

Effects of propofol on human cholangiocarcinoma and the associated mechanisms

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Abstract. Cholangiocarcinoma (CCA) is the most common type of biliary duct malignancy. Propofol is a fast-acting intravenous anesthetic, which also exerts an anti-cancer effect. The aim of the current study was to explore the effects of propofol on human CCA and the associated mechanisms *in vitro*. The results indicated that as concentration (0, 1, 5 and 10 $\mu\text{g/ml}$) of propofol and treatment time (24, 48 and 72 h) increased, the cell inhibition rate of human CCA QBC939 cells increased. Furthermore, treatment with various concentrations of propofol for 48 h resulted in a decrease in migration and invasion capacity in QBC939 cells. Propofol also induced the apoptosis of QBC939 cells and cell cycle arrest in G1 phase. Propofol treatment increased the expression level of Bax and decreased that of Bcl-2. In addition, the effects of propofol on gene expression were evaluated, including Wnt3 α , β -catenin, Snail1 and c-myc in the Wnt/ β -catenin signaling pathway. It was identified that as the concentration of propofol increased, the expression of these genes decreased. In conclusion, the current results indicate that propofol is a promising therapeutic agent for the treatment of CCA.

Introduction

At present, cholangiocarcinoma (CCA) is the most prevalent bile duct malignancy in the clinic and is a fatal malignancy originating from biliary epithelial cells (1,2). In recent decades, the incidence of CCA has increased (3,4). Surgery is the preferred treatment for CCA, but it is also carries risks and there are numerous complications following surgery (5). Therefore, postoperative care and rehabilitation training for patients with postoperative CCA is very important. Currently, the prognosis of patients with CCA remains unsatisfactory, with a 5-year survival rate of ~5% (6). In addition, CCA is generally resistant to chemotherapeutic drugs, and is prone

to relapse and metastasis (7). Therefore, it is of great clinical significance to identify highly effective therapeutic drugs with minimal side effects and to clarify the mechanism of action, in order to improve the therapeutic effect and prolong the survival of patients with CCA.

Propofol (2, 6-diisopropylphenyl), an alkyl acid, is a fast-acting intravenous anesthetic. It is a widely used drug for induction and maintenance of anesthesia in the clinic (8). Propofol injection has the characteristics of rapid distribution (half-life of 2-4 min) and rapid elimination (half-life of 30-60 min) (9). Numerous studies have demonstrated the superiority of propofol over volatile agents, because propofol does not suppress the immune system in a cancerous environment (10-14). In recent decades, numerous studies have demonstrated that propofol has a variety of other effects, including possible anti-cancer actions (15-18). However, to the best of our knowledge, the effect and mechanism of propofol on CCA cells remains unclear. Therefore, the current study aimed to investigate the effect and molecular mechanism of propofol on CCA cancer *in vitro*.

Materials and methods

Materials. The human CCA cell line QBC939 was obtained from American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). All primary antibodies [anti- β -actin (cat. no. 4970), anti-CyclinE (cat. no. 20808), anti-Bax (cat. no. 5023), anti-Bcl-2 (cat. no. 4223), anti-Wnt3 α (cat. no. 2721), anti-Snail1 (cat. no. 3879), anti-c-myc (cat. no. 13987) and anti- β -catenin (cat. no. 8480)] were acquired from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture and treatment. Human CCA QBC939 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were incubated in a humidified incubator at 37°C with 5% CO₂. Propofol was dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C. QBC939 cells were treated with various concentrations of propofol (0, 1, 5 or 10 $\mu\text{g/ml}$) for 24, 48 or 72 h at 37°C (19,20). Then, the cells were subjected to assays as described below.

MTT assay. Cells were collected at logarithmic phase, inoculated in a 96-well plate with 1×10^4 cells/well and incubated at

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37°C with 5% CO₂ for 12 h. Then, the cells were treated with various concentrations of propofol (0, 1, 5 or 10 µg/ml) for 24, 48 or 72 h at 37°C. Subsequently, MTT (20 µl; 5 mg/ml) was added to each well, and the cells were incubated for a further 4 h. The formazan crystals were dissolved in 150 µl DMSO and stirred slowly for 10 min. The optical density (OD) of each sample was determined at the wavelength of 570 nm with an immunoassay analyzer. The cell inhibition rate=(1-OD value of treatment group/OD value of control) x100%. All experiments were performed in triplicate and repeated three times.

