Scientific, Inc.) and MMP levels were assessed by measuring the reduction in the red/green fluorescence intensity ratio.

**Reactive oxygen species (ROS) determination.** Stably transfected cells were uniformly seeded into a 24-well plate with  $5 \times 10^4$  cells per well and cultured for 16-18 h. MitoSOX™ Red stock solution (5 mmol/l; Thermo Fisher Scientific, Inc.) was

prepared in DMSO under light protection, then diluted in phosphate buffered saline buffer (1:1,000) to produce a MitoSOX™ Red working solution (5  $\mu$ mol/l). Next, the MitoSOX™ Red working solution was added to the well at 300  $\mu$ l/well and incubated at 37°C for 10 min in the dark. The culture solution was aspirated and the cells were washed three times. Then, 500  $\mu$ l preheated phosphate buffered saline buffer was added and the plate was immediately placed under a fluorescence microscope (EVOS M5000; Thermo Fisher Scientific, Inc.) for observation and image capture.

**Dual luciferase reporter (DLR) analysis.** In the DLR assay, the binding site between miR-211-5p and SIRT1 was predicted using bioinformatics analysis tools, including StarBase (<https://rnasysu.com/encori/>), PITA ([https://tools4mirs.org/software/target\\_prediction/pita/](https://tools4mirs.org/software/target_prediction/pita/)) and miRmap (<https://mirmap.ezlab.org/>) databases. Based on the predicted binding site, the target gene SIRT1-wild-type (SIRT1-WT) vector and mutant (SIRT1-MUT) vector with miR-211-5p binding sites were designed through an online bioinformation analysis website. The DLR vector plasmid was constructed by Sangon Biotech Co., Ltd. Cells were transfected with the aforementioned vectors and miR-211-5p mimic/mimic-NC using Lipofectamine as the transfection reagent for 24 h at 37°C for 24 h. Luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega Corporation) according to the manufacturer's instructions. *Renilla* luciferase activity was used for normalization.

**Statistical analysis.** Data were analyzed and mapped using GraphpadPrism9 (version 9.5.0; Dotmatics). The diagrams were made using Photoshop (Adobe Systems, Inc.). All data were presented as means  $\pm$  standard deviation; independent samples t-test was used for pairwise comparisons and a one-way analysis of variance followed by Tukey's post hoc test was used for multiple comparisons.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

*Sevoflurane exposure hinders the viability of glioma cells and induces apoptosis.* To explore whether the influences

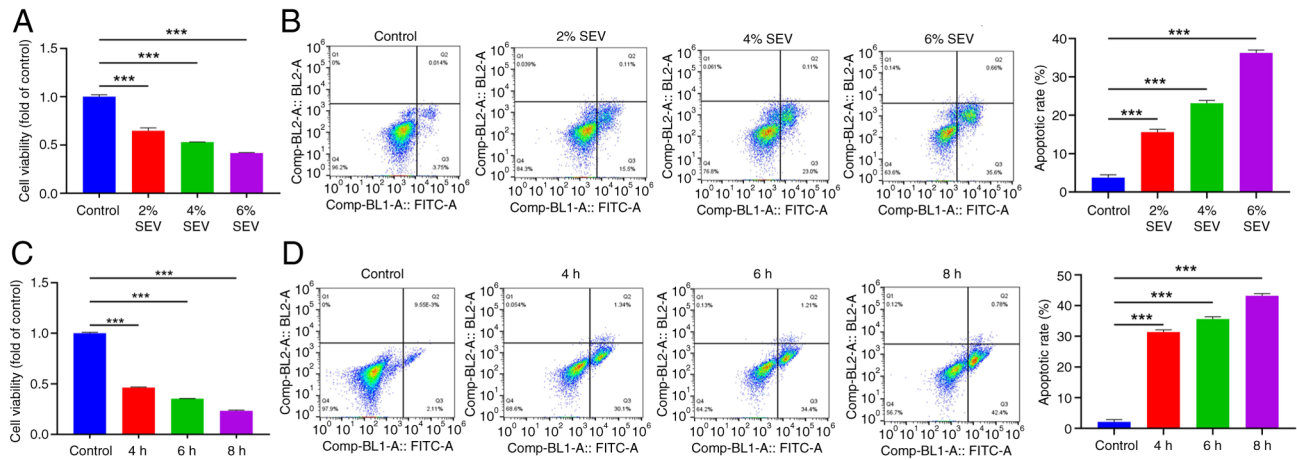


Figure 1. SEV exposure hinders the viability of glioma cells and induces apoptosis. (A) MTT assay was performed to detect SEV at different concentrations (0, 2, 4 and 6%) on the U251 cell viability. (B) Flow cytometry was performed to detect SEV at different concentrations (0, 2, 4 and 6%) on apoptosis of U251 cells. (C) MTT assay was performed to detect SEV at different times (0, 4, 6 and 8 h) on the U251 cells viability. (D) Flow cytometry was performed to detect SEV at different times (0, 4, 6 and 8 h) on apoptosis of U251 cells. \*\*\* $P < 0.001$ ,  $n = 3$ . SEV, sevoflurane.

