



Effect of mTOR Inhibitor Combined with Photodynamic Therapy on the Regulation of Cancer Stem Cells through Reactive Oxygen Species

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ABSTRACT

To explore the mechanism of mammalian target of rapamycin (mTOR) inhibitor combined with photodynamic therapy to regulate cancer stem cells through reactive oxygen species (ROS). A2780 cell line was purchased from the American Type Culture Collection and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 50 μg/mL penicillin and 50 μg/mL streptomycin, maintained at 37°C and 5% CO₂ in a humid incubator environment. Quantitative analysis of ROS was performed using flow cytometry and median fluorescence intensity detection. Cell proliferation was detected by MTT method. Combined treatment promoted the production of ROS. The cell viability of the combined treatment group was lower than that of the control group at 24h, 48h and 72h (P<0.05). Compared with the control group, the expression level of E-cadherin mRNA in the combined treatment group increased (P<0.05), and the expression levels of N-cadherin and vimentin decreased (P<0.05). Compared with the control group, the apoptosis rate of the combined treatment group increased (P<0.05). Compared with the control group, the expression levels of Bax, caspase-3 and caspase-9 were higher in the combined treatment group (P<0.05), and the expression levels of Bcl-2, PI3K, Akt and mTOR were lower (P<0.05). mTOR inhibitor combined with photodynamic therapy increased the generation of ROS to inhibit PI3K/Akt/mTOR signal transduction, increase the expression levels of pro-apoptotic proteins Bax, caspase-3 and caspase-9, inhibit the expression level of anti-apoptotic protein Bcl-2 and the epithelial-mesenchymal transition of cancer stem cells, reduce the proliferation ability of cancer stem cells, and increase the apoptosis of cancer stem cells.

Article Information

Received 19 July 2021

Revised 01 October 2021

Accepted 18 April 2022

Available online 15 July 2022
(early access)

Key words

mTOR inhibitor, Photodynamic therapy, Reactive oxygen species, Cancer stem cells, Proliferation, Apoptosis

INTRODUCTION

Ovarian cancer is the most common malignant tumor in female genital tract. According to cell origin, about 30 different types of ovarian cancer are defined, such as epithelial ovarian cancer, germ cell ovarian cancer and stromal cell ovarian cancer (Kawakami *et al.*, 2020). Among them, epithelial ovarian cancer is the most common type, accounting for about 90% of ovarian malignant tumors. Studies from different groups show that epithelial-mesenchymal transition and cancer stem cells

are closely related to the chemical resistance, metastasis and tumor recurrence of epithelial ovarian cancer patients (Daniel *et al.*, 2019). PI3K/Akt/mTOR is an important intracellular pathway to regulate cell cycle and proliferation. Everolimus is an oral mTOR inhibitor. Inhibition of mTOR signaling can reduce cell proliferation and enhance apoptosis and endocrine sensitivity of tumor cells (Carayol *et al.*, 2010). Everolimus has been approved by the U.S. Food and Drug Administration for the treatment of various tumors. Studies have confirmed the clinical benefits of everolimus for ovarian cancer patients. Photodynamic therapy involves the photoinduced activation of photosensitizers which produces reactive substances that can cause cell damage (Qian *et al.*, 2019). After absorbing light, the photosensitizer is activated from its ground state to a short-lived singlet excited state, and can undergo intersystem cross-conversion to a long-lived triplet state. In triplet state, the photosensitizer can transfer electrons to surrounding substrate molecules (such as solvents, biomolecules or oxygen) through type I reaction, or transfer energy to oxygen molecules through

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0030-9923/2022/0001-0001 \$ 9.00/0



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type II reaction (Yang *et al.*, 2020). This leads to a series of cytotoxic reactive species. Therefore, photodynamic therapy is expected to be a curative and non-invasive treatment for many solid tumors. More and more evidences show that ROS plays a role in cell signal transduction. These signals are transmitted in tissues to coordinate various cellular processes. At physiological dose, ROS maintains the balance of cell nutrition and cytokines (Sharif *et al.*, 2019). However, under some specific circumstances, the slight change of ROS level may have a profound impact on the fate of stem cells, directly inducing the differentiation of cancer stem cells or inducing the heterogeneity of cancer stem cells in tumors (Bai *et al.*, 2020). In addition, ROS is related to the level of many biological processes, including but not limited to gene expression, protein translation and protein or nucleic acid interaction (Nascimento-Filho *et al.*, 2019). Our research aims to explore the mechanism of mTOR inhibitor combined with photodynamic therapy to regulate cancer stem cells through ROS.