Western blotting. QBC939 cells were treated with various concentrations of propofol (0, 1, 5 or 10 µg/ml) for 48 h at 37°C. Then the cells were washed with PBS three times and lysed on ice in radioimmunoprecipitation buffer (cat. no. P0013B; Beyotime Institute of Biotechnology, Nanjing, China) with 1 mM PMSF for 30 min at 4°C. Protein was collected and stored -20°C. Bicinchoninic protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used to detect protein concentration. Equal amounts of protein (30 µg/lane) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Then, the membrane was blocked at room temperature for 2 h with 5% skimmed milk in PBS with 0.1% Tween-20 (PBST) and incubated with primary antibodies (β-actin, CyclinE, Bcl-2, Bax, Wnt3a, β-catenin, Snail1 and c-myc; all 1:1,000; Cell Signaling Technology, Inc.) overnight at 4°C. The following day, the membrane was washed four times in 1X PBST (10 min/wash) and incubated with anti-rabbit immunoglobulin G horseradish peroxidase-coupled secondary antibodies (cat. no. 7074; 1:1,000; Cell Signaling Technology, Inc.) for 2 h at room temperature. Proteins were detected using SignalFire™ Plus ECL Reagent (cat. no. 12630; Cell Signaling Technology, Inc.) and imaged. β-actin was used as an internal control.

Transwell assay. To investigate the effects of propofol on QBC939 cell migration and invasion, a 24-well Transwell plate (8-µm pore size) was used. The chamber inserts were coated with or without 200 mg/ml of BD Matrigel™ Matrix (BD Biosciences, Franklin Lakes, NJ, USA) for the invasion and migration assay, respectively. Logarithmic phase QBC939 cells were inoculated into 6-well plates (1x10⁴ cells/well) and placed in a constant temperature incubator for routine culture. When the cells reached 70-80% confluence, they were treated with various concentrations of propofol for 48 h. Then, 100 µl DMEM containing 10% FBS was added to the upper chamber for 1 h. Subsequently, the cells were digested with 0.25% trypsin and resuspended in DMEM to prepare a single cell suspension. The cell density was adjusted to 10⁶ cells/ml. DMEM (0.5 ml) containing 10% FBS was added to the lower chamber, and 100 µl cell suspension was added to the upper chamber of each insert. The plates were cultured at 37°C with 5% CO₂ for 24 h. Then, cells that had not migrated or invaded from the upper chamber to the lower chamber were gently wiped away with a clean cotton swab. The cells on the lower chamber were stained with 0.5 ml 0.1% crystal violet at room temperature for 20 min. Five fields of view were observed for each chamber by a light microscope and the mean value was calculated.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from QBC939 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The RNA concentration was detected using NanoDrop 2000 (Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNAs using the PrimeScript RT Reagent kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. qPCR was performed using QuantiFast SYBR Green PCR kit (Qiagen, Inc., Valencia, CA, USA) and a CFX Connect Real-Time system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Amplification conditions were as follows: 10 min at 95°C, followed by 35 cycles of 15 sec at 95°C and 40 sec at 55°C. The primer sequences used for RT-qPCR were listed in Table I. Relative gene expression was analyzed using the 2^{-ΔΔC_q} method (21).

Flow cytometry analysis. QBC939 cells were collected in logarithmic growth phase, and the cell suspension density was adjusted and inoculated into 6-well plates at 1x10⁵ cells/well. Subsequently, cells were treated with various concentrations of propofol for 48 h. Then, the cells were fixed with 70% methanol at -20°C overnight, washed with PBS twice, stained with propidium iodide (PI; cat. no. 4087; Cell Signaling Technology, Inc.) and incubated at 4°C for 30 min in the dark. Flow cytometry was performed to detect cell-cycle distribution. For detection of cell apoptosis, cells were stained with Annexin V and PI for 15 min at room temperature in the dark prior to flow cytometry. Data were analyzed using WinMDI (version 2.5; Purdue University Cytometry Laboratories, West Lafayette, IN, USA).

