Influence of carboplatin on the proliferation and apoptosis of ovarian cancer cells through mTOR/p70s6k signaling pathway

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Summary

Purpose: To investigate the influence of carboplatin on the proliferation and apoptosis of ovarian cancer cells through mTOR/p70S6K signaling pathway.

Methods: The mRNA and protein expressions were detected via Western blotting and RT-PCR to study whether the mTOR/p70S6K signaling pathway was activated in OVCAR-3 and Caov-3 ovarian cancer cell lines. After cells were treated with different concentrations of carboplatin, the mRNA and protein expressions of mTOR, p70S6K and 4E-BP1 were detected via RT-PCR and Western blotting. OVCAR-3 cells were treated with 20 and 50 μM carboplatin for 4 hrs, and then apoptosis was analyzed and assessed. OVCAR-3 cells were treated with different concentrations of carboplatin (20, 50, 100, 150 and 200 μM) for 24 and 48 hrs, respectively.

Results: The mTOR signaling pathway was activated in OVCAR-3 and Caov-3 ovarian cancer cell lines. The mRNA level of mTOR in Caov-3 cells was higher, but that of p70S6K was lower. Carboplatin significantly reduced the mRNA expression of mTOR (p<0.01), whereas the mRNA expressions of p70S6K and 4E-BP1 in carboplatin-treated cells were increased in a dose-dependent manner (p<0.01). Carboplatin inhibited the mTOR protein expression in a dose-dependent manner (p<0.01). The proliferation of OVCAR-3 cells exposed to carboplatin was reduced compared with that of untreated cells (p<0.01), and the inhibitory effect of carboplatin on the proliferation of OVCAR-3 cells was time- and dose-dependent.

Conclusion: The mTOR/p70S6K pathway was activated in ovarian cancer. Carboplatin could rapidly inhibit the expression of mTOR, and the phosphorylation of its major downstream effectors p70S6K and 4E-binding protein 1 (4E-BP1) arrested cells in G0/G1 phase and induced ovarian cancer cell apoptosis.

Key words: hepatocellular carcinoma, oxidoredox-nitro domain containing protein 1, prognosis

Introduction

Ovarian cancer is one of the most common cancers in developing countries, especially in China [1]. However, the pathogenesis of this disease is still not fully understood [2]. Although treatment strategies have been improved, the prognosis of patients with ovarian cancer is still poor. In addition, ovarian cancer cells are known to be resistant to chemotherapeutic drugs, resulting in significant decline in 5-year survival of patients with ovarian cancer. It is obvious that better understanding the molecular mechanisms of occurrence and progression of ovarian cancer can help improve the prognosis of patients with this malignancy.

Carboplatin is widely used in a variety of human cancers in the clinic, including ovarian cancer, lung cancer, head-neck cancer, and cervical cancer [3-5]. Unlike cisplatin, carboplatin is a cisplatin analogue with few adverse reactions [6,7]. Therefore, carboplatin is considered as a means of inhibiting the cancer cell growth via death receptors [8,9].
Mammalian target of rapamycin (mTOR) signaling pathway is often activated in human cancers [10-15]. Ribosomal S6 kinases S6K1 and S6K2 and 4E-binding protein 1 (4E-BP1) are activated and phosphorylated by mTOR[16-19]. These two events lead to the increase in ribosomal biosynthesis and the selective translation of specific messenger ribonucleic acid (mRNA) groups [20-22].

Recently, mTOR has been considered as an important and attractive target for the treatment of cancer [20,21]. The potential application of mTOR inhibitors in the treatment of various cancers, including renal cancer, esophageal cancer, prostate cancer, breast cancer, pancreatic cancer and lung cancer, has been studied extensively [22-24]. However, mTOR/p70S6K signaling pathway in ovarian cancer has not been studied so far, so it is necessary to study the role of mTOR/p70S6K signaling pathway as a new possible target of anticancer drugs in ovarian cancer. In this study, the effects of carboplatin on activated mTOR and its major downstream members in ovarian cancer cell lines were studied, and changes in mRNA and protein expression levels, cell cycle and apoptosis in ovarian cancer cells were detected. It is concluded that the mTOR/p70S6K signaling pathway is constitutively activated in ovarian cancer cell lines. In addition, carboplatin can specifically block the mTOR-mediated signaling pathway, arrest cells in G0/G1 phase, induce apoptosis, and improve the sensitivity of ovarian cancer OVCAR-3 cells to carboplatin.

