RETRACTED ARTICLE: Sevoflurane suppresses migration and invasion of glioma cells by regulating miR-146b-5p and MMP16

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Introduction

Glioma is a primary brain tumor with poor prognosis and there are many strategies for the treatment of glioma [1]. Surgical resection is the main treatment for patients with primary and advanced glioma [2]. Although novel surgical techniques have gained great development, the dissemination and metastasis of cancer are increased because of the release of cancer cells into circulation during the surgery. Thus, it is urgent to explore novel strategies to prevent cancer cell dissemination and metastasis for improving cancer prognosis after resection treatment.

Migration and invasion contribute to patients’ death in glioma, and blocking cancer cell metastasis has been indicated as a promising avenue for therapeutic treatment [3]. A previous study has reported that the anesthetic technique and anesthetics have important impacts on cell migration and invasion in different cancers. However, the underlying mechanism that allows anesthetics-mediated progression of glioma cells remains elusive.

Methods: Sevoflurane (Sev), a class of common anesthetics, was used to expose to U87-MG and U251 cells. The expressions of microRNA-146b-5p (miR-146b-5p) and matrix metalloproteinase 16 (MMP16) were measured by quantitative real-time polymerase chain reaction or western blot. Transfection was performed in glioma cells with miR-146b-5p inhibitor, inhibitor negative control, MMP16 overexpression vector, empty vector, small interfering RNA against MMP16 or scramble. Cell migration and invasion were analyzed by the trans-well assay. The interaction between miR-146b-5p and MMP16 was explored by luciferase activity and RNA immunoprecipitation assays.

Results: Sev treatment inhibited migration and invasion of glioma cells. The expression of miR-146b-5p was enhanced and MMP16 protein was decreased in glioma cells after exposure of Sev. Knockdown of miR-146b-5p or overexpression of MMP16 reversed Sev-induced inhibition of migration and invasion of glioma cells. Moreover, MMP16 was indicated as a target of miR-146b-5p and its silencing attenuated the regulatory role of miR-146b-5p abrogation in Sev-treated glioma cells.

Conclusion: Sev impeded cell migration and invasion through regulating miR-146b-5p and MMP16 in glioma, indicating a novel theories foundation for the application of anesthetics like Sev in glioma.

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is known about whether Sev modulates miR-146b-5p in glioma progression. In the present study, we measured the effect of Sev on migration and invasion of glioma cells and explored whether miR-146b-5p is involved in the regulatory effect of Sev.

Materials and methods

Cell culture and exposure to Sev

The human glioma cell lines, U87-MG and U251, were obtained from American Tissue Culture Collection (Manassas, VA, USA) and grown in Dulbecco’s Modified Eagle Medium (DMEM; Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Gibco), 1% penicillin/streptomycin (Gibco) in an incubator with 5% CO₂ at 37°C. For Sev exposure, U87-MG and U251 cells at log phase were seeded onto plates and cultured overnight. Then cell plates were placed in a climate chamber connected to an anesthesia machine (GE Healthcare Life Sciences, Chalfont, UK). Anesthetic vaporizer (Bisen, Guangzhou, China) was used to supply Sev into the chamber and the concentrations of Sev were continuously monitored by a gas monitor (Drager, Lübeck, Germany). The cells were exposed to different concentrations (1.7%, 3.4% or 5.1%) of Sev for 6 h, and then cultured in normal conditions for 24 h before further analyses.

Cell transfection

pcDNA3.1-MMP16 overexpression vector (MMP16), pcDNA3.1 empty vector (vector), small interfering RNA (siRNA) against MMP16 (siMMP16), siRNA control (scramble), miR-146b-5p mimic (miR-146b-5p), mimic negative control (NC), miR-146b-5p inhibitor (anti-miR-146b-5p) and inhibitor negative control (anti-NC) were synthesized by Genepharma (Shanghai, China). U87-MG and U251 cells were transfected with 100 ng pmirGLO constructs and 20 nM miR-146b-5p or NC. After the post-transfection for 48 h, cells were collected via the centrifugation at 12,000 g for 10 min and quantified by using the BCA protein assay kit (Beyotime, Shanghai, China). The supernatant of each group was collected via the centrifugation at 12,000 g for 10 min, separated via SDS-PAGE gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Subsequently, the membranes were blocked with 5% non-fat milk for 1 h at room temperature and then interacted with primary antibodies overnight at 4°C, followed by incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. The antibodies against MMP16 (ab73877), GAPDH (ab181602) and secondary antibody (ab6721) were purchased from Abcam (Cambridge, MA, USA). After washed with PBS for three times, the protein signaling was visualized using enhanced chemiluminescence chromogenic substrate (Beyotime) and the relative protein levels were normalized to GAPDH level.

