Propofol Regulates the Proliferation and Migration of Lung Cancer Cells Through Atr Signaling Pathway

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Abstract

Aims: Here we aim to investigate the regulation of propofol on DNA damage caused by replication fork arrest in esophageal squamous cell carcinoma cells.

Methods: A549 and NCI-H460 cells were treated with propofol and hydroxyurea (HU) in vitro. CCK-8 assay was used to examine cell proliferation. Transwell assay was employed to investigate cell migration and invasion abilities. Western blotting was carried out to study the activities of ATR signals. Laser confocal microscopy was utilized to study the formation of p-RPA32 foci.

Results: Propofol treatment promoted the apoptosis and suppresses the proliferation, migration and invasion, possibly by increasing the sensitivity of A549 and NCI-H460 cells against DNA damage. Propofol treatment enhanced the sensitivity of A549 and NCI-H460 cells to damages caused by replication fork arrest, as well as the activity of ATR signaling pathway. Propofol regulated the sensitivity of A549 and NCI-H460 cells to DNA replication damage by affecting the level of H3K27me3.

Conclusions: The present study demonstrates that propofol up-regulates the expression of H3K27me3 in lung cancer cells, promotes the recruitment of exonuclease MUS81 in stagnant replication fork, induces apoptosis caused by DNA damage, and thus inhibits the proliferation and metastasis of tumor cells.

Introduction

Epidemiological studies show that the incidence of cancer-related diseases is increasing rapidly all over the world, posing a great threat to human health [1, 2]. Although surgery can effectively remove most of the primary tumor lesions, most patients still die of tumor metastasis and recurrence [3]. Anesthesia is an important part of surgery, and whether anesthesia can reduce the postoperative recurrence of tumor has become a research hotspot [4]. Anesthetic drugs play important roles in regulating the perioperative interaction between immunity and tumors [5]. Although anesthetic drugs have shown their effects on tumor biological functions, there is no clear conclusions at the level of clinical research, and high-quality randomized clinical trials are still needed to improve the evidence.

Propofol is a short acting intravenous anesthetic commonly used in clinic. Its chemical name is 2,6-diisopropylphenol and its molecular formula is C₁₂H₁₈O. It is used for the induction and maintenance of general anesthesia [6]. Propofol is often used together with epidural or spinal anesthesia, or with analgesics, muscle relaxants and inhalation anesthetics [7, 8]. Evidence shows that propofol can regulate the occurrence and development of tumor cells. Retrospective study shows that, compared with inhalation anesthesia, total intravenous anesthesia by propofol can improve the overall survival of patients with various types of tumors [9]. Different from inhalation anesthetics and other intravenous anesthetics, propofol can inhibit the proliferation, migration, invasion and angiogenesis of various tumor cells by acting on different cell signal pathways, and restore or enhance the sensitivity of tumor cells to chemotherapeutic drugs [10]. At present, the function and mechanism of propofol on non-small cell lung cancer (NSCLC) are not clear.

ATR signaling pathway is one of the key detection points in the process of DNA replication [11]. When the DNA replication process encounters other molecular events, such as drug stimulation, protooncogene transcription and dNTP depletion, it will cause short-term stagnation of DNA, resulting in single stranded DNA exposure [12]. At this time, the timely activation of ATR signaling pathway will promote the repair of single stranded DNA and stabilize the DNA replication fork. After the stimulus is withdrawn, the replication fork will restart to ensure the survival of cells [13]. In recent years, evidence shows that propofol can inhibit the proliferation and metastasis of a variety of tumor cells in vivo and in vitro, which suggests that propofol anesthesia in surgery is conducive to inhibit the postoperative recurrence in tumor patients [14]. In addition, propofol can also inhibit cell apoptosis and promote cell survival in ischemia-induced hypoxic injury, suggesting that propofol may affect DNA replication damage [15]. By now, it is unsure whether propofol affects DNA damage repair and ATR signaling pathway in tumor cells.

In the present study, we investigate the effect and action mechanism of propofol on the proliferation and metastasis of NSCLC.

Materials And Methods

Cells

Lung cancer A549 and NCI-H460 cell lines (Cell Bank, Chinese Academy of Sciences, Shanghai, China) were resuscitated and cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 IU/ml streptomycin under 37°C and 70% humidity in the presence of 5% CO₂. The cell lines were passaged every three days and logarithmic growth cells were collected for experiments.

