Sevoflurane inhibits the malignant potential of head and neck squamous cell carcinoma via activating the hypoxia-inducible factor-1α signaling pathway *in vitro*

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Abstract. Sevoflurane, an inhalational anesthetic, is extensively used during oral cancer surgery. However, the effect of sevoflurane on head and neck squamous cell carcinoma (HNSCC) remains unclear. The objective of the present study was to investigate the effects of sevoflurane on the proliferation, apoptosis and invasion in HNSCC cell lines and the underlying molecular mechanism. The Cell Counting Kit-8 assay was used to evaluate cell proliferation. Apoptosis was analyzed by flow cytometry. Cell invasion was evaluated using the Transwell invasion assay. The expression levels of Akt, p-Akt (Ser473), hypoxia-inducible factor-1α (HIF-1α), Fas and Bcl-2 were measured by western blotting. Significant inhibition of cell proliferation and induction of apoptosis were observed in FaDu and CAL-27 cells following sevoflurane treatment. The expression of Akt, p-Akt (Ser473) and Bcl-2 was supressed, while that of Fas was significantly increased, which was partly associated with the activation of the HIF-1α pathway. In addition, the results revealed a statistically significant inhibition of cell invasion in the FaDu cell line following exposure to 2 and 4% sevoflurane for 2, 4, 6 and 8 h. Therefore, the present study demonstrated that sevoflurane decreased the malignant behavior of HNSCC cell lines *in vitro*, which was associated with activation of the HIF-1α pathway.

Introduction

Head and neck cancer (HNC) ranks as the 6th most common type of cancer worldwide, with the vast majority being head and neck squamous cell carcinomas (HNSCC) (1). Despite advances in diagnostic and treatment methods over the past two decades, the overall survival rate of HNSCC remains poor (5-year survival rate of ~50%) (2). Surgical resection is a commonly used treatment for patients with HNSCC (3). However, tumor metastasis often occurs after HNSCC surgery, and is the leading cause of death in HNSCC patients (4). During cancer resection, a number of factors may affect the risk of metastatic recurrence. These factors include combinations of the surgery *per se*, anesthetic drugs or techniques, acute pain and opioid analgesia (5), suggesting that anesthetic management may have the potential to minimize cancer recurrence.

Balanced anesthesia (inhalational combined with intravenous anesthesia) is the most widely used type of anesthesia in head and neck tumor surgery (6). Compared with total intravenous anesthesia, balanced anesthesia enables a better haemodynamic stability during surgery and a faster and safer recovery of consciousness after surgery (7), which is particularly beneficial for elderly HNSCC patients (8). However, the risks during the surgery are often overlooked. Sevoflurane, a volatile anesthetic agent that is widely used during HNSCC surgery. The duration of oral cancer surgery is often >6 h for complete resection of the primary tumor (9,10) and flap reconstruction (11,12), which means that sevoflurane may interact with the HNSCC cells for a considerable length of time. However, the effect of sevoflurane on HNSCC cells remains unclear. Therefore, the focus of the present study was the potential interaction between HNSCC cells and the common inhalational anaesthetic sevoflurane.

The effects of sevoflurane on HNSCC cell proliferation, apoptosis and invasion were investigated using two different types of HNSCC cells in an *in vitro* model, in order to elucidate the molecular mechanism underlying the effects of sevoflurane on HNSCC cells.

Materials and methods

Cell culture. The CAL-27 and FaDu HNSCC cell lines were obtained from the Institutes of Biomedical Sciences, Fudan University (Shanghai, China). The HNSCC cells were grown in Dulbecco's modified Eagle's medium (DMEM) supple-

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mented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C. All experiments were performed with cells in the logarithmic phase of growth.

Drugs. Sevoflurane was purchased from Maruishi Pharmaceutical Co., Ltd. (Osaka, Japan). The hypoxia-inducible factor-1α (HIF-1α) inhibitor YC-1 was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). For *in vitro* studies, YC-1 was dissolved in dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA, St. Louis, MO, USA) at the indicated concentrations.

Sevoflurane exposure. FaDu and CAL-27 cells were exposed to sevoflurane *in vitro* according to the experimental protocol previously described (13,14). Cell culture plates were placed in an air-tight glass chamber with inflow and outflow connectors. The chamber atmosphere was maintained continuously saturated with water at 37˚C. The entrance port of the chamber was connected to an anesthetic machine (Cicero-EM 8060; Dräger, Lübeck, Germany). Sevoflurane was delivered into the chamber by a sevoflurane vaporizer (Sevoflurane®; Abbott Laboratories, Abott Park, IL, USA) attached to the anesthesia machine. A Datex infrared gas analyser (Puritan-Bennett, Tewksbury, MA, USA) was used to continuously monitor the delivered CO_2, O_2 and sevoflurane concentrations.