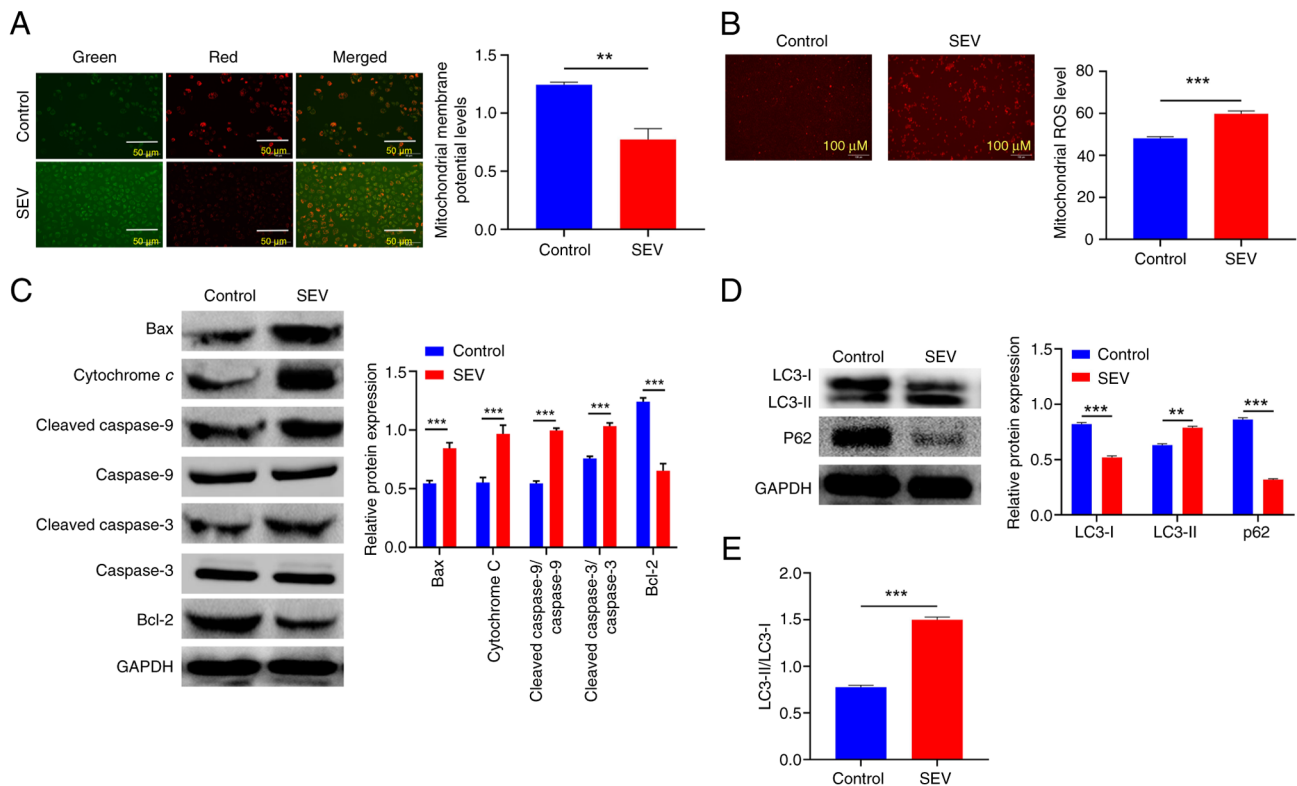


Figure 2. Sevoflurane induces apoptosis of glioma cells by mitochondrial apoptosis pathway. (A) JC-1 staining was performed to detect MMP level. (B) MitoSOX™ Red fluorescence staining was used to detect mitochondrial reactive oxygen species levels. (C) Western blotting was performed to detect the expression of Bax, cytochrome *c*, cleaved-caspase-9/caspase-9, cleaved-caspase-3/caspase-3 and Bcl-2 proteins. (D) Western blotting was performed to detect the expression of LC3-I, LC3-II and p62 proteins; (E) LC3-II/I ratio. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ,  $n = 3$ . MMP, mitochondrial membrane potential; Bax, bcl-2-associated X protein; Bcl-2, B-cell lymphoma-2.

of SEV on glioma cells were concentration-dependent and time-dependent, the present study examined the influences of SEV treatment on glioma cells at different concentrations and times. The results of MTT and flow cytometry revealed that SEV reduced U251 cell viability and promoted apoptosis in a concentration-dependent manner (Fig. 1A and B;  $P < 0.001$ ). Also, SEV decreased U251 cell viability and accelerated apoptosis (Fig. 1C and D) in a time-dependent manner

( $P < 0.001$ ). Therefore, the present study selected the optimal concentration of 6% in the concentration gradient and the optimal induction time of 8 h in the time gradient as the test concentration for subsequent experiments.

*Sevoflurane induces apoptosis of glioma cells by mitochondrial apoptosis pathway.* Further, MMP levels were detected to determine whether SEV can accelerate apoptosis