MATERIALS AND METHODS

Cell culture

The A2780 cell line was purchased from American Center for Type Culture Collection. All cell culture reagents were purchased from Thermo Fisher Scientific Technology Company. The A2780 cell line was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin and 50 µg/mL streptomycin, and maintained in a humid incubator at 37°C and 5% CO₂.

Preparation of alginate nanoparticles loaded with methylene blue to enhance photodynamic therapy

The aqueous solution of methylene blue (1 mL, 5 mg/mL) and sodium alginate (1 mL, 10 mg/mL) was mixed and sonicated (Sonicator 3000, Misonix, Farmingdale, New York) on an ice bath for 5 min. 1 ml of this mixture was emulsified in 2 mL of 2.5% w/v sodium docusate chloroform solution by ultrasonic treatment at 18-21 W for 5 min. The water-in-oil emulsion was further emulsified into 2.5% w/v aqueous solution of polyvinyl alcohol by ultrasonic treatment on an ice bath of 18-21 W for 5 min to obtain the water in oil emulsion. 5ml of 60% w/v calcium chloride aqueous solution was gradually added to this final emulsion. Chloroform was evaporated by stirring the emulsion overnight under ambient conditions and under vacuum for 2 h. The nanoparticles were washed four times with deionized water by ultracentrifugation (at 4°C, Beckman, Palo Alto, Calif.) at 35,000 rpm for 30 min to remove excess polyvinyl alcohol and unencapsulated methylene blue. After the final washing, the precipitate

was resuspended in deionized water and centrifuged (1000 rpm, 10 min) to remove any large aggregates. The supernatant was lyophilized using Freezone 4.5 freeze drying system (Labconco, Kansas City, MO.).

ROS assay

2×10⁵ A2780 cells were treated with solvent control, and mTOR inhibitor combined with photodynamic therapy respectively. After 48h of treatment, the cells were separated, centrifuged and resuspended in 1xPBS. CellROX® green reagent (Life Technologies, Scoresby, Victoria, Australia) was added to cells to a final concentration of 5 µM. The mixture was incubated at 37°C for 30 min according to the manufacturer's procedure. The fluorescence was detected by FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA), and was excited at ~485 nm and emitted at ~520 nm.

Cell viability assay

MTT assay was performed to examine the effect of mTOR inhibitor combined with photodynamic therapy on cell viability. A2780 cells were inoculated into a 96-well culture plate at a density of 8,000 cells/well. After incubation for 24h, 48h and 72h with different treatments, the cell viability was determined by the decrease of MTT. The absorbance was measured at wavelengths of 560 nm (MTT formazan) and 670 nm (background) using a Synergy™H4 hybridization microplate reader (BioTek, Winooski, VT, USA). The experiment was carried out in triplicate.

Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted with TRIzol reagent (Invitrogen, USA). Isolated RNA was reverse transcribed into cDNA using Prime Script RT kit (Takara Biotechnology, China). Primers of E-cadherin, N-cadherin, vimentin and GAPDH (control) were synthesized by Invitrogen (Mulgrave, Victoria, Australia). The quantitative PCR was performed using IQ5 real-time PCR system (Bio-Rad, USA) with a reaction volume of 20 µL. The obtained values were used as threshold cycles for each gene, and were standardized with respect to the reference gene (GAPDH). The 2-ΔΔCT method was used to calculate the relative changes of each gene in different cell lines.

Quantitative apoptosis of flow cytometry

24h after treating cells with mTOR inhibitor combined with photodynamic therapy, A2780 cells were treated with trypsin and washed twice with cold PBS. The cells were resuspended in a volume of 1×10⁵ cells/mL containing 5 µL A0nnexin V: PE and 5 µL 7- amino actinomycin D (7-AAD), with a total volume of 100 µL. Cells were gently

mixed and incubated in the dark at room temperature for 15m. Binding buffer (250 μ L.mu.l) was added to the sample, and the number of apoptotic cells was quantified by flow cytometry.