Cell survival analysis. The viability of CAL-27 and FaDu cells was evaluated using the Cell Counting Kit-8 (CCK-8) assay. In brief, the cells ($5x10^3$) were plated in 96-well cell culture plates in DMEM with 10% FBS in a final volume of 0.1 ml. At 60% confluence, the cells were treated with 2 and 4% sevoflurane for 2, 4, 6 and 8 h, then placed in a CO_2 incubator for an additional 24-h culture. Cell survival was assessed by addition of 10 µl CCK-8 solution to 100 µl of medium for another 3 h in 37˚C prior to reading the absorbance at 450 nm using a Bio-Rad microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Three independent experiments were performed.

Apoptosis analysis by Annexin V/propidium iodide (PI) flow cytometry. FaDu and CAL-27 cells were cultured in 6-well plates ($1x10^6$ cells/well). After treatment with 2 and 4% sevoflurane for 2, 4, 6 and 8 h, the cells were placed in a CO_2 incubator for an additional 24-h culture. Thereafter, the apoptotic percentage of cells was measured by flow cytometry analysis using an Annexin V-fluorescein isothiocyanate (FITC) Apoptosis kit (Becton-Dickinson, Franklin Lakes, NJ, USA). The cells were washed twice with cold phosphate-buffered saline (PBS), and resuspended in 400 µl with 1X binding buffer at a concentration of $1x10^6$ cells/ml. This binding buffer was supplemented with 5 µl of Annexin V-FITC and incubated at room temperature in the dark for 15 min. PI (10 µl) was then added and incubated at 4˚C in the dark for 5 min. The cells were then immediately analyzed with a flow cytometer (BD FACSCanto II; BD Biosciences, Franklin Lakes, NJ, USA). All assays were repeated at least three times.

Transwell invasion assay. FaDu cells were seeded in 24-well plates ($2x10^5$ cells/well). After being exposed to different concentrations of sevoflurane for 2, 4, 6 and 8 h, the cells were placed in a CO_2 incubator for an additional 24 h of culture. Thereafter, the Transwell chambers, which incorporated a polycarbonate filter membrane (diameter 6.5 mm, pore size 8 µm; Corning Costar, Corning, NY, USA), were used to evaluate cell invasiveness. The polycarbonate filters at the bottom of the Transwell chamber were coated with 100 µl Matrigel (BD Biosciences) and air-dried in a laminar hood overnight. The cells were harvested and then inoculated into the upper compartment of the Transwell chambers ($2x10^5$ cells/well). DMEM with FBS was added into the lower compartment (600 µl/well). The cells were cultured at 37˚C in 5% CO_2 atmosphere for 24 h. Cells that did not penetrate the polycarbonate membrane at the bottom of the chamber were gently wiped off using a cotton swab. The membrane was then removed, fixed with 4% paraformaldehyde for 15 min and stained with 0.5% crystal violet. Five fields of vision were randomly selected under a microscope (Olympus IX51; Olympus, Tokyo, Japan), and the number of cells that penetrated the membrane was counted.

Western blotting. FaDu cells were cultured in 6-well plate ($1x10^6$ cells/well). After treatment with 2 and 4% sevoflurane for 2, 4 and 8 h, the cells were placed in a CO_2 incubator for an additional 24-h culture. Thereafter, the cell lysates were collected. The proteins were quantified using a bicinchoninic acid assay kit (Pierce, Rockford, IL, USA). The proteins (20 µg) were separated with 10% SDS-PAGE gels and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed milk and incubated overnight at 4˚C with primary antibodies: Anti-GAPDH (rabbit; 1:1,000; ab37168), HIF-1α (mouse; 1:500; ab113642) and Bcl-2 (rabbit; 1:500; ab194583) were all purchased from Abcam (Cambridge, UK); the Fas, anti-phospho-Akt (Ser473; rabbit; 1:1,000; cat. no. 9271) and anti-Akt (rabbit; 1:1,000; cat. no. 9272) antibodies were all obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The immune complexes were detected through incubation of the membrane with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:200; cat. no. 111-035-003) or goat anti-mouse antibody (1:200; cat. no. 115-035-003) (both from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h at room temperature and subsequent exposure of the membrane to enhanced chemiluminescence reagents (EMD Millipore).

HIF-1α inhibitor treatment. To investigate the effect of YC-1 (HIF-1α inhibitor) on sevoflurane-mediated apoptosis, FaDu cells were pretreated with 100 µM YC-1 for 8 h prior to exposing the cells to 2 and 4% sevoflurane for 4 h. The protein expression levels of HIF-1α, Fas and Bcl-2 were detected at 24 h post-sevoflurane treatment by western blotting.