Methods

Cell lines and cell culture

Two ovarian cancer cell lines OVCAR-3 and Caov-3 were purchased by the State Key Laboratory of Molecular Oncology, Chinese Academy of Medical Sciences. Both cell lines were cultured in the Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS) and double antibodies under 5% CO2 at 37°C.

Experimental reagents and equipment

Streptavidin-peroxidase (SP) kit was purchased from Santa Cruz, USA, TRIZol reagent was purchased from Invitrogen, Carlsbad, USA, alfalfa mosaic virus (AMV) First Strand DNA synthesis kit. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. mTOR, p70S6K and 4E-BP1 were analyzed via PCR, and oligonucleotide primers used were as follows: mTOR (195 bp): forward primer: 5'CGCTGTACATCCCTTTATCG-3', reverse primer: 5'-ATGTCAAAACACCTCCACC-3'. p70S6K (188 bp): forward primer: 5'-TACTTCGGGTACCTTGTAAG-3', reverse primer: 5'-GATGAAAGGATGCTTACT-3'. 4E-BP1 (135 bp): forward primer: 5'-ACCGAAATCTCTGATGGAG-3', reverse primer: 5'-CCTGCTATATCTGAGTAA-3', forward primer: 5'-ACCCTTTTCTCTGCAGTAA-3', reverse primer: 5'-AGTCCACACTGACCTTGTTG-3'. PCR conditions were as follows: 95°C for 3 min, 95°C for 30 s, 54°C (mTOR), 49°C (p70S6K) and 56°C (4E-BP1) for 30 s, a total of 50 cycles, 72°C for 1 min and extension at 72°C for 5 min finally. The amplification products were subjected to agarose gel electrophoresis, observed and photographed under an ultraviolet lamp.

Western blotting

Ovarian cancer OVCAR-3 cells treated with carboplatin at different concentrations (0, 20 and 50 μM) were collected, and lysed in cold lysis buffer for 20 min. After centrifugation at 12,000 rpm for 5 min, the supernatant was collected as the total cell protein extract. The protein concentration was determined using the Bradford method. The protein extract received 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to obtain mTOR, and 10% SDS-PAGE to obtain p70S6K. Phosphorylated (p)-p70S6K, Akt1/2 and β-actin were separated from the total cell protein extract. The protein was transferred onto a nitrocellulose membrane using a semi-dry transfer. The membrane was washed 3 times at 5-min intervals, and sealed using 5% skim milk-phosphate buffered saline with Tween-20 (PBS) at room temperature for 2 hrs. After that, anti-mTOR, p70S6K, p-p70S6K, 4E-BP1, p-4E-BP1, AKT1/2 and β-actin antibodies diluted by 1% skim milk-PBST were added, respectively, and appropriate horseradish peroxidase (HRP)-labeled secondary antibody was added for incubation. Finally, the band of specific protein on nitrocellulose membrane was detected using diaminobenzidine (DAB) solution according to the manufacturer’s instructions. After the above incubation, the membrane was washed with PBS 5 times.