Statistical analysis. All quantitative data are presented as the mean ± standard deviation. All experiments were repeated three times. Differences between multiple groups were compared by one-way analysis of variance followed by Tukey's test, and differences between two groups were compared by Student's t-test. GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to perform statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Propofol inhibits QBC939 cell proliferation. In order to study the anti-tumor effect of propofol on human CCA cells, an MTT assay was performed. Following treatment with various concentrations of propofol for 24, 48 or 72 h, respectively, inhibition of proliferation appeared to increase as concentration of propofol in QBC939 cells and the duration of the incubation increased (Fig. 1).

Propofol inhibits migration and invasion capacity in QBC939 cells. The effects of propofol on migration and invasion of QBC939 cells were examined by Transwell assay. The results indicated that migration and invasion of cells decreased gradually with increased concentrations of propofol compared with the control (Fig. 2). Doses of 1, 5 and 10 µg/ml significantly decreased migration and invasion compared with the 0 µg/ml group.

Table I. Primer sequences for polymerase chain reaction.

Gene	Primer (5'-3')	
	Forward	Reverse
Bcl-2	TTGGATCAGGGAGTTGGAAG	TGTCCCTACCAACCAGAAGG
Bax	CGTCCACCAAGAAGCTGAGCG	CGTCCACCAAGAAGCTGAGCG
Cyclin E	AGCCAGCCTTGGGACAATAAT	GAGCCTCTGGATGGTGAAT
Wnt3a	GGCTCCTCTCGGATACCTCT	GGGCATGATCTCCACGTAG
β -catenin	AACAGGGTCTGGGACATTAGTC	CGAAAGCCAATCAAACACAAAC
Snail1	GGTTCTTCTGCGCTACTGCTG	GTCGTAGGGCTGCTGGAAGG
c-myc	CATCAGCACAACTACGCAGC	GCTGGTGCATTTTCGGTTGT
GAPDH	CTTTGGTATCGTGGAAGGACTC	GTAGAGGCAGGGATGATGTTCT

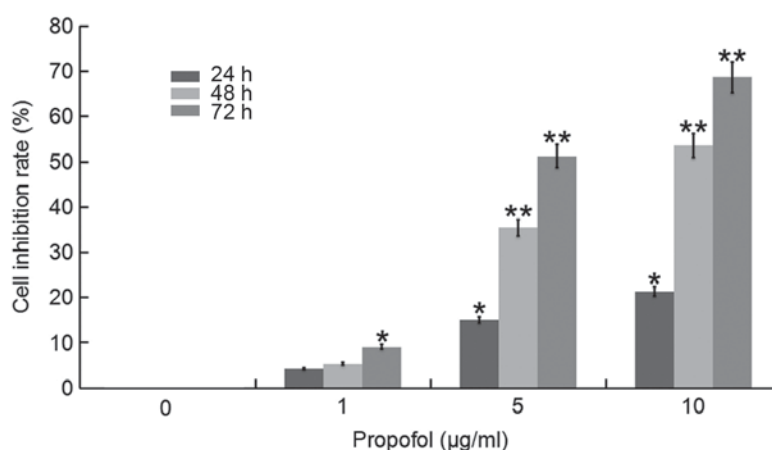


Figure 1. Effect of propofol on QBC939 cell proliferation. QBC939 cells were treated with different concentrations of propofol (0, 1, 5 and 10 $\mu\text{g/ml}$) for 24, 48 and 72 h, respectively. Relative proliferation inhibition rate of cells was detected by MTT assay. Data are presented as the mean \pm standard deviation. * $P < 0.05$, ** $P < 0.01$ vs. 0 $\mu\text{g/ml}$ propofol treatment group.