Logarithmic growth A549 and NCI-H460 cells (2×10^5) were seeded into 24-well plates containing antibiotics-free RPMI-1640 medium with 10% FBS. When the cells reached more than 70% confluency, they were transfected with scrambled RNA sequence (negative control (NC) group) and MUS81 interference sequence (siRNA-MUS81 group) (HanBio, Shanghai, China) according to the manufacturer's manual. In a vial, 1.5 μ l 20 pmol/ μ l NC or siR-MUS81 (HanBio, Shanghai, China) was mixed with 50 μ L Opti Mem medium (Thermo Fisher Scientific, Waltham, MA, USA). In the other vial, 1 μ L Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) was mixed with 50 μ L Opti Mem medium. After five minutes, the mixtures in the two vials were combined and incubated for 20 minutes at room temperature. Then, the mixtures were added onto cells in respective groups. Six hours later, the medium was replaced with RPMI-1640 medium containing 10% FBS. After cultivation for 48 hours, the cells were collected for further assays.

CCK-8 assay

The cells were seeded into 96-well plates at a density of 1×10^4 /well, and cultured in RPMI-1640 medium with 10% FBS. Cells were divided into propofol group and control group (3 replicates each). Propofol group was treated with 0.2, 0.5, 1 or 2 mM hydroxyurea (HU) for 24 h, and control group was treated with equal volumes of DMSO. After replacing the medium, the cells were cultured for additional 24 h. Then, 20

μl CCK-8 reagent (5 g/L; Beyotime, Shanghai, China) was added before incubation at 37°C for 2 h. The absorbance of each well was measured at 490 nm for plotting cell proliferation curves.

Transwell assay

To test invasion ability, Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was thawed at 4°C overnight and diluted with serum-free RPMI-1640 medium (dilution 1:2). The mixture (50 μl) was evenly smeared into the upper chamber (Corning, Corning, NY, USA) and incubated at 37°C for 1 h before solidification. To test migration ability, Matrigel was not needed in the upper chamber. Cells (2×10^5) from each group were seeded into the upper chamber containing 200 μl serum-free RPMI-1640 medium. In addition, 600 μl RPMI-1640 medium supplemented with 10% FBS was added into the lower chamber. After 24 h, the chamber was removed and the cells in the upper chamber were wiped off. After being fixed with 4% formaldehyde for 10 min, the membrane was stained using Giemsa method for microscopic observation of 5 random fields (200×). The number of transwell cells was calculated for the evaluation of cell invasion and migration ability. All procedures were carried out on ice with pipetting tips being cooled at 4°C.

Flow cytometry

Cells in each group (1×10^6) were washed twice with precooled phosphate-buffered saline. BD Cycletest Plus DNA Reagent Kit (BD Biosciences, Franklin Lakes, NJ, USA) was used to determine cell cycle according to the manufacturer's manual. The result was analyzed using ModFit software version 3.2 (Verity Software House, Topsham, ME, USA).

Cells in each group (1×10^6) were washed with pre-cooled phosphate-buffered saline twice and subjected to flow cytometry using ANXN V FITC APOPTOSIS DTEC KIT I (BD Biosciences, Franklin Lakes, NJ, USA) following the manufacturer's manual to detect cell apoptotic rate. Cells with ANNEXIN V-positive values were early apoptotic cells, those with PI-positive values were necrotic cells, and those with double positive values were late apoptotic cells.

Laser confocal microscopy

NC group and propofol group of cells (1×10^6) were seeded onto petri-dishes (diameter, 6 cm) and incubated at 37°C and under 5% CO₂ for 24 h. Then, HU was used to stimulate the cells for 24 h. After discarding medium, the cells were washed with phosphate-buffered saline (PBS) for three times, and fixed with 4% formaldehyde for 10 min. After addition of 1.25 M glycine for neutralization, the fixing solution was discarded. After washing with cold PBS, the cells were perforated with 1% Triton X100 at room temperature for 5 min. After additional washing with PBS for 2 times, the cells were incubated with p-RPA32 at 4°C overnight, followed by washing with PBS. Then, fluorescence secondary antibody was added for an incubation of 2 h. After additional washing with PBS for 3 times, the formation of foci was observed under a laser scanning confocal microscope (SP8; Leica, Wetzlar, Germany).