Western blot analysis

The A2780 cells were washed with PBS after being treated with mTOR inhibitor combined with photodynamic therapy for 24h, and were lysed with radioimmunoassay buffer (50 mmol 4-(2- hydroxyethyl) piperazine -1-ethanesulfonic acid, pH 7.5), 150 mM sodium chloride, 10% glycerol and 1.5 mM magnesium chloride, 1% TritonTMX-100 with pH 8.0, 1 mM ethylenediamine tetraacetic acid, 10 mM sodium pyrophosphate and 10 mM sodium fluoride, as well as a mixture of protease and phosphatase inhibitor. Then, 3,000 \times g was centrifuged for 10 min at 4°C. The protein concentration was measured using PierceTM dicinnonic acid protein assay kit (Thermofisher Scientific Inc.), and the protein sample was denatured at 95°C for 5 min. The same amount of protein sample (30 μ g) was added to 7%–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis microgel. The protein was transferred to polyvinylidene fluoride membrane at 4°C and 400 mA for 2h. The membrane was sealed with skim milk for 1h, probed with the first antibody

at 4°C overnight, and incubated with the corresponding second antibody. The visualization was performed using Bio-Rad ChemiDocTMXRS system (Bio-Rad Laboratories Inc., Hercules, CA, USA), and the blots were analyzed using Image Lab 3.0 (Bio-Rad Laboratories Inc.). The protein level was standardized as the matching optical density value of β -actin.

Statistical analysis

All experiments are repeated in triplicate. The data is expressed as the mean \pm standard deviation (SD) of continuous variables. T-test of students and one-way ANOVA with after-the-fact Tukey test are used to compare different groups of data. P <0.05 was considered to have statistical significance.

RESULTS AND DISCUSSION

Table I shows the effect of MTOR inhibitor combined with photodynamic therapy on ROS content, cell proliferation, epithelial and mesenchymal proteins, pro-apoptotic and anti-apoptotic proteins, PI3K/Akt/mTOR proteins and apoptosis rate of cancer stem cells.

Table I. Effect of MTOR inhibitor combined with photodynamic therapy on ROS content (determined by flow cytometry), cell proliferation (determined by MTT assay), mRNA expression level of epithelial and mesenchymal proteins (determined by qRT-PCR), apoptosis rate (determined by flow cytometry), pro-apoptotic and anti-apoptotic proteins (determined by Western Blot analysis) and expression level of PI3K/Akt/mTOR proteins (determined by Western Blot analysis).

Determination method		Control (n=3)	Combined treatment (n=3)	t value	p value
ROS content	Fluorescence intensity	168.79 \pm 16.38	2587.32 \pm 224.56	18.624	<0.001
Cell proliferation	24h (%)	95.34 \pm 3.26	83.13 \pm 4.33	6.597	0.021
	48h (%)	93.46 \pm 5.23	74.33 \pm 3.85	13.264	0.015
	72h (%)	92.58 \pm 4.37	65.87 \pm 2.65	15.234	<0.001
mRNA expression level of epithelial and mesenchymal proteins	E-cadherin	0.78 \pm 0.15	3.24 \pm 0.13	12.521	<0.001
	N-cadherin	3.95 \pm 0.15	1.14 \pm 0.12	8.624	<0.001
	Vimentin	4.86 \pm 0.13	1.13 \pm 0.15	9.624	<0.001
Apoptosis by flow cytometry	apoptosis rate	2.75 \pm 0.23	18.34 \pm 2.27	13.264	0.015
Pro-apoptotic and anti-apoptotic proteins	Bax	1.33 \pm 0.14	3.58 \pm 0.32	12.564	0.013
	Bcl-2	2.15 \pm 0.34	0.89 \pm 0.13	8.654	0.012
	caspace-3	1.08 \pm 0.16	4.37 \pm 0.25	6.254	0.011
	caspace-9	1.13 \pm 0.17	4.26 \pm 0.23	8.326	0.008
Expression level of PI3K/Akt/mTOR proteins	PI3K	3.25 \pm 0.14	1.33 \pm 0.26	15.654	<0.001
	Akt	3.87 \pm 0.25	1.08 \pm 0.14	13.264	<0.001
	mTOR	4.16 \pm 0.29	1.26 \pm 0.13	11.235	<0.001

