Propofol Regulates ADAM8 in Pancreatic Cancer Cells by Targeting SP1

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Abstract

Background

Propofol is a commonly used anesthetic with controversial effects on cancer cells. A growing number of studies have demonstrated that low concentrations of propofol are associated with tumor suppression, but deeper insights into its underlying mechanism are needed. The study detailed herein focuses upon the effect of propofol on pancreatic cells and the mechanism by which propofol down-regulated ADAM8 expression.

Methods

Treatment pancreatic cell line Panc-1 was performed with different concentrations of propofol. MTT assay and flow cytometry were used to assess cell proliferation and the cell cycle. A wound healing assay was used to evaluate the migration capacity of Panc-1 cells. Quantitative real-time PCR (qRT-PCR) and western blot were used to analyze specificity protein 1 (SP1) and a disintegrin and metalloproteinase 8 (ADAM8) expression. Luciferase assay was performed to determine the transcriptional activity of SP1. RNA interference (RNAi) was used to explore whether propofol regulated ADAM8 in Panc-1 cells by targeting SP1.

Results

Propofol significantly inhibited the proliferation, migration and invasion of pancreatic cancer cells and decreased the percentage of cells in S-phase. Furthermore, propofol failed to regulate ADAM8 expression in Panc-1 cells with SP1 knockdown. Luciferase assays demonstrated that propofol repressed the transcriptional activity of SP1, while ADAM8 was a direct target of SP1.

Conclusions

These results suggest that propofol affect biological behavior of pancreatic cancer cells through ADAM8 by targeting SP1.

Introduction

According to the official journal of the American Cancer Society, there were approximately 18.1 million new cases of cancer and 9.6 million deaths from cancer worldwide in 2018^[1]. The number of pancreatic cancer patients increased by 459,000, of which 432,000 died of pancreatic cancer^[2]. It is difficult to detect pancreatic cancer in the early stages, hence patients are often stricken with advanced pancreatic cancer at the time of diagnosis, which contributes largely to five-year overall survival rate being less than <5%^[3]. Currently, surgical resection combined with chemotherapy is the only way to treat pancreatic cancer, but surgical stress can affect the immune and neuroendocrine systems and induce inadvertent seeding of tumor cells during surgery, which is the main cause of tumor recurrence^[4]. Anesthesia management is

an essential part of the perioperative period and it has been found that anesthetics could participate in different physiological and pathophysiological functions of cells, such as cell proliferation, angiogenesis and apoptosis^[5]. Recently, a meta-analysis has shown that propofol-based total intravenous anesthesia (TIVA) can observably improve recurrence-free survival rate (pooled HR, 0.78; 95% CI, 0.65 to 0.94; $P < 0.01$) and overall survival rate (pooled HR, 0.76; 95% CI, 0.63 to 0.92; $P < 0.01$) for various cancers^[6], suggesting that propofol may be involved in tumor suppression.

ADAM8 (Disintegrin and metalloproteinase 8) is a type I transmembrane (TM) glycoprotein whose expression levels in normal tissue is typically low and limited to a few distinct cell types in the lymphatic organs as components of the immune system^[7] and the central nervous system^[8]. Only under the pathological stimuli, ADAM8 can be induced to significant protein levels in diseases including osteosarcoma, colorectal cancer, gastric cancer and pancreatic cancer, which makes ADAM8 potentially relevant for pathophysiology. Once upregulated, ADAM8 is proteolytically active and results in enhanced shedding of cell adhesion molecules, cytokine receptors and extracellular matrix (ECM) components^[9]. In our previous study, we found that propofol could downregulate ADAM8 expression under hypoxic conditions^[10], which partially inhibits the activity of ADAM8 (this was not observed when compared with the control drug Batimastat, BB-94)^[10, 11]. For these reasons it is possible that other mechanisms participate in the effect of propofol on pancreatic cancer through ADAM8.

Specificity protein 1 (SP1) is a widely studied transcription factor, which regulates target genes by binding to GC-boxes with the consensus sequence 5' -G/T-GGGCGG-G/A-G/A-C/T-3' or 5' -G/T-G/A-GGCG-G/T-G/A-G/A-C/T-3' on their promoter regions^[12]. SP1 not only affects tumor suppressors but also regulates oncogenes, suggesting that it may play an important role in the development of tumor and metastasis process. Recent studies have also demonstrated that SP1 has an impact on tumor invasion and metastasis. In oral squamous cell carcinoma (OSCC), SP1 promotes cell invasion and migration by upregulating Annexin A2 transcription^[13]. The study demonstrated that knockdown of SP1/Syncytin1 axis inhibits the proliferation and metastasis through the AKT and ERK1/2 signaling pathways in non-small cell lung cancer^[14]. The study detailed herein investigates whether SP1 mediates the effect of ADAM8 upon pancreatic cancer cells following treatment with propofol.

