Propofol suppresses growth, migration and invasion of A549 cells by down-regulation of miR-372

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Abstract

Background: Propofol, a commonly used intravenous anesthetic during cancer resection surgery, has been found to exhibit tumor inhibitory effects in vitro and in vivo. The role of propofol in lung cancer has been previously reported, whereas its action mechanism remains unclear. This study further investigated the effects of propofol on lung cancer A549 cell growth, migration and invasion, as well as the underlying mechanisms.

Methods: Cell viability, proliferation, migration, invasion and apoptosis were assessed by CCK-8 assay, BrdU assay, two chamber transwell assay and flow cytometry, respectively. The regulatory effect of propofol on microRNA-372 (miR-372) expression in A549 cells was analyzed by qRT-PCR. Cell transfection was used to change the expression of miR-372. The protein expression of key factors involving in cell proliferation, apoptosis, migration and invasion, as well as Wnt/β-catenin and mTOR pathways were analyzed by western blotting.

Results: Propofol inhibited lung cancer A549 cell viability, proliferation, migration, and invasion, but promoted cell apoptosis. Moreover, miR-372 was down-regulated in propofol-treated A549 cells. Overexpression of miR-372 abrogated the effects of propofol on proliferation, migration, invasion and apoptosis of A549 cells. Knockdown of miR-372 had opposite effects. Furthermore, propofol suppressed Wnt/β-catenin and mTOR signaling pathways by down-regulating miR-372.

Conclusion: Propofol inhibits growth, migration and invasion of lung cancer A549 cells at least in part by down-regulating miR-372 and then inactivating Wnt/β-catenin and mTOR pathways.

Keywords: Lung cancer, Propofol, microRNA-372, Wnt/β-catenin pathway, mTOR signaling pathway

Introduction

Lung cancer is the most leading cause of cancer-related deaths all around the world, which accounts for approximately 1.8 million new cases and 1.2 million deaths each year [1, 2]. The 5-year survival rates of patients with lung cancer vary from 4 to 17% depending on different histological features and disease stage [3]. Unfortunately, advances in diagnosis and therapeutic strategies, including percutaneous lung biopsy, tumor marker detection, surgical, medicine, and radiological intervention, still doesn’t effectively improved the long-term survival rate of lung cancer patients [4, 5]. Novel and more effective therapeutic medicines are urgently needed.

Propofol (2, 6-diisopropylphenol) is one of the most widely accepted and commonly used intravenous sedative hypnotic agents. Increasing evidences show that propofol not only has various anesthetic advantages, but also possesses a number of non-anesthetic effects [6]. Hsing et al. showed that propofol could inhibit endotoxic inflammation by decreasing reactive oxygen species (ROS) generation [7]. Cui et al. indicated that propofol could prevent oxygen and glucose deprivation-induced autophagic cell death of PC-12 cells and cerebral ischemia-reperfusion injury in rats [8]. Interestingly, a possible correlation between propofol and cancer has been observed in recent years, which revealed that propofol could exert tumor suppressive or tumor promoting effects in different cancers [9, 10]. In terms of lung cancer, Liu et al. proved that propofol inhibited the growth and epithelial-mesenchymal transition...
MicroRNAs are a class of small and endogenous RNA transcripts in cells without protein-coding activity [12]. Some miRNAs have been found to be aberrantly expressed in lung cancer patients, indicating that these miRNAs may play important roles in the pathogenesis of lung cancer [13]. Many links between lung cancer and miRNAs have been reported, including low expression of miR-21 and high expression of miR-92 [14], as well as tumor suppressor function of miR-34 [15]. In addition, miR-372 is frequently up-regulated in patients with lung cancer [16], hepatocellular carcinoma [17], and colorectal cancer [18]. Wang et al. reported that Up-regulation of miR-372 promoted growth and metastasis of lung squamous cell carcinoma cells, while down-regulation of miR-372 inhibited cell growth and metastasis [16].

In this study, we further investigated the effects of propofol on proliferation, apoptosis, migration, and invasion of lung cancer A549 cells. To clarify the underlying molecular mechanism of tumor suppressive roles of propofol in A549 cells, we detected the expression of miR-372 and analyzed the mediating effects of miR-372 on growth and metastasis of A549 cells.

Materials and methods

Cell culture and treatment

Human lung cancer cell line A549 (American Type Culture Collection, ATCC, Manassas, VA, USA) was cultured in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO, USA) containing 1% (v/v) 1× antibiotic-antimycotic mixture (Thermo Fisher Scientific, Waltham, MA, USA) and 10% (v/v) fetal serum albumin (FBS, Sigma-Aldrich) at 37 °C in a humidity incubator with 5% CO₂ and 95% air.

