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Original Article

Sevoflurane induces cortical neurons apoptosis in mice via microRNA-21/PTEN/AKT pathway

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Abstract: Previous reports have shown that neonatal exposure to sevoflurane is neurotoxic, causing severe cortical neurons injury, which results in learning and memory deficits. However, the precise molecular mechanism has not yet been elucidated. Recent studies have demonstrated that microRNAs (miRNAs) serve a regulatory function in many injury models including the neurons injury. The aim of present study is to investigate whether the miRNAs are involved in sevoflurane-induced cortical neurons injury. Here, we established a mice model of neurons injury through exposure to sevoflurane and performed the miRNA microarray to analyze miRNAs expression in cortical tissues. The apoptosis of cortical neurons was detected by Western Blot and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay. Validation analysis of microRNA-21 (miR-21) level in serum and cortical tissues was performed using quantitative reverse transcription PCR (qRT-PCR). The primary mice cortical neurons isolated from neonatal mice were used as cellular model in our study. Then, the effect of miR-21 on cell viability and apoptosis was determined using the cell counting kit-8 (CCK-8), Western Blot, caspase-3 activity kit and flow cytometry, respectively. Furthermore, the target gene of miR-21 and the downstream signaling pathway were also investigated. Our results showed that sevoflurane significantly increased the numbers of TUNEL positive cells and sevoflurane exposure led to a significant elevation in levels of cleaved caspase-3, cleaved-PARP and Bax whereas marked decrease in expression of Bcl-2. Microarray data revealed that 15 miRNAs were upregulated and 25 miRNAs were downregulated, and miR-21 was one of the miRNAs being most significantly downregulated after sevoflurane treatment. Subsequently, overexpression of miR-21 protected primary mice cortical neurons injury by inhibiting sevoflurane induced apoptosis. Moreover, phosphatase and tensin homolog deleted on chromosome ten (PTEN) was proved to be a direct and functional target of miR-21 by the dual luciferase reporter assay. Furthermore, we also found that the anti-apoptotic effects of miR-21 are dependent on the activation of PTEN/Akt pathway. Our results suggest that sevoflurane induced cortical neurons injury by downregulating the expression of miR-21, which induces apoptosis through inactivating the PTEN/AKT/mTOR signaling pathway.

Keywords: Sevoflurane, cortical neurons injury, MicroRNA-21, PTEN/AKT/mTOR signaling pathway

Introduction

Sevoflurane, a volatile anesthetic, is widely used in clinical settings, especially in pediatric anesthesia. Some studies reported that sevoflurane administration produced impairment in learning and memory that persists for weeks or months in young and aged rats [1, 2]. Furthermore, children exposed to sevoflurane in early life have a higher incidence of learning deficits [3]. Although it has been well documented that sevoflurane could affect cell survival and potentiate neuronal apoptosis both *in vivo* and *in vitro* [4, 5], limited studies have pay attention on the effect of sevoflurane on cortical neurons.

MicroRNAs (miRNAs) are endogenous, noncoding, single-stranded RNAs about 20~23 nucleotides in length. They can regulate hundreds of gene expression via complementarity between the miRNA seed sequence and the 3'untranslated region (3'UTR) of its target gene, resulting in degradation of target mRNAs and/or inhibition of mRNAs translation [6]. Recent reports showed that a large number of miRNAs are expressed in the central nervous system (CNS) [7-9]. Interestingly, some studies have demon-

strated that the involvement of miRNAs in several types of CNS injuries including cortical neurons injury. For example, Goto et al. demonstrated that sevoflurane combined with propofol anesthesia may lead to alterations in expression levels of miRNAs in the hippocampus of rats [10]. Cai et al. found that miR-27a protected hippocampal neurons against oxygen-glucose deprivation-induced injuries by modulation of forkhead box protein O1 (FOXO1) and apoptosis-related gene caspase-3 expression [11]. A study performed by Wang et al. showed that miR-93 antagomir attenuated hydrogen peroxide (H_2O_2)-induced cortical neurons injury through regulating the expression of the nuclear factor erythroid 2-related factor (Nrf2) and heme oxygenase-1 (HO-1) [12]. Thus, we hypothesize that the sevoflurane induces cortical neurons injury in mice via modulation of miRNAs.

In this study, we performed miRNA arrays to detect the expression patterns of miRNAs in a mice cortical neurons injury model. Moreover, we carried out studies to investigate the roles and molecular mechanisms of miR-21 regulation involved in sevoflurane induced cortical neurons apoptosis in the primary rat cortical neurons. Our findings indicated that miR-21/PTEN/AKT/mTOR axis play an important role in the sevoflurane induced cortical neurons injury, suggesting miR-21 may be a potential therapeutic target for sevoflurane induced learning and memory deficits.

Materials and methods

Experimental animals and sevoflurane exposure

All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of the People's Hospital of Suzhou New District. Twenty 15-month-old male C57BL/6J mice were used for this study. These mice were allowed to acclimate in the animal care facility for 1 week before the experiment, with free access to food and water, and kept in a room with controlled temperature (21 ± 2°C) and humidity (60 ± 5%) on a 12-h light/dark cycle. These mice were randomly divided into two groups: the sham anesthesia (control group, n = 10) and the sevoflurane anesthesia (sevoflurane group, n = 10). Mice randomised to the SEV group were exposed to 2.5% sevoflurane carried by 2 L/min 100% oxygen for 2 h in an anaesthesia chamber, whereas the sham anesthesia group received 2 L/min 100% oxygen for 2 h in an identical chamber. Oxygen, carbon dioxide and sevoflurane concentrations in the exhalation port of the chamber were monitored using the DatexTM infrared analyzer (Capnomac, Helsinki, Finland). At the end of the procedures, samples were immediately harvested. Blood samples were collected by cardiac puncture and centrifuged at 3000 g for 10 min to abstract serum, which was stored at -80°C. Cortical tissues were frozen in liquid nitrogen or fixed in 10% formaldehyde, routinely dehydrated, and paraffin embedded for TUNEL assay.

Neuronal cell culture and treatment

Cortical tissue from forty postnatal day 1 (P1) C57BL/6J mice was dissected and cut into pieces under sterile conditions. Cortical tissues were dissociated with 0.125% trypsin and 0.01% DNase I (Sigma, St. Louis, MO, USA) at 37°C and then was gently resuspended in Dulbecco's Modified Eagle's Medium with serum (Gibco-BRL, GrandIsland, NY, USA) and centrifuged. After adjusting concentration, the cells were plated onto culture dishes or culture plates in advance coated with 0.1 mg/ml poly-L-lysine (Sigma). The cultures were incubated into a humidified atmosphere saturated with 95% air-5% CO_2 at 37°C for overnight and then the medium was changed into neuron-defined serum-free Neuorbasal medium supplemented with 2% B27 (Gibco-BRL) and 0.5 mML-glutamine (Gibco-BRL). Cultured cortical neurons were exposed with 2.5% sevoflurane treatment as described previously [13]. Briefly, neurons were placed in an airtight chamber gassed with 2.5% sevoflurane in the carrying gases, then the chamber was sealed after 15-min gassing and kept sealed for 2 h at 37°C and the concentration of sevoflurane in the chamber was confirmed by the DatexTM infrared analyzer (Capnomac, Helsinki, Finland). After sevoflurane or sham exposure, cell samples were harvested for further expriments.