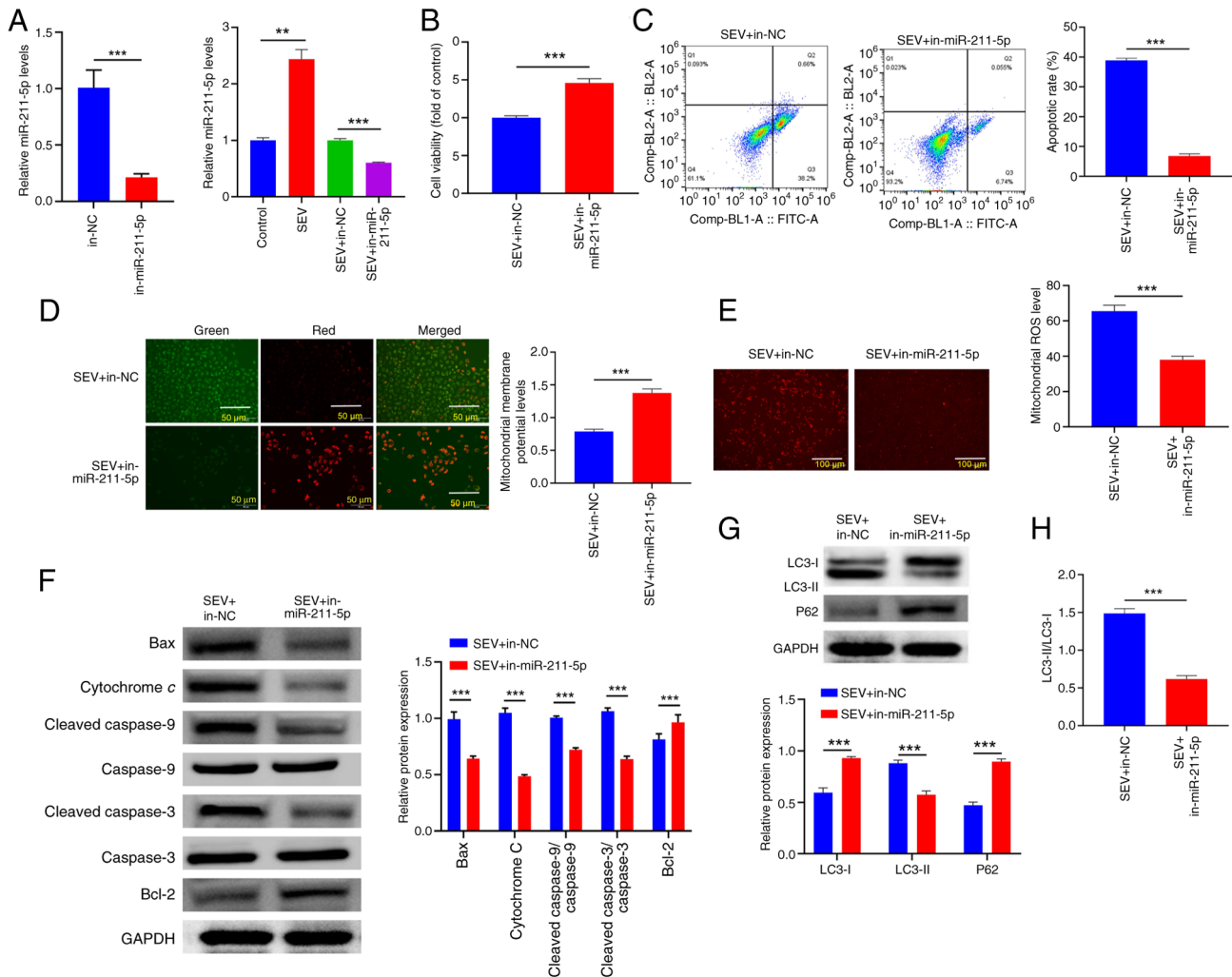


Figure 3. SEV exposure promotes apoptosis of glioma cells through upregulation of miR-211-5p expression, mediating mitochondrial apoptosis pathway. (A) U251 cells were treated with SEV and miR-211-5p mimic/mimic NC, followed by reverse transcription-quantitative PCR was performed to detect miR-211-5p in cells. (B) MTT method was performed to evaluate cell viability, while (C) flow cytometry was performed to detect cell apoptosis. (D) JC-1 staining was performed to detect MMP level. (E) MitoSOX™ Red fluorescence staining was used to detect mitochondrial reactive oxygen species levels. (F) Western blotting was used to detect the expression of Bcl-2, Bax, cytochrome *c*, cleaved-caspase-9/caspase-9 and cleaved-caspase-3/caspase-3 proteins. (G) Western blotting was used to detect the expression of LC3-I, LC3-II and p62 proteins. (H) LC3-II/I ratio. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ,  $n = 3$ . SEV, sevoflurane; miR, microRNA; NC, negative control; MMP, mitochondrial membrane potential; ROS, reactive oxygen species; Bcl-2, B-cell lymphoma-2; Bax, bcl-2-associated X protein.

by mediating the mitochondrial apoptosis pathway. As a key marker of mitochondrial function, MMP is a critical driver of ATP synthesis and a decline in MMP can lead to reduced mitochondrial productivity and even cell death. The present study results showed that after treating U251 cells with SEV, the red fluorescence intensity, representing high MMP, was weakened. Conversely, the green fluorescence intensity, representing low MMP, was enhanced under the same fluorescence intensity ( $P < 0.01$ ). This indicated that SEV interfered with and destroyed MMP, thereby activating the intrinsic apoptosis pathway (Fig. 2A). Since ROS is a major mediator of the mitochondria-mediated apoptosis pathway (19), the present study further investigated mitochondrial ROS levels in U251 cells. This revealed enhanced red fluorescence intensity ( $P < 0.001$ ) after treating U251 cells with SEV, suggesting that SEV increased mitochondrial ROS levels (Fig. 2B). Afterwards, exploration was further performed on the expression of proteins associated with the mitochondrial

apoptosis pathway. The results showed that the expression levels of Bax, cytochrome *c*, cleaved-caspase-9/caspase-9 and cleaved-caspase-3/caspase-3 were significantly increased after the treatment of U251 cells with SEV ( $P < 0.01$ ). The expression level of Bcl-2 was significantly decreased, indicating that SEV promoted mitochondrial apoptosis by regulating the proteins associated with the mitochondrial apoptosis pathway (Fig. 2C). Since the mitochondrial apoptosis pathway was closely related to autophagy, the expression of autophagy-related proteins was further examined. According to the data, U251 cells treated by SEV showed higher LC3-II levels, lower p62 and LC3-I levels. Additionally, the conversion rate of LC3-I to LC3-II was increased (Fig. 2D and E;  $P < 0.01$ ). These results suggested that SEV could induce autophagy of glioma cells. Taken together, these results supported the hypothesis that SEV could induce apoptosis of U251 cells through a mitochondria-dependent apoptosis pathway.