Materials And Methods

Cell culture and reagent

Human pancreatic cancer cell line Panc-1 was purchased from American Type Culture Collection (ATCC). Panc-1 cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco Laboratory, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS, Gibco) in humidified atmosphere containing 5% CO₂ at 37°C. Knockdown SP1 cell line was established by infection of cells with shSP1 lentivirus. The lentiviral plasmids which targeted SP1 (shSP1) were purchased from Vigene Biosciences (Shangdong, China) and the control shRNA sequence (shCtrl) were purchased from Sigma-Aldrich (Merck KGaA,

Darmstadt, Germany). To exclude the influence of lipid emulsion, pure propofol was obtained from Sigma Chemical (St Louis, MO, USA).

Cell growth assay

Cell growth assay was assessed using the thiazolyl blue tetrazolium bromide (MTT) assay. Cells were seeded in 96-well plates (6×10^3 cells/well) with propofol for 48 hours. MTT solution (Sigma, St. Louis, MO, USA) was added and incubated for four hours at 37°C. Then, 200 µL dimethyl sulfoxide was used to dissolve the precipitation. The absorption values were measured at 490 nm using Multiskan Spectrum (Thermo Scientific, USA).

Wound healing assay

In a 6-well plate, Panc-1 cells (1×10^6 cells/well) were added to DMEM media, and incubated overnight in order to create a monolayer of cells. A scratch was made in the middle of the well with a pipette tip and the debris was washed away, then new media was added to the wells. Using an optical microscope, the cells were imaged and the initial area of the scratch for the field of view was determined using the area (the length multiplied by the average width, 3 fields of view were measured) devoid of cells. The plate was incubated at 37°C for 24 hours, after which the same field of view was imaged and the area devoid of cells was recalculated using the same methodology. The final area of the scratch wound was divided by the initial area, which yielded the percentage wound remaining of the initial area covered by migrating cells over the 24 hours culture period.

Cell cycle analysis

Cells were incubated with propofol at indicated doses for 24 hours, then washed with cold PBS. Subsequently the cells were fixed using cold 75% ethanol overnight at 4°C, washed with cold PBS and stained in PI for 30 min at 37°C prior to being analyzed by flow cytometry.

Total RNA isolation and quantitative real-time PCR

Total RNA was isolated from cell lines with TRIzol (Invitrogen Life Technologies, Carlsbad, USA) according to the manufacturer's protocol. A NanoDrop spectrophotometer (Thermo Scientific, USA) was used to measure RNA concentration. Reverse transcription reactions were performed with PrimeScript RT Reagent kit (Takara, Dalian, China), and quantitative real-time PCR (RT-PCR) was performed using FastStart Universal SYBR Green Master (Roche, Mannheim, Germany) and the Step One Plus Real-time PCR system (Applied Biosystems, Singapore). According to the manufacturer's protocol, the thermocycling conditions were as follows: pre-denaturation, 95°C for 30 sec; amplified reaction, 95°C for 5 sec, 60°C for 20 sec, 40 cycles; dissociation curve, 95°C for 60 sec, 55°C for 30 sec, 95°C for 30 sec. Expression was normalized to β -actin level. The $2^{-\Delta\Delta C_t}$ method was used for relative quantification. All experiments were performed in biological triplicates, each with technical triplicates (n=3). The primer sequences were provided as follow:

ADAM8: fw: 5'-ACAATG CAG AGT TCC AGA TGC-3'; rev:5'-GGA CCA CAC GGAAGT TGA GTT-3';

Sp1: fw:5'-CGGAATTCATGAGCGACCAAGATCACTCCATG-3'; rev: 5'-CGGAATTCTTGGACCCATGCTACCTTGCATCC-3',

GAPDH: fw: 5'-GTC AGT GGT GGA CCTGAC CT-3', rev: 5'-TGG TGC TCA GTT TAG CCC AGG-3'.