The combined treatment group had increased ROS content ($P<0.05$), indicating that combined treatment promoted ROS generation (Table I). The cell viability of the combined treatment group decreased at 24h, 48h and 72h ($P<0.05$), indicating that the combined treatment inhibited the proliferation of cancer stem cells. The mRNA expression level of E-cadherin in the combined treatment group increased ($P<0.05$), while the mRNA expression levels of N-cadherin and vimentin decreased ($P<0.05$), indicating that combined treatment inhibited epithelial-mesenchymal transition. The apoptosis rate of the combined treatment group was higher than that of the control group ($P<0.05$), indicating that combined treatment promoted the apoptosis of cancer stem cells. The expression levels of Bax, caspase-3 and caspase-9 in the combined treatment group increased ($P<0.05$), while the expression level of Bcl-2 decreased ($P<0.05$), indicating that combined treatment promoted the expression of pro-apoptotic protein and inhibited the expression of anti-apoptotic protein. Compared with the control group, the expression levels of PI3K, Akt and mTOR protein in the combined treatment group decreased ($P<0.05$), which indicated that combined treatment interfered the proliferation and apoptosis of cancer stem cells by inhibiting PI3K/Akt/mTOR signaling pathway.

DISCUSSION

Photodynamic therapy is an effective and promising cancer treatment method. It directly generates ROS through photochemical reaction. This kind of oxygen-dependent exogenous ROS has anti-cancer stem cells effect. In addition, photodynamic therapy may increase ROS generation by changing metabolism, endoplasmic reticulum stress or mitochondrial membrane potential. Type I reactions can generate free radicals by transferring electrons or H atoms to molecules other than oxygen. Because I-type reaction is less sensitive to local oxygen concentration, changing the generation mechanism of free radicals from II-type to I-type may overcome an important limitation of photodynamic therapy. Previous studies have shown that a closed environment can ensure photosensitizer molecules, and the interaction between photosensitizer and substrate near static electricity and reduced oxygen concentration can promote electron transfer, thus promoting type I chemical reaction (Park *et al.*, 2020). To create these conditions and promote the type I chemical reaction, we encapsulate a widely used photosensitizer methylene blue in polymer surfactant nanoparticle system, which is composed of sodium alginate and sodium docusate. Encapsulation of methylene blue in

nanoparticles will allow cationic methylene blue molecules and anions to interact electrostatically with polymer and surfactant molecules, and promote charge transfer and free radical formation. In addition, encapsulation in nanoparticles will lead to local high concentration of methylene blue molecules, thus promoting the formation of dye dimer in ground state and excited state.

As is known to all, ROS in photodynamic therapy has a short half-life, high reactivity and limited diffusion distance. Therefore, the main target location of photodynamic therapy is usually subcellular localization of photosensitizer, which helps us explain how photodynamic therapy affects the characteristics of cancer stem cells, including differentiation, self-renewal, apoptosis, autophagy and immunogenicity (Favi *et al.*, 2020). Broadly speaking, excessive ROS will damage the redox system, cause oxidative damage to DNA and other molecules, change mitochondrial permeability, activate unfolded protein reaction, autophagy and cancer stem cells at rest. Therefore, understanding the molecular mechanism of ROS affecting cancer stem cells is helpful to improve the efficiency of photodynamic therapy and prevent tumor recurrence and metastasis. Under normal circumstances, about 90% of ROS in the body is generated by mitochondrial electron transfer chain. During hypoxia, light stimulation, ischemia-reperfusion, aging and mitochondrial respiratory depression, ROS generated by ETC increased (Myers *et al.*, 2019). Over 90% of oxygen in mitochondria is reduced to water molecules by cytochrome oxidase, while only 0.1%-0.2% of O_2 forms ROS through electron flow (mainly through electron transport chain complexes I and III). Our research showed that mTOR inhibitor combined with photodynamic therapy could promote the generation of cancer stem cells ROS.

The activation of invasion and metastasis of cancer cells is one of the characteristics of changing the normal function of cells to obtain enhanced malignant growth, and it is also the main obstacle for human beings to overcome cancer. Epithelial-mesenchymal transition refers to the biological process in which epithelial cells are transformed into cells with interstitial phenotype under special physiological or pathological conditions. That is to say, epithelial cells lose their original phenotype connected with basement membrane, and gain resistance to interstitial cell phenotype, such as the ability to undergo apoptosis or degrade extracellular matrix, and higher migration and invasion ability (Chang *et al.*, 2020). The weakening of tumor cell adhesion and the enhancement of tumor cell movement are the basis of invasion and metastasis. Epithelial mesenchymal transition provides conditions for invasion and metastasis of epithelial-derived tumor cells. Therefore, epithelial-mesenchymal transition is closely