Statistical analysis. All data are presented as the mean ± standard error of the mean. Data were examined using an analysis of variance and the least significant differences method for multi-sample comparisons. $P<0.05$ was considered to indicate statistically significant differences.

Results

Sevoflurane inhibits HNSCC cell survival. In order to determine the antiproliferative potency of single exposure of human HNSCC cells to sevoflurane, the CCK-8 assay was performed. Both the FaDu and CAL-27 cell lines exhibited growth

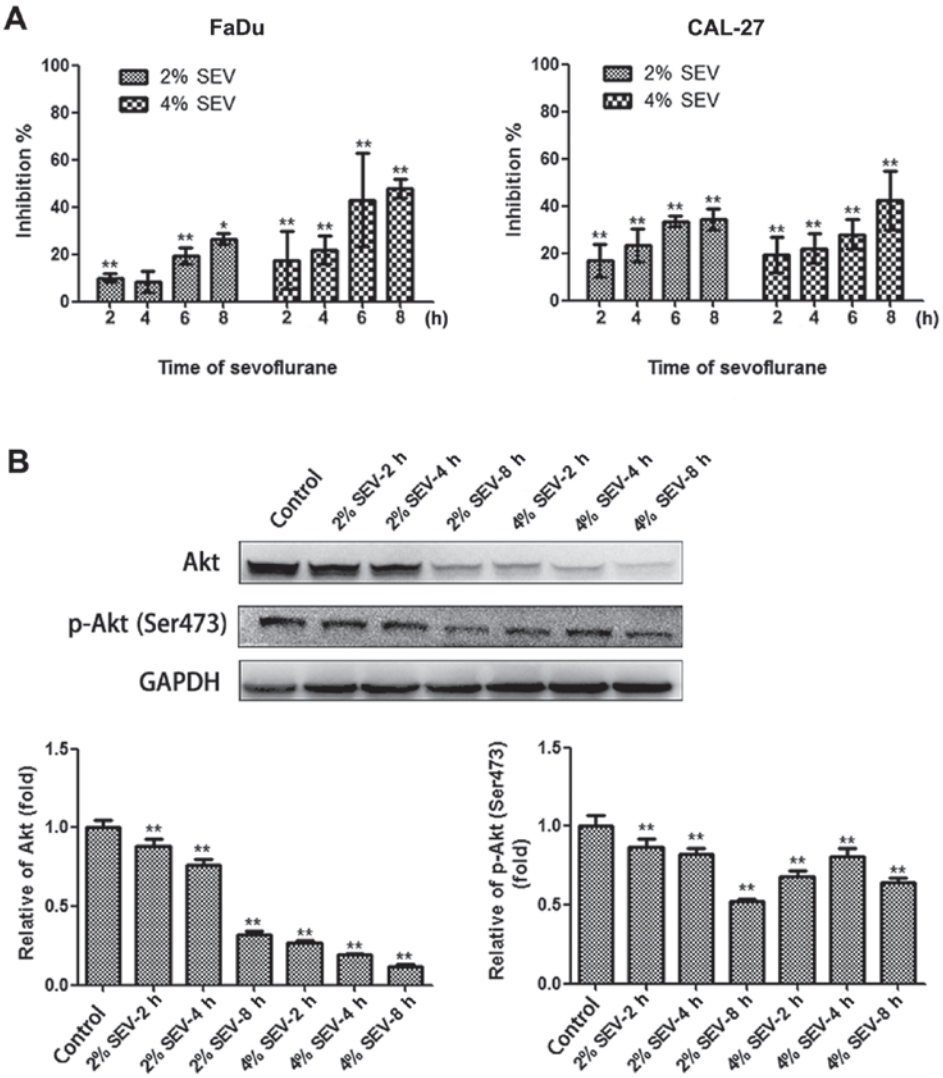

Figure 1. (A) Growth inhibition of FaDu and CAL-27 cells by 2 and 4% sevoflurane. The head and neck squamous cell carcinoma (HNSCC) cells FaDu and CAL-27 were exposed to sevoflurane for 2, 4, 6 and 8 h and were analyzed with the Cell Counting Kit-8 assay. Data are expressed as the percentage inhibition of cell growth. (B) Dose-dependent effect of sevoflurane on the activation of Akt in FaDu cells. The cells were exposed to 2 and 4% sevoflurane for 2, 4 and 8 h. After treatment, cell lysates were examined by western blotting to determine the protein levels of Akt and p-Akt (Ser473). GAPDH was used as the loading control. The results are presented as means ± standard deviation from three independent experiments. *P<0.05 and **P<0.01, respectively, using the Student's t-test for the comparison between the cells treated with sevoflurane and the corresponding control group. SEV, sevoflurane.

alterations following a single exposure to 2 and 4% sevoflurane. Our data demonstrated that the incubation of FaDu and CAL-27 cells with sevoflurane resulted in a time- and concentration-dependent elevation of cell inhibition (Fig. 1A).

