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In vitro study on blocking mTOR signaling pathway in EGFR-TKI resistance NSCLC

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ABSTRACT

Objective: To investigate the effect and mechanism of inhibitor everolimus on EGFR-TKI resistance NSCLC. **Methods:** MTT assay was used to detect proliferation of human non-small cell lung cancer cell line A549. Flow cytometry was used to detect the changes of apoptosis and cycle distribution in each group after 24 h and 48 h. RT-PCR was used to detect the changes of PTEN and 4EBP1 expression levels after 48 h of monotherapy and combination therapy. **Results:** MTT assay showed that everolimus had dose-dependent inhibition against growth of A549 cells. Flow cytometry showed when everolimus could induce apoptosis and induce G0/G1 phase cell cycle arrest, which was time-dependent ($P<0.05$). RT-PCR showed everolimus could increase PTEN and 4EBP1 expression. **Conclusions:** mTOR inhibitor everolimus has an inhibitory effect on EGFR-TKI resistant NSCLC, which cannot reverse the resistance effect of EGFR-TKI resistant cell line A549. The relationship between EGFR/AKT signaling pathway and the mTOR signaling pathway and the mechanism in non-small cell lung cancer need further study.

1. Introduction

Lung cancer is one of the world's most common cancer which is harmful to human health. The vast majority of lung cancer is non-small cell lung cancer (NSCLC)[1,2]. Although there have been great development of diagnosis and therapy, the prognosis of lung cancer is not very well and has a high mortality. Epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) has been applied to the treatment of NSCLC and improved the survival rate of some patients. However, it has limitation in its scope for patients with EGFR-TKI resistance, so we need to explore new treatments for lung cancer. It is discovered in recent years that target of rapamycin (mTOR) signaling pathway activation is closely related to tumor resistance mechanisms. Everolimus as mTOR inhibitor has attracted more and more attention[3-5]. This study aimed to investigate the effect of mTOR inhibitor on the proliferation and apoptosis of EGFR-TKI resistance NSCLC and provide experimental data for clinical application.

2. Materials and methods

2.1. Tumor Cell Lines

Human non-small cell lung cancer cell line A549 were purchased from cell bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

2.2. Reagents and instruments

RPMI 1640 medium was purchased from Sigma, tetramethylrhodamine tetrazolium salt (MTT), formazan were

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purchased from Gibco, fetal bovine serum, trypsin digestion enzymes were purchased from Sigma. PTEN and 4EBP1 are rabbit anti-human polyclonal antibody, propidium chloride was purchased from Beijing Solarbio company, Everolimus was a gift from Novartis; It was dissolved in DMSO at 10 μmol/L,-20 ℃, 5% CO_2, 37 ℃. Saturated humidity ordinary cell incubator which was purchased from Shanghai Huaxian Medical Nuclear Instrument Co., Ltd. PCR amplification was purchased from Bio-Rad company. Flow cytometry was purchased from Beckman Coulter Company. Electric oven thermostat blast DHG-9246A type purchased from Beijing Liuyi Instrument Factory, centrifuge was purchased from the Eppendorf company, German.

2.3. Cell growth inhibition assay

Human non-small cell lung cancer cell line A549 was placed in 5% CO_2, 37 ℃. After cell attachment, culture medium was changed and culture was continued. RPMI1640 medium containing 10% fetal calf serum was used to stop the digestion, and the original culture medium was discarded when the A549 cells adherent reached to 80%. It was washed 2 times with PBS then was added with 0.25% trypsin solution, the passage was cultured according to a certain proportion. The experiment was carried out when cells were in the logarithmic growth phase. A549 cells suspension in the logarithmic growth phase was adjusted to 6×10^4 cells/mL, and was cultured with 200 μL cell suspension per well in 96-well plates to set up a normal cell control group and 5 drug experimental group.