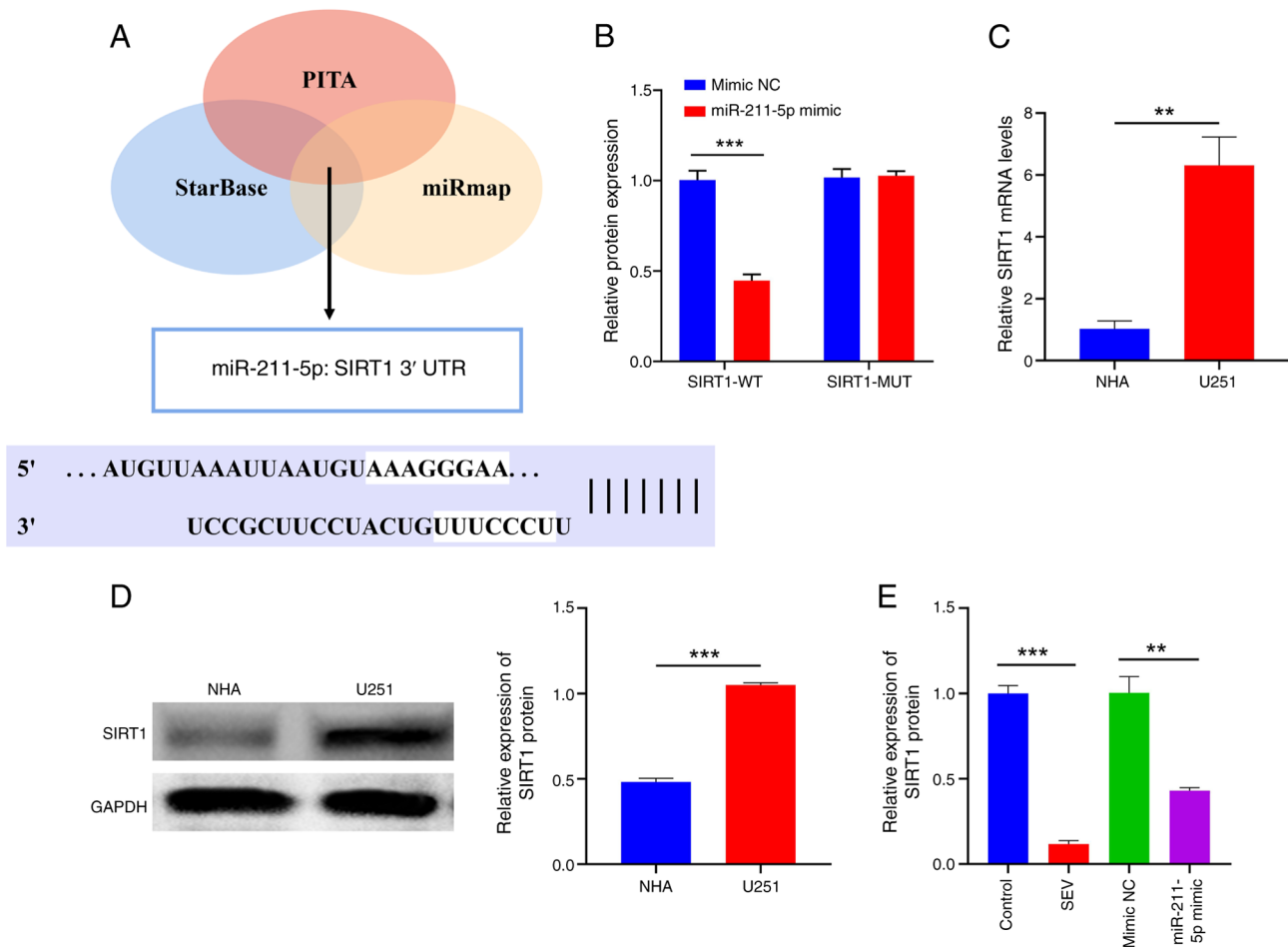


Figure 4. SIRT1 acts as the target of miR-211-5p. (A) Bioinformatics tools, including StarBase, PITA and miRmap, were used to predict the binding site between miR-211-5p and SIRT1. (B) The luciferase activity was used to evaluate the interaction between miR-211-5p and SIRT1. (C) Reverse transcription-quantitative PCR and (D) Western blotting showed SIRT1 expression in NHA and U251 cells. (E) Reverse transcription-quantitative PCR showed SIRT1 expression in U251 cells under different treatments. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ,  $n = 3$ . SIRT1, silent information regulator 1; miR, microRNA; NHA, normal human astrocytes; WT, wild type; MUT, mutant; NC, negative control.