Subsequently, the mechanisms underlying the reduction in cell proliferation caused by sevoflurane were investigated. It was previously suggested that the p-Akt levels were increased in response to isoflurane, which was associated with enhanced renal cancer growth and malignant potential (15). Therefore, the total and p-Akt levels in FaDu cells treated with various concentrations and action times of sevoflurane were evaluated by western blotting. A marked downregulation of both total and p-Akt levels was observed in FaDu cells (Fig. 1B).

Sevoflurane induces apoptosis of HNSCC cells. To investigate the apoptosis percentage, the cells were treated with 2 and 4% sevoflurane for 2, 4, 6 and 8 h, and the percentage of apoptotic cells was measured by flow cytometry. As shown in Fig. 2, the ratio of early apoptosis was 3.2% in the non-treated control FaDu cells and 3.5% in the non-treated control CAL-27 cells. Following sevoflurane treatment, the ratio of apoptotic cells increased in both cell lines (Fig. 2A and B). The effect of sevoflurane-induced FaDu and CAL-27 cell apoptosis was concentration- and time-dependent. Moreover, the results were further confirmed by detection of the protein levels of Fas and Bcl-2 in FaDu cells. As shown in Fig. 4, western blotting revealed that the expression of Fas was significantly increased following sevoflurane treatment, particularly with 2 or 4% sevoflurane for 8 h. Furthermore, the expression of Bcl-2 was downregulated when the cells were exposed to 2 and 4% sevoflurane for 8 h (Fig. 4).

Sevoflurane inhibits FaDu cell invasion. To investigate the effect of sevoflurane on the invasion of FaDu cells, the effect of sevoflurane on the invasive ability of cancer cells was evaluated by an invasion assay using a BioCoat Matrigel Invasion