Experimental methods

Treatment of ovarian cancer cells with carboplatin

Ovarian cancer OVCAR-3 cells and Caov-3 cells digested by trypsin were incubated for 24 hrs, and continued to be incubated for 6 hrs after deprivation of serum. After that, serum-starved cells were treated with different concentrations of carboplatin in the medium containing 10% FBS.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from ovarian cancer cell lines using TRIZol reagent and reversely transcribed into complementary DNA (cDNA) using the AMV First Strand DNA synthesis kit. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. mTOR, p70S6K and 4E-BP1 were analyzed via PCR, and oligonucleotide primers used were as follows: GAPDH (570 bp): forward primer: 5'-GACAAGGACTGCTTACT-3', reverse primer: 5'-ATGTCAAAACACCTCCACC-3'. 4E-BP1 (153 bp): forward primer: 5'-ACCGAAATCTCTGATGGAG-3', reverse primer: 5'-CCTGCTATATCTGAGTAA-3', forward primer: 5'-AGTCCACACTGACCTTGTTG-3'. PCR conditions were as follows: 95°C for 3 min, 95°C for 30 s, 54°C (mTOR), 49°C (p70S6K) and 56°C (4E-BP1) for 30 s, a total of 50 cycles, 72°C for 1 min and extension at 72°C for 5 min finally. The amplification products were subjected to agarose gel electrophoresis, observed and photographed under an ultraviolet lamp.
Influence of carboplatin on ovarian cancer cells

Cell cycle analysis

The effects of carboplatin on mTOR and cycle progression of ovarian cancer OVCAR-3 cells were investigated via propidium iodide (PI) staining, respectively. DNA content was determined using the Becton Dickinson FACScan flow cytometer. Cells treated with different concentrations of carboplatin were fixed in 70% cold ethanol and then kept at 4°C until staining. Fixed cells were incubated with RNase (50 μg/mL) at 37°C for 30 min, and then 5 μL PI (50 μg/mL) was added into the cell suspension, followed by incubation in the dark at room temperature for another 30 min and analysis.

Determination of apoptosis

Ovarian cancer OVCAR-3 cells digested using trypsin and treated with carboplatin were washed with cold PBS and resuspended. Annexin V-FITC at a final concentration of 1 μg/mL and 250 ng PI were added into the mixture containing 100 μL cell suspension and binding buffer. After cells were vortexed and incubated in the dark at room temperature for 15 min, 400 μL binding buffer was added into the mixture for flow cytometry using the Becton Dickinson FACScan flow cytometer.

Determination of cell proliferation

Water-soluble tetrazolium-8 (WST-8) can be reduced to soluble formazan by dehydrogenase in mitochondria, and it is superior to methyl thiazolyl tetrazolium (MTT) in analyzing cell proliferation and is little toxic to cells. Cell proliferation was determined using WST-8 dye according to the manufacturer’s instructions. Briefly, cells were inoculated into a 96-well plate (5×10³ well), incubated at 37°C for 24 hrs, and starved using serum for 6 hrs. After that, cells were treated with 10% FBS and increasing concentrations of carboplatin for 24 or 48 hrs. After 10 μL WST-8 dye was added into each well, cells were incubated at 37°C for 2 hrs, and finally the optical density (OD) was read at 450 nm.

Statistics

One-way analysis of variance (ANOVA) was performed for data analysis using SPSS 17.0 software, and semi-quantitative values of three independently repeated Western blotting assays were statistically analyzed via densitometry using TotalLab 2.0 software. Data were presented as mean±standard deviation and p<0.05 suggested that the difference was significant.

Results

mTOR and its downstream targets in ovarian cancer cell lines

The mRNA and protein expressions were detected via Western blotting and RT-PCR to study

![Figure 1. Protein expressions of mTOR and p-p70S6K in different cell lines. A: Protein expressions of mTOR, p70S6K, p-p70S6K and β-actin (control) in both ovarian cancer cell lines were analyzed via Western blotting. B: Semi-quantitative values of three independently repeated Western blotting assays were statistically analyzed via densitometry using TotalLab 2.0 software, and data are presented as mean±standard deviation. N=3, p<0.05 vs OVCAR-3 cells.](image)

