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Isoflurane promotes proliferation and invasion of cervical carcinoma cells via downregulation of miR-375 expression

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Summary

Objective: Isoflurane is a widely used volatile anesthetic. The aim of the current study was to evaluate effects of isoflurane on the behavior of human cervical cancer (CC) cells and the molecular mechanisms related with this activity. *Materials and Methods:* The effects of isoflurane on CC cell proliferation, apoptosis, and invasion were detected by CCK-8 assay, flow cytometry, and matrigel invasion assay. Real-time PCR was used to assess miR-375 expression. MiR-375 mimics were transfected into CC cells to upregulate miR-375 levels. *Results:* Isoflurane significantly promoted CC cell proliferation and invasion, and suppressed cell apoptosis. Isoflurane also efficiently decreased miR-375 expression. Moreover, transfection of miR-375 mimics reversed the effects of isoflurane on the biological behavior of CC cells. *Conclusions:* The present data indicates that isoflurane could promote CC cell viability and invasion and inhibit cell apoptosis through the downregulation of miR-375.

Key words: Isoflurane; Cervical cancer; miR-375; Proliferation; Invasion.

Introduction

Cervical cancer (CC), the second most frequently diagnosed gynecological cancer, is a serious threat to women's health worldwide [1]. Despite recent development in diagnostic technologies and treatment modalities, many CC patients are diagnosed at the advanced stage, and the long-term survival of these patients is still unsatisfactory. It is urgent to elucidate the molecular mechanisms underlying CC and investigate new therapeutic strategies to improve the prognosis of CC patients.

MicroRNAs (miRs) are short (19-25 nucleotides in length), non-coding, single-stranded RNAs that negatively regulate target gene expression at the post-transcriptional level [2]. It is now clear that miRs play important roles in multiple biological processes related to carcinogenesis and cancer progression [3]. Upregulation of oncogenic miRs and downregulation of tumor suppressive miRs have been reported in a wide range of cancers including CC [4]. As a potential tumor suppressor, miR-375 has been revealed to be downregulated in many human malignancies, such as laryngeal squamous cell carcinoma [5], papillary thyroid carcinoma [6], esophageal squamous cell carcinoma [7], lung cancer [8], gastric cancer [9], hepatocellular carcinoma [10], colorectal cancer [11], and pancreatic ductal adenocarcinoma [12]. Wang *et al.* confirmed decreased miR-375 expression in CC patients and its association with lymph node metastasis [13]. Over-expression of miR-375 was able to suppress CC cell proliferation, block G1-to-S cell-cycle transition, increase cell apoptosis, and inhibit cell migration and invasion [13, 14]. Moreover, miR-375 promoted radiosensitivity of CC cells in vitro [15]. These findings indicated that miR-375 might be implicated in CC development and could act as a therapeutic target.

Currently, surgery remains the first-line treatment for the majority of CC patients. Extensive studies have demonstrated that events within the perioperative period, including anesthetic technique and analgesic drugs, significantly influence the outcomes of cancer patients (recurrence, metastasis, and prognosis) [16, 17]. Isoflurane is a widely used volatile anesthetic. Some recent studies showed that isoflurane could promote malignant biological behaviors of non-small cell lung cancer (NSCLC) [18], prostate cancer [19], ovarian cancer [20], and renal cancer cells [21]. However, little information is available regarding the potential effects of isoflurane in CC cells. The aims of this study were to investigate the effects of isoflurane on the biological behavior of CC cancer cells, as well as to assess the related molecular mechanisms.

Materials and Methods

Human CC cell lines HeLa and SiHa were obtained and cultured in DMEM supplemented with 10 % fetal bovine serum at 37°C in an atmosphere consisting of 5% CO_2.

Isoflurane gas exposure was performed as previously described. [19, 21]. Briefly, HeLa and SiHa cells were placed in purposely-built chambers connected to calibrated flow

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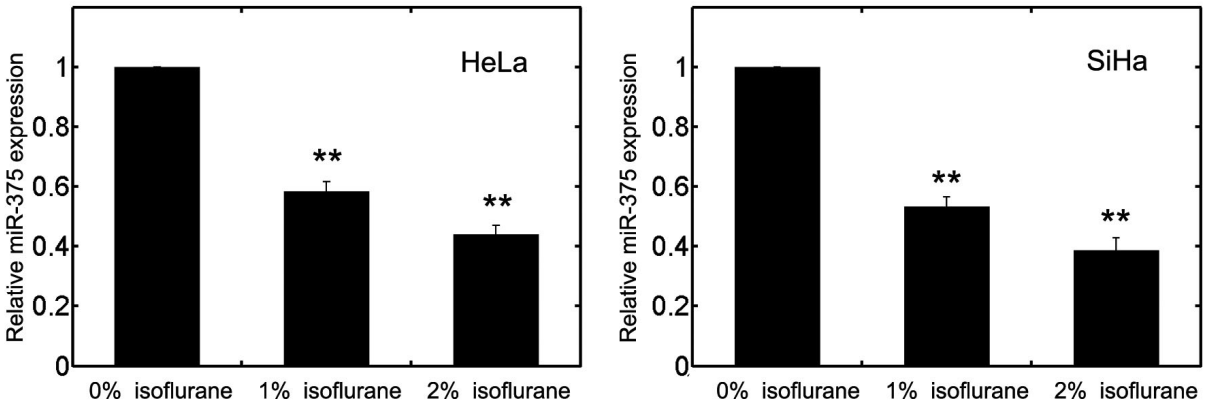