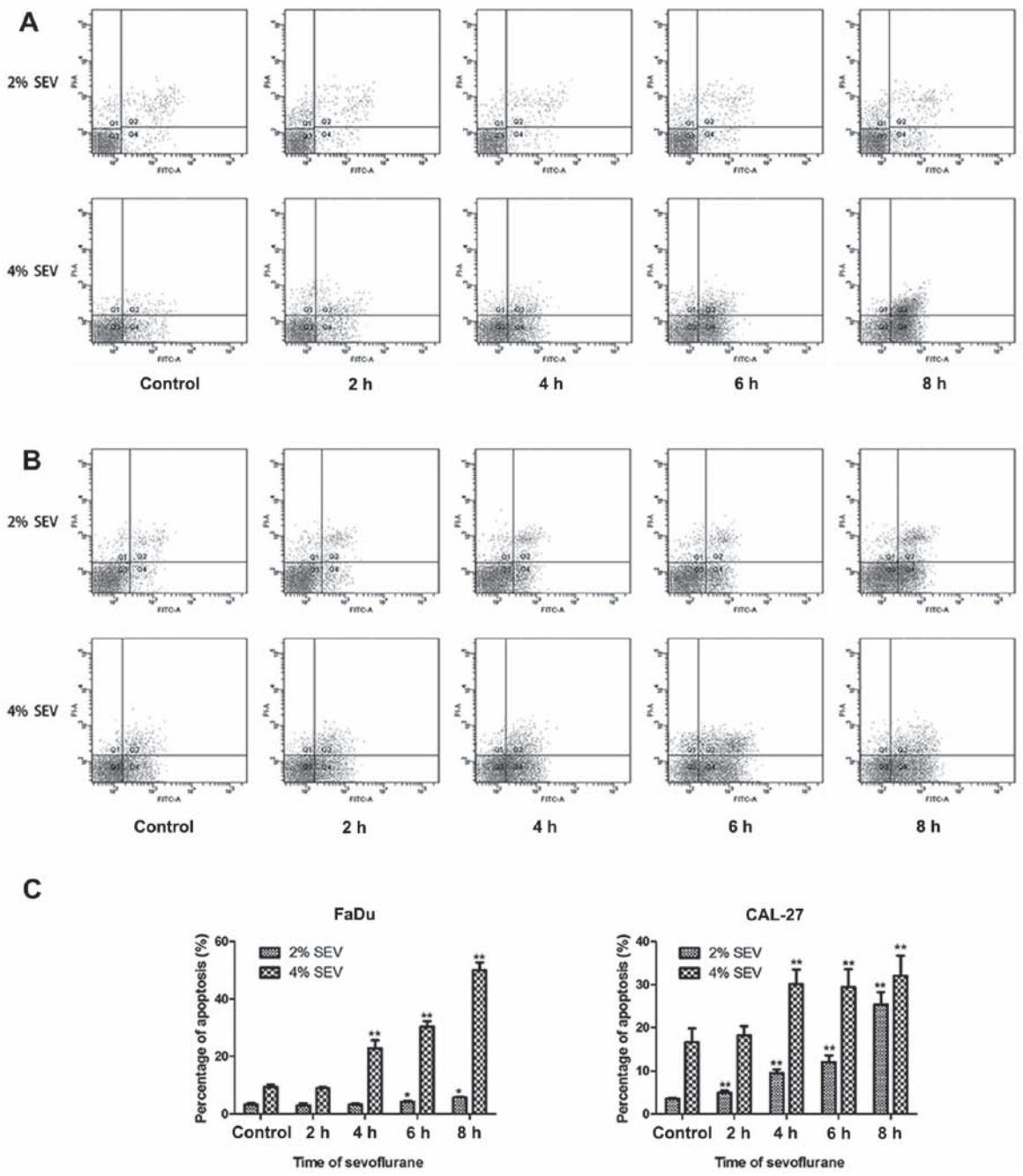

Figure 2. Effect of sevoflurane on apoptosis of FaDu and CAL-27 cells. (A) FaDu and (B) CAL-27 cells were treated with 2 and 4% sevoflurane for 2, 4, 6 and 8 h. The percentage of apoptotic cells was analyzed by Annexin V/propidium iodide staining. (C) The bars represented the percentage of apoptotic FaDu and CAL-27 cells at different time-points and concentrations of sevoflurane. Data are presented as means ± standard deviation of three independent experiments. *P<0.05 and **P<0.01 using the Student's t-test for the comparison between cells treated with sevoflurane and the corresponding control group. SEV, sevoflurane.

Chamber kit *in vitro*. The number of invading FaDu cells was significantly decreased at 6 and 8 h in the groups treated with 2% sevoflurane compared with that in the control group (number of invading cells: 246±37 in controls, 210±29.3 with 2% sevoflurane for 6 h and 173±14.6 with 2% sevoflurane for 8 h) (Fig. 3A and B). There was a significant difference at 4 h between the groups treated with 4% sevoflurane and the control group (number of invading cells: 256±12.8 in controls, 82±13.8 with 4% sevoflurane for 4 h, 55±8.2 with 4% sevoflurane for 6 h and 26±9.8 with 4% sevoflurane for 8 h) (Fig. 3A and B). These results demonstrated that sevoflurane decreased cell invasion in the FaDu cell line.

Sevoflurane induces HIF-1α expression in a time- and concentration-dependent manner. To evaluate the effect of sevoflurane on HIF-1α protein expression, FaDu cells were exposed to 2 and 4% sevoflurane for 2, 4 and 8 h, and were then harvested for immunoblotting after 24 h of incubation. Immunoblotting data revealed a significant increase in HIF-1α protein levels in a time- and concentration-dependent manner when cells were exposed to 2 and 4% sevoflurane (Fig. 4).

Effect of the HIF-1α inhibitor YC-1 on the sevoflurane-induced apoptosis signaling pathway. To further explore whether the sevoflurane upregulation of Fas expression occurred via