MiRNA microarray

Total RNA was isolated from cortical tissues using a miRNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. The

purity and quantity of total RNA were assessed by NanoDrop ND-1000 Spectrophotometry (Thermo Scientific, USA) and Agilent's 2100 Bioanalyzer. Then, total RNA (200ng) was labeled with miRCURY™ Hy3™/Hy5™ Power labeling kit and hybridized to the miRCURY LNA™ Array (v.18.0) (Agilent Technologies), incubated, washed and scanned in Agilent high resolution microarray scanner. Data was analyzed using GeneSpring software (Agilent Technologies, USA). The heat map of the 40 microRNAs most obvious differences was created using a method of hierarchical clustering by GeneSpring GX, version 7.3 (Agilent Technologies, California, United Stages).

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA and miRNA were isolated from the cortical tissues, cortical neurons and serum samples with TRizol reagent (Invitrogen, Corp., Carlsbad, CA, USA) and miRNeasy Mini Kit (Qiagen, Valencia, CA), respectively, according to the manufacturers' protocols. For detection of miRNAs and mRNAs, cDNA synthesis was performed from total RNA using the PrimeScript reverse transcription reagent Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Quantitative analysis of the change in expression levels were performed using SYBR Premix Ex Taq™ (TaKaRa, Dalian, China) at ABI 7500 system. Quantitative normalization was performed on the expression of RNU6 small nucleolar RNA or GAPDH genes for miRNAs and mRNA, respectively. The sequences of primers used (Sangon Biotech Co., Ltd., Shanghai, China) were as follows: miR-21, forward, 5'-ACGTTGTGTAGCTTATCAGACTG-3', and reverse, 5'-AATGGTTGTTCTCCACACTCTC-3'; and U6, forward 5'-CTCGCTTCGGCAGCAC-3' and reverse 5'-AACGCTTCACGAATTTGCGT-3'; PTEN mRNA forward: 5'-AAGCTGGAAAGGGACGAACT-3', reverse: 5'-ACACATAGCGCCTCTGACTG-3' and GAPDH mRNA forward: 5'-CCTCTCTCTAATCAGCCCTCTG-3', reverse: 5'-AGAAGGCTGGGGCTCATTTG-3'. Relative expression levels between samples were calculated using $2^{-\Delta\Delta CT}$ method as previously described [14]. Experiments were conducted in triplicate.

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)

TUNEL was carried out using a commercial kit according to the instructions of the manufacturer (Roche, South San Francisco, CA, USA) as described previously. Briefly, after deparaffinization, the sections were incubated for 15 min at RT with a 20 μg/ml Proteinase K (Gibco BRL). The slides were rinsed twice times with PBS before being incubated in TUNEL reaction mixture for 60 min at 37°C. Further incubation with HRP-streptavidin reagent (1:200) in PBS was performed for 30 min at RT. After rinsing with PBS three times for 5 min, sections were counterstained with hematoxylin. Then, the percentage of apoptotic cells was calculated by counting TUNEL-positive cells in five randomly selected fields on each slide.

Cell transfection

The cortical neurons were seeded in 6-well plates and grown overnight. Then, they were transiently transfected with miR-21 mimics, miR-21 inhibitor, and corresponding negative controls (RiboBio, Guangzhou, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The transfected cells were growth-arrested by incubation with serum-free medium 12 h before sevoflurane exposure.

Cell viability

Cell viability was determined by CCK8 assay (Beyotime, Shanghai, China), followed by manufacturer's instructions. Briefly, cortical neurons were seeded in 96-well plates at a density of 5000 cells/well, and subjected to the various treatments as we described above. At the end of the exposure, 10 μL CCK8 solution was added to each well and incubated for 1 h at 37°C. The OD_{450} values were measured with a microplate reader (Thermo Labsystems, Vantaa, Finland).

Cell apoptosis

The cortical neurons were harvested 48 h after treatment by trypsinization. After washing twice with cold PBS, the cells were resuspended in 1 × Annexin V binding buffer and then stained with propidium iodide and Annexin V using the Annexin V-FITC apoptosis detection kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's protocol. The apoptosis ratio was analyzed with a flow cytometry (FACScan; BD Biosciences) equipped with a Cell Quest software.