In control group MTT dissolve solution was added, while in drug groups different concentrations of everolimus 200 μL was added (A: 0.1 nmol/L, B: 1 nmol/L, C: 10 nmol/L, D: 100 nmol/L, E: 1 000 nmol/L).After culturing for 48 h, 10 μL 5 mg/ml MTT solution was added to each well, and reincubated for 4 h. The supernatant were abandoned after blue-purple formazan was formed. It was washed in PBS for 2 times and was added with 150 μL DMSO solution to dissolve formazan. It was divided into two parts after 4 h. A: the absorbance (A) value was measured in automatic microplate reader detector at 570 nm wavelength to calculate the killing rate. The killing rate (%) = [1 - (A effect target cell well -A effect cell well) / A target cell well] × 100%.B: 0.3-0.5 mL propidium chloride dyeing solution was added. After flow cytometry detection,the rate of apoptosis was calculated with. Each independent experiment was repeated three times.

2.4. RT-PCR detection

A549 cells were collected by TRIzol (Invitrogen Company) extraction of total RNA in tissues. cDNA was synthesized according to instructions. The cDNA was used as a template for PCR amplification, and GADPH was used as an internal reference. PTEN reaction conditions: after 95 ℃ 5 min, 95 ℃ 30 s → 62 ℃ 30 s → 72 ℃ 30 s, amplified for 35 cycles; 72 ℃ 7 min, size of the products: 232 bp. PTEN reaction conditions: after 95 ℃ 5 min, 95 ℃ 30 s → 62 ℃ 30 s → 72 ℃ 30 s, amplified for 35 cycles; 72 ℃ 7 min, size of the products: 232 bp.4EBP1 reaction conditions: after 95 ℃ 5 min, 95 ℃ 30 s → 53 ℃ 30 s → 72 ℃ 30 s, amplified for 35 cycles; 72 ℃ 7 min, size of the products: 167 bp. 1.5% agarose gel electrophoresis and EB staining were carried out. GADPH was used as an internal reference to calculate the relative content of gene PTEN and 4EBP1 mRNA. Results were expressed by percentage ratio of the central point density PTEN, 4EBP1 in GADPH bands.

2.5. Statistical analysis

All data was analyzed with SPSS 17.0 software. Data were expressed as mean±SD values. Mean comparison were compared with t test. $P<0.05$ was regarded as statistical significance.

3. Results

3.1. Impact of everolimus on proliferation of A549 cells

After treatment with different concentrations of everolimus, 24 h inhibition rates of A549 cells were (7.8±2.3)%, (15.2±2.5)%, (21.2±3.4)%, (42.1±4.8)%, (67.0±6.4)%, 48 h inhibition rates were (15.6±2.6)%, (29.8±2.9)%, (34.3±4.3)%, (56.8±6.5)%, (80.7±7.8)%. There were statistically significant difference in the inhibition rate between different drug concentrations and different action time ($P<0.05$).

3.2. Impact of everolimus on A549 apoptosis

After treatment with different concentrations of everolimus, 24 h apoptosis rate of A549 cells were (1.1±0.2)%, (1.7±0.2)%, (2.2±0.2)%, (3.4±0.3)%, (5.6±0.3)%, 48 h apoptosis rates were (2.1±0.2)%, (3.4±0.3)%, (6.3±0.4)%, (10.7±0.5)%, (16.5±0.7)%. There were statistically significant difference in the inhibition rate between different drug concentrations and different action time ($P<0.05$).

3.3. Impact of everolimus on A549 cells PTEN, 4EBP1 mRNA expression levels

PTEN and 4EBP1 expression in the experimental group were

significantly higher than control group, and the expression was increased with everolimus drug concentration (Table 1, Figure 1).

Table 1
PTEN, 4EBP1 mRNA expression levels in each group.

	PTEN	4EBP1
Control group	0.159±0.171	0.089±0.034
Experimental group A	0.221±0.223*	0.113±0.044*
Experimental group B	0.334±0.242*△	0.134±0.051*
Experimental C	0.429±0.279*△○	0.167±0.062*△
Experimental D	0.487±0.310*△○	0.198±0.058*△○
Experimental E	0.525±0.329*△○◇	0.211±0.059*△○

Note: * Compared with the control group, $P<0.05$, △ Compared with the experimental group A, $P<0.05$, ○ compared with the experimental group B, $P<0.05$, ◇ compared with experimental group C, $P<0.05$.