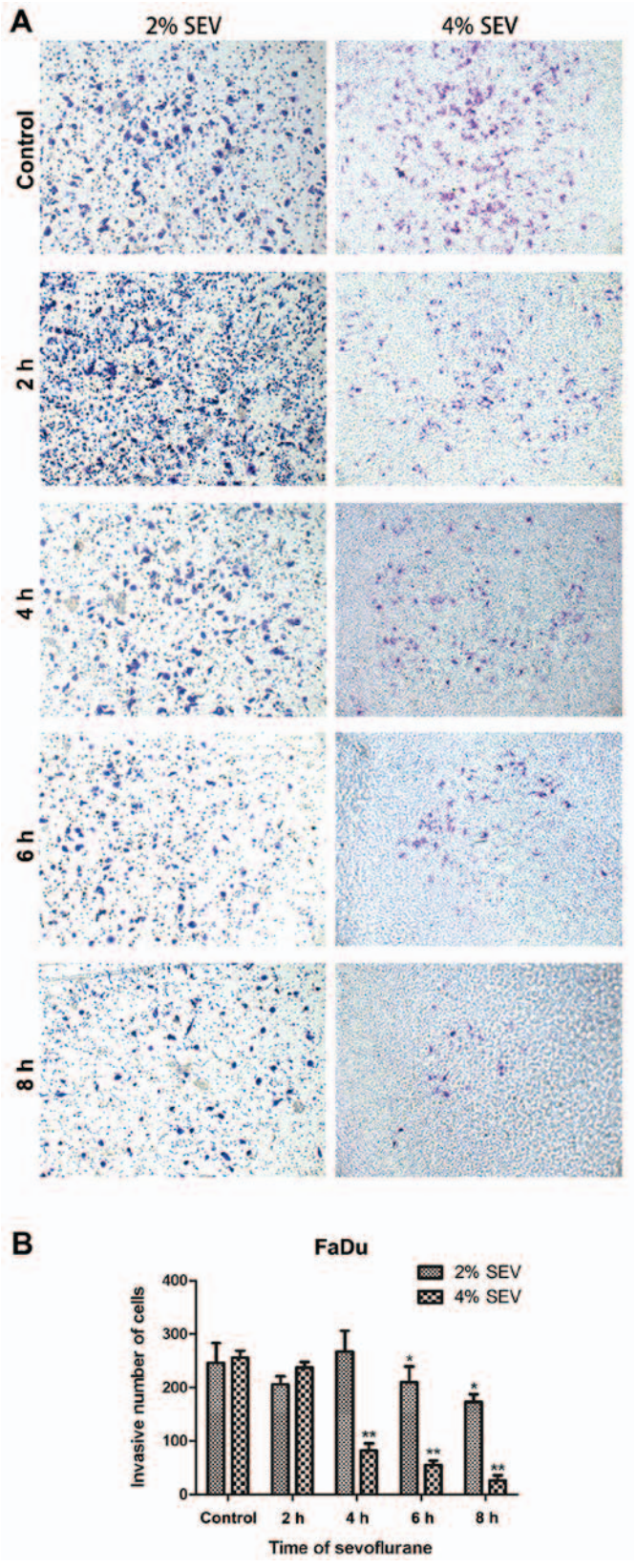

Figure 3. Effect of sevoflurane on the invasion of FaDu cells. (A) The cells were exposed to different concentrations (2 and 4%) of sevoflurane for 2, 4, 6 and 8 h, and the number of invading cells was evaluated by the Transwell invasion assay at 24 h post-sevoflurane treatment (magnification, x200). (B) Representation of the invading cells at different time-points and at different concentrations of sevoflurane. Data are presented as the means ± standard deviation of three independent experiments. *P<0.05 and **P<0.01 using the Student's t-test for the comparison between the cells treated with the indicated concentrations of sevoflurane and the control groups. SEV, sevoflurane.

activating the HIF-1α signaling pathway, FaDu cells were pretreated with the HIF-1α inhibitor YC-1 (100 μM) for 8 h prior to exposing the cells to 2 and 4% sevoflurane for 4 h. The western blotting results revealed that the combination treatment of YC-1 with 2 and 4% sevoflurane was able to downregulate the level of HIF-1α and inhibit the expression of Fas more effectively compared with sevoflurane treatment or YC-1 treatment alone; however, there was no obvious effect of YC-1 on Bcl-2 (Fig. 5). The results suggested that increased Fas expression was partly associated with activation of the HIF-1α pathway.

Discussion

Whether the anesthetic procedures affect the outcome after cancer surgery has been a research focus. Retrospective data have demonstrated that the choice of anaesthesia technique for cancer surgery may affect the risk of cancer recurrence and metastasis (4,16-18). Sevoflurane as an inhalational anesthetic that is extensively used during HNSCC surgery as anesthesia maintenance or induction. However, the risks during the operative period are often overlooked. The duration of oral cancer surgery is often >6 h, and during this time the effect of sevoflurane on tumor cells is unknown. It has been reported that sevoflurane may induce T-lymphocyte (19), H4 human neuroglioma cell (20) and Jurkat T cell (21) apoptosis. Moreover, the anticancer effect of sevoflurane has been previously reported. *In vitro* studies demonstrated that sevoflurane exerted an inhibitory effect on growth and promoted apoptosis of SW620 colon cancer (22), Caco-2 laryngeal cancer (23) and A549 lung adenocarcinoma cells (24). However, the effect of sevoflurane on the proliferation, apoptosis, cell cycle, migration and invasion of HNSCC cells remains unclear, and the underlying molecular mechanisms require further elucidation.