Western blot

Cells were lysed by RIPA buffer (Dingguo, Beijing, China) to extract total protein. 50µg of extracted protein was separated using 10%SDS-PAGE and electroblotted on PVDF-membranes. The membranes were incubated with ADAM8 (ab255608, 1:1000, Abcam, Cambridge, UK), SP1(ab231778, 1:1000, Abcam, Cambridge, UK), β-actin (ab8226, 1:1000, Abcam, Cambridge, UK) antibodies overnight at 4°C prior to incubation with goat anti-rabbit HRP-conjugated antibody (ab181662, 1:2000, Abcam, Cambridge, UK). The imaging of proteins was performed using the Oddyssey system (Li-Cor biosciences, Lincoln, USA).

Lentiviral infection

Lentivirus vectors for SP1 knockdown (shSP1) were purchased from Vigene Biosciences (P100029, Shangdong, China). A total of 50,000 cells/well were seeded in 6-well plates. The shSP1 lentivirus or negative control lentivirus (shCtrl) was added to the cells in the presence of 5µL Polybrene (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) to increase the efficiency of infection. After 96h, the transduced cells were selected with 1 µg/mL puromycin for 2 months. Then selected cells were treated with different concentrations of Propofol.

Luciferase reporter assays

Transfection and luciferase reporter assay were performed as previously described^[15]. Wild-type or mutant ADAM8, which contains mutations at the 3'-UTR SP1 binding sites, and synthesized promoter mimic or vector were co-transfected for 48 hours, then harvested prior to determining the luciferase activity, which was measured using a dual-luciferase reporter assay system (Promega, Fitchburg, WI, USA).

Statistical analysis

All values were presented as mean ± standard deviation (SD). All results were from three independent experiments. SPSS 20.0 software was used for statistical analysis. One-way ANOVA followed by Duncan's multiple range test and Student's *t* test were performed to assess variation among experimental groups. The threshold for significance was set at $P < 0.05$.

Results

Propofol inhibits the proliferation of pancreatic cancer cells

Firstly, the effect of 0 µg/mL, 5 µg/mL, 10 µg/mL and 20 µg/mL propofol concentration upon Panc-1 cell proliferation was monitored using MTT assays. As shown in Fig. 1, propofol could suppress the proliferation of Panc-1 cells in a dose-dependent manner. It was observed that the 10 µg/mL propofol caused the lowest survival rate. This data revealed that propofol treatment could inhibit the growth of pancreatic cancer cells.

Propofol decreases the number of pancreatic cancer cells in S-phase

Next, the potential influence of propofol on the cell cycle of Panc-1 cells was determined. Panc-1 cells were treated with 0 µg/mL, 5 µg/mL, 10 µg/mL and 20 µg/mL propofol and measured the cell cycle distribution of Panc-1 cells in different phases using a flow cytometer. This data indicated that propofol had an impact upon the cell cycle of Panc-1 cells and the number of pancreatic cancer cells at S-phase gradually reduced as the concentration of propofol increased (Fig. 2A,B). Furthermore, whilst the percentage of cells at G1-phase appeared to be increased, there was no statistical difference. This data demonstrated that cells were blocked at S-phase, resulting in a relative decrease in cell DNA synthesis and replication.

Propofol inhibits migration of pancreatic cancer cells

In addition to the inhibitory effects on cell proliferation, we investigated the potential impacts of propofol on malignant behavior related to cancer metastasis in Panc-1 cells. For this purpose, wound healing assays were employed, which demonstrated that propofol treatment significantly suppressed the migration capacity of Panc-1 cells (Fig. 3A,B). At the same time, the wounds healed slower at higher concentrations of propofol.

Propofol inhibits expression of ADAM8

To investigate the effects of propofol on ADAM8, we extracted mRNA and protein from Panc-1 cells treated with 0 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL propofol. We found that propofol downregulated transcriptional and translational levels of ADAM8 in a dose-dependent manner (Fig. 4A,B,C).