Figure 1. — Isoflurane suppresses miR-375 expression in both HeLa and SiHa cells. $^{**}p < 0.01$ compared with the control group without isoflurane treatment.

meters and an in-line vaporizer used to deliver the desired composition of isoflurane in 21% oxygen and 5% CO_2 balanced with nitrogen. Cells were exposed to different concentrations of isoflurane (0, 1.0 or 2.0%) for two hours at 37°C, and then returned to a standard incubator containing humidified air and 5% CO_2. Cells were analyzed 12 hours after isoflurane gas exposure.

CC cells were plated in 96-well plates (5×10^3 cells/well), and cell viability was measured using the cell counting kit-8 (CCK-8) every 24 hours according to the manufacturer's protocol. Briefly, 10 μL of CCK-8 reagents were added into each well and the plates were incubated for one hour at 37°C. The OD values were measured with a microplate reader at the 450-nm wavelength.

Apoptosis was assessed using Annexin V-FITC/PI double staining kit. After isoflurane exposure, CC cells were washed twice with cold PBS and resuspended in 1× binding buffer at 1×10^5 cells/mL. Then 10 μL of annexin V-FITC and 5 μL of propidium iodide (PI) were added to 200 μL of cells and incubated at 4°C in the dark for 30 minutes. The cells were examined by flow cytometry and the data were analyzed using the FlowJo v7.6 software package.

Cell invasion assay was performed using Transwell chambers coated with Matrigel. Briefly, HeLa and SiHa cells were seeded in the upper chambers with serum-free DMEM, while DMEM containing 20% FBS was added to the lower chambers. Twenty-four hours later, cells that invaded through the Matrigel were fixed with 95 % ethanol and stained with crystal violet. The number of invaded cells was counted in five randomly selected microscopic fields.

After isoflurane treatment, CC cells were collected and washed with PBS. Total RNA was extracted using the Trizol reagent according to the manufacturer's protocol, and was reverse transcribed into cDNA using primescript RT reagent . qRT-PCR was performed on a 7500 RT-PCR system using SYBR premix kit, with U6 snRNA as an internal control. For miR-375, the primers were as follows: forward, 5'-AGC CGT TTG TTC GTT CGG CT-3' and reverse, 5'-GTG CAG GGT CCG AGG T-3'. For U6, the primers were as follows: forward 5'-CTC GCT TCG GCA GCA CA-3', and reverse, 5'-AAC GCT TCA CGA ATT TGC GT-3'. All samples were run in triplicate, and the relative amount of miR-375 to U6 was calculated according to the $2^{-\Delta Ct}$ method.

miR-375 mimics and negative control (NC) were purchased. CC cells were seeded in 24-well plates at 3×10^5 cells/well and incubated overnight. Transfection was performed using lipofectamine 2000 transfection reagent. The cells were harvested for further analysis 48 hours after transfection.

All experiments were performed in triplicate, and the data were expressed as mean ± SD. Data were analyzed via Student's *t*-test or one-way analysis of variance (ANOVA), using Statistical package 19.0. All tests were two-tailed, and the significance level was set at $p < 0.05$.

Results

As shown in Figure 1, treatment with 1% or 2% isoflurane significantly decreased the levels of miR-375 in HeLa cells compared to the untreated cells ($p < 0.01$). Similar results were observed in SiHa cells ($p < 0.01$, Figure 1). These results suggested that isoflurane could downregulate the expression of miR-375 in CC cells. Two percent isoflurane was used to investigate the effects of isoflurane on the biological behavior of CC cells in further analyzes.

The authors observed upregulated miR-375 expression after miR-375 mimics transfection in CC cells ($p < 0.01$, Figure 2A). CCK-8 assay was performed to determine cell viability and the results showed that cell viability in both HeLa and SiHa cells was significantly increased by isoflurane compared to the control groups ($p < 0.01$, Figure 2B).