related to tumor invasion and metastasis. The related molecular mechanism of epithelial-mesenchymal transition in tumor cells is the loss of epithelial morphology and the acquisition of related markers (including E-cadherin, desmosomal smooth muscle protein, Muc-1, cytokeratin 18, occludins, claudins and ZO-1) and mesenchymal markers (including N-cadherin, Vimentin, fibronectin, vitronectin, α smooth muscle actin and FSP1). Among all the factors that promote tumor cell migration, ROS plays a key role by activating the signal that causes cell migration, and epithelial-mesenchymal transition is the first step of tumor cell migration activated by ROS (Hibdon *et al.*, 2019; Ohata *et al.*, 2019; Liang *et al.*, 2020). Our research showed that mTOR inhibitor combined with photodynamic therapy inhibited epithelial-mesenchymal transition of cancer stem cells and reduced the proliferation ability of cancer stem cells during ROS generation.

Apoptosis is carried out by members of caspase family. It can be activated by two main pathways, namely, the extrinsic death receptor pathway and the intrinsic mitochondrial/cytochrome C mediated pathway. These two pathways are interrelated and are triggered by activating caspase-3, 6 and 7 (Digomann *et al.*, 2019). In the death receptor pathway, the combination of extracellular death ligand and members of tumor necrosis factor and nerve growth factor receptor superfamily can induce caspase-8 activation, and then activate caspase-3 and caspase-7, which leads to further caspase activation events and eventually leads to cell death. As we all know, the destruction of mitochondria and the release of cytochrome C can trigger the process of apoptosis. The release of cytochrome c in mitochondria is triggered by pro-apoptotic members of Bcl-2 family, but the anti-apoptotic members of Bcl-2 family who are antagonized by anti-apoptotic members can be inhibited by post-translational modification and/or increase of PUMA expression. PUMA is an important regulator of p53-mediated apoptosis (Che *et al.*, 2021). In addition, the stimulation of apoptosis of cytochrome C released by mitochondria is the main inducement of caspase activation. In our study, we found that the combination of mTOR inhibitor and photodynamic therapy activated caspase-9. The activated caspase-9 activated caspase-3 in turn. Activated caspase-3 eventually induced apoptosis and decreased Bcl-2 level.

PI3K/Akt/mTOR is an important intracellular pathway to regulate cell cycle, rest and proliferation. Many somatic mutations causing homologues of phosphorylase and tensin, Akt1 and mTOR, are found in ovarian cancer, which induce the enhancement of PI3K/Akt/mTOR signal. Overactivation of PI3K/Akt/mTOR signal is related to cancer metastasis and chemical resistance. According to reports, PI3K regulates G1 cell cycle and apoptosis

of ovarian cancer by activating Akt/mTOR/p70S6K1 signaling, while the inhibition of PI3K can destroy the proliferation of ovarian cancer cells and trigger cell death (Kahraman *et al.*, 2019). More importantly, it is found that inhibition of PI3K/Akt/mTOR signal makes chemoresistant ovarian cancer cells re-sensitive to chemotherapy drugs. These findings establish PI3K/Akt/mTOR signaling as an attractive therapeutic target for ovarian cancer treatment. PI3K/Akt/mTOR signal transduction pathway is the main pathway to promote cell growth, movement, protein synthesis, survival and metabolism, which can respond to hormones, growth factors and nutrients (Yau *et al.*, 2019). PI3K activates serine/threonine kinase Akt, and will lead to phosphorylation and activation of serine/threonine kinase mTOR through cascade reaction of regulators in vivo. In this study, the inhibition of PI3K/Akt/mTOR contributed to the apoptosis induction of mTOR inhibitor combined with photodynamic therapy.

CONCLUSIONS

We proved that mTOR inhibitor combined with photodynamic therapy could increase ROS generation, inhibited PI3K/Akt/mTOR signal transduction, increased the expression levels of pro-apoptotic proteins Bax, caspase-3 and caspase-9, and inhibited the expression levels of anti-apoptotic protein Bcl-2. It could also inhibit epithelial-mesenchymal transition of cancer stem cells, reduce proliferation ability of cancer stem cells and increase apoptosis of cancer stem cells. The combination of photodynamic therapy and mTOR inhibitor has shown good experimental results. However, how to use photodynamic therapy safely and effectively to activate immunity is still a difficult problem in clinical treatment design. Based on these considerations, further research is needed to expand the clinical application of photodynamic therapy regardless of its anti-tumor effect.

Statement of conflict of interest

The authors have declared no conflict of interest.

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