Analysis and identification of the ADAM8 promoter region and transcription factors

In the UCSC database, the promoter region of ADAM8 was predicted to be located at chr10: 133,262,422-133,264,422 (GRCh38), and the luciferase reporter vectors containing the indicated genomic fragments of the ADAM8 gene was constructed. The result of the dual-luciferase assay showed that the luciferase activity was significantly increased in Panc-1 cells transfected promoter mimic constructed gene compared with that in Panc-1 cells transfected with the pscheck2 vector (Fig. 5A). To further investigate the potential regulators involved in ADAM8 expression, potential transcription factor binding sites in the ADAM8 promoter were identified using three online software packages: Pubmed (<https://pmlegacy.ncbi.nlm.nih.gov/gene/101>), JASPAR (<http://jaspar.genereg.net/>) and GeneCards

(<http://genecards.org>), binding sites for transcription factor SP1 and ZBTB40 were found in the promoter region of ADAM8 (Fig. 5B). Of the two potential transcription factors, only SP1 binding markedly enhanced luciferase activity driven by the promoter region of ADAM8 gene due to the activity of SP1 (Fig. 5C). These results led us to hypothesize that SP1 may function as a transcription factor targeted by propofol.

Propofol potentially targets SP1 to regulate ADAM8

To investigate whether propofol suppresses biological behavior through ADAM8 by targeting SP1, Panc-1 cells were treated with 0 μ g/mL, 5 μ g/mL, 10 μ g/mL, 20 μ g/mL propofol and the results of the dual-luciferase assay showed that the luciferase activity was significantly inhibited in a dose-dependent manner, and it was observed that the concentration of 10 μ g/mL propofol caused the lowest activity (Fig. 6A). We further established Panc-1-SP1-shRNA cell lines and Panc-1-NC-shRNA cell line which was negative control. Three Panc-1-SP1-shRNA cell lines were established and the one in which SP1 expression was the least at both the mRNA and protein levels was selected (Fig. 6B, C, D). The protein and mRNA were extracted from the Panc-1-SP1-shRNA2 cell line and Panc-1-NC-shRNA cell line to see whether expression of ADAM8 was influenced. This data showed that expression of ADAM8 (Fig. 6E, F, G) was modified at different concentrations of propofol in the control group, while expression of ADAM8 (Fig. 6H, I, J) was not influenced in the experimental group.

Discussion

In this study, propofol has been confirmed to inhibit proliferation, block the cell cycle at the S-phase and suppress the metastasis of pancreatic cancer cells. To provide deeper insight into the molecular mechanism, several transcription factors for ADAM8 were investigated. Interestingly, SP1 was verified to regulate ADAM8 expression, which was affected by propofol treatment in Panc-1 cells. In fact, previous studies have shown that propofol not only affects epigenetic pathways, such as those involving histone acetylation, miRNA and lncRNA^[16-18]; but also modulates genetic signaling pathways, including the SLUG, MAPK, Nrf2 and NF- κ B pathway^[19-22]. The data contained herein demonstrated that propofol exerted an inhibitory effect on pancreatic cancer cell proliferation, migration and cell cycle through ADAM8 by targeting SP1.

Propofol is a commonly used intravenous sedative-hypnotic agent. Apart from its multiple anesthetic advantages, propofol exerts a number of non-anesthetic effects; accumulating evidence shows that it affects cancer development by direct and indirect ways. A number of studies have indicated that propofol suppresses the malignancy of a variety of human cancers, such as hepatocellular carcinoma (HCC)^[23], breast cancer^[24], and lung cancer^[25]. Moreover, some studies have suggested a possible correlation between propofol and chemotherapy, though the results are undefined^[26]. Previous studies conducted by our research group have shown that propofol inhibits pancreatic tumor growth via ADAM8^[10] and further found that propofol specifically inhibited ADAM8 expression and activation in response to hypoxia in pancreatic cancer^[11]. The results of present study are consistent with these previous reports.

ADAM8 is a proteolytically active member of the ADAM8 protease family. Increase expression of ADAM8 was observed in breast cancer^[27], lung adenocarcinoma^[28] and pancreatic cancer^[29]. ADAM8 has been shown to cleave important extracellular matrix (ECM) components of the tumor stroma such as growth factors or cell surface protein^[30]. Epidermal growth factor has been demonstrated to reduce cell attachment, cell-cell interaction, and cell spreading; but suppressed expressions of cyclin A, D1 and cdk2^[31]. Cyclins play an important role in cell proliferation, pluripotency and cell fate specification. The presented herein demonstrated that propofol inhibited the migration and proliferation of Panc-1 cells, and decreased the number of cancer cells in S-phase. Since DNA synthesis and replication is an important part of S-phase, the decreasing percentage of cells on S-phase could indicate that propofol suppresses the pancreatic cancer cells in this way, which is a prospect that requires further study.