![Figure 2. mRNA levels of mTOR and its downstream effectors in ovarian cancer OVCAR-3 cells. The mRNA levels of mTOR and its two downstream effectors (p70S6K and 4EBP1) in cells treated with different concentrations of carboplatin were analyzed via RT-PCR. Semi-quantitative detection was performed for mRNA levels of mTOR, p70S6K and 4EBP1, respectively. Semi-quantitative values of three independently repeated assays were statistically analyzed via densitometry using BandScan 5.0 software, and data are presented as mean±standard deviation. P<0.05 vs ovarian cancer OVCAR-3 cells without carboplatin treatment.](image)
whether the mTOR/p70S6K signaling pathway was activated in both ovarian cancer cell lines. Results of Western blotting showed high protein expressions of mTOR and p-p70S6K, and the p70S6K protein expressions were weaker in both ovarian cancer cell lines. There were statistically significant differences in the protein expression levels of mTOR and p70S6K between the two cell lines. In other words, mTOR (p<0.05) and p70S6K (p<0.01) protein levels in OVCAR-3 cells were higher than those in Caov-3 cells. In addition, results of RT-PCR showed that the mTOR signaling pathway was activated in both ovarian cancer cell lines. The mRNA level of mTOR in Caov-3 cells was higher, but that of p70S6K was lower (Figure 1).

Changes in mRNA and protein expressions in carboplatin-treated ovarian cancer OVCAR-3 cells

After cells were treated with different concentrations of carboplatin, the mRNA and protein expressions of mTOR, p70S6K and 4E-BP1 were detected via RT-PCR and Western blotting, respectively. Carboplatin significantly reduced the mRNA expression of mTOR (p<0.01), whereas the mRNA expressions of p70S6K and 4E-BP1 in carboplatin-treated cells were increased in a dose-dependent manner (p<0.01) (Figure 2). These results indicated that carboplatin effectively inhibited the mTOR mRNA level and increased the non-phosphorylation levels of two downstream effectors (p70S6K and 4E-BP1).

Results of Western blotting showed that carboplatin inhibited the mTOR protein expression in a dose-dependent manner (p<0.01) (Figure 3). Twenty μM carboplatin significantly blocked phosphorylation after action for 6 hrs, promoting non-phosphorylation of p70S6K (p<0.01). Similarly, the effect of carboplatin on phosphorylation status of 4E-BP1 was detected using antibodies of 4E-BP1 and p-4E-BP1. As shown in Figure 3, the phosphorylation level of 4E-BP1 was lower, indicating that carboplatin inhibited phosphorylation of 4E-BP1 in OVCAR-3 cells (p<0.01). However, carboplatin had no effect on the upstream factor of mTOR, Akt (p>0.05).

Cell cycle analysis

After treatment with carboplatin, the proportion of cells in G0/G1 phase was significantly higher than that in untreated cells (p<0.01). After treatment with 20 and 50 μM carboplatin for 24 hrs, the proportions of cells in G0/G1 phase were increased from 63.23% to 72.81% (p<0.05) and 78.38% (p<0.05), respectively, and the difference...
was statistically significant between low and high concentrations of carboplatin (p<0.01), indicating that carboplatin can effectively block the ovarian cancer OVCAR-3 cells in G0/G1 phase. On the other hand, the proportion of cells in S phase was decreased compared with that in untreated cells (p<0.01).

To detect whether small-interfering RNA (siRNA) in mTOR influenced the cycle of ovarian cancer cells, ovarian cancer OVCAR-3 cells were transfected for 48 hrs, and non-transfected cells and cells transfected with control siRNA were used as controls. To determine whether siRNA in mTOR and carboplatin had a synergistic effect, cells transfected with mTOR siRNA for 24 hrs were exposed to 20 μM carboplatin for 24 hrs. Analysis of the results of flow cytometry showed that mTOR siRNA inhibited the cell cycle progression, resulting in a significant increase in the proportion of cells in G0/G1 phase from 63.90% to 75.45% (p<0.01). Control siRNA had no effect on the cycle of ovarian cancer OVCAR-5 cells. In addition, the proportion of cells in G0/G1 phase in cells treated with mTOR and carboplatin siRNA (79.05%) was higher than that in cells treated with mTOR siRNA only (75.45%), and the difference was statistically significant (p<0.05) (Figure 4), suggesting that mTOR siRNA and carboplatin have a synergistic effect on the cell cycle.