Propofol induces QBC939 cell apoptosis. To investigate whether propofol inhibited the proliferation of QBC939 cells via inducing apoptosis, flow cytometry was performed to detect cell apoptosis. Following treatment with various concentrations of propofol for 48 h, the apoptosis rate of QBC939 cells appeared to increase as concentration of propofol increased (Fig. 3A and B). Furthermore, RT-qPCR and western blot analysis were conducted to determine the expression levels of apoptosis-associated genes, Bax and Bcl-2. The results indicated that with an increase of propofol concentration, the Bax mRNA and protein level in QBC939 cells gradually increased, while the mRNA and protein level of Bcl-2 decreased (Fig. 3C-E).

Propofol induces cell-cycle arrest in QBC939 cells. To demonstrate the possible mechanism of propofol-induced cell growth inhibition in QBC939 cells, cell cycle progression was also analyzed. QBC939 cells were treated with different concentrations of propofol for 48 h and then the cell cycle distribution was analyzed using flow cytometry (data not shown). The results indicated that the percentage of G1 phase of QBC939 cells appeared to increase as concentration of propofol increased (Fig. 4A). In addition, the mRNA and protein expression of CyclinE, an important regulator of G1

and S phases in the cell cycle, was detected. It was identified that the expression of CyclinE was gradually decreased at the mRNA and protein level in QBC939 cells as propofol concentration increased compared with the control (Fig. 4B and C).

Propofol inhibits the Wnt/ β -catenin signaling pathway. QBC939 cells were treated with various concentrations of propofol for 48 h, then the Wnt/ β -catenin signaling pathway was analyzed. RT-qPCR and western blot assays indicated that the expression of Wnt3a, β -catenin, Snail1 and c-myc was gradually decreased at both the protein (Fig. 5A) and mRNA (Fig. 5B-E) level in QBC939 cells as propofol concentration increased compared with the control.

Discussion

CCA is a rapidly growing and lethal cancer that is usually incurable unless the primary tumor and any metastases are removed completely by surgery (22). As surgical treatment is prone to complications and seriously affects the quality of life of patients, in the postoperative period, it is necessary to strengthen the rehabilitation of patients with CCA from psychological and dietary perspectives (23,24). Following CCA surgery, patients should follow their doctor's advice and

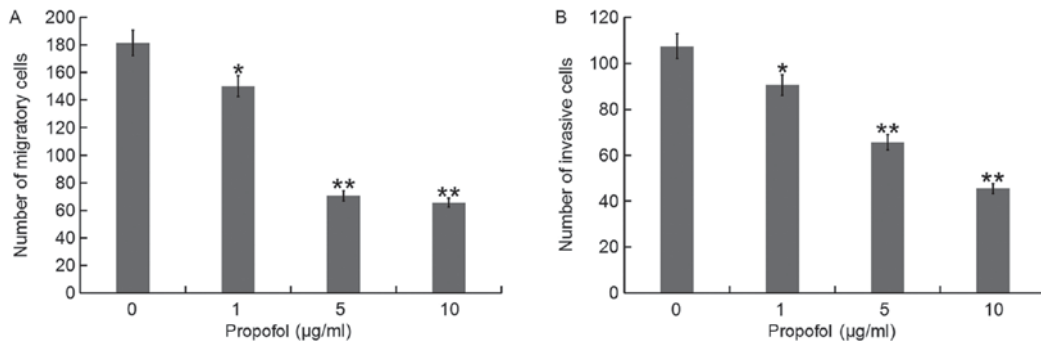


Figure 2. Effect of propofol on QBC939 cell migration and invasion capacity. QBC939 cells were treated with different concentrations of propofol (0, 1, 5 and 10 µg/ml) for 48 h. The migration and invasion of cells in different groups was determined by Transwell assay. The number of (A) migratory and (B) invasive cells was determined in triplicate. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. 0 µg/ml propofol treatment group.

