Propofol Mediates Pancreatic Cancer Cell Activity Through the Repression of ADAM8 via SP1

Yutong Gao
Guizhou University

Yu Zhou
Zunyi Medical University

Chunlin Wang
Guiyang Medical University

Klarke Sample
Guizhou Provincial People's Hospital

Xiangdi Yu (✉ Xiangdi_Yu@163.com)
Guizhou Provincial People's Hospital

Yaacov Ben-David
Guiyang Medical University

Research Article

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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 and when used as an intravenous anesthesia improved recurrence-free survival rates for many cancers, but deeper insights into its underlying mechanism are needed.

Methods

The study detailed herein focuses upon the effect of propofol on pancreatic cancer cells and the mechanism by which propofol reduces ADAM8 expression. The ability of propofol to impact the proliferation, migration and cell cycle of a pancreatic cancer cell line was assessed \textit{in vitro}. This was mechanistically explored following the identification of SP1 binding sites within ADAM8, which enabled the regulatory effects of SP1 on ADAM8 following propofol treatment to be further explored.

Results

This study was able to show that propofol significantly inhibited the proliferation, migration and invasion of pancreatic cancer cells and decreased the percentage of cells in S-phase. Propofol treatment was also shown to repress ADAM8 and SP1 expression, but was unable to affect ADAM8 expression following knockdown of SP1. Moreover, a direct physical interaction between SP1 and ADAM8 was verified using Co-immunoprecipitation and dual-luciferase reporter assays.

Conclusion

These results suggest that propofol represses pathological biological behaviors associated with pancreatic cancer cells through the suppression of SP1, which in turn results in lower ADAM8 mRNA expression and protein levels.

Introduction

In 2018 the pancreatic cancer diagnoses worldwide and 432,000 deaths resulting from pancreatic cancer (1). It is difficult to detect pancreatic cancer in the early stages, hence patients are often stricken with advanced disease at the time of diagnosis, which contributes to the five-year overall survival rate being less than $< 5\%$ (2). Currently, surgical resection combined with chemotherapy is the predominant method for the treatment of 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 (3). Anesthesia management is an essential part of the perioperative period and it has been
found that anesthetics participate in different physiological and pathophysiological functions of cells, such as cell proliferation, angiogenesis and apoptosis (4). 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 (5), suggesting that propofol may be involved in tumor suppression. Hence, this study aims to address some of the mechanisms associated with this phenomenon.

‘A disintegrin and metalloproteinase 8’ (ADAM8) 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 (6) and the central nervous system (7). Only under the pathological stimuli is ADAM8 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 (8). In our previous study, we found that propofol could downregulate ADAM8 expression under hypoxic conditions (9), which partially inhibits the activity of ADAM8 (this was not observed when compared with the control drug Batimastat, BB-94)(9, 10). 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-C/T-3’ within their promoter regions (11). 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 (12). Additionally, it has been 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 (13). 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 lentiviral transduction

The 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 a humidified atmosphere containing 5% CO₂ at 37°C. A knockdown SP1 cell line was established via the transfection of cells with shSP1 lentivirus. The lentiviral plasmids that targeted SP1 (shSP1) were purchased from VigeneBiosciences (Shangdong, China) and the control shRNA sequence (shCtrl) were purchased from Sigma-Aldrich (MerckKGaA, Darmstadt, Germany). For the transduction, 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; MerckKGaA, Darmstadt, Germany). After 96 hours, the transduced cells were selected with 1 µg/mL puromycin. Subsequently, the selected cells were treated with different concentrations of Propofol. Importantly, pure propofol was obtained from Sigma Chemical to exclude the influence of lipid emulsion (St Louis, MO, USA).

**Cell growth assay**

Cell growth was assessed using the thiazolyl blue tetrazolium bromide (MTT) assay. Cells were seeded in 96-well plates (6×10³ cells/well) with propofol for 48 hours. MTT solution (Sigma, St. Louis, MO, USA) was added and incubated for four hours at 37°C prior to the precipitation being dissolved in 200 µL dimethyl sulfoxide. The absorption values were measured at 490 nm using a Multiskan Spectrum (Thermo Scientific, USA).

**Wound healing assay**

In a 6-well plate, Panc-1 cells (1×10⁶ cells/well) were added to DMEM 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 prior to the addition of new media 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 devoid of cells (the length multiplied by the average width, 3 fields of view were measured). 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, and used to determine 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 minutes 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 using TRIzol (Invitrogen Life Technologies, Carlsbad, USA) according to the manufacturer’s protocol. A NanoDrop spectrophotometer (Thermo Scientific, USA) was used to measure the 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 seconds; amplified reaction, 95°C for 5 seconds, 60°C for 20 seconds, 40 cycles; dissociation curve, 95°C for 60 seconds, 55°C for 30 seconds, 95°C for 30 seconds. The \(2^{-\Delta\Delta Ct}\) method was used for relative quantification against the
expression levels of β-actin. All experiments were performed in biological triplicates, each with technical triplicates (n = 3). The following primer sequences were used for the aforementioned reactions:

ADAM8: fw: 5′-ACAATGCAGAGTTCCAGATGC-3′,
rev: 5′-GGA CCA CAC GGAAGT TGA GTT-3′.