Western blotting

Cells in each group (1×10^6) were lysed with 1 ml precooled Radio-Immunoprecipitation Assay (RIPA) lysis buffer (Beyotime, Shanghai, China) for 30 min on ice. The mixture was centrifuged at 12,000 rpm and 4°C for 10 min. The supernatant was used to determine protein concentration by bicinchoninic acid (BCA) protein concentration determination kit (Beyotime, Shanghai, China). The samples were then mixed with 5× sodium dodecyl sulfate loading buffer before denaturation in boiling water bath for 10 min. Afterwards, the samples (20 µg) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 100 V. The resolved proteins were transferred to polyvinylidene difluoride membranes on ice (250 mA, 1 h) and blocked with 5% skimmed milk at room temperature for 1 h. Then, the membranes were incubated with rabbit anti-human ATR (1:1000), p-ATR (1:1000), RPA32 (1:1000), p-RPA32 (1:1000), MUS81 (1:1000), H3K27me3 (1:1000) (Cell Signaling Technology, Danvers, MA, UK) or mouse anti-human GAPDH monoclonal antibody (1:4000; Beyotime, Shanghai, China) polyclonal primary antibodies at 4°C overnight. After extensive washing with phosphate-buffered saline with Tween 20 (0.1%) for 5 times of 5 min, the membranes were incubated with goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:4000; Cell Signaling Technology, Danvers, MA, UK) for 1 h at room temperature before washing with phosphate-buffered saline with Tween 20 for 5 times of 5 min. Then, the membrane was developed with enhanced chemiluminescence detection kit (Beyotime, Shanghai, China) for imaging. Image lab v3.0 software (Bio-Rad, Hercules, CA, USA) was used to acquire and analyze imaging signals. The relative contents of target proteins were expressed against GAPDH.

Statistical analysis

The results were analyzed using SPSS 18.0 statistical software (IBM, Armonk, NY, USA). The data were expressed as means $\pm$ standard deviations and underwent normality test. If the data fit normal distribution and the variance was homogeneous, multigroup measurement data were analyzed using one-way ANOVA and Dunnett's test, while comparison between two groups was carried out using paired Student's t-test. If the data were not normal distribution or the variance was not uniform, multigroup measurement data were analyzed using Kruskal-Wallis test and Tamhane's T2 or Dunnett's T3 method, while comparison between two groups was carried out using Mann-Whitney test. $P < 0.05$ indicated statistically significant differences.

Results

Propofol treatment promotes the apoptosis and suppresses the proliferation, migration and invasion, possibly by increasing the sensitivity of A549 and NCI-H460 cells against DNA damage

To test the biological function of propofol in repairing DNA damage in lung cancer cells, A549 and NCI-H460 cells were incubated with propofol at 37°C for 24 h, and then treated with different concentrations of HU for 24 h. CCK-8 assay showed that the absorbance of propofol groups of A549 and NCI-H460 cells

was significantly lower than that of negative control groups ($P < 0.05$; Fig. 1A and B). Flow cytometry showed that the percentages of G1-phase cells in propofol groups of A549 and NCI-H460 cells were significantly higher than those in negative control groups ($P < 0.05$; Fig. 1C and D). In addition, the apoptosis of propofol groups of A549 and NCI-H460 cells was enhanced compared with negative control groups (Fig. 1E and F). Transwell assay showed that the numbers of both A549 and NCI-H460 cells with migration or invasion in propofol group were significantly reduced than those in negative control group ($P < 0.05$; Fig. 1G). These results indicate that propofol treatment promotes the apoptosis and suppresses the proliferation, migration and invasion, possibly by increasing the sensitivity of A549 and NCI-H460 cells against DNA damage.