Luciferase reporter assay

The putative binding sites of miR-146b-5p and MMP16 were predicted by TargetScan (http://www.targetscan.org/vert_72/). The 3’-UTR of MMP16 containing wild-type (wt) or mutant (mut) miR-146b-5p binding site was amplified and cloned into pmirGLO vectors (Promega) to generate MMP16-wt or MMP16-mut, respectively. For luciferase reporter assay, U87-MG and U251 cells were seeded in 24-well plates and co-transfected with the 100 ng pmirGLO constructs and 20 nM miR-146-5p or NC. After the post-transfection for 48 h,
luciferase activity was analyzed with a dual-luciferase assay kit (Promega) according to the manufacturer’s instructions.

**RNA immunoprecipitation (RIP)**

RIP assay was performed by using Magna RNA immunoprecipitation kit (Millipore) according to the manufacturer’s protocols. U87-MG and U251 cells transfected with miR-146b-5p or NC were lysed in RIP immunoprecipitation buffer. The cell lysis was incubated with magnetic beads coated with antibody against Ago2. The IgG and input were used as controls. The enrichment of MMP16 on beads was analyzed by qRT-PCR.

**Statistical analysis**

All data were presented as the means ± SD from three independent experiments and analyzed via Student’s t test or one-way analysis of variance (ANOVA) followed by the Brown–Forsythe test using GraphPad Prism 7 (GraphPad Inc., San Diego, CA, USA). The p values less than .05 were considered as statistically significantly different.
Results

Sev exposure inhibits migration and invasion of glioma cells

To investigate the effect of Sev on glioma cell migration and invasion, U87-MG and U251 cells were treated with different concentrations of Sev for 6 h and then cultured for 24 h, followed by placing into a trans-well chamber. After the incubation for 8 h, the number of migratory or invasive cells was counted. Compared with those in the control group, cell migration was obviously inhibited in U87-MG and U251 cells after treatment of Sev in a concentration-dependent manner.
Similarly, exposure of Sev also led to a progressive loss of the invasive ability of U87-MG and U251 cells in a concentration-dependent manner (Figure 1(B)).

Sev exposure enhances the level of miR-146b-5p in glioma cells

To explore the potential mechanism, the expression of miR-146b-5p was measured in glioma cells after treatment of different concentrations of Sev. The data of qRT-PCR revealed that the expression of miR-146b-5p was significantly elevated in U87-MG cells after treatment of Sev in a dose-dependent manner (Figure 2(A)). Likewise, the U251 cells treated with Sev also displayed an obvious increase of miR-146b-5p level compared with those of the control group (Figure 2(B)). The cells treated with 5.1% Sev were used for subsequent experiments due to the most significant alteration in this group. In addition, we selected five miRNAs (miR-150-5p, miR-223-3p, miR-146b-5p, miR-182-5p and miR-489-3p) down-regulated in glioma and detected the expression levels in U251 cells after treated with 5.1% sevoflurane using qRT-PCR. The results revealed that miR-146b-5p was significantly altered in five differentially expressed miRNAs, so miR-146b-5p was selected for further study (Supplementary Figure).

Knockdown of miR-146b-5p reversed Sev-induced inhibition of migration and invasion of glioma cells

To evaluate whether miR-146b-5p was involved in an inhibitive role of Sev in glioma progression, U87-MG and U251 cells were transfected with anti-miR-146b-5p or anti-NC and then exposed to 5.1% Sev for 6 h. As a result, the abundance of miR-146b-5p was markedly decreased in U87-MG and U251 cells transfected with anti-miR-146b-5p compared with that in anti-NC group after treatment of Sev (Figure 3(A)). Then trans-well analysis was performed to elucidate the effect of miR-146b-5p on migration and invasion. Results showed that knockdown of miR-146b-5p reversed Sev-mediated suppression of migration in U87-MG and U251 cells (Figure 3(B)). Moreover, the suppressive effect of Sev on cell invasion was attenuated by abrogation of miR-146b-5p in U87-MG and U251 cells (Figure 3(C)).