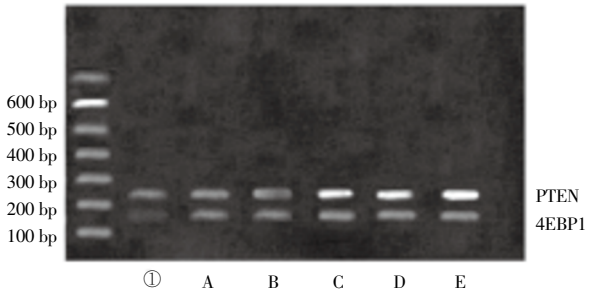

Figure 1. PTEN, 4EBP1 mRNA expression levels in each group.

4. Discussion

The specific mechanism of non-small cell lung cancer resistance for EGFR-TKI is very complex. Currently a viable solution is to blocking tumor cell proliferation signal transduction pathway. mTOR is a key regulator of cyclin synthesis, which has two signaling pathways, one is PI3K/AKT/mTOR pathway, another is AKT/TSC1-TSC2/mTOR pathway. Both promote cell proliferation by accelerating the G1-S cell cycle conversion; several studies suggest that mTOR signaling pathway plays an important role in tumor formation. Everolimus as a new mTOR inhibitor, and the mechanism of anti-tumor action is still unclear. Studies suggest that everolimus combined with high-dose cyclophosphamide can inhibit angiogenesis. It provides a new treatment for EGFR-TKI resistance by everolimus blocking mTOR signaling pathway[6-10].

In this study, A549 cell line is EGFR wild-type. Early studies suggest that EGFR-TKI is ineffective on wild-type EGFR NSCLC using A549 cells meet the requirements of the study. In this study, the experiment was performed in vitro. After the EGFR-TKI primary resistant cell lines exposure to everolimus, the result showed that everolimus can significantly inhibit A549 cells, which shows time and dose-dependent and easy to induce apoptosis, the results is similar to relevant research. Research shows everolimus is a safe and effective inhibitor of tumor growth, but some studies suggest that everolimus did not affect cell cycle, but directly induce apoptosis. PTEN is considered to be an important tumor suppressor gene which is the first tumor suppressor gene with phosphatase activity currently. It is widely mutant in malignant tumors. It can induce p21, p27, p57 expression and restrict cell division by inhibiting the activation of AKT protein overexpression and inhibit tumor cell adhesion by blocking integrin signaling pathway, it can also reduce VEGF and other vascular inflammatory cytokines.

4EBP1 is the endogenous cap-dependent translation inhibitor[11,12]. This study showed that PTEN, 4EBP1 expressions increased with everolimus drug concentration increase. It's mechanism is everolimus can block mTOR signaling pathway, prompt PTEN overactive and inhibit the pathway related genes upstream and downstream, inhibit chemokine receptor expression[13-16]. 4EBP1 is the negative regulator gene of mTOR signaling pathway downstream. Studies suggest that 4EBP1 can reverse elF-4E[17-20], induced inactivation of mTOR signaling pathway, resulting in the effect of depressing carcinoma[21,22]. Therefore, mTOR inhibitors have been applied in varying degrees of treatment for various malignancies in recent years,which has its unique advantages as a multi-targeted anticancer drugs.

mTOR inhibitors can inhibit proliferation and promote apoptosis for EGFR-TKI resistance non-small cell lung cancer cells, but the safety and clinical efficacy of mTOR inhibitors is still uncertain. It is believe that with continued research of mTOR inhibitors, it can provide new strategies and methods for the clinical treatment of lung cancer[23-25].

In summary, everolimus inhibits the growth of A549 cells, which is related with its cycle arrest and induction of apoptosis; mTOR signaling pathway plays an important role in tumor formation. On this basis, further study of the mTOR signaling pathway and its upstream and downstream genes to understand the pathogenesis of cancer. It can prove the pathogenesis and targeted therapy for lung cancer through further study of everolimus effect and action mechanism.

Conflict of interest statement

We declare that we have no conflict of interest.

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