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Original Research

Effect and mechanism of rapamycin on proliferation and apoptosis of human lung cancer cells

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Abstract: The purpose of this study was to investigate the effects of RAPA on the proliferation and the expression of p53, Bcl-2 and Bax proteins in cultured human small cell lung cancer (NCI-H446) cells, and to explore the possible mechanism of RAPA-treated NCI-H446 cells with different concentrations of RAPA-treated NCI-H446 cells. The proliferation of NCI-H446 cells in all groups was assayed by the CCK-8 method. FITC-Annexin V/PI double staining method was used to determine the apoptosis of NCI-H446 cells. The immunohistochemical SP method was used to detect the expression of p53, Bcl-2 and Bax. Expression of p53, Bcl-2 and Bax mRNA was detected by RT-PCR. The results showed that, after 48h treatment, the proliferation of NCI-H446 cells treated with 5ng/mL, 10ng/mL and 15ng/mL RAPA decreased significantly ($P < 0.05$) and the proliferation inhibition rate increased significantly ($P < 0.05$) compared with the control group, and the proliferation inhibition rate had a dose-dependent relationship with RAPA. Compared with the control group, the apoptosis rate of NCI-H446 cells treated with 5ng/mL, 10ng/mL and 15ng/mL RAPA increased significantly ($P < 0.05$), and there was a dose-dependent relationship between the apoptosis rate and RAPA. The expression of Bcl-2 protein and mRNA was higher in the control group, while the expression of p53 and Bax protein and mRNA was lower. The expression of Bcl-2 protein and mRNA decreased and the expression of p53 and Bax protein and mRNA increased gradually with the increase of concentration and the prolongation of action time in 5ng/mL, 10ng/mL and 15ng/mL RAPA groups. In the control group, the intracellular Ca^{2+} concentration was constant, and there was no significant change with time; while in the 5ng / mL, 10ng / mL, and 15ng / mL RAPA group, the intracellular Ca^{2+} concentration in the RAPA group increased significantly after 12 h of administration ($P <0.05$); After that, with the prolonged action time of the medicine, the intracellular Ca^{2+} concentration in the 5ng / mL, 10ng / mL, and 15ng / mL RAPA group decreased, but at 72h, the effect was 5ng / mL, 10ng / mL, and 15ng / mL RAPA. The intracellular Ca^{2+} fluorescence intensity in the group was still significantly higher than that in the control group ($P <0.05$). In conclusion, RAPA can induce apoptosis of NCI-H446 cells by down-regulating Bcl-2 gene expression, up-regulating P53 and Bax gene expression, and increasing intracellular Ca^{2+} concentration and its apoptosis induction effect have timeliness and dose-effect.

Key words: Lung cancer; Rapamycin; Cell proliferation; Apoptosis; Bcl-2; p53; Bax.

Introduction

Lung cancer is a disease characterized by the uncontrolled growth of cells in lung tissue. If left untreated, cell growth can spread beyond the lungs to a process called metastasis to surround tissues or other organs. Most lung cancers, called primary lung cancers, are carcinomas that originate in the lining tissue. The main types of lung cancer are small-cell lung cancer (SCLC), also known as atmospheric cellular cancer, and non-small cell lung cancer (NSCLC). The most common symptoms are cough (along with bloody sputum), weight loss, and shortness of breath. The most common cause of lung cancer (90%) is exposure to secondhand smoke for a long time (1-2).

The percentage of lung cancers in non-smokers is 15% and is often due to a combination of genetic factors, radon gas, asbestos, and air pollution are related to third-party cigarette smoke. Lung cancer may be detected on chest radiography and computerized cross-section. Diagnosis can be confirmed by a biopsy, often through bronchoscopy or a guide. Long-term treatment and outcome depend on the type of cancer, the stage (severity of the spread), and the overall health of the person, which is measured by the state of function. Lung cancer is a common primary malignant tumor of the lung, with high morbidity and mortality, which poses a great threat to people's physical and mental health. The surgical treatment effect of small cell lung cancer is poor. Although it is sensitive to chemotherapy medicines, it will soon develop medicine resistance (1,2). Anti-tumor medicine therapy is one of the main methods for the clinical treatment of lung cancer. However, there are many anti-tumor medicines and their anti-tumor effects are also different. Rapamycin (RAPA) is a new immunosuppressive agent, which was found to have a good anti-rejection effect in liver transplantation earlier (3,4). In recent years, with the gradual application of RAPA in

clinical practice, it has been found that RAPA also has certain anti-tumor characteristics, which can inhibit the growth and metastasis of liver cancer (5), nasopharyngeal carcinoma (6), osteosarcoma (7) and other cells. However, there are few researches on the effect and mechanism of RAPA on small cell lung cancer.