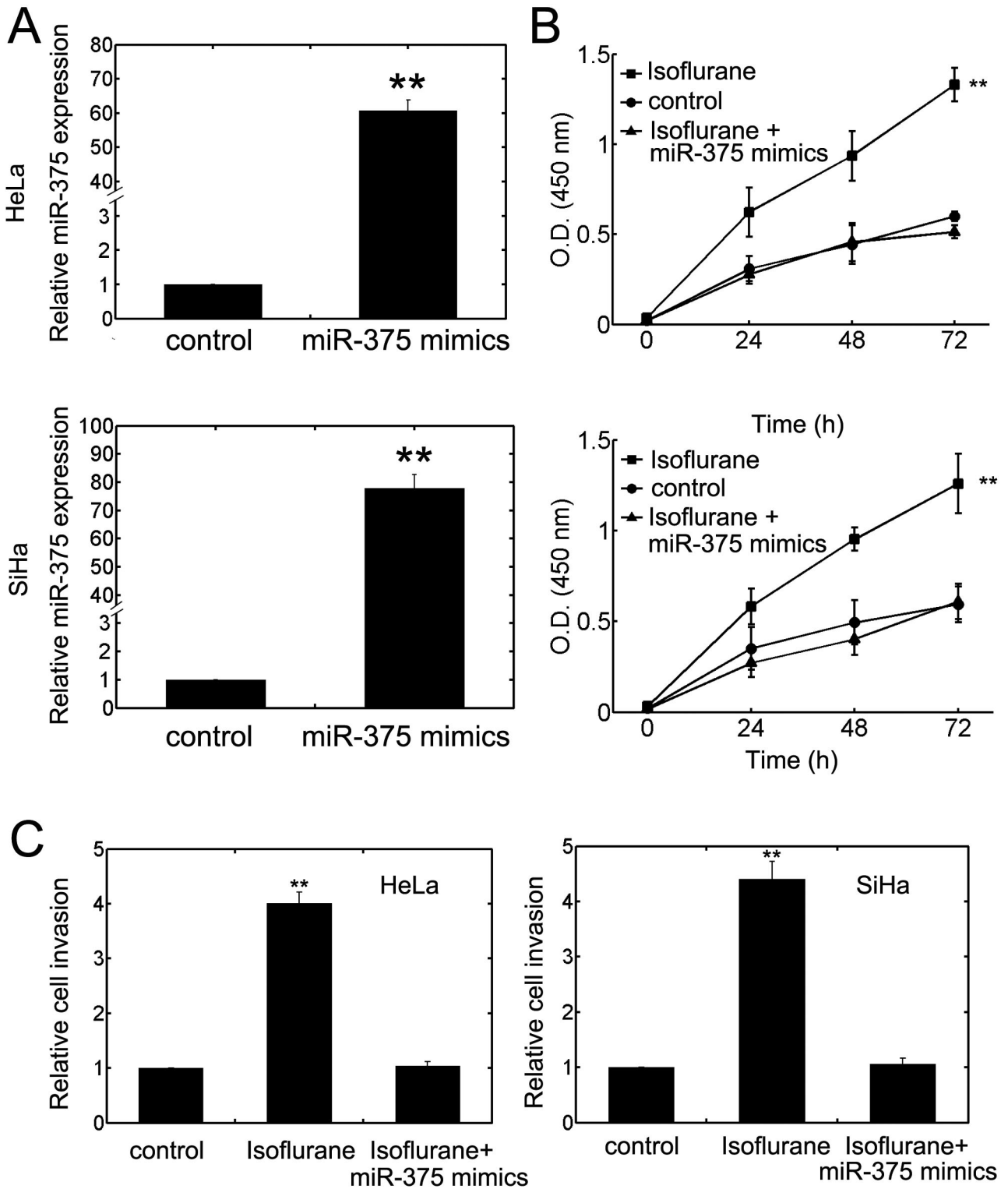

Figure 2. — Isoflurane promoted cervical cancer (CC) cell proliferation and invasion, while miR-375 overexpression could abolish these effects. MiR-375 mimics transfection significantly increase the expression of miR-375 in both HeLa and SiHa cells. ** $p < 0.01$ *vs.* control. CCK8 assay shows that cell viability is significantly increased by isoflurane, which could be reversed by miR-375 mimics transfection. ** $p < 0.01$ *vs.* control. Transwell invasion assay indicated increased CC cell invasion after isoflurane treatment, while miR-375 overexpression reversed these effects. ** $p < 0.01$ *vs.* control.