*Sevoflurane exposure promotes apoptosis of glioma cells through upregulation of miR-211-5p expression, mediating mitochondrial apoptosis pathway.* To further clarify whether SEV could mediate the role of miR-211-5p in glioma, RT-qPCR was used to detect the regulatory influences of SEV on miR-211-5p in glioma cells. The results revealed that SEV upregulated miR-211-5p expression. Subsequently, miR-211-5p expression was interfered with in the presence of SEV exposure and transfection efficiency was confirmed by RT-qPCR (Fig. 3A;  $P < 0.01$ ). The results of MTT and flow cytometry assays showed that downregulation of miR-211-5p reversed the inhibitory influence of SEV on the viability of glioma cells and the promotion of apoptosis (Fig. 3B and C;  $P < 0.001$ ). In addition, downregulation of miR-211-5p reversed the influence of SEV on mitochondrial apoptotic pathway-associated proteins, MMP, ROS and autophagy in glioma cells (Fig. 3D-H;  $P < 0.01$ ). To sum up, these data showed that SEV played a role in the malignant phenotype of glioma cells by inducing miR-211-5p.

*SIRT1 serves as the target of miR-211-5p.* In addition, the present study searched for possible downstream mRNA of miR-211-5p. The binding site (Fig. 4A) between miR-211-5p and SIRT1 was predicted by the StarBase, PITA and miRmap

databases. This finding was further confirmed by a DLR gene assay. The data showed that the upregulation of miR-211-5p could depress the luciferase activity of SIRT1-WT, while the activity of SIRT1-MUT was almost unaffected under the same conditions (Fig. 4B) ( $P < 0.001$ ). In addition, SIRT1 was highly expressed in U251 cells compared to normal cells (Fig. 4C and D;  $P < 0.01$ ). Notably, overexpression of miR-211-5p or SEV depressed SIRT1 expression in U251 cells (Fig. 4D). Furthermore, downregulation of miR-211-5p eliminated the negative influence of SEV on SIRT1 expression (Fig. 4E;  $P < 0.01$ ). In summary, SIRT1 was the direct target of miR-211-5p and SEV could depress SIRT1 by upregulating miR-211-5p.

*SIRT1 mediates SEV mitochondria-dependent apoptosis pathway to induce the apoptosis of glioma cells.* To clarify whether SIRT1 mediated the effect of SEV on glioma cells, the present study upregulated SIRT1 expression in the presence of SEV and the transfection efficiency was proved by RT-qPCR (Fig. 5A). The effects of SIRT1 on the proliferation and apoptosis of glioma cells were then observed. The data demonstrated that upregulation of SIRT1 reversed the effects of SEV on the viability and apoptosis of glioma