**Determination of apoptosis**

To detect whether carboplatin could affect ovarian cancer cell apoptosis, ovarian cancer OVCAR-3 cells were treated with 20 and 50 μM carboplatin for 4 hrs, respectively, and then apoptosis was assessed and analyzed. Cells treated with apoptotic agent and untreated cells were used as positive and negative controls, respectively. The results revealed that carboplatin significantly induced apoptosis of ovarian cancer OVCAR-3 cells in a dose-dependent manner. After action of 20 and 50 μM carboplatin for 4 hrs, the proportion of Annexin V/PI-positive cells (II) was increased (20 μM, 19.11%, 50 μM, 43.54%) (p<0.01) compared with that in untreated cells (negative control: 4.62%; Figure 5).


Effect of carboplatin on ovarian cancer cell proliferation

To determine the effect of carboplatin on ovarian cancer cell proliferation, ovarian cancer OVCAR-3 cells were treated with different concentrations of carboplatin (20, 50, 100, 150 and 200 μM) for 24 and 48 hrs, respectively. As shown in Figure 6, the proliferation of ovarian cancer OVCAR-3 cells exposed to carboplatin was reduced compared with that of untreated cells (p<0.01), and the inhibitory effect of carboplatin on the proliferation of ovarian cancer OVCAR-3 cells was time- and dose-dependent.

Discussion

Abnormal activation of mTOR has been found in a variety of cancers, including human ovarian cancer, breast cancer, hepatocellular carcinoma, and squamous cell carcinoma of the head and neck. However, there have been no studies on the role of mTOR in ovarian cancer so far. In this study it was found that mTOR in poorly-differentiated ovarian cancer OVCAR-3 cells was overexpressed, and the mTOR expression level was higher than that in highly-differentiated Caov-3 cells (p<0.05). On the other hand, high expressions of p-p70S6K and p-4E-BP1 were detected in both cell lines. Therefore, the mTOR/p70S6K signaling pathway was activated in both ovarian cancer cell lines.

In mammals, mTOR is regulated by the kinase cascade consisting of pI3K, pI3K-dependent kinase 1 and Akt [23]. mTOR can promote the phosphorylation of downstream effectors p70S6K and 4E-BP1, thereby stimulating the translation of proteins required for the cell cycle from G1 to S phase [24]. Previous reports have confirmed that carboplatin can inhibit mTOR and phosphorylation of p70S6K and 4E-BP1 in a variety of cancers [25] except ovarian cancer. The results of this study showed that 20 μM carboplatin significantly inhibited the phosphorylation of p70S6K and 4E-BP1. Carboplatin significantly blocked ovarian cancer cells in G0/G1 phase, leading to a significant increase in the proportion of cells in G0/G1 phase. Moreover, 50 μM carboplatin significantly increased the proportion of Annexin V/PI-positive ovarian cancer cells, so that the apoptosis rate of carboplatin-treated cells was 10-fold higher than that of untreated cells. However, the expression of Akt was not affected by carboplatin, suggesting that Akt is an upstream factor of mTOR, and carboplatin is a specific inhibitor of mTOR.

mTOR is a kind of central signal integrator that receives signals from growth factors, nutrients, etc. and is also a central controller for eukaryotic cell growth and proliferation [26]. It has been proved that activated mTOR has functions of promoting not only tumor growth, but also tumor invasion and metastasis [27].

In this study, it was found that the mTOR/p70S6K signaling pathway was activated in ovarian cancer, which may be an important mechanism of ovarian cancer cell survival and proliferation. Further studies are ongoing to investigate the potential application of mTOR/p70S6K pathway as a specific therapeutic target for ovarian cancer and the sensitivity of ovarian cancer cells in vivo to mTOR inhibitors.

Authors’ contributions

HZ wrote the manuscript and helped with cell culture. HZ contributed to treatment of ovarian cancer cells with carboplatin. HL and ZX performed PCR. ZD and EZ performed western blot. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the ethics committee of Chinese PLA General Hospital.

Conflict of interests

The authors declare no conflict of interests.

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