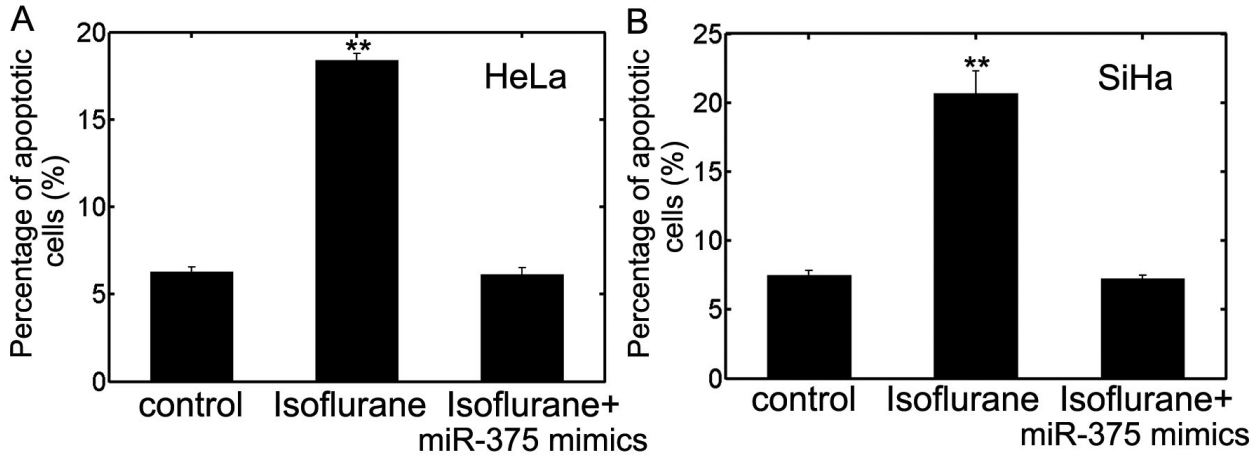

Figure 3. — Isoflurane significantly suppresses cell apoptosis in both HeLa and SiHa cells, while miR-375 overexpression reverses these effects. ** $p < 0.01$ *vs.* control.

Matrigel invasion assay also revealed that isoflurane significantly promoted HeLa and SiHa cell invasion ($p < 0.01$, Figure 2C). Further, miR-375 overexpression abolished isoflurane-induced promotion of CC cell proliferation and invasion (Figures 2B-2C).

As indicated in Figure 3, the percentages of apoptotic cells were significantly downregulated by administration of isoflurane compared to the control group ($p < 0.01$) in both HeLa and SiHa cells. However, miR-375 overexpression reversed isoflurane-induced suppression of CC cell apoptosis. These results suggested that isoflurane inhibited CC cell apoptosis, which could be reversed by miR-375.

Discussion

There is increasing evidence in recent years, suggesting that anesthetic technique may affect long-term outcomes of cancer patients after surgery [17, 22]. In vitro experiments also showed that anesthetic drugs could affect the malignant phenotypes of cancer cells [23, 24]. In the present study, the authors investigated the influence of isoflurane on the behavior of CC cells and the role of miR-375 in these effects. They found that isoflurane decreased miR-375 expression in HeLa and SiHa cells. Functional assays indicated that isoflurane promoted CC cell viability and invasion and inhibited cell apoptosis, and these effects could be reversed by miR-375 overexpression. To the best of the authors' knowledge, this study revealed the oncogenic properties of isoflurane through downregulation of miR-375 in CC for the first time.

The present findings were consistent with the results of other studies. For example, Zhang *et al.* revealed that isoflurane promoted NSCLC cell proliferation, migration and invasion [18]. Huang *et al.* reported that isoflurane increased prostate cancer cell proliferation, migration, and chemoresistance to docetaxel [19]. Moreover, isoflurane increased the malignant potential (proliferation, migration, and angiogenesis) of ovarian cancer and renal cancer cells [20, 21]. Thus, the oncogenic properties of isoflurane were not specific in CC, and the effects of isoflurane on the biological behavior of other cancer cells deserve further study.

Mounting evidence has demonstrated that miRs are important regulators in CC progression [25-27]. Decreased miR-375 expression and its tumor suppressive function has been confirmed in CC [13, 14]. To clarify the mechanism underlying isoflurane-induced promotion of malignant phenotypes in CC cells, the effect of isoflurane on miR-375 expression was examined. The present authors found that isoflurane suppressed the expression of miR-375 in CC cells. More importantly, upregulation of miR-375 via miR-375 mimics transfection reversed the effect of isoflurane on CC cell proliferation, apoptosis, and invasion, indicating that the oncogenic function of isoflurane in CC may be partly due to its inhibition of miR-375 expression. Generally, miRs exert their function through regulating target gene expression. In terms of tumor suppressive miR-375, several related pathways have been corroborated, including PDPK1/RPS6KA3[28], PDK-1-AKT[29], p53[15], JAK2/STAT[30], MAP3K8/ERK[31], and Notch pathway [32]. Thus, the potential regulatory circuitry afforded by miR-375 may be enormous, and the target genes involved in miR-375-mediated CC progression need to be further elucidated in future studies.

In conclusion, the present study indicated that isoflurane could promote CC cell viability and invasion and inhibit cell apoptosis. Downregulation of miR-375 might contribute to the oncogenic activity of isoflurane. Future studies are needed to validate its clinical relevance.

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