Human lung epithelial cell line BEAS-2B was obtained from Cell Bank of Chinese Academy of Science (Shanghai, China) and cultured in BEGM Bullet kit (Clonetics Corporation, Walkersville, MD, USA) at 37 °C in a humidity incubator with 5% CO₂ and 95% air.

Cells were treated by propofol (Sigma-Aldrich) from 2 to 10 μg/mL for 48 h in this research.

CCK-8 assay

A549 or BEAS-2B cells were seeded in 96-well plate (Thermo Fisher Scientific) with 5 × 10³ cells/well. After 2–10 μg/mL propofol treatment for 48 h, 10 μL CCK-8 solution was added into the culture medium of each well. The plates were further incubated for 1 h at 37 °C. Then, the absorbance of each well was measured at 450 nm using the Microplate Reader (Bio-Rad, Hercules, CA, USA). Cell viability (%) was calculated by average absorbance of propofol treatment group/average absorbance of control group × 100%.

Proliferation assay

Transfected or non-transfected A549 cells spread to the bottom of the dish with diameter of 3.5 cm were incubated for 24 h. Bromodeoxyuridine (BrdU, Sigma-Aldrich) was added into the culture medium at the concentration of 1 mg/ml before 8 μg/mL propofol treatment by 3 h. After that, cells were successively incubated with rat anti-BrdU antibody (ab6326) and goat anti rat IgG (ab150157, Abcam Biotechnology, Cambridge, MA, USA). Subsequently, the rate of BrdU-positive cells in each group were observed and counted under the fluorescence microscope (Nikon, Japan) from 10 selected visual fields.

Apoptosis assay

Cell apoptosis was determined by propidium iodide (PI) and fluorescein isothiocyanate (FITC)-conjugated Annexin V staining and flow cytometry analysis. Briefly, transfected or non-transfected A549 cells were seeded into 6-well plate (Thermos Fisher Scientific) with 1 × 10⁵ cells/well. After 8 μg/mL propofol treatment for 48 h, cells were washed twice with phosphate buffer saline (PBS) and suspended in binding buffer containing Annexin V-FITC for 15 min at room temperature in the dark. Then, PI solution was added into the cell suspension and cell suspension was incubated at room temperature for 10 min in the dark. Followed by washing twice with PBS, the rate of apoptotic cells was measured using flow cytometry analysis with FACSscan (Beckman Coulter, Fullerton, CA, USA). Data were analyzed by using FlowJo software.

Migration and invasion assay

The migration and invasion of A549 cells were determined by two chamber transwell assay (Corning Incorporation, New York, NY, USA). Briefly, after relevant treatment or transfection, 1 × 10⁵ A549 cells were suspended in 200 μl serum-free RPMI-1640 medium and added into the upper chamber. 600 μl complete RPMI-1640 medium was added into the lower chamber. After incubation for 48 h at 37 °C, cells were fixed with methanol immediately. Non-traversed cells in upper chamber were removed using cotton swab carefully and traversed cells in lower chamber was stained using crystal violet and counted under microscope. Cell migration (%) was calculated by average migrated cells in propofol treatment group/average migrated cells in control group × 100%.

Cell invasion was conducted similarly with the cell migration assay except that the upper side of the polycarbonate film was spread with Matrigel (500 ng/μL; BD Biosciences, Franklin Lakes, NJ, USA). Cell invasion (%) was calculated by average invaded cells in propofol
treatment group/average invaded cells in control group × 100%.

Cell transfection
miR-372 mimic, miR-372 inhibitor and their negative control (NC) were synthesized by GenePharma Corporation (Shanghai, China). Cell transfection was conducted using lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instruction.

qRT-PCR analysis
Total RNA in A549 cells was extracted from cells using Trizol reagent (Life Technologies). The Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II with the TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA, USA) were used for determining the expression of miR-372. The expression of U6 acted as internal control. Data were calculated by 2\(-\Delta\Delta\text{Ct}\) method [19].