MMP16 is down-regulated and its overexpression attenuated Sev-induced inhibition of migration and invasion of glioma cells

Seeing that MMP16 is a key factor of cell metastasis in cancers, the abundance of MMP16 was next measured in U87-MG and U251 cells after treatment of varying concentrations of Sev. Compared with the control group, treatment of Sev resulted in a progressive reduction of MMP16 protein level in U87-MG and U251 cells in a concentration-dependent manner (Figure 4(A)). To confirm the role of MMP16 in glioma progression, cells were transfected with MMP16 overexpression vector or vector prior to exposure to 5.1% Sev. After the transfection, the abundance of MMP16 protein was effectively restored in U87-MG and U251 cells with transfection of MMP16 than that in empty vector group (Figure 4(B)). Subsequently, the effect of MMP16 on cell migration and

Figure 4. Role of MMP16 immigration and invasion of glioma cells after exposure to sevoflurane. (A) The protein expression of MMP16 was measured in U87-MG and U251 cells after treatment of different concentrations of sevoflurane by western blot. (B) The abundance of MMP16 protein was detected in U87-MG and U251 cells transfected with MMP16 or vector after treatment of 5.1% sevoflurane by western blot. Cell migration (C) and invasion (D) were examined in U87-MG and U251 cells transfected with MMP16 or vector after treatment of 5.1% sevoflurane by trans-well assay. *p<.05, compared with Control group or 5.1% sevoflurane + vector group.
invasion was investigated in Sev-treated glioma cells. Transwell analysis revealed that overexpression of MMP16 significantly alleviated Sev-mediated inhibition of migration and invasion in U87-MG and U251 cells (Figure 4(C,D)).

**MMP16 is a target of miR-146b-5p**

In order to explore the potential association of miR-146b-5p and MMP16, the putative binding sites of them were predicted by TargetScan online (Figure 5(A)). To validate this prediction, we constructed the wt or mut luciferase reporter vector and analyzed the luciferase activity after transfection. Results showed that luciferase activity was significantly decreased in U87-MG and U251 cells transfected with MMP16-wt and miR-146b-5p compared with that in the NC group, whereas it showed little change in MMP16-mut group (Figure 5(B)). Moreover, RIP assay was also conducted to identify this interaction, which displayed that enrichment of MMP16 was obviously enhanced in U87-MG and U251 cells transfected with miR-146b-5p compared with that in NC group. *p < .05, compared with NC or anti-NC group.

Figure 5: Relationship of miR-146b-5p and MMP16. (A) The putative binding sites of miR-146b-5p and MMP16 were predicted by TargetScan. (B) Luciferase activity was analyzed in U87-MG and U251 cells co-transfected with MMP16-wt or MMP16-mut and miR-146b-5p or NC. (C) The enrichment of MMP16 was measured in U87-MG and U251 cells transfected with miR-146b-5p or NC after Ago2 or IgG RIP. (D) The effect of miR-146b-5p on MMP16 protein expression was investigated in U87-MG and U251 cells transfected with NC, miR-146b-5p, anti-NC or anti-miR-146b-5p by western blot. *p < .05, compared with NC or anti-NC group.
In addition, the effect of miR-146b-5p on MMP16 protein expression was investigated in glioma cells. Western blot analysis revealed that the abundance of MMP16 protein was evidently reduced by overexpression of miR-146b-5p in U87-MG and U251 cells, while it was increased by knockdown of miR-146b-5p (Figure 5(D)).

**Interference of MMP16 weakens the regulatory effect of miR-146b-5p knockdown on migration and invasion in Sev-treated glioma cells**

To explore whether miR-146b-5p-mediated migration and invasion were regulated by MMP16, glioma cells were transfected with anti-NC, anti-miR-146b-5p, anti-miR-146b-5p and scramble or siMMP16 and then treated with 5.1% Sev. The data of western blot analysis showed that down-regulation of miR-146b-5p led to a strong increase of MMP16 protein level in U87-MG and U251 cells after treatment of Sev, which was weakened by the interference of MM16 (Figure 6(A)). Moreover, trans-well assay exhibited that silencing of MMP16 attenuated exhaustion of miR-146b-5p-mediated increase of migratory ability in Sev-treated U87-MG and U251 cells (Figure 6(B)). Similarly, MMP16 interference also alleviated the depletion of miR-146b-5p-induced enhanced number of invasive cells in U87-MG and U251 cells after the treatment of Sev (Figure 6(C)).