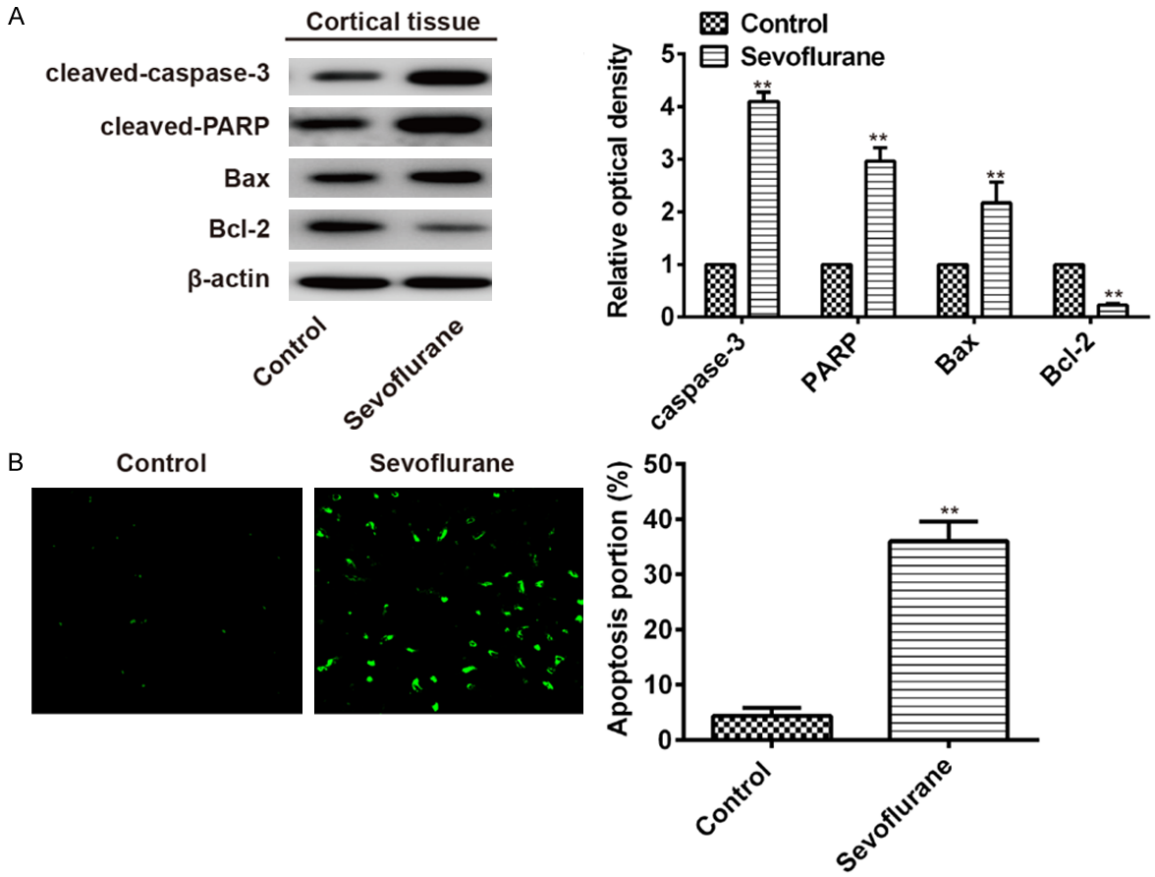

Figure 1. Sevoflurane exposure induces neurons apoptosis in mice cortical tissues. A. The levels of apoptosis proteins (cleaved-caspase-3, cleaved-PARP, Bax and Bcl-2) in mice cortical tissues were measured by Western Blot. B. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was used to analyze neuronal apoptosis after sevoflurane exposure. Data represent the mean ± SD of three independent experiments. $^{**}p < 0.01$ vs. Control group.

Caspase-3 activity assay

The cortical neurons were seeded in 6-well plates at a density of 1×10^5 cells/cm^2 for 24 h. Following treatment for 48 h, caspase-3 activity in cell lysates was determined using a Caspase-3 Activity Assay kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocol.

Luciferase assays

The 3'-UTR of PTEN, with wild-type or mutant (Mut) binding sites for miR-21, was amplified and cloned into the pGL3 vector (Promega, Madison, WI, USA) to generate the plasmid pGL3-WT-PTEN-3'-UTR or pGL3-Mut-PTEN-3'-UTR, respectively. For the luciferase reporter assay, HEK293 cells were co-transfected with the luciferase reporter vectors and miR-21 mimics, miR-21 inhibitor or corresponding negative control using Lipofectamine 2000 reagent. The pRL-TK plasmid (Promega, Madison, USA) was used as a normalizing control. After 48 h of incubation, luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

Western blot assay

Total proteins were extracted from tissues and cells using radioimmunoprecipitation assay (RIPA) lysis buffer (Sigma, St Louis, MO, USA) and quantified with a Bicin Choninic Acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). 40 μg of protein were separated by 10% SDS-PAGE (Bio-Rad, Hercules, CA) and transferred onto a polyvinylidene difluoride membrane (Millipore, USA). The blots were incubated with the previously described antibodies specific for Bcl-2 (dilution, 1:1,000), Bax (dilution, 1:1,000),

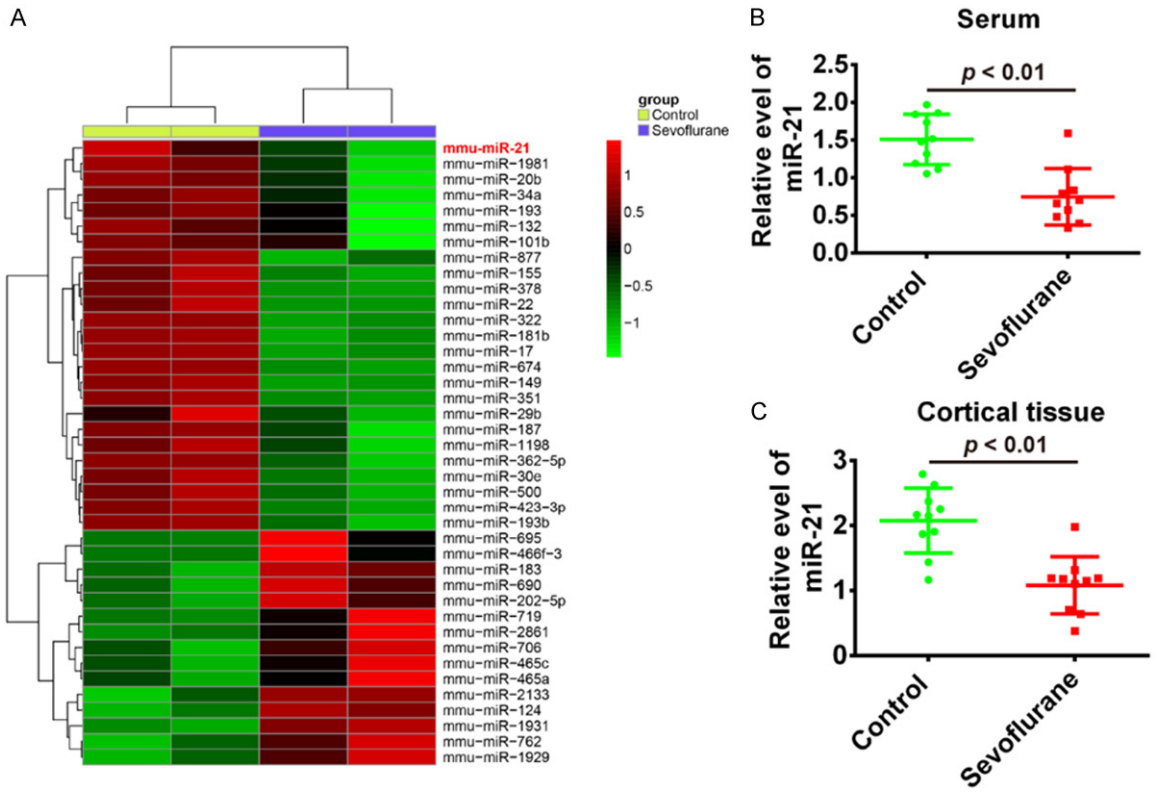

Figure 2. Selection of differently expressed microRNAs during sevoflurane induced cortical neurons injury. A. Heatmap of normalized expression levels of miRNAs in cortical tissues from mice exposed with sevoflurane (n = 2). Rows: Groups; Columns: microRNAs; Color key indicates microRNA expression value, red: Highest, green: Lowest. B, C. qRT-PCR was performed to determine the expression levels of miR-21 in serum samples and cortical tissues in sevoflurane group (n = 10) and control group (n = 10). Data represent the mean ± SD of three independent experiments. $p < 0.01$ vs. Control group.

cleaved caspase-3 (dilution, 1:1,000), cleaved-PARP (dilution, 1:1,000), PTEN (dilution, 1:1,000), AKT (dilution, 1:1,000), p-AKT (dilution, 1:1,000), mTOR (dilution, 1:1,000) and p-mTOR (dilution, 1:1,000) and β-actin (dilution, 1:2,000). All antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The protein bands were visualized by ECL detection reagent (GE Healthcare Life Sciences, Piscataway, NJ, USA). The intensity of protein fragments was quantified with the Quantity One software (4.5.0 basic, Bio-Rad).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Results are presented as the mean ± standard deviation. Differences were analyzed with the Student's t-test between two groups or with one-way ANOVA among multiple groups. A *p*-value of less than 0.05 was considered statistically significant.