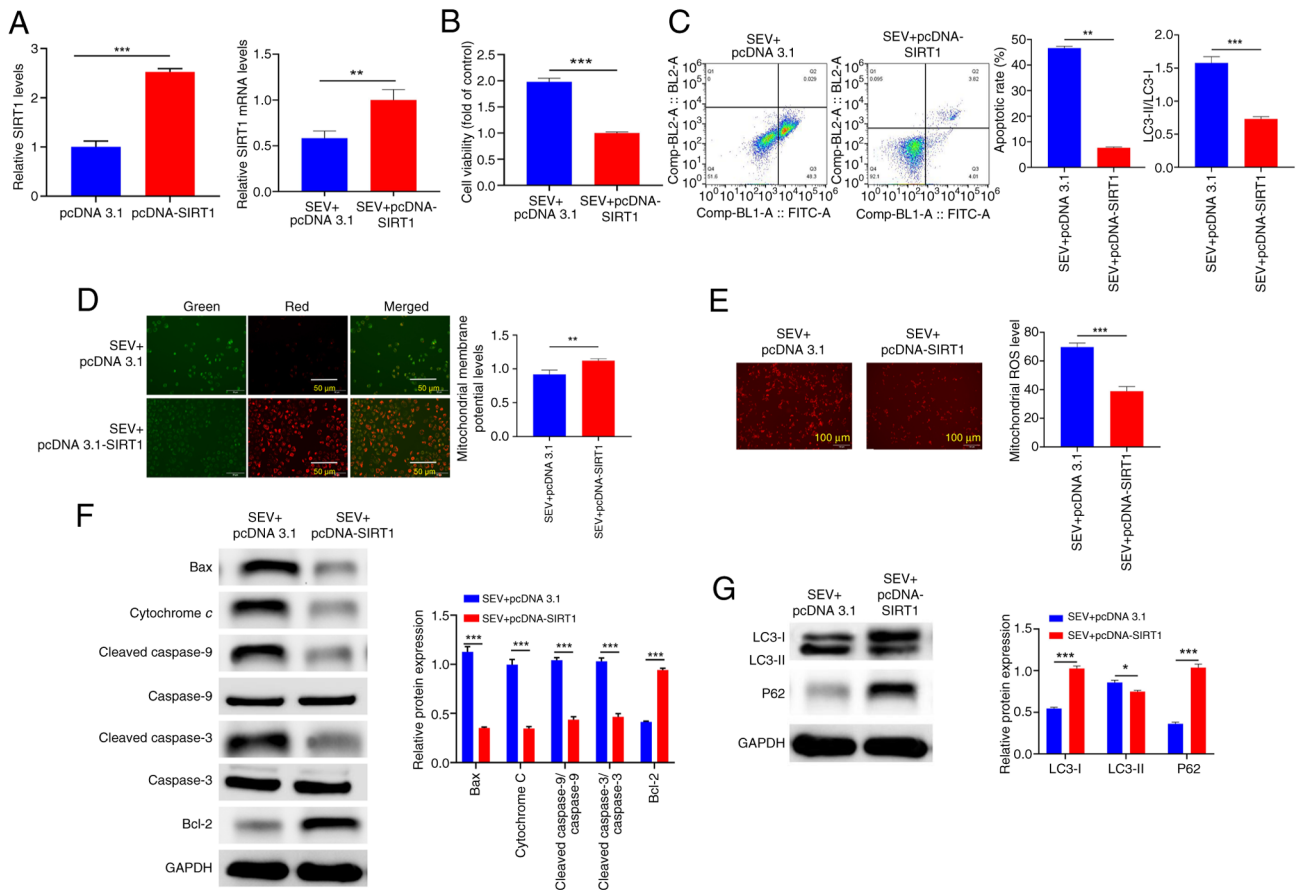


Figure 5. SIRT1 mediates sevoflurane mitochondria-dependent apoptosis pathway to induce the apoptosis of glioma cells. (A) The transfection efficiency of SIRT1 in U251 cells was elevated by reverse transcription-quantitative PCR. (B) Cell viability was assessed using the MTT method, and (C) flow cytometry detection of cell apoptosis. (D) JC-1 staining to detect MMP level. (E) MitoSOX™ Red fluorescence staining was used to detect mitochondrial reactive oxygen species levels. (F) Western blotting detection of Bcl-2, Bax, cytochrome c, cleaved-caspase-9/caspase-9, cleaved-caspase-3/caspase-3, and Bcl-2 protein expression. (G) Western blotting detection of LC3-I, LC3-II and p62 protein expression. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ,  $n = 3$ . SIRT1, silent information regulator 1; MMP, mitochondrial membrane potential; Bcl-2, B-cell lymphoma-2; Bax, bcl-2-associated X protein.

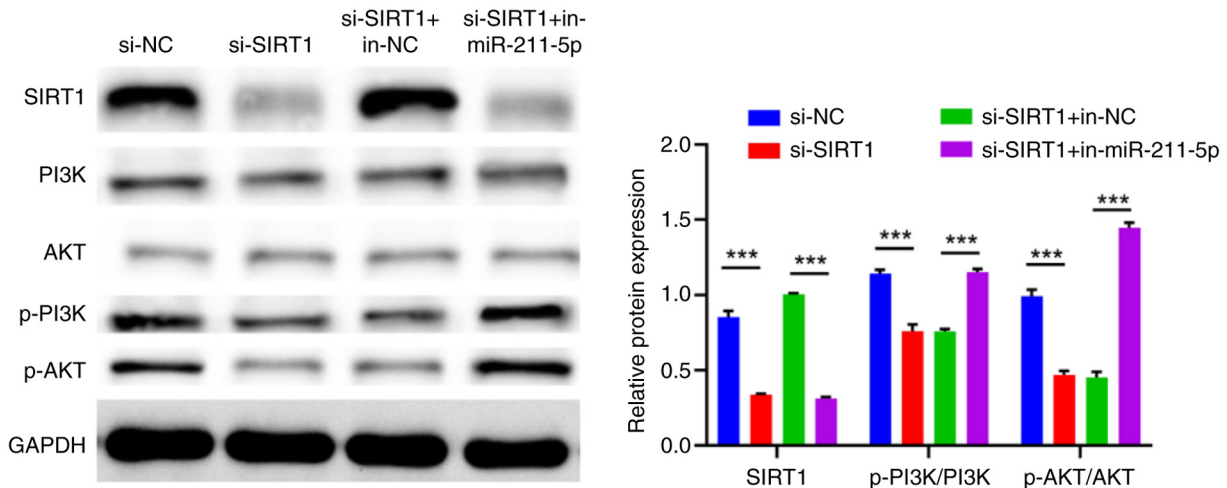


Figure 6. miR-211-5p inactivates the PI3K/AKT pathway by targeting SIRT1. Western blotting was used to detect silenced SIRT1 and miR-211-5p expression in U251 cells and the protein levels of p-PI3K/PI3K and p-AKT/AKT. \*\*\* $P < 0.001$ ,  $n = 3$ . SIRT1, silent information regulator 1; miR, microRNA; p-, phosphorylated; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; si, small interfering; NC, negative control.

cells (Fig. 5B and C;  $P < 0.01$ ). Additionally, upregulation of SIRT1 reversed the effects of SEV on mitochondrial apoptotic pathway-associated proteins, MMP, ROS and

autophagy in glioma cells (Fig. 5D-G;  $P < 0.05$ ). These results suggested that the role of SEV in glioma may depend on the miR-211-5p/SIRT1 signal axis activation.

*miR-211-5p inactivates the PI3K/AKT pathway by targeting SIRT1.* According to previous studies, the driver of the PI3K/AKT signaling pathway was significantly associated with inhibition of the endogenous apoptotic pathway in glioma cells (12,25). The experimental results of the present study showed that after SIRT1 expression was knocked down, p-PI3K/PI3K and p-AKT/AKT protein were decreased in U251 cells ( $P<0.001$ ), suggesting that the downregulation of SIRT1 inhibited the PI3K/AKT pathway activation in U251 cells (Fig. 6). In addition, inhibition of miR-211-5p could promote SIRT1 expression ( $P<0.001$ ) and then activate the PI3K/AKT pathway (Fig. 6). In summary, miR-211-5p could depress the PI3K/AKT pathway via targeting SIRT1.