In the present study, the effect of clinically relevant concentrations of sevoflurane on HNSCC cells was examined. FaDu and CAL-27 are two widely used HNSCC cell lines. Sevoflurane obviously affected the viability of FaDu and CAL-27 cells *in vitro* (Fig. 1A). Upon investigating the mechanisms underlying the reduction in cell proliferation caused by sevoflurane, it was observed that Akt and p-Akt (Ser473) were inhibited by sevoflurane treatment. Thus, it was hypothesized that sevoflurane exerts its the antitumor effect partially through the PI3K/Akt pathway.

The molecular mechanism of apoptosis induction in HNSCC cells after a single exposure to sevoflurane in clinically useful concentrations was also investigated. Under our experimental conditions, the ratio of apoptotic cells significantly increased in both the FaDu and CAL-27 cell lines. Fas and its ligand, FasL, are cell surface receptors that belong to the tumor necrosis factor receptor family. Interaction of the Fas receptor on cells with FasL results in ligand-mediated cell death (25-28). Fas-mediated cell death has also been implicated in the regulation of tumor development, growth and progression. Downregulation of Fas or impaired Fas signaling have been correlated with tumor progression (27,29-32). Bcl-2 is an anti-apoptotic member of the Bcl-2 family, which prevents apoptosis by inhibiting the release of mitochondrial apoptogenic factors into the cytoplasm (33). In the present study, the data demonstrated that sevoflurane upregulated the levels

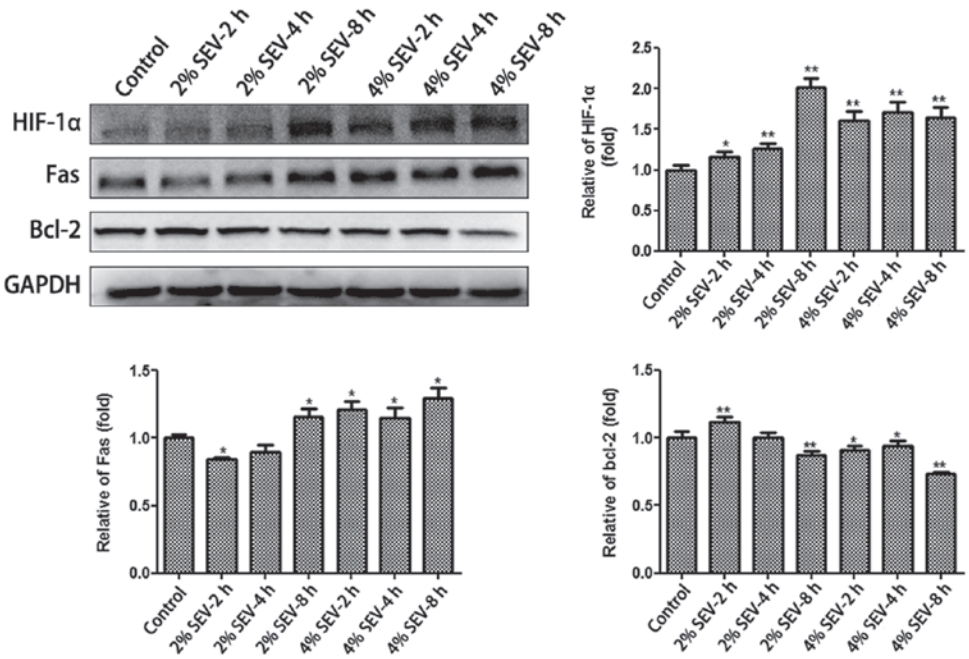

Figure 4. Dose-dependent effect of sevoflurane on the expression of hypoxia-inducible factor-1α (HIF-1α), Fas and Bcl-2 in FaDu cells. The cells were exposed to 2 and 4% sevoflurane for 2, 4 and 8 h. After treatment, cell lysates were examined by western blotting for the protein levels of HIF-1α, Fas and Bcl-2. GAPDH was used as the loading control. The results are presented as the means ± standard deviation from three independent experiments. *P<0.05 and **P<0.01 using the Student's t-test for comparison between cells treated with sevoflurane and the corresponding control group.