Results

Sevoflurane exposure induces neurons apoptosis in rat cortical tissues

Previous studies showed that sevoflurane exposure led to neuronal apoptosis in a variety of adolescent animal models [4, 5], which may contribute to the deficits in hippocampus-dependent learning and memory [15-18]. To determine whether sevoflurane can induce apoptosis of cortical neurons, we examined the levels of markers of apoptosis including cleavaged caspase-3, cleaved-PARP, Bcl-2 and Bax in rat cortical tissues at 2 h after the mice were exposed to 2.5% sevoflurane. Western blot analysis showed that sevoflurane treatment led to a significant elevation in the levels of apoptotic proteins cleaved-caspase-3, cleaved-PARP and Bax, whereas marked decrease in expression of Bcl-2 (**Figure 1A**). We also analyzed the apoptosis in cortical tissues using TUNEL staining assay. As shown in **Figure**

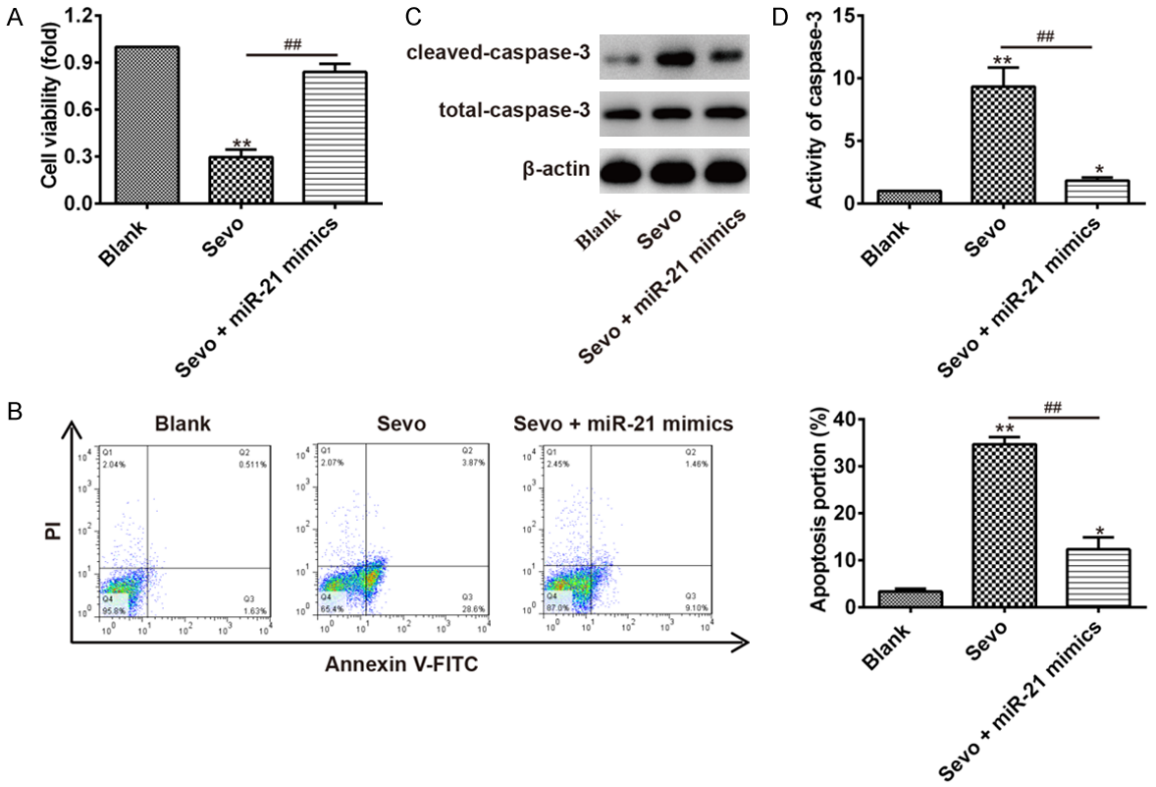

Figure 3. Overexpression of miR-21 attenuated sevoflurane-induced apoptosis in primary mice cortical neurons. Primary cortical neurons from newborn mice were transfected with miR-21 mimics at 12 h prior to sevoflurane exposure. After 48 h transfection, cells were harvested for cell activity analysis and apoptosis estimate. A. Cell viability was measured by CCK-8 assay. Data represent the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$ vs. Blank group, ##$p < 0.01$ vs. Sevo group. B. The apoptosis was detected by flow cytometry. Data represent the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$ vs. Blank group; ##$p < 0.01$ vs. Sevo group. C. The expressions of cleaved caspase-3 and total caspase-3 were determined by Western Blot. D. Caspase-3 activity was analyzed by a commercial Caspase-3 activity kit. Data represent the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$ vs. Blank group, ##$p < 0.01$ vs. Sevo group.

1B, sevoflurane significantly increased TUNEL staining-positive cells in cortical tissues compared with control group. These results suggest that sevoflurane induces cortical neurons injury, which may be associated with learning and memory deficits.

Selection of differently expressed microRNAs during sevoflurane induced cortical neurons injury

It has been reported that miRNAs play important roles in several types of CNS injuries, particularly cortical neurons injury [12, 19-23]. And, a recent study reported that sevoflurane exposure induced neural apoptosis by modulation of miR-34c [24]. Thus, we assume that sevoflurane induced cortical neurons injury through regulation of miRNAs. To determine the potential involvement of miRNAs in cortical neurons injury induced by sevoflurane in mice, we performed the miRNA microarray to investigate miRNAs expression in injury *cortical tissues*. The miRNA microarray identified 15 miRNAs that were up-regulated and 25 miRNAs that were down-regulated in sevoflurane group, compared with the control group (**Figure 2A**). Among the aberrantly expressed miRNAs, miR-21 was one of miRNAs being most significantly downregulated, and many studies revealed that downregulation of miR-21 promoted apoptosis in various types of cells [25, 26]. Interestingly, a previous research showed that miR-21 was found to be downregulated in rat lung tissues following sevoflurane anesthesia [27]. Therefore, we focused on miR-21 in cortical neurons injury for further study.