SP1 is involved in basal transcriptional regulation in various genes. SP1 contains three highly homologous C2H2 regions, which exhibit direct binding to DNA, thus enhancing gene transcription^[32]. In this study, it was demonstrated that propofol could not inhibit Panc-1 cell migration and ADAM8 expression in Panc-1 cells following SP1 knockdown by shRNA, therefore it is likely that SP1 directly mediates the expression and therefore functions of ADAM8 following propofol treatment (supplementary results). These findings contribute to the expansion of knowledge in the field of perioperative anesthetics and their effects upon tumor cells.

In conclusion, the data from this study suggests that propofol plays a critical role in inhibiting the proliferation and migration of pancreatic cancer cells and also blocks the cell cycle of pancreatic cancer cells at S-phase by potentially targeting SP1 to regulate ADAM8.

Declarations

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Availability of data and materials

All data and materials are available without restriction. Researchers can obtain data by contacting the corresponding authors.

Authors' contribution

XY and YBD contributed to the conception, design of the study. YG, CW and YZ contributed to perform the experiments, data acquisition and interpretation. KMS was involved in bioinformation analysis. YG drafted the manuscript. XY, YBD and KMS reviewed the manuscript critically. All authors contributed to the interpretation of the findings, and reviewed, edited and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Figures

Figure 1

Propofol inhibits proliferation of pancreatic cancer cells. Panc-1 cells were exposed to different concentrations of propofol (0 μg/mL, 5 μg/mL, 10 μg/mL, 20 μg/mL). The MTT assay was used to assess cell proliferation. Experiments in this figure were performed in triplicate and displayed using the mean ± SD. *P < 0.05; **P < 0.01.

Figure 2

Propofol blocks the cell cycle of pancreatic cancer cells on S-phase. After panc-1 cells were treated with different concentrations of propofol (0 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL) for 24 hours. (A) Flow cytometry was used to measure the distribution of cell cycle and the results were analyzed using flowjo software. (B) The percentage of panc-1 cells on S-phase were decreased after propofol treatment. Experiments in this figure were performed in triplicate and displayed using the mean $\pm$ SD. * $P < 0.05$; ** $P < 0.05$.

Figure 3

Propofol inhibits migration of pancreatic cancer cells. Panc-1 cells were exposed to 5 µg/mL, 10 µg/mL, 20 µg/mL propofol compared with untreated cells for 24 hours. (A) Then cell migration was detected using wound healing assays and the relative migrated surface was analyzed by Image J software. (B)

The relative migrated surface reduced significantly after propofol treatment. Experiments in this figure were performed in triplicate and displayed using the mean $\pm$ SD. *P<0.05; **P<0.005.

Figure 4

Propofol inhibits ADAM8 expression both at translation and transcription level.(A)The protein levels of ADAM8in panc-1 cells treated with 0 μ g/mL, 5 μ g/mL, 10 μ g/mL, 20 μ g/mL propofol were detected using western blotting. (B) The results were measured by Image J software. (C) qPCR was used for the detection of mRNA level of ADAM8 in panc-1 cells treated with 0 μ g/mL, 5 μ g/mL, 10 μ g/mL, 20 μ g/mL propofol. All experiments in this figure were performed in triplicate and displayed using mean $\pm$ SD. *P<0.05; **P<0.005.

Figure 5

The analysis and identification of ADAM8 core promotor. (A) Dual luciferase reporter assays were used to determine the core activity region of ADAM8. (B) Prediction of transcription factors binding sites in the ADAM8 promoter region using Pubmed,JASPARand GeneCards. (C) The influence of transcription factors on ADAM8 promoters were determined by dual luciferase reporter assays and displayed using the mean $\pm$ SD.*P<0.05; ***P<0.001.

Figure 6

Propofol potentially targets SP1 to regulate ADAM8. (A) Panc-1 cells were treated with 0 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL propofol. The dual-luciferase assay was used for measuring the luciferase activity (A). The protein and mRNA levels of SP1 in three panc-1 cell lines transfected with SP1 knockdown plasmids (Panc-1-SP1-shRNA) and in panc-1 cells were detected by western blot and qPCR (B, C, D). (E) The protein and mRNA levels of ADAM8 in the Panc-1-NC-shRNA2 cell line and Panc-1-SP1-

shRNA cell line exposed to 0µg/mL, 5µg/mL, 10µg/mL, 20µg/mL propofol and displayed using mean±SD (E, F, G, H, I J). *P<0.05; **P<0.005; ***P<0.001.