Sp1: fw: 5′-CGGAATTCTTGACCAAGATCAGCTCCATG-3′,
rev: 5′-CGGAATTCTTGGACCCATGCTACCTTCATCC-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 using RIPA buffer (Dingguo, Beijing, China) to extract total protein. 50µg of the extracted protein used for 10% SDS-PAGE and subsequently 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 Oddessy system (Li-Cor biosciences, Lincoln, USA).

**Dual-luciferase reporter assays**

The transfection and luciferase reporter assay were performed as previously described (14). Wild-type or mutant ADAM8, which contains mutations at the 3'-UTR SP1 binding sites, and a 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).

**Co-immunoprecipitation assay**

Cell lysates were prepared by using mRIPA buffer (50mM Tris-HCl, pH 7.8, 150mM NaCl, 5mM EDTA, 0.1% Triton-X100, 0.05% NP-40), and total protein was extracted from Panc-1 cells. Subsequently, the lysates were rotated and incubated overnight at 4°C with 2µg of the anti-ADAM8 or anti-SP1, antibody alongside a negative control containing 2µg of a rabbit IgG antibody, supernatant without any antibody (Input) was used as a positive control. After incubation, the mixture was incubated with Protein A or G Sepharose agarose beads at 4°C for 3 hours. The beads were collected and sequentially washed five times with RIPA lysis buffer (1ml), then analyzed by western blotting using anti-ADAM8 or anti-SP1 antibody correspondingly. The intensity of the specific bands was estimated by Image J2X software package. The assays were repeated at least three times.

**Statistical analysis**
Results

Propofol inhibits the proliferation of pancreatic cancer cells

Firstly, the effect of 0µg/mL (negative control), 5µg/mL, 10µg/mL and 20µg/mL propofol concentration upon Panc-1 cell proliferation was monitored using MTT assays. As shown in Figure. 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 0µg/mL, 5µg/mL, 10µg/mL and 20µg/mL propofol and the cell cycle distribution of Panc-1 cells in different phases was measured using a flow cytometer. This data indicated that propofol had an impact upon the cell cycle of Panc-1 cells because there was a significant gradual reduction of the number of pancreatic cancer cells at S-phase as the concentration of propofol increased (Figure. 2A, B). Whilst the number of cells at G1-phase appeared to be increased, there was not a statistically significant difference between the percentages of G1 cells. This data demonstrated that cells were blocked at S-phase, which could indicate 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 migrative capacity of Panc-1 cells (Figure. 3A, B) i.e. 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 0µg/mL, 5µg/mL, 10µg/mL, 20µg/mL propofol. We found that propofol caused decreased mRNA expression and protein levels of ADAM8 in a dose-dependent manner (Figure. 4A, B, C).

Verification of the direct interaction between SP1 and ADAM8
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 were constructed. To 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) (15), binding sites for transcription factor SP1 and ZBTB40 were found in the promoter region of ADAM8 (Figure. 5A). Of the two potential transcription factors, only SP1 mimics markedly enhanced luciferase activity (Figure. 5B). Co-IP experiments were performed in order to further explore whether SP1 binds the promoter regions of ADAM8. The results showed that SP1 and ADAM8 were detected in the corresponding precipitated protein complexes, indicating that SP1 interacted directly with ADAM8 (Figure. 5C). These results led prompted the hypothesis 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 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL propofol and the results of the dual-luciferase assay showed that the luciferase activity was significantly inhibited in a dose-dependent manner, it was observed that the concentration of 10 µg/mL propofol caused the lowest activity (Figure. 6A). Additionally, Panc-1-NC-shRNA (the negative control) and three Panc-1-SP1-shRNA cell lines were established, the one that expressed the least SP1 at both the mRNA and protein levels was selected (Figure. 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 by propofol in the absence of SP1. This data showed that expression of ADAM8 (Figure. 6E, F, G) was modified at different concentrations of propofol in the control group, while expression of ADAM8 (Figure. 6H, I, J) was not influenced in the experimental group.