To validate the expression of miR-21 obtained from miRNA microarray assay, we analyzed the

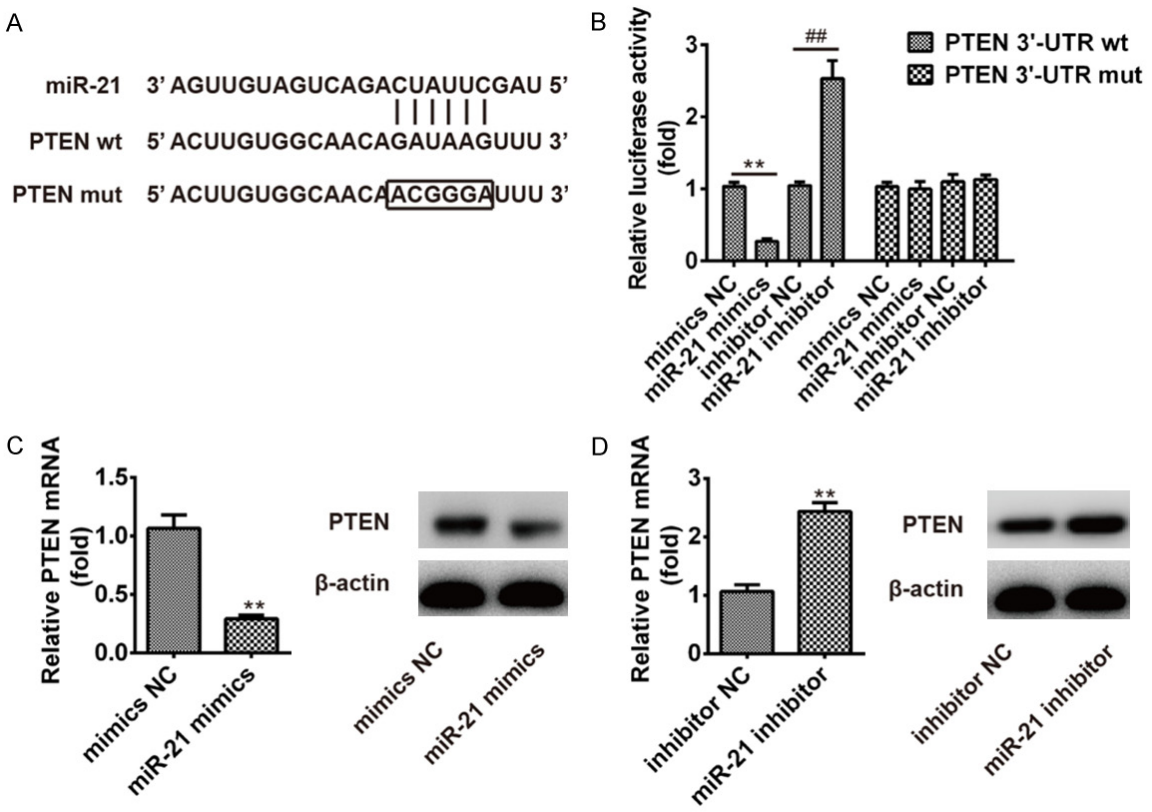

Figure 4. PTEN is a target of miR-21. A. miR-21-binding sequences in the 3'-UTR of PTEN and mutated sites in 3'-UTR of PTEN. B. miR-21 mimic suppressed the luciferase activities of constructs containing the 3'-UTR segment of PTEN, while miR-21 inhibitor significantly increased the luciferase activities of constructs containing the 3'-UTR segment of PTEN (n = 3). Data represent the mean ± SD of three independent experiments. $^{**}p < 0.01$ vs. mimics NC; $^{\#\#}p < 0.01$ vs. inhibitor NC. C, D. The expression of PTEN was detected by qRT-PCR and Western Blot after treatment with miR-21 mimics or miR-21 inhibitor. Data represent the mean ± SD of three independent experiments. $^{**}p < 0.01$ vs. mimic NC or inhibitor NC.

miR-21 expression in 10 pairs of serum samples and cortical tissues from mice treat with/without sevoflurane by qRT-PCR. As shown in **Figure 2B**, the miR-21 expression levels in 10 serum samples from mice exposed to sevoflurane were significantly lower in relative to controls. Consistent with the results displayed in **Figure 2B**, the expression of miR-21 was dramatically down-regulated in cortical tissues from mice exposed to sevoflurane, compared with controls (**Figure 2C**). These results indicate that miR-21 may be involved in the cortical neurons injury induced by sevoflurane.

Overexpression of miR-21 attenuated sevoflurane-induced cortical neurons injury in vitro

To evaluate the role of miR-21 in sevoflurane-induced cortical neurons injury, a sevoflurane-induced primary cortical neurons model was established as described previously [28]. Primary cortical neurons were transfected with miR-21 mimics at 12 h prior to sevoflurane exposure. After transfection for 48 h, the effect of the miR-21 mimics on cell viability and apoptosis under sevoflurane exposure was analyzed. CCK-8 results showed that sevoflurane inhibited cell viability, and its inhibitory effect was reversed when miR-21 was overexpressed (**Figure 3A**). Furthermore, flow cytometry data indicated that the promotion of apoptosis caused by sevoflurane was attenuated after miR-21 mimics transfection (**Figure 3B**). Next, we examined the expression of cleaved caspase-3 and caspase-3 activity. The results indicated that both the level of cleaved caspase-3 protein and caspase-3 activity were markedly increased by sevoflurane exposure, but overexpression of miR-21 significantly reversed the pro-apoptotic effects of sevoflurane (**Figure 3C**, **3D**), suggesting that cell apoptosis induced by sevoflurane was mediated by downregulation of miR-21.

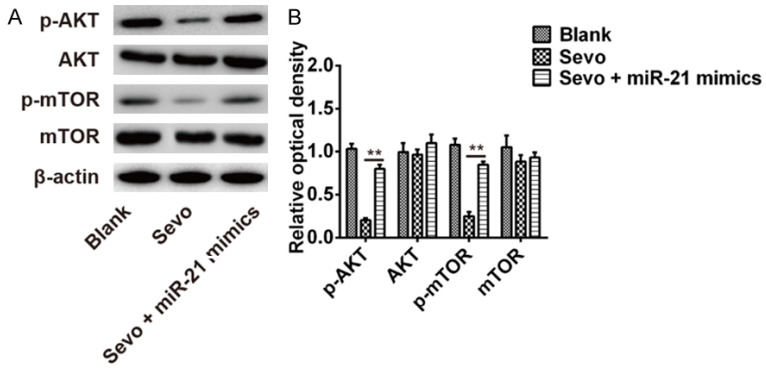

Figure 5. Sevoflurane inhibited the activation of PTEN/AKT/mTOR pathway via downregulation of miR-21. Primary cortical neurons from newborn mice were transfected with miR-21 mimics at 12 h prior to sevoflurane exposure. After 48 h transfection, cells were harvested for Western Blot. A. The expressions of p-AKT, AKT, p-mTOR and mTOR were detected by Western Blot. B. The bands were semi-quantitatively analyzed by using Image J software, normalized to β-actin density. Data represent the mean ± SD of three independent experiments. $^{**}p < 0.01$ vs. Sevo group.