## Discussion

SEV is one of the most commonly used inhaled anesthetics in clinical practice and its anti-cancer benefits have been reported. Recently, there have been a number of studies on how SEV can treat glioma (20,21,26). As reported by an earlier study, SEV inhibited the malignant phenotype of glioma cells by regulating the miR-218-5p/DEK proto-oncogene/ $\beta$ -Catenin axis (27). However, until now, only a few articles have addressed the effects and molecular regulatory mechanisms of SEV on mitochondrial apoptosis. Therefore, the present study focused on how SEV affected the induction of apoptosis in glioma cells based on the mitochondrial apoptosis pathway.

The regulatory balance between cancer cell viability and apoptosis is the basis of tumorigenesis and the imbalance of apoptosis is the terminal marker of cancer occurrence and progression (28). Researchers have indicated that SEV can induce apoptosis of a number of tumor cells such as lung cancer cells (29), colon cancer cells (30) and glioma cells (27). However, there is no uniform concentration standard and time standard for SEV induction in these researches. Therefore, the present study examined the impact of SEV on glioma cells using varying concentration and time gradients. Consistent with a study on the function of SEV on breast cancer cells (14), SEV significantly reduced cell viability and increased apoptosis in a dose-dependent and time-dependent manner. The optimal induction concentration and time were 6% and 8 h, respectively, indicating the protective effect of SEV on glioma.

Mitochondria play a central role in the process of apoptosis because both intrinsic and extrinsic pathways can converge at the mitochondrial level (31). A number of studies identify a close correlation between mitochondrial dysfunction and the apoptosis of glioma cells (32). For example, Wang *et al* (33) confirmed that Embelin induced apoptosis and cell cycle arrest of brain glioma cells through the mitochondrial pathway. Gu *et al* (6) verified that Jinsufenol promoted apoptosis by activating the mitochondrial apoptotic pathway in glioma. It is noteworthy that SEV can initiate mitochondrial apoptotic pathway, induce cytotoxicity and cause cell apoptosis (18,19). Therefore, the present study hypothesized that SEV could also initiate mitochondrial apoptotic pathway in glioma, thereby causing cell apoptosis. The intrinsic mitochondria-mediated pathway is characterized by depolarization of MMP. MMP, an indicator of mitochondrial membrane permeability, is reduced in early apoptosis, leading to the activation of caspase 9 (7).

In the present study, SEV interfered with and destroyed MMP. Some studies show that mitochondria are the main producers of ROS. Notably, excessive ROS can lead to the loss of respiratory chain functional complexes, resulting in the reduction of biological energy, ultimately leading to cell death (34,35). In the present study, SEV increased ROS accumulation in mitochondria. Mitochondria-mediated apoptosis has been reported to be associated with apoptotic pathway-related proteins. When mitochondria are damaged, cytochrome *c* in the inter-membrane space will be released into the cytoplasm. Cytochrome *c* interacts with apoptotic protease activating factor-1, which binds and activates caspase 9, promoting the formation of apoptosome and ultimately leading to cell apoptosis (31). In addition, the mitochondrial apoptotic pathway is directly and strictly regulated by the balance of pro-apoptotic and anti-apoptotic Bcl-2 family proteins. Following apoptosis stimulation, Bax will specifically translocate to the mitochondrial membrane to form homologous oligomers with other Bax proteins there. This process damages the MMP, increases the permeability of the mitochondrial outer membrane and promotes the release of apoptosis factors into the cytoplasm. Accordingly, the effects of SEV on endogenous mitochondria-mediated pathway-associated proteins were examined in the present study. The examination results showed that SEV could increase the expressions of apoptotic proteins (Bax, cytochrome *c*, Cleaved-caspase-9/caspase-9, Cleaved-caspase-3/caspase-3). In addition, the anti-apoptotic protein (Bcl-2) was decreased, suggesting that SEV promoted mitochondrial apoptosis by regulating proteins associated with the mitochondrial apoptosis pathway. These findings imply that SEV may cause the apoptosis of glioma cells by initiating the mitochondrial apoptosis pathway.

The homeostasis of mitochondria seems to have nothing to do with autophagy, but in fact they are inextricably linked. The relationship between the two is crucial for the state and survival of mitochondria and tumor cells (5). The autophagy mechanism enables cells to selectively eliminate excess or redundant mitochondria, thereby preserving mitochondrial function and maintaining cellular homeostasis (36). In the process of autophagy activation, LC3-I is transformed into LC3-II. Thereafter, LC3-II is connected with p62, integrated into the autophagosome and finally degraded into the autophagolysosome (37). In the present study, glioma cells treated with SEV showed higher LC3-II levels and lower p62 and LC3-I levels. Also, the conversion rate of LC3-I to LC3-II increased significantly, suggesting that SEV induced autophagy activity in glioma cells. Overall, SEV can induce apoptosis through a series of mechanisms, including the loss of MMP, the production of ROS, increased expression of apoptotic proteins, decreased expression of anti-apoptotic proteins, as well as the blocking of mitochondrial autophagy.