Propofol treatment enhances the sensitivity of A549 and NCI-H460 cells to damages caused by replication fork arrest, as well as the activity of ATR signaling pathway

Subsequently, we studied the regulation of DNA damage in A549 and NCI-H460 cells by propofol at molecular level, using Western blotting and laser confocal microscopy. Western blotting showed that after HU treatment, p-ATR and p-RPA32 expression levels in A549 and NCI-H460 cells treated with propofol were significantly higher than those in negative control groups ($P < 0.05$; Fig. 2A and B). Laser confocal microscopy showed that the numbers of p-RPA32 foci in A549 and NCI-H460 cells treated with propofol were significantly higher than those in negative control groups ($P < 0.05$; Fig. 2C). The results suggest that propofol treatment enhances the sensitivity of A549 and NCI-H460 cells to damages caused by replication fork stagnation, as well as the activity of ATR signaling pathway.

Propofol regulates the sensitivity of A549 and NCI-H460 cells to DNA replication damage by affecting the level of H3K27me3

The damage and repair after replication fork stagnation are closely related to the level of H3K27me3, which can recruit exonuclease MUS81 to the replication fork, resulting in DNA damage. Western blotting showed that H3K27me3 protein levels in A549 and NCI-H460 cells treated with propofol were significantly higher than those in NC groups ($P < 0.05$; Fig. 3A and B). After HU treatment, MUS81 expression in chromatin was elevated in both A549 and NCI-H460 cells ($P < 0.05$; Fig. 3C and D). Flow cytometry showed that the apoptotic rates of A549 and NCI-H460 cells treated with both propofol and H3K27me3 inhibitor were significantly lower than those in propofol groups ($P < 0.05$; Fig. 3E). Western blotting showed that the phosphorylation levels of ATR signaling pathway proteins ATR and RPA32 in HU-induced A549 and NCI-H460 cells treated with both propofol and H3K27me3 inhibitor were lower than those in propofol group ($P < 0.05$; Fig. 3F and G). The results indicate that propofol regulates the sensitivity of A549 and NCI-H460 cells to DNA replication damage by affecting the level of H3K27me3.

Discussion

Studies show that strong DNA damage repair ability of tumor cells plays important roles in the recurrence and distant metastasis [16, 17]. The inhibitory effect of propofol on tumor cell growth and metastasis has gradually attracted extensive attention. Propofol can significantly inhibit the proliferation and invasion of

a variety of tumors, such as human cervical cancer, fibrosarcoma and osteosarcoma [18, 19]. Moreover, 1 - 5 ng/ml propofol is able to directly inhibit the invasion abilities of human cervical cancer HeLa, fibrosarcoma HT1080, osteosarcoma HOS and melanoma 7951 cell lines [20, 21]. DNA replication pressure is an important factor affecting cell survival. In the process of cell division, DNA will accurately complete a replication and distribute it to the offspring cells. Any factor affecting DNA replication will induce the stagnation and damage of DNA replication fork [22, 23]. If not recovered in time, the replication fork will collapse, resulting in DNA damage that affects cell survival. Propofol has been reported to inhibit tumor cell proliferation and regulate cell cycle, suggesting that propofol may be involved in DNA synthesis. In the present study, we found that propofol was able to significantly promote the sensitivity of esophageal squamous cell carcinoma cells to HU, a replication fork arrest drug, in vitro. After propofol treatment, HU was able to significantly induce the apoptosis of esophageal squamous cell carcinoma cells. In addition, the distant metastasis ability of esophageal tumor cells in propofol treatment group was also significantly reduced after HU treatment. It is reported that after HU-induced DNA arrest, the replication fork will flip under the action of RPA and RAD51 proteins to form a Holliday junction structure and maintain the stability of the replication fork [24]. If the stagnation factor persists, the replication fork will cause single strand and double strand damage under the action of exonuclease, which will activate ATR signal pathway and promote DNA repair [25]. In the present study, we discovered that under HU treatment, p-RPA32 and p-ATR signaling pathway in esophageal squamous cell carcinoma in propofol group was significantly higher than that in control group. Laser confocal microscopy results also showed that the foci formed by p-RPA32 and p-ATR were increased significantly, suggesting that intracellular DNA damage was increased. These data suggest that propofol promotes the sensitivity of esophageal squamous cell carcinoma to HU. We believe that propofol can promote the sensitivity of esophageal squamous cell carcinoma cells to replication fork, so as to inhibit their proliferation and metastasis.