PTEN is a direct target of miR-21 in primary cortical neurons

Previous studies have reported that PTEN, a well-known pro-apoptotic gene, was a direct target of miR-21 in several cell types [29-31]. However, the association between miR-21 and PTEN in cortical neurons remains to be clarified. According to bioinformatics analysis, PTEN was potentially a downstream target of miR-21. The binding sites between miR-21 and PTEN were illustrated in **Figure 4A**. To confirm that miR-21 could interact with 3'-UTR of PTEN through the complementary sequence, a luciferase reporter assay was performed. The results showed that overexpression of miR-21 significantly decreased the luciferase activity of wt-PTEN-3'-UTR, whereas knockdown of miR-21 increased the luciferase activity. Likewise, cells co-transfected with miR-21 mimics, miR-21 inhibitor and PTEN-mut-3'-UTR showed no obvious change in the luciferase activity (**Figure 4B**). We further examined whether miR-21 could modulate the expression of PTEN in cortical neurons. The results of qRT-PCR showed that overexpression of miR-21 in cortical neurons significantly reduced the expression of PTEN, whereas knockdown of miR-21 increased the expression of PTEN at protein and mRNA level (**Figure 4C**, **4D**). Taken together, these data may suggest that downregulation of miR-21 exerts its pro-apoptotic effect by targeting PTEN in sevoflurane-induced cell model.

Sevoflurane inhibited the activation of PTEN/AKT/mTOR pathway via downregulation of miR-21

PTEN has been widely accepted as a significant negative regulator of PI3K/Akt/mTOR signaling pathway [32, 33], which has been reported to be related to the neurons apoptosis in the damaged central nervous system [34, 35]. Based on above data, we speculated whether the pro-apoptotic effect of miR-21 in sevoflurane-induced apoptosis is via regulating PTEN/AKT/mTOR pathway in cortical neurons. Therefore, we performed Western blot analysis to determine the expression level of AKT, phosphorylated-Akt (p-Akt), mTOR and phosphorylated-mTOR (p-mTOR) in sevoflurane-treated cortical neurons. We confirmed that p-Akt and p-mTOR protein expression were significantly decreased in cortical neurons following sevoflurane treatment, but not the total amounts of two proteins. Conversely, overexpression of miR-21 significantly reversed the inhibitory effect of sevoflurane on the protein levels of p-Akt and p-mTOR (**Figure 5A**, **5B**). These results suggest that sevoflurane exposure blocked the PTEN/AKT/mTOR pathway, whereas miR-21 inhibitor transfection reactivated this pathway.

Discussion

In the present study, by using miRNA microarray analyses for the first time, we found that a large set of miRNAs were significantly deregulated in sevoflurane induced injury mice model and the most downregulated miR-21 in cortical tissues for further study. Subsequently, our data showed that overexpression of miR-21 inhibited sevoflurane induced primary cortical neurons apoptosis through reactivation of PTEN/AKT/mTOR pathway. Our findings firstly demonstrated that miR-21 was involved in cortical neurons injury caused by sevoflurane, providing a new therapeutic approach for neurons injury.

Sevoflurane, a widely used inhalation anesthetic, is generally considered safe and effective in

pediatric anesthesia. Nevertheless, there is growing concern about the neurological adverse effects of this agent. Repeated sevoflurane exposure increases the risk of developing a learning disability [36]. Indeed, 6 h of sevoflurane anesthesia administered to newborn rats critically impairs long-term cognitive ability [37]. Zhang et al. showed that sevoflurane induces cortical neurons injury in primary rat when the concentration is >4% [38]. Chen et al. found that sevoflurane exposure in aged rats led to endoplasmic reticulum stress-induced neurons injury, which resulted in learning and memory deficits [39]. Thus it is important to understand the molecular mechanism of sevoflurane induced neurons injury.

Neural apoptosis is the major cause for sevoflurane-induced cognitive impairment [15, 40, 41]. For example, Chen et al. found that sevoflurane triggered endoplasmic reticulum (ER) stress and lead to neuronal cell apoptosis in the hippocampus, and ultimately developed cognition impairment [39]. A study performed by Deng et al. found that exposure to anesthetics during a susceptive neurodevelopmental period may lead to neuronal apoptosis and subsequent learning difficulties [42]. A recent study from Feng et al. demonstrated that sevoflurane exposure led to neuronal apoptosis and pathological alterations in the hippocampus of neonatal rats [16]. Thus, reducing neuronal apoptosis in the neofetal brain is key for improving sevoflurane induced memory impairment. In the present study, we found that that 2.5% sevoflurane exposure could increase the TUNEL-positive cells, accompany with the increased cleaved-caspase-3, cleaved-PARP and Bax expressions and decreased Bcl-2 expression in cortical tissue. These results suggest that sevoflurane exposure induces neurons apoptosis in the cortical tissues by regulating the expression of apoptosis proteins, then leading to neurons lost and cognition impairment.

Recent studies have demonstrated miRNAs play an important role in regulating the neuronal apoptosis in several models. For example, Jiang et al. found that miR-34a negatively regulated anesthesia-induced hippocampal apoptosis and memory impairment through fibroblast growth factor receptor 1 (FGFR1) in young animals [43]. Xu et al. showed that knockdown of miR-124 reduced ketamine-induced apoptosis in hippocampal CA1 neurons [44]. In our study, by using miRNA microarray analyses, we found that miR-21 was downregulated in mice cortical tissues after sevoflurane exposure and overexpression of miR-21 inhibited sevoflurane-induced apoptosis in primary cortical neurons. These results indicate that cortical neurons apoptosis induced by sevoflurane is mediated by downregulation of miR-21, and overexpression of miR-21 may have a protective effect in sevoflurane induced cortical neurons apoptosis.

It is well-known that miR-21 exerts anti-apoptotic effect by targeting pro-apoptotic genes in many diseases [45-48]. PTEN, a classical pro-apoptotic gene, was one of validated targets of miR-21 in several cell types [49-51]. Wang et al. found that inhibition of miR-21 can promote cell apoptosis by upregulating PTEN expression in neuroblastoma cells [52]. Hu et al. reportedthat knockdown of miR-21 significantly increased apoptosis by targeting PTEN in rats after contusion spinal cord injury (SCI) [23]. Notably, Han et al. demonstrated that miR-21 alleviated apoptosis of cortical neurons through promoting PTEN/Akt signaling pathway in vitro after experimental traumatic brain injury [53]. These prompted us to investigate whether miR-21 exerts its anti-apoptotic through regulation of PTEN in sevoflurane induced cortical neurons injury. As expected, PTEN was proved to be a functional target of miR-21 and negatively regulated by miR-21 in the primary rat cortical neurons. More importantly, PTEN could change Akt phosphorylation status to regulate the downstream of Akt signaling pathway [54], which has been reported that plays an important role in the neurons apoptosis in various injury model [55, 56]. Consistent with previous report [53], our study also revealed that the PTEN/Akt signaling pathway was modulated by miR-21. Our study indicated that miR-21 was involved in sevoflurane-induced cortical neurons apoptosis through PTEN/AKT pathway.

In conclusion, this study indicated that sevoflurane significantly induced cortical neurons injury via miR-21/PTEN/AKT/mTOR pathway. Our findings provide new insights into the potential molecular mechanisms of sevoflurane induced learning and memory deficits and indicated that exogenous overexpression of miR-21 may prove to be a promising strategy for sevoflurane induced cortical neurons injury therapies.

Disclosure of conflict of interest

None.