With the advances in genomics and proteomics, more and more miRNAs have been confirmed to be abnormally expressed during the development of glioma, such as miR-19 (38), miR-21 and miR-26a (39). They contribute to the pathological process of glioma by regulating the biological function of glioma cells. It is noteworthy that SEV plays a role in tumor progression through regulation of miRNA (20,26). Based on this, the present study hypothesized that SEV may be involved in the progression of glioma through regulation of

miRNA, which encouraged the further exploration of SEV and related miRNA. As a mature sequence of miR-211, miR-211-5p has been identified to be under-expressed in gliomas and involved in the regulation of apoptosis pathways in glioma cells (11). Notably, the present study indicated that SEV could promote miR-211-5p expression. In addition, downregulation of miR-211-5p reversed the effects of SEV on glioma cells, suggesting that SEV was involved in the progression of glioma through regulation of miR-211-5p expression.

A previous study reveals that miRNA, as part of the RNA-induced silencing complex, can regulate the expression of a number of genes by partially supplementing the 3' untranslated region of the target mRNA (40). In the present study, SIRT1 was confirmed as a direct target gene of miR-211-5p through bioinformatics websites and DLR gene assays. SIRT1 has been widely recognized as a key epigenetic regulator involved in a number of biological processes, including metabolic reprogramming, maintenance of genomic stability, autophagy, senescence, mitochondrial apoptosis and tumorigenesis (9). It has been shown that SIRT1 highly expresses in glioma tissues and cell lines and that patients with higher SIRT1 expression have a poorer prognosis (41). Moreover, silencing SIRT1 gene can significantly promote the apoptosis of glioma cells and inhibit the proliferation, invasion and metastasis of glioma cells (10). However, whether SEV is involved in the progression of glioma cells through the miR-211-5p/SIRT1 axis is unclear. Therefore, the present study conducted a functional rescue experiment and discovered that SEV inhibited the expression of SIRT1 by upregulating miR-211-5p. SEV then mediated the mitochondrial apoptosis pathway to induce apoptosis of glioma cells. More importantly, the present study also validated that miR-211-5p inactivated the PI3K/AKT signaling pathway by targeting SIRT1. According to a previous study, SIRT1 may be a promoter of cancer cell death mediated by mitochondrial apoptosis through the PI3K/AKT signaling pathway (42). Hence, it was hypothesized that SEV was involved in the malignant progression of tumor cells through the mitochondrial apoptosis pathway, which was closely related to the regulation of miR-211-5p/SIRT1/PI3K/AKT signaling axis.

Furthermore, the present study also focused on the role of autophagy in SEV-induced apoptosis of glioma cells. Autophagy is a process by which cells maintain intracellular balance by breaking down their own organelles and proteins. Its regulation mainly involves a series of genes and proteins, including mTOR, Beclin 1 and LC3. Inhibition of mTOR activity can promote the autophagy process of cells and then foster the occurrence of apoptosis. AKT is reported to activate Rheb protein by inhibiting the function of the tuberous sclerosis complex 1/tuberous sclerosis complex 2. The activated Rheb protein further enables mTOR to form the active mTOR complex 1, which mediates cell growth and metabolic responses (43,44). Therefore, the present study hypothesized that SEV may promote autophagy and eventually induce apoptosis by inhibiting the activity of the mTOR signaling pathway.

The present study confirmed that SEV induced apoptosis in glioma cells via the mitochondrial pathway, highlighting its potential role in cancer treatment beyond surgery. Clinically, SEV can be repurposed as an adjuvant therapy for glioma, inducing apoptosis through the miR-211-5p/SIRT1/PI3K/AKT

axis. miR-211-5p can also serve as a biomarker for treatment response. However, further research is still needed. Future studies need to validate these results in animal models and clinical trials to establish safety and efficacy. Additionally, exploring the effects of SEV on tumor migration, invasion and immune response can provide a comprehensive understanding of anti-cancer potential of SEV. In the future, the present study will consider including glioma samples of different grades to more fully evaluate the role of SIRT1 in glioma development and its feasibility as a potential therapeutic target. The present study hypothesized this will provide greater insight into our research and the field of glioma therapy. While the findings of the present study provided a scientific basis for the potential application of SEV in the treatment of glioma, more research and evaluation is needed before it can be translated into clinical practice. This includes ensuring that the dosing strategy is safe, effective and takes into account individual patient differences and the need for a combination treatment regimen. In addition, the findings were acquired based on cell experiments and further validation is necessary in animal models.

The present study revealed that SEV could induce apoptosis through a series of events, including the loss of MMP, the production of ROS, the activation of the mitochondrial apoptosis pathway and the blocking of mitochondrial autophagy. Furthermore, it also revealed that SEV could induce apoptosis of glioma cells through the mitochondrial apoptosis pathway and was related to the regulation of miR-211-5p/SIRT1/PI3K/AKT signaling axis. The results may enrich the application function of SEV and provide some new evidences for the efficacy of SEV in the treatment of glioma. However, there are certain limitations in the present study. The research was conducted on glioma cell lines, which may not fully reflect the tumor microenvironment *in vivo*. The long-term effects of SEV were not assessed, and the lack of validation in animal models limits its clinical relevance. Additionally, other glioma-related pathways and tumor behaviors, such as migration and invasion, were not explored. Future studies should address these aspects to provide a more comprehensive understanding.

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## Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

## Authors' contributions

HW and HP contributed to conception and design; GC, SZ and HQ made contributions to acquisition of data; XZ, AY and XS analysed and interpreted data; HW and HP were involved in drafting the manuscript and revising it critically for important

intellectual content. HW, GC and HP confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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