Western blotting
RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Basel, Switzerland) was used to extract the total proteins in A549 cells after relevant treatment or transfection. After quantification by using BCA assay (Beyotime Biotechnology, Shanghai, China), 30 μg of proteins in each group were electrophoresed by SDS-PAGE and transferred onto the PVDF membranes. The primary antibodies of p53 (ab131442), p16 (ab118459), Cyclin D1 (ab134175), Bcl-2 (ab32124), Bax (ab32503), cleaved-Caspase-3 (ab32042) cleaved-Caspase-9 (ab2324), metalloproteinase-9 (MMP-9, ab73734), Vimentin (ab8978), Wnt3a (ab28472), β-catenin (ab32572), p70S6K (ab2571, phospho T389), p70S6K (ab9366), p-mTOR (ab84400, phospho S2448), mTOR (ab2732), and β-actin (ab8226) as well as the appropriate secondary antibodies were all obtained from Abcam Biotechnology. PVDF membranes were incubated with the primary antibody at 4 °C overnight and secondary antibodies at room temperature for 2 h in the dark. After the membrane surface was covered by 200 μL Immobilon Western chemiluminescent HRP substrate (Millipore, Massachusetts, USA), the signals were captured, and the intensities of the bands were quantified by Image Lab™ software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis
All experiments in this research were repeated three times. Results of multiple experiments were presented as mean ± standard deviation (SD). Graphpad Prism version 6.0 software (GraphPad Software, San Diego California, USA) was conducted to statistical analysis. P-values were calculated using one-way analysis of variance, two-way analysis of variance or student t test. In all figures, the P < 0.05 was considered to indicate a statistically significant result.

Results
Propofol suppressed A549 cell growth, but induced cell apoptosis
Firstly, the effects of propofol on viability, proliferation, and apoptosis of A549 cells were evaluated. Results in Fig. 1a showed that propofol suppressed the viability of A549 cells in a dose-dependent manner (P < 0.05, P < 0.01 or P < 0.001). Figure 1b displayed that 2–8 μg/mL propofol treatment had no significant effects on BEAS-2B cell viability, while 10 μg/mL propofol treatment remarkably reduced the viability of BEAS-2B cells (P < 0.05). 8 μg/mL propofol treatment was chosen for further experiments. Figure 1c presented that the BrdU-positive cells were notably reduced after 8 μg/mL propofol treatment (P < 0.01). The expressions of anti-proliferative proteins, p53 and p16 were both up-regulated, while the expression of pro-proliferative protein Cyclin D1 was down-regulated in A549 cells after 8 μg/mL propofol treatment (P < 0.001, Fig. 1d). In addition, 8 μg/mL propofol treatment significantly promoted A549 cell apoptosis (P < 0.001, Fig. 1e). The expression of anti-apoptotic protein Bcl-2 was reduced, while the expressions of pro-apoptotic proteins Bax, cleaved-Caspase-3 and cleaved-Caspase-9 were enhanced in A549 cells after 8 μg/mL propofol treatment (P < 0.01 or P < 0.001, Fig. 1f). Taken together, these results suggested that propofol could effectively suppress A549 cell growth, but induced cell apoptosis.

Propofol inhibited the migration and invasion of A549 cells
Then, the effects of propofol on migration and invasion of A549 cells were studied. Results showed that 8 μg/mL propofol treatment significantly suppressed the migration and invasion of A549 cells (P < 0.05 or P < 0.01, Fig. 2a and b). The protein expressions of MMP-9 and Vimentin in propofol-treated A549 cells were both decreased (P < 0.05 or P < 0.01, Fig. 2c and d). These findings indicated that propofol could inhibit the migration and invasion of A549 cells.

Propofol down-regulated the expression of miR-372 in A549 cells
The expression of miR-372 in A549 cells after 8 μg/mL propofol treatment was evaluated using qRT-PCR. Figure 3 displayed that 8 μg/mL propofol treatment significantly decreased the expression of miR-372 in A549 cells (P < 0.01), which indicating that miR-372 might participate in the effects of propofol on A549 cells.
Propofol suppressed A549 cell proliferation and induced cell apoptosis by down-regulating miR-372