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References

[1] Culley DJ, Baxter M, Yukhananov R and Crosby G. The memory effects of general anesthesia persist for weeks in young and aged rats. Anesth Analg 2003; 96: 1004-1009, table of contents.

[2] Liu XS, Xue QS, Zeng QW, Li Q, Liu J, Feng XM and Yu BW. Sevoflurane impairs memory consolidation in rats, possibly through inhibiting phosphorylation of glycogen synthase kinase-3beta in the hippocampus. Neurobiol Learn Mem 2010; 94: 461-467.

[3] Wilder RT, Flick RP, Sprung J, Katusic SK, Barbaresi WJ, Mickelson C, Gleich SJ, Schroeder DR, Weaver AL and Warner DO. Early exposure to anesthesia and learning disabilities in a population-based birth cohort. Anesthesiology 2009; 110: 796-804.

[4] Kvolik S, Dobrosevic B, Marczi S, Prlic L and Glavas-Obrovac L. Different apoptosis ratios and gene expressions in two human cell lines after sevoflurane anaesthesia. Acta Anaesthesiol Scand 2009; 53: 1192-1199.

[5] Dong Y, Zhang G, Zhang B, Moir RD, Xia W, Marcantonio ER, Culley DJ, Crosby G, Tanzi RE and Xie Z. The common inhalational anesthetic sevoflurane induces apoptosis and increases beta-amyloid protein levels. Arch Neurol 2009; 66: 620-631.

[6] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.

[7] Xie H, Zhao Y, Zhou Y, Liu L, Liu Y, Wang D, Zhang S and Yang M. MiR-9 Regulates the Expression of BACE1 in dementia induced by chronic brain hypoperfusion in rats. Cell Physiol Biochem 2017; 42: 1213-1226.

[8] Sun ZZ, Lv ZY, Tian WJ and Yang Y. MicroRNA-132 protects hippocampal neurons against oxygen-glucose deprivation-induced apoptosis. Int J Immunopathol Pharmacol 2017; 30: 253-263.

[9] Su S, Shao J, Zhao Q, Ren X, Cai W, Li L, Bai Q, Chen X, Xu B, Wang J, Cao J and Zang W. MiR-30b attenuates neuropathic pain by regulating voltage-gated sodium channel Nav1.3 in rats. Front Mol Neurosci 2017; 10: 126.

[10] Goto G, Hori Y, Ishikawa M, Tanaka S and Sakamoto A. Changes in the gene expression levels of microRNAs in the rat hippocampus by sevoflurane and propofol anesthesia. Mol Med Rep 2014; 9: 1715-1722.

[11] Cai Q, Wang T, Yang WJ and Fen X. Protective mechanisms of microRNA-27a against oxygen-glucose deprivation-induced injuries in hippocampal neurons. Neural Regen Res 2016; 11: 1285-1292.

[12] Wang P, Liang X, Lu Y, Zhao X and Liang J. MicroRNA-93 downregulation ameliorates cerebral ischemic injury through the Nrf2/HO-1 defense pathway. Neurochem Res 2016; 41: 2627-2635.

[13] Fang F, Lin W, Ling X, Song R, Liu Q, Lai B and Cang J. The hippocampal cyclin D1 expression is involved in postoperative cognitive dysfunction after sevoflurane exposure in aged mice. Life Sci 2016; 160: 34-40.

[14] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402-408.

[15] Ge HW, Hu WW, Ma LL and Kong FJ. Endoplasmic reticulum stress pathway mediates isoflurane-induced neuroapoptosis and cognitive impairments in aged rats. Physiol Behav 2015; 151: 16-23.

[16] Feng X, Liu JJ, Zhou X, Song FH, Yang XY, Chen XS, Huang WQ, Zhou LH and Ye JH. Single sevoflurane exposure decreases neuronal nitric oxide synthase levels in the hippocampus of developing rats. Br J Anaesth 2012; 109: 225-233.

[17] Zhang X, Xue Z and Sun A. Subclinical concentration of sevoflurane potentiates neuronal apoptosis in the developing C57BL/6 mouse brain. Neurosci Lett 2008; 447: 109-114.

[18] Satomoto M, Satoh Y, Terui K, Miyao H, Takishima K, Ito M and Imaki J. Neonatal exposure to sevoflurane induces abnormal social behaviors and deficits in fear conditioning in mice. Anesthesiology 2009; 110: 628-637.

[19] Lei P, Li Y, Chen X, Yang S and Zhang J. Microarray based analysis of microRNA expression in rat cerebral cortex after traumatic brain injury. Brain Res 2009; 1284: 191-201.

[20] Redell JB, Liu Y and Dash PK. Traumatic brain injury alters expression of hippocampal microRNAs: potential regulators of multiple pathophysiological processes. J Neurosci Res 2009; 87: 1435-1448.

[21] Zhang L, Dong LY, Li YJ, Hong Z and Wei WS. miR-21 represses FasL in microglia and protects against microglia-mediated neuronal cell death following hypoxia/ischemia. Glia 2012; 60: 1888-1895.

[22] Buller B, Liu X, Wang X, Zhang RL, Zhang L, Hozeska-Solgot A, Chopp M and Zhang ZG. MicroRNA-21 protects neurons from ischemic death. FEBS J 2010; 277: 4299-4307.