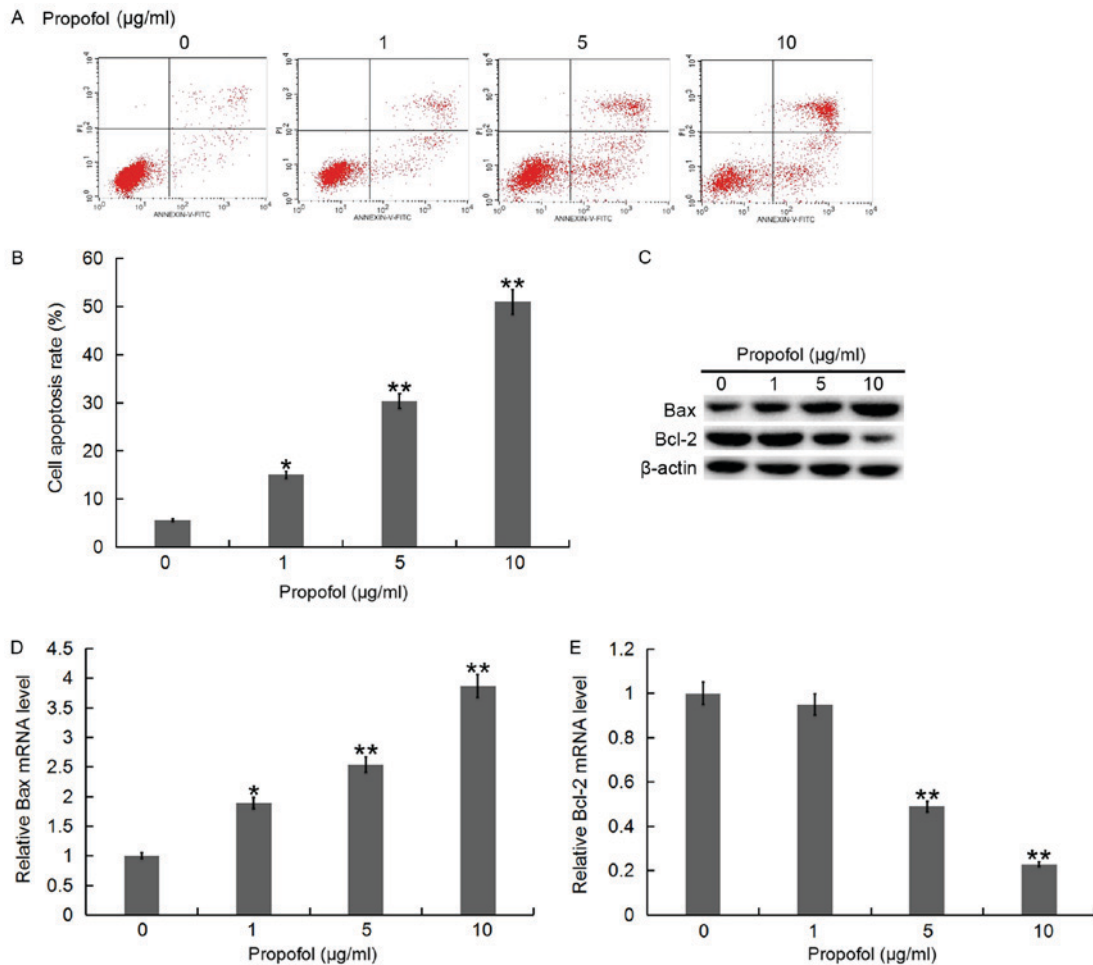


Figure 3. Effect of propofol on QBC939 cell apoptosis. QBC939 cells were treated with various concentrations of propofol (0, 1, 5 and 10 µg/ml) for 48 h. (A and B) QBC939 cell apoptosis was measured by flow cytometry and data were analyzed. (C) A western blot assay detected the protein expression levels of Bcl-2 and Bax. (D and E) An RT-qPCR assay detected the mRNA expression levels of (D) Bax and (E) Bcl-2. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. 0 µg/ml propofol treatment group.

regularly attend check-ups at their hospital, participate in light physical activity and avoid prolonged periods of sitting or low activity to facilitate the recovery of body function (25). However, the majority of CCA patients are diagnosed at an advanced stage, when surgery is not possible (26). Therefore, CCA treatment is still a major clinical challenge, and exploring new and effective drugs and strategies for the treatment of CCA is of great clinical significance.