Under normal circumstances, the content of p53 in cells is very low. Under the stimulation of DNA damage or other conditions, the content of p53 in cells will increase. When p53 mutates, it loses its anti-cancer function and turns into an oncogene that promotes cell malignant transformation (8). It is now clear that p53 is a negative regulatory factor in the cell growth cycle, which is related to important biological functions such as cell cycle regulation, DNA repair, cell differentiation, apoptosis and so on, and plays an important role in inhibiting the growth of cancer cells (9). P53 is an upstream regulatory gene of bcl-2 and bax, which can regulate cell apoptosis together with bcl-2 and Bax. Wild-type p53 can down-regulate the expression of bc1-2 and up-regulate the expression of bax gene, thus regulating cell apoptosis. Bcl-2 is a gene that specifically inhibits apoptosis. Overexpression of Bcl-2 can inhibit cell apoptosis and prolong and expand cell survival, which is one of the important mechanisms of tumor occurrence (10,11).

The purpose of this study was to investigate the effects of RAPA on the proliferation and the expression of p53, Bcl-2 and Bax proteins in cultured human small cell lung cancer (NCI-H446) cells, and to explore the possible mechanism of RAPA-treated NCI-H446 cells with different concentrations of RAPA-treated NCI-H446 cells.

Materials and Methods

Cell culture

Small cell lung cancer (NCI-H446) cells were cultured in RPMI1640 medium containing 10% FBS fetal bovine serum in a cell incubator at 37 ℃ and 5% CO2.

Cell grouping, medicine treatment

NCI-H446 cells were cultured in RPMI 1640 medium containing 10% calf serum, 100U/ml penicillin and 100U/ml streptomycin respectively. The cells were cultured at 37 ℃, saturated with 5%CO_2 and digested with 0.25% trypsin. RAPA was dissolved with DMSO and then diluted with RPMI-1640 culture medium. The final working concentration of RAPA in each treatment group was 5ng/mL, 10 ng/mL and 15 ng/mL respectively, and a control group (0 ng/ml RAPA) was set up.

The proliferation of NCI-H446 cell was assayed by CCK-8 method

NCI-H446 cells in the logarithmic growth phase were selected, digested into single-cell suspension with pancreatin solution, and inoculated on 96-well plate. The control group (RAPA, 0 ng/ml) and RAPA group were set up five multiple pores respectively, and 200μL of NCI-H446 cell suspension was dripped into each well for overnight. The control group was given 200μL of fresh medium, and the RAPA group was given 200μL of medium containing different concentrations (5ng/mL, 10 ng/mL, 15 ng/mL) of RAPA respectively, and cultured in an incubator under 37 ℃ and 5% carbon dioxide. After 48 hours of incubation, 20μL CCK-8 solution was dripped into each well in a dark environment and then incubated for another 3 hours. The absorbance of each well (wavelength 450 nm) was measured by an enzyme-labeled instrument. The proliferation inhibition rate of NCI-H446 cells in each group was calculated. Cell proliferation inhibition rate (%) = (absorbance value of control group-absorbance value of RAPA group)/ absorbance value of control group × 100%.

FITC-Annexin V/PI double staining method was used to determine the apoptosis of NCI-H446 cells

Adjust the cell concentration to 5.0×10^5/ml. After 24 hours of culture, each group was added with different concentrations of medicine-containing culture solution, and 6 multiple pores were set up respectively. After 24, 48 and 72 hours of culture, cells were collected. NCI-H446 cells were digested with trypsin without EDTA. NCI-H446 cells were washed twice with PBS solution and centrifuged for 5 min. NCI-H446 cells were collected and mixed with FITC-Annexin V/PI kit. The cells were incubated again for 20 min in light and room temperature. The apoptosis rate of NCI-H446 cells in each group was determined by flow cytometry.