[23] Hu JZ, Huang JH, Zeng L, Wang G, Cao M and Lu HB. Anti-apoptotic effect of microRNA-21 after contusion spinal cord injury in rats. J Neurotrauma 2013; 30: 1349-1360.
[24] Zhou X, Xian D, Xia J, Tang Y, Li W, Chen X, Zhou Z, Lu D and Feng X. MicroRNA-34c is regulated by p53 and is involved in sevoflurane-induced apoptosis in the developing rat brain potentially via the mitochondrial pathway. Mol Med Rep 2017; 15: 2204-2212.
[25] Cui M, Zhang M, Liu HF and Wang JP. Effects of microRNA-21 targeting PITX2 on proliferation and apoptosis of pituitary tumor cells. Eur Rev Med Pharmacol Sci 2017; 21: 2995-3004.
[26] Shi L, He Y, Bai B and Chen M. [Effects of microRNA-21 inhibitor on apoptosis of type II alveolar epithelial cells in rats with hyperoxia-induced acute lung injury]. Zhonghua Wei Zhong Bing Ji Jiu Yi Xue 2017; 29: 244-248.
[27] Tanaka S, Ishikawa M, Arai M, Genda Y and Sakamoto A. Changes in microRNA expression in rat lungs caused by sevoflurane anesthesia: a TaqMan(R) low-density array study. Biomed Res 2012; 33: 255-263.
[28] Wei H, Kang B, Wei W, Liang G, Meng QC, Li Y and Eckenhoff RG. Isoflurane and sevoflurane affect cell survival and BCL-2/BAX ratio differently. Brain Res 2005; 1037: 139-147.
[29] Lu X, Sun C, Zheng D, Liu R, Wei X and Wu Z. [The miR-21 attenuates hepatocyte hypoxia/reoxygenation injury via inhibiting PTEN/PI3K/AKT signaling pathway]. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 2017; 33: 497-502.
[30] Fang H, Xie J, Zhang M, Zhao Z, Wan Y and Yao Y. miRNA-21 promotes proliferation and invasion of triple-negative breast cancer cells through targeting PTEN. Am J Transl Res 2017; 9: 953-961.
[31] Shi B, Deng W, Long X, Zhao R, Wang Y, Chen W, Xu G, Sheng J, Wang D and Cao S. miR-21 increases c-kit+ cardiac stem cell proliferation in vitro through PTEN/PI3K/Akt signaling. PeerJ 2017; 5: e2859.
[32] Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP and Mak TW. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell 1998; 95: 29-39.
[33] Lee JE, Lim MS, Park JH, Park CH and Koh HC. PTEN promotes dopaminergic neuronal differentiation through regulation of ERK-dependent inhibition of S6K signaling in human neural stem cells. Stem Cells Transl Med 2016; 5: 1319-1329.
[34] Park KK, Liu K, Hu Y, Kanter JL and He Z. PTEN/mTOR and axon regeneration. Exp Neurol 2010; 223: 45-50.
[35] Zhu H, Xie R, Liu X, Shou J, Gu W, Gu S and Che X. MicroRNA-494 improves functional recovery and inhibits apoptosis by modulating PTEN/AKT/mTOR pathway in rats after spinal cord injury. Biomed Pharmacother 2017; 92: 879-887.
[36] Amrock LG, Starner ML, Murphy KL and Baxter MG. Long-term effects of single or multiple neonatal sevoflurane exposures on rat hippocampal ultrastructure. Anesthesiology 2015; 122: 87-95.
[37] Tao G, Luo Y, Xue Q, Li G, Tan Y, Xiao J and Yu B. Docosahexaenoic acid rescues synaptogenesis impairment and long-term memory deficits caused by postnatal multiple sevoflurane exposures. Biomed Res Int 2016; 2016: 4062579.
[38] Zhang DX, Zhang LM, Zhao XC and Sun W. Neuroprotective effects of erythropoietin against sevoflurane-induced neuronal apoptosis in primary rat cortical neurons involving the EPOR-Erk1/2-Nrf2/Bach1 signal pathway. Biomed Pharmacother 2017; 87: 332-341.
[39] Chen G, Gong M, Yan M and Zhang X. Sevoflurane induces endoplasmic reticulum stress mediated apoptosis in hippocampal neurons of aging rats. PLoS One 2013; 8: e57870.
[40] Satomoto M. [Neonatal exposure to sevoflurane induces abnormal social behaviors and deficits in fear conditioning in mice]. Masui 2010; 59 Suppl: S196-205.
[41] Xie Z, Dong Y, Maeda U, Moir R, Inouye SK, Culley DJ, Crosby G and Tanzi RE. Isoflurane-induced apoptosis: a potential pathogenic link between delirium and dementia. J Gerontol A Biol Sci Med Sci 2006; 61: 1300-1306.
[42] Deng M, Hofacer RD, Jiang C, Joseph B, Hughes EA, Jia B, Danzer SC and Loepke AW. Brain regional vulnerability to anaesthesia-induced neuroapoptosis shifts with age at exposure and extends into adulthood for some regions. Br J Anaesth 2014; 113: 443-451.
[43] Jiang XL, Du BX, Chen J, Liu L, Shao WB and Song J. MicroRNA-34a negatively regulates anesthesia-induced hippocampal apoptosis and memory impairment through FGFR1. Int J Clin Exp Pathol 2014; 7: 6760-6767.
[44] Xu H, Zhang J, Zhou W, Feng Y, Teng S and Song X. The role of miR-124 in modulating hippocampal neurotoxicity induced by ketamine anesthesia. Int J Neurosci 2015; 125: 213-220.
[45] Di Y, Lei Y, Yu F, Changfeng F, Song W and Xuming M. MicroRNAs expression and function in cerebral ischemia reperfusion injury. J Mol Neurosci 2014; 53: 242-250.
[46] Wei C, Li L, Kim IK, Sun P and Gupta S. NF-kappaB mediated miR-21 regulation in cardiomyocytes apoptosis under oxidative stress. Free Radic Res 2014; 48: 282-291.
[47] Yang Q, Yang K and Li A. microRNA-21 protects against ischemia-reperfusion and hypoxia-re-

perfusion-induced cardiocyte apoptosis via the phosphatase and tensin homolog/Akt-dependent mechanism. Mol Med Rep 2014; 9: 2213-2220.

[48] Zeng J, Xiong Y, Li G, Liu M, He T, Tang Y, Chen Y, Cai L, Jiang R and Tao J. MiR-21 is overexpressed in response to high glucose and protects endothelial cells from apoptosis. Exp Clin Endocrinol Diabetes 2013; 121: 425-430.

[49] Meng F, Henson R, Lang M, Wehbe H, Maheshwari S, Mendell JT, Jiang J, Schmittgen TD and Patel T. Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. Gastroenterology 2006; 130: 2113-2129.

[50] Ji R, Cheng Y, Yue J, Yang J, Liu X, Chen H, Dean DB and Zhang C. MicroRNA expression signature and antisense-mediated depletion reveal an essential role of MicroRNA in vascular neointimal lesion formation. Circ Res 2007; 100: 1579-1588.

[51] Haverty PM, Fridlyand J, Li L, Getz G, Beroukhim R, Lohr S, Wu TD, Cavet G, Zhang Z and Chant J. High-resolution genomic and expression analyses of copy number alterations in breast tumors. Genes Chromosomes Cancer 2008; 47: 530-542.

[52] Wang Z, Yao W, Li K, Zheng N, Zheng C, Zhao X and Zheng S. Reduction of miR-21 induces SK-N-SH cell apoptosis and inhibits proliferation via PTEN/PDCD4. Oncol Lett 2017; 13: 4727-4733.

[53] Han Z, Chen F, Ge X, Tan J, Lei P and Zhang J. miR-21 alleviated apoptosis of cortical neurons through promoting PTEN-Akt signaling pathway in vitro after experimental traumatic brain injury. Brain Res 2014; 1582: 12-20.

[54] Wang G, Jiang X, Pu H, Zhang W, An C, Hu X, Liou AK, Leak RK, Gao Y and Chen J. Scriptaid, a novel histone deacetylase inhibitor, protects against traumatic brain injury via modulation of PTEN and AKT pathway: scriptaid protects against TBI via AKT. Neurotherapeutics 2013; 10: 124-142.

[55] Kim GS, Cho S, Nelson JW, Zipfel GJ and Han BH. TrkB agonist antibody pretreatment enhances neuronal survival and long-term sensory motor function following hypoxic ischemic injury in neonatal rats. PLoS One 2014; 9: e88962.

[56] Liu H, Huang X, Liu X, Xiao S, Zhang Y, Xiang T, Shen X, Wang G and Sheng B. miR-21 promotes human nucleus pulposus cell proliferation through PTEN/AKT signaling. Int J Mol Sci 2014; 15: 4007-4018.