Propofol is one of many anesthetics and has been extensively studied. Previous studies have suggested that it has an anti-cancer effect in addition to its anesthetic effect. Research has demonstrated that propofol could inhibit cancer cell proliferation, migration and invasion, and thus exert anti-tumor function (27-30). Previous studies have identified that propofol could inhibit the development of numerous cancer types, including pancreatic (31), breast (32), colon (33), gastric (34)

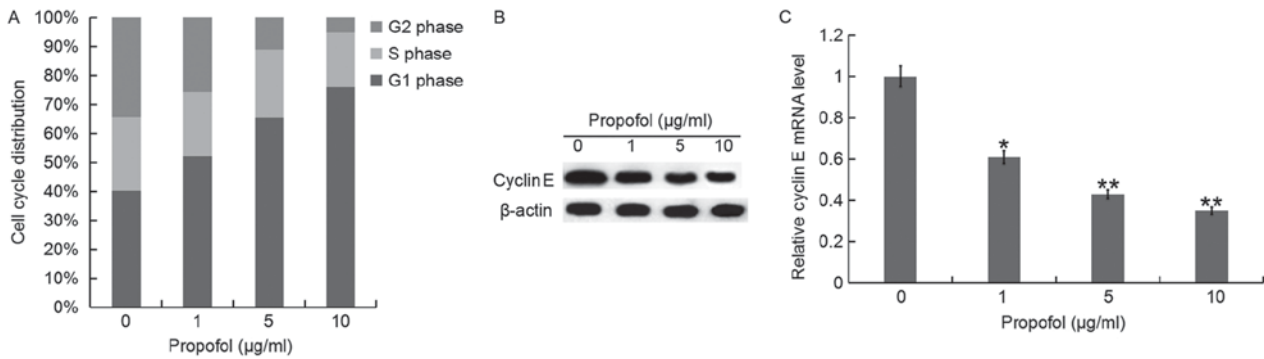


Figure 4. Effect of propofol on QBC939 cell cycle. QBC939 cells were treated with various concentrations of propofol (0, 1, 5 and 10 µg/ml) for 48 h. (A) QBC939 cell-cycle progression was examined by propidium iodide staining and flow cytometry. (B) A western blot assay detected the protein expression level of CyclinE. (C) An RT-qPCR assay detected the mRNA expression level of CyclinE. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. 0 µg/ml propofol treatment group.