Histone modification can regulate the transcriptional activity of genes by affecting chromatin opening. H3K27me3 is a modified marker of gene transcription inhibition, and its high enrichment state in chromatin promoter region and enhancer region can change the open and closed states of chromatin and turn off gene transcription [26, 27]. Studies show that H3K27me3 is abnormally elevated in solid tumors, resulting in abnormal gene transcriptional activity, so as to regulate tumor progression [28, 29]. H3K27me3 participates in the regulation of tumor suppressor gene expression in triple-negative breast cancer cells [30]. Histone modification can regulate DNA replication, damage repair and chromatin state by recruiting chromatin remodeling factors, nucleases and DNA repair proteins. For example, H3K27me3 has been reported to recruit nuclease MUS81 to participate in the excision of stagnant replication forks [31]. Here, we found that propofol treatment increased the expression of H3K27me3 and the enrichment of MUS81 protein in chromatin, and H3K27me3 inhibitor suppressed the regulation of propofol on HU sensitivity of esophageal cancer cells. These results suggested that propofol participated in the regulation of replication fork stability through H3K27me3/MUS81 pathway, enhanced the sensitivity of esophageal squamous cell carcinoma cells to DNA damage caused by replication fork stagnation, and promoted the apoptosis of esophageal squamous cell carcinoma. EZH2, KDM6A and KDM6B are the main regulatory factors of H3K27me3, and the way in which propofol affects H3K27me3 still needs to be

further studied. In addition, whether there are other histone modifications at the replication fork and whether propofol affects other modifications and nuclease recruitment still need to be further investigated.

In conclusion, the present study demonstrates that propofol can up-regulate the level of H3K27me3 in esophageal squamous cell carcinoma cells, enhance the enrichment of MUS81 at the stagnant replication fork, induce DNA-damaging apoptosis, and inhibit the proliferation and metastasis of esophageal squamous cell carcinoma.

Declarations

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions

YZ and WW contributed to the design of the study. YZ, DL, HL and YM performed the experiments. YZ and DL analyzed the data. YZ and WW interpreted results and prepared the manuscript. The final version of the manuscript has been read and approved by all authors.

Ethical approval and consent to participate

All procedures performed in the current study were approved by the Ethics Committee of Jinan University. Written informed consent was obtained from all patients or their families.

Consent for publication

Written informed consents for publication of any associated data and accompanying images were obtained from all patients or their parents, guardians or next of kin.

Conflict of interest

The author declares that there is no financial or any conflict of interests related to this paper.

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Figures

Figure 1

Propofol promotes the sensitivity of lung cancer cells to hydroxyurea (HU) in vitro. (A-B) Proliferation of (A) A549 and (B) NCI-H460 cells studied by CCK-8 assay. *P < 0.05 compared with NC group. (C-D) Cell cycles of (C) A549 and (D) NCI-H460 cells studied by flow cytometry. *P < 0.05 compared with NC group. (E-F) Apoptosis of (E) A549 and (F) NCI-H460 cells induced by HU, as studied by flow cytometry. (G)

Migration and invasion of A549 and NCI-H460 cells studied by Transwell assay. *P < 0.05 compared with NC group.

Figure 2

Propofol promotes the sensitivity of A549 and NCI-H460 cells to hydroxyurea (HU). (A) Propofol enhances the phosphorylation of ATR and RPA32 proteins in ATR signal pathway. (B) Foci formation of RPA protein

in A549 and NCI-H460 cells after induction by HU.

Figure 3

Propofol affects the sensitivity of A549 and NCI-H460 cells to HU through H3K27me3/MUS81 pathway. (A-B) H3K27me3 protein levels in (A) A549 and (B) NCI-H460 cells treated with propofol. * $P < 0.05$ compared with NC group. (C-D) MUS81 protein expression in cytoplasm and chromatin of (C) A549 and (D) NCI-H460 cells after HU treatment. (E) Apoptotic rates of A549 and NCI-H460 cells treated with propofol alone or both propofol and H3K27me3 (EZH2) inhibitor. * $P < 0.05$ compared with NC group. (F-G) Phosphorylation levels of ATR signaling pathway proteins ATR and RPA32 in HU-induced (F) A549 and (G) NCI-H460 cells treated with propofol alone or both propofol and H3K27me3 (EZH2) inhibitor.