To analyze the roles of miR-372 in propofol-induced A549 cell proliferation inhibition and cell apoptosis, miR-372 mimic or miR-372 inhibitor was transfected into A549 cells to overexpress or knockdown miR-372. Results showed that miR-372 mimic transfection dramatically enhanced the expression of miR-372, while miR-372 inhibitor noticeably reduced the expression of miR-372 in A549 cells (P < 0.05 or P < 0.001, Fig. 4a). Figure 4b displayed that miR-372 overexpression reversed the anti-proliferative effect of propofol on A549 cells (P < 0.01), while miR-372 knockdown enhanced the anti-proliferative effect of propofol on A549 cells (P < 0.05). Compared to propofol+NC group, the protein expressions of p53 and p16 in A549 cells were decreased in propofol+miR-372 mimic group and increased in propofol+miR-372 inhibitor group (P < 0.05 or P < 0.001, Fig. 4c). The protein expression of Cyclin D1 in A549 cells was enhanced in propofol+miR-372 mimic group and reduced in propofol+miR-372 inhibitor group.
relative to propofol+NC group \((P < 0.05\) or \(P < 0.001\), Fig. 4c). Moreover, miR-372 overexpression suppressed the apoptotic-promoting effect of propofol, while miR-372 silence potentiated the apoptotic-promoting effect of propofol on A549 cells \((P < 0.01\), Fig. 4d). Western blotting illustrated that compared to propofol+NC group, the protein expressions of Bax, cleaved-Caspase 3 and cleaved-Caspase 9 in A549 cells were all reduced in propofol+miR-372 mimic group and enhanced in propofol+miR-372 inhibitor group (Fig. 4e). The protein expression of Bcl-2 in A549 cells was enhanced in propofol+miR-372 mimic group and reduced in propofol+miR-372 inhibitor group, relative to propofol+NC group (Fig. 4e). Taken together, these above findings suggested that propofol suppressed A549 cell proliferation and induced cell apoptosis might be via down-regulating miR-372.
Propofol suppressed A549 cell migration and invasion by down-regulating miR-372

The roles of miR-372 in propofol-induced A549 cell migration and invasion inhibition were also explored. Figure 5a and b showed that the anti-cancer effects of propofol weakened the propofol-induced A549 cell migration and invasion inhibition, while miR-372 knockdown promoted the propofol-induced A549 cell migration and invasion inhibition ($P < 0.05$ or $P < 0.01$). Compared to propofol+NC group, the protein expressions of MMP-9 and Vimentin in A549 cells were enhanced in propofol+miR-372 mimic group and reduced in propofol+miR-372 inhibitor group ($P < 0.05$ or $P < 0.01$, Fig. 5c and d). These findings suggested that miR-372 was also involved in the propofol-induced A549 cell migration and invasion inhibition.

Propofol suppressed Wnt/β-catenin and mTOR signaling pathways in A549 cells by down-regulating miR-372

Wnt/β-catenin and mTOR signaling pathways were found to be involved in the anti-cancer effects of propofol [20, 21]. So, we assessed the effects of propofol and miR-372 on Wnt/β-catenin and mTOR pathways in A549 cells. Results showed that the protein expressions of Wnt3α, p/t-β-catenin, p/t-p70S6K and p/t-mTOR in A549 cells were all down-regulated after 8 μg/mL propofol treatment ($P < 0.05$ or $P < 0.01$, Fig. 6a and b), which suggested that propofol could inactivate Wnt/β-catenin and mTOR pathways in A549 cells. Moreover, compared to propofol+NC group, the protein expressions of Wnt3α, p/t-β-catenin, p/t-p70S6K and p/t-mTOR in A549 cells were enhanced in propofol+miR-372 mimic group and reduced in propofol+miR-372 inhibitor group ($P < 0.05$ or $P < 0.001$), which indicated that propofol inactivated Wnt/β-catenin and mTOR pathways in A549 cells might be via down-regulating miR-372.

Discussion

Lung cancer is the most common and lethal cancer with metastasis potential [2]. Propofol is a widely used intravenous anesthetic [20]. In this study, we found that propofol suppressed lung cancer A549 cell viability, proliferation, migration and invasion, but promoted cell apoptosis. Mechanistically, we revealed that propofol down-regulated the expression of miR-372 in A549 cells. miR-372 participated in the effects of propofol on A549 cell proliferation, migration, invasion and apoptosis. Furthermore, we pointed out that propofol inactivated Wnt/β-catenin and mTOR pathways in A549 cells by down-regulating miR-372.