**Discussion**

In this study, propofol was confirmed to inhibit proliferation, block the cell cycle at the S-phase and suppress the migratory capabilities 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 IncRNA (16, 17); but also modulates genetic signaling pathways, including the SLUG, MAKP, Nrf2 and NF-κB pathway (18). 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) (19), breast cancer (20), and lung cancer (21). Moreover, some studies have suggested a possible correlation between propofol and chemotherapy, though the mechanism remains undefined (22). Previous studies conducted by our research group have shown that propofol inhibits pancreatic tumor growth via ADAM8 (9) and further found that propofol specifically inhibited ADAM8 expression and activation in response to hypoxia in pancreatic cancer (10). The results of this 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 (23), lung adenocarcinoma (24) and pancreatic cancer (25). ADAM8 has been shown to cleave important extracellular matrix (ECM) components of the tumor stroma such as growth factors or cell surface proteins (26). 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 (27). Cyclins play an important role in cell proliferation, pluripotency and cell fate specification. Since DNA synthesis and replication is an important part of S-phase, the decreased percentage of cells in S-phase identified in this study could indicate that propofol suppresses the pancreatic cancer cells in the aforementioned way, which is a prospect that requires further study.

SP1 is involved in basal transcriptional regulation of various genes. SP1 contains three highly homologous C2H2 regions, which exhibit direct binding to DNA, thus enhancing gene transcription (28). In this study, it was demonstrated that SP1 interacted directly with ADAM8, and propofol could not inhibit Panc-1 cell migration and ADAM8 expression in Panc-1 cells following SP1 knockdown by shRNA. Additionally, luciferase activity was reduced with increasing concentrations of propofol in cells transfected with reporter vectors and SP1 mimics. These results suggest that SP1 directly mediates the expression and therefore functions of ADAM8 following propofol treatment. 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 targeting SP1 to regulate ADAM8.

**Declarations**

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Not applicable.

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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.

References


Figures

![Figure 1](image)
Propofol inhibits the proliferation of pancreatic cancer cells. Panc-1 cells were exposed to different concentrations of propofol (0μg/mL, 5μg/mL, 10μg/mL, 20μg/mL). The MTT assay was used to assess cell proliferation. The experiments in this figure were performed in triplicate and displayed using the mean ±SD.*P<0.05; **P<0.005.

**Figure 2**

Propofol blocks the cell cycle of pancreatic cancer cells at S-phase. After Panc-1 cells were treated with different concentrations of propofol (0μg/mL, 5μg/mL, 10μg/mL, 20μg/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 at S-phase was decreased after propofol treatment. The experiments in this figure were performed in triplicate and displayed using the mean ±SD.*P<0.05; **P<0.005.
Figure 3

Propofol inhibits migration of pancreatic cancer cells. Panc-1 cells were exposed to 5μg/mL, 10μg/mL, 20μg/mL propofol and compared to untreated cells for 24 hours. (A) cell migration was detected using wound healing assays and the relative migrated surface was analyzed by Image J software. (B) The relative rate of wound healing was significantly decreased after propofol treatment (i.e. propofol inhibited migration). The experiments in this figure were performed in triplicate and displayed using the mean ±SD. *P<0.05; **P<0.005.

Figure 4

Propofol decreases ADAM8 both mRNA expression and protein levels. (A) The protein levels of ADAM8 in panc-1 cells were treated with 0μg/mL, 5μg/mL, 10μg/mL, 20μg/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 0μg/mL, 5μg/mL, 10μg/mL, 20μg/mL.
propofol. All experiments in this figure were performed in triplicate, the samples derive from the same experiment and that gels were processed in parallel, and displayed using mean ±SD. *P<0.05; **P<0.005.

**Figure 5**

Verification of the direct interaction between SP1 and ADAM8. (A) Prediction of transcription factor binding sites in the ADAM8 promoter region using Pubmed, JASPAR and GeneCards. (B) The influence of transcription factors on ADAM8 promoters were determined by dual luciferase reporter assays, the samples derive from the same experiment and that gels were processed in parallel, and displayed using the mean ± SD. *P<0.05; ***P<0.001. (C) Co-IP assay was performed to detect the interaction between SP1 and ADAM8 in Panc-1 cells.
Figure 6

Propofol potentially targets SP1 to regulate ADAM8. (i) Panc-1 cells were treated with 0μg/mL, 5μg/mL, 10μg/mL and 20μg/mL propofol. The dual-luciferase assay was used for measuring the luciferase activity (A). The protein and mRNA levels of SP1 in three groups of Panc-1 cells transfected with SP1 knockdown plasmids (Panc-1-SP1-shRNA) and in control Panc-1 cells were detected by western blot and qPCR (B, C, D). (ii) The protein and mRNA levels of ADAM8 in the Panc-1-NC-shRNA2 cell line and Panc-1-
SP1-shRNA cell line were exposed to 0µg/mL, 5µg/mL, 10µg/mL, 20µg/mL propofol, the samples derive from the same experiment and that gels were processed in parallel, and displayed using mean ±SD (E, F, G, H, I J). *P<0.05; **P<0.005; ***P<0.001.