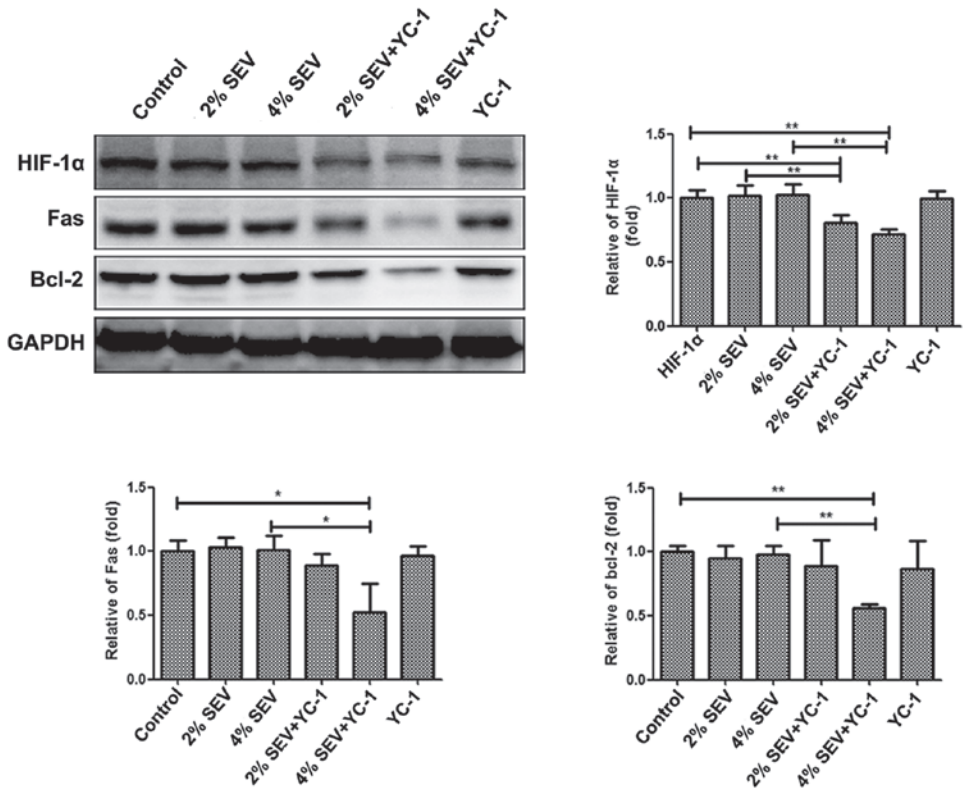

Figure 5. Effect of the hypoxia-inducible factor-1α (HIF-1α) inhibitor YC-1 on the sevoflurane-induced apoptosis signaling pathway in FaDu cells. Cells were pretreated with HIF-1α inhibitor YC-1 (100 μM) for 8 h and then treated with 2 and 4% sevoflurane for 4 h. The expression of HIF-1α, Fas and Bcl-2 in the FaDu cells in each group were detected by western blotting at 24 h post-sevoflurane treatment. *P<0.05 and **P<0.01 using the Student's t-test for comparison between the cells treated with sevoflurane and the corresponding control group.

of Fas and downregulated the levels of Bcl-2 in FaDu cells. In addition, it was observed that HIF-1α was noticeably increased when the cells were exposed to sevoflurane. HIF-1α is a dimeric transcription factor that mediates various cellular

responses to hypoxia and may be upregulated by sevoflurane and isoflurane (34-37). Furthermore, the increase in the level of Fas was abolished when cells were pretreated with the HIF-1α inhibitor YC-1 (Fig. 5). There was no change in Bcl-2 when YC-1 was used. These findings suggest that sevoflurane-mediated cell apoptosis was associated with activation of the Fas/FasL signaling pathway, which may be regulated by HIF-1α.

The development of cancer invasion is a complex cascade of events involving tumor dissemination from the primary site to distant organs. During this process, the degradation of extracellular matrix, which poses a biochemical and mechanical barrier to cell movement, has been shown to be an important biological process in the metastasis of cancer cells (38,39). Recent research reported that sevoflurane may attenuate the migration and invasion in U87MG glioma cells (40) and LoVo colon cancer cells (41) *in vitro*. In the present study, exposure to sevoflurane at clinically relevant concentrations significantly inhibited the invasion of FaDu cells (Fig. 3). However, cancer metastasis is not only regulated by the direct effects of the general anesthetic on the tumor cells, but is also affected by the effects of the anesthetic on immune cells (42).

In conclusion, the results of the present study demonstrated that sevoflurane inhibited proliferation, invasion and migration, and induced apoptosis in HNSCC cells. Moreover, the results of the study indicated that the antiproliferative effect of sevoflurane was associated with downregulating the expression of p-Akt (Ser473), and that the resulting cell apoptosis was associated with activation of the Fas/FasL signaling pathway, which may be regulated by HIF-1α. Finally, some potential limitations of the present study should be mentioned: *In vivo* research should also be conducted to veify the findings of the present study, as it more accurately simulates the tumor environment and provides more objective results of tumor growth; the molecular mechanism of the effect sevoflurane on HNSCC should also be fully elucidated to reach definitive conclusions.

Acknowledgements

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