**Discussion**

The available evidence has indicated that tumor cell migration and invasion contribute to cancer dissemination and malignance [16]. A previous study suggested that application of inhalation anesthetics halothane, Sev and isoflurane plays an anti-cancer role in human cancer cell lines [17]. Sever et al. indicated that Sev exposure inhibited migration and invasion in colorectal cancer and lung cancer [6,8]. In this study, we investigated the inhibitive effect of Sev on migration and invasion of glioma cells and first provided the regulatory network of Sev/miR-146b-5p/MMP16 in glioma.

The former finding revealed that Sev or thiopental decreased migration and MMP2 activity in glioma cells [9]. Moreover, Yi et al. reported that Sev treatment repressed cell migration and invasion in U251 glioma cells [18]. In agreement with these researches, this study also revealed that Sev treatment led to inhibition of migration and invasion of glioma cells. Nevertheless, the underlying mechanism that allows Sev to regulate metastasis of glioma remains largely unclear. Accruing works revealed that Sev exposure could result in dysregulation of miRNAs in many conditions. For example, Sev suppressed the proliferation of breast cancer cells via regulating miR-203 [19]. Furthermore, Sev upregulated miR-637 to suppress migration and invasion of glioma...
cells [18]. To figure out whether Sev-induced loss of migration and invasion was mediated by miRNA, we measured the miRNA level in glioma cells after treatment of Sev. Results displayed high expression of miR-146b-5p in Sev-treated U87-MG and U251 cells compared with that in the control group, which uncovered that miR-146b-5p might play an essential role in Sev-mediated metastasis of glioma.

miR-146b-5p has been indicated as a tumor suppressor in many types of cancers, including non-small cell lung cancer and gallbladder carcinoma [13,20,21]. Therefore, we hypothesized miR-146b-5p might also serve as a tumor suppressor in glioma. Here we revealed that knockdown of miR-146b-5p reversed Sev-induced reduction of migration and invasion in glioma cells, indicating Sev plays anti-metastasis role in glioma by regulating miR-146b-5p. These findings also suggested that abrogation of miR-146b-5p contributed to migration and invasion of glioma cells, which was also consistent with former effort [22]. Functional miRNAs are known to regulate targets expressions by binding their 3’ UTR.

Previous efforts have reported that miR-146b-5p could exhibit suppressive role in migration and invasion in renal cancer and pancreatic cancer by regulating MMP16 [23,24]. In addition, the interaction between miR-146b-5p and MMP16 was also reported in glioma cells [25,26]. In this study, we also assumed that MMP16 was associated with miR-146b-5p in glioma progression. We conducted the luciferase activity and RIP assays. The interaction between miR-146b-5p and MMP16 was validated by loss of luciferase activity in MMP16-wt and miR-146b-5p group and enhanced enrichment of MMP16 in miR-146b-5p and Ago2 RIP group.

MMPs play pivotal roles in progression, migration and metastasis in human cancers [27]. Former works have demonstrated that Sev could decrease the expression of MMPs, such as MMP2 and MMP9 [9,28]. MMP16, another member of MMPs family, was also reported to facilitate migration and invasion of glioma cells by acting as a functional target of miRNAs [26,29]. In this research, MMP16 protein expression was reduced in glioma cells after treatment of Sev and its overexpression alleviated Sev-induced inhibition of migration and invasion of glioma cells. This also revealed the pro-metastasis role of MMP16, which is accordant with previous reporters. Besides, trans-well analysis revealed that interference of MMP16 weakened the regulatory effect of miR-146b-5p down-regulation on Sev-mediated migration and invasion in glioma cells. These results reflected that Sev regulated migration and invasion of glioma cells by modulating miR-146b-5p and MMP16 expression. The emerging evidence suggested that in vivo experiments are responsible for better understanding the glioma biology and related mechanism [30]. Hence, an animal model should be established to explore the role and mechanism of Sev in glioma in further study.

In conclusion, the present study showed that inhalable anesthetic, Sev, blocked migration and invasion of glioma cells and this effect was mediated by miR-146a-5p and MMP16. These findings indicate a novel theories foundation of Sev in glioma and may cause significant clinical implications for the application of volatile anesthetic agents in glioma during surgical resection.

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