As one of the most extensively used intravenous anesthetic medicines, propofol exerts multiple advantages in clinical anesthesia [22]. In addition to the anesthetic effect, propofol also has been found to exert cardio-protective [23], anti-inflammatory [24] and anti-tumor effects [25]. In this research, we revealed that the growth and metastasis of lung cancer A549 cells were both inhibited by propofol. The pro-proliferative protein, Cyclin D1, cell migration- and invasion-related proteins, MMP-9 and Vimentin, as well as anti-apoptotic protein Bcl-2 were all down-regulated by propofol treatment. The anti-proliferative proteins, p53 and p16, as well as pro-apoptotic proteins, Bax, cleaved-Caspase-3, and cleaved-Caspase-9 were all up-regulated by propofol treatment. These results were consistent with the previous studies. For example, Liu et al. demonstrated that propofol suppressed viability, migration and invasion of A549 cells, increased E-cadherin expression, but decreased N-cadherin, Vimentin and Snail expression in A549 cells [11]. Additionally, Ye et al. and Wu et al. indicated that propofol suppressed invasion of human lung cancer A549 cells by down-regulating aquaporin-3 (AQP-3), MMP-2, and MMP-9 and inhibiting p38 MAPK signaling [26, 27]. Furthermore, Cui et al. pointed out that propofol induced endoplasmic reticulum stress and apoptosis of lung cancer H460 cells and also decreased tumor size and weight of established xenografted tumors [28].

Some studies showed that a number of miRNAs might be involved in the effects of propofol on cancers, such as miR-1284 [11], miR-142 [29], and miR-143 [30]. One of the most important findings in this study was that miR-372 participated in the effects of propofol on lung cancer A549 cells. miR-372 has been found to exert tumor promoting roles in human gastric cancer [31] and testicular germ cell tumor [32]. Moreover, miR-372 up-regulation was correlated with advanced tumor node metastasis (TNM) stage of hepatocellular carcinoma patients [17]. Wang et al. found that miR-372 was significantly overexpressed in both lung squamous cell carcinoma tissues and cell lines [16]. They proved that miR-372 overexpression...
enhanced lung cancer cell proliferation and invasion, while miR-372 silence inhibited cell growth, migration, and invasion. In the current research, we found that propofol down-regulated the expression of miR-372 in A549 cells. Up-regulation of miR-372 abrogated the effects of propofol on A549 cells, while down-regulation of miR-372 enhanced the anti-tumor effects of propofol. These finding suggested that
Propofol exerted anti-tumor effects on lung cancer cells also by down-regulating miR-372.

To further analyze the mechanism of anti-tumor effects of propofol on A549 cells, we investigated the activation of Wnt/β-catenin and mTOR signaling pathways in A549 cells. Wnt/β-catenin signaling pathway has been demonstrated to be activated in lung cancer [33, 34]. Inhibition of Wnt/β-catenin pathway has been found to contribute to the suppression of lung cancer [33, 34]. miR-371-373 cluster was showed to be positively correlated with Wnt/β-catenin signaling activity in several human cancer cell lines [35]. In this study, we identified miR-372 as a novel regulator of the canonical Wnt/β-catenin signaling pathway in lung cancer A549 cells. Targeting mTOR pathway was considered as a therapeutic target for lung cancer treatment [36, 37]. The inhibited phosphorylation of mTOR and p70S6K was conducive to the suppressed treatment with miR-372 mimic.
proliferation of A549 cells [38]. In this study, we revealed that Wnt/β-catenin and mTOR/p70S6K pathways were both inhibited by propofol. Overexpression of miR-372 abrogated the effects of propofol on Wnt/β-catenin and mTOR/p70S6K pathways, while knockdown of miR-372 had opposite effects. These findings suggested that propofol exerted anti-tumor effects on lung cancer A549 cells might be through down-regulating miR-372 and then inactivating Wnt/β-catenin and mTOR signaling pathways.

Conclusions
To sum up, our research further confirmed the anti-tumor effects of propofol on lung cancer cell growth and metastasis. Propofol suppressed growth, migration and invasion of A549 cells at least partially via down-regulation of miR-372 and inactivation of Wnt/β-catenin and mTOR pathways (Fig. 7). This study will be helpful for further understanding the molecular mechanisms of anti-tumor effects of propofol on lung carcinoma and provide theoretical basis for deeply exploring the treatment of lung cancer by using propofol.

Fig. 6 Propofol suppressed Wnt/β-catenin and mTOR signaling pathways in A549 cells by down-regulating miR-372. After 8 μg/mL propofol treatment and/or miR-372 mimic (or miR-372 inhibitor) transfection, the protein expressions of (a) Wnt3a, p-β-catenin, t-β-catenin, (b) p-p70S6K, t-p70S6K, p-mTOR and t-mTOR in A549 cells were evaluated using western blotting. *P < 0.05, **P < 0.01, ***P < 0.001

Fig. 7 Proposed pathway of anti-tumor effects of propofol on lung cancer A549 cells
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Availability of data and materials
The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
DG conceived the study; HS and DG carried out the experiments and wrote the paper. Both authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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