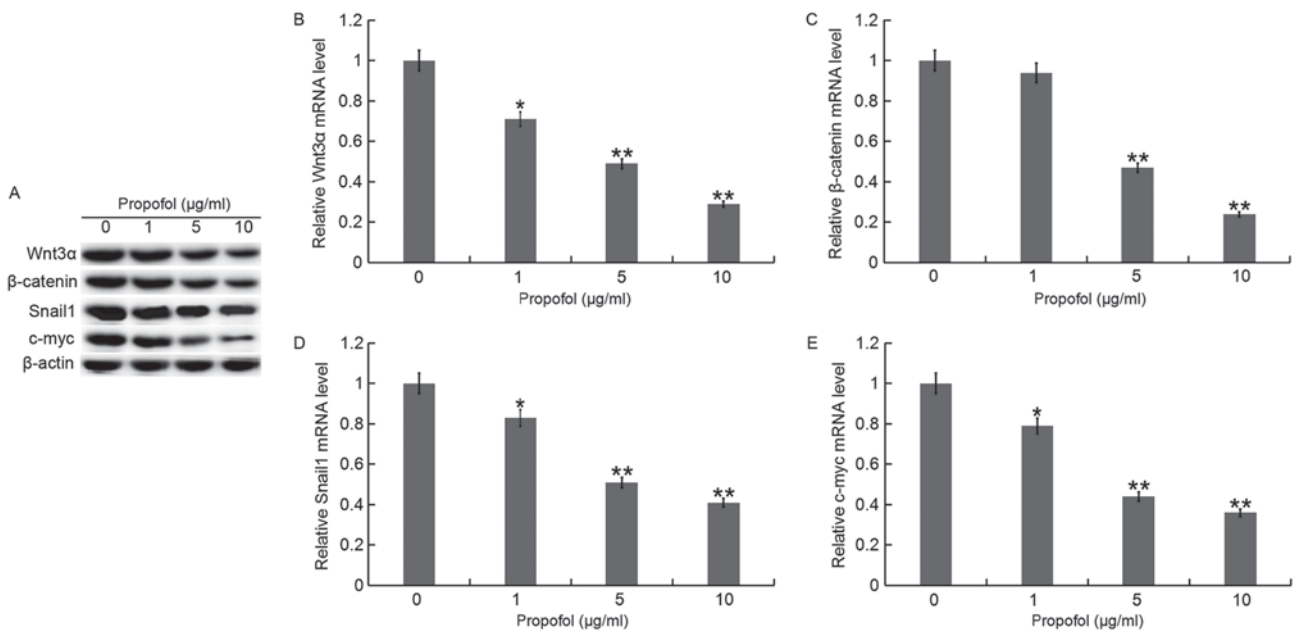


Figure 5. Effect of propofol on the Wnt/β-catenin signaling pathway in QBC939 cells. QBC939 cells were treated with various concentrations of propofol (0, 1, 5 and 10 µg/ml) for 48 h. (A) A western blot assay measured the protein expression levels of Wnt3α, β-catenin, Snail1 and c-myc. (B-E) An RT-qPCR assay was used to detect the mRNA levels of (B) Wnt3α, (C) β-catenin, (D) Snail1 and (E) c-myc. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. 0 µg/ml propofol treatment group.

and cervical (35) cancer. Zhang *et al* (36) demonstrated that propofol promotes the proliferation and invasion of gallbladder cancer cells by activating Nrf2. By contrast, Liu *et al* (37) indicated that propofol inhibits the proliferation and invasion of pancreatic cells by modulating the microRNA-21/Slug signaling pathway. However, there are few reports on the role of propofol in CCA, and the precise mechanism is still unknown. Therefore, the aim of the current study was to explore the effect of propofol on CCA QBC939 cells and its mechanism of action.

In the current study, an MTT assay demonstrated that as concentration of propofol and treatment time increased, the cell inhibition rate of QBC939 cells increased. Next, QBC939 cells were treated with different concentrations of propofol, and a Transwell assay was used to detect cell migration and invasion. Compared with the control group, experimental groups exhibited a significant decrease in the number of migratory and invasive cells. Therefore, these results indicated

that propofol significantly inhibits the proliferation, invasion and migration of QBC939 cells, providing a theoretical basis for using propofol as a therapeutic drug for CCA.

Furthermore, flow cytometry was performed to analyze the apoptosis of QBC939 cells, and it was identified that the apoptosis rate of QBC939 cells appeared to increase as concentration of propofol increased. The effect of propofol on apoptosis-associated proteins in QBC939 cells was evaluated by RT-qPCR and western blot assays. The results suggested that propofol inhibited the expression of the anti-apoptotic protein Bcl-2 and promoted the expression of the pro-apoptotic protein Bax. Another important finding in the current study was that propofol arrested cell cycle in the G1 phase, and it may be associated with a decrease in the protein level of CyclinE.

In recent decades, it has been reported that the Wnt/β-catenin signaling pathway is closely associated with the

occurrence of cancer. The Wnt/ β -catenin signaling pathway plays an important regulatory role in the proliferation, survival and metastasis of tumor cells (38,39). To further explore the molecular mechanism of propofol-induced apoptosis in human CCA QBC939 cells, the effect of propofol on certain genes (Wnt3 α , β -catenin, Snail and c-myc) in the Wnt/ β -catenin signaling pathway was examined. It was identified that as the concentration of propofol increased, the expression of Wnt3 α , β -catenin, Snail and c-myc gradually decreased. In future experiments, the association between propofol and this pathway will be studied in greater detail.

In summary, the current results indicate that propofol inhibits the proliferation, migration and invasion of QBC939 cells, and induces apoptosis and cell cycle arrest. Its mechanism of action may be associated with the Wnt/ β -catenin signaling pathway. This could provide a target and experimental basis for clinical treatment of CCA. However, the current study is a preliminary study of the effects of propofol on CCA and more detailed research in this area is required. Future studies will aim to investigate the effects of propofol on other CCA cell lines and study the molecular mechanisms of propofol in depth.

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

ZZ collaborated to design the study. MZ and SW assessed and analyzed the data. All authors, including CW, collaborated to interpret the results and develop the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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