Immunohistochemical SP method was used to detect the expression of p53, Bcl-2 and Bax

The cell concentration was adjusted to 1.0×10^5/ml, and the cells were seeded on a slide-containing culture plate. When the cells adhere well to the growth, add different concentrations of medicine-containing culture solution, and set up three duplicate wells each. After 24, 48, and 72 hours of incubation, the slides were removed and fixed with 4% paraformaldehyde. Immunohistochemical detection was performed in strict accordance with the SP kit instructions. The known positive was used as a positive control for the photo, and PBS was used as a negative control instead of the primary antibody. The positive staining of p53, Bcl-2 and Bax proteins showed yellow or brownish-yellow particles in the cytoplasm. Four visual fields were selected for the positive rate determination in each case.

Expression of p53, Bcl-2 and Bax mRNA was detected by RT-PCR

Adjust the cell concentration to 1.0×10^6/ml, inoculate it into the culture plate, add different concentrations of medicine-containing culture solution after the cells adhere to the wall, and set up 3 multiple pores in each group. After 24, 48 and 72 hours of culture, the cells were collected and semi-quantitative analysis of Bcl-2 and Bax mRNA expression was carried out according to the method of literature (13,14). P53 forward primer:5′-GTACCGTATGAGCCACCTGAG-3', p53 reverse primer: 5′-CGTCCCAGAAGATTCCCAC-3′; Bcl-2 upstream primer: 5'-GTGGTCCACCTGACCCTC-CG3', downstream primer: 5'-CATCCCAGCCTCCGTTATCC-3'; Bax upstream primer D'-ATGGTCACG-GTCCAACCACC-3', downstream primer: 5'-ATGGTCACG-GTCCAACCACC-3'; β-actin upstream primer: 5'-ATGGARTCCTGTGGCATCCA-3', downstream primer: 5'-CGCTCAGGAGGAGCAATGAT-3'

Changes of intracellular Ca^{2+}concentration were measured by laser confocal microscopy

The cells were inoculated into the culture plate with 1.0×10^4/ml concentration. After adhering to the culture plate, different concentrations of RAPA (0 ng/ml, 5ng/mL, 10 ng/mL, 15 ng/mL) were added in the culture solution, and cultured for 12, 24, 48, 72 hours. Refer to reference (12) to detect the change of intracellular Ca^{2+} concentration.

Statistical treatment

The data were analyzed by SPSS 25 software. The measurement data were expressed by means of mean ± standard deviation, the analysis of variance was used for the comparison between groups, and the LSD t-test was used for the comparison between the two groups. $P < 0.05$ was statistically significant.

Results

Comparison of the proliferation of NCI-H446 cells

After 48h treatment, the proliferation of NCI-H446 cells treated with 5ng/mL, 10ng/mL and 15ng/mL RAPA decreased significantly ($P < 0.05$) and the proliferation inhibition rate increased significantly ($P < 0.05$) compared with the control group, and the proliferation inhibition rate had a dose-dependent relationship with RAPA, as shown in Figure 1.

Comparison of apoptosis in NCI-H446 cells

Compared with the control group, the apoptosis rate of NCI-H446 cells treated with 5ng/mL, 10ng/mL and 15ng/mL RAPA increased significantly ($P < 0.05$), and there was a dose-dependent relationship between the apoptosis rate and RAPA, as shown in Table 1.

Effect of RAPA on Expression of p53, Bcl-2 and Bax Proteins in Cells

The expression of Bcl-2 protein was higher in the control group, while the expression of p53 and Bax protein was lower. The expression of Bcl-2 decreased and the expression of p53 and Bax increased gradually with the increase of concentration and the prolongation of action time in 5ng/mL, 10ng/mL and 15ng/mL RAPA groups. See Figures 2, 3 and 4.

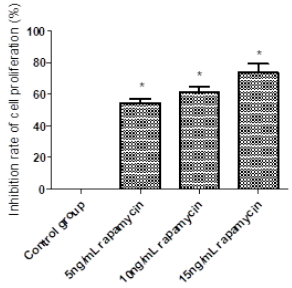

Figure 1. Comparison of the proliferation of NCI-H446 cells in each group (n=5). Compared with the control group, *P<0.05.

Effect of RAPA on the expression of p53, Bcl-2 and Bax mRNA

In the control group, the expression of Bcl-2 was higher, but the expression of Bax mRNA was lower; In 5 ng/mL, 10 ng/mL and 15 ng/mL RAPA groups, the expression of Bcl-2 mRNA decreased, while the expression of p53 and Bax mRNA increased gradually with the increase of concentration and the prolongation of action time. See Figures 5, 6 and 7.

Changes of intracellular Ca^{2+} concentration after RAPA treatment

In the control group, the intracellular Ca^{2+} concentration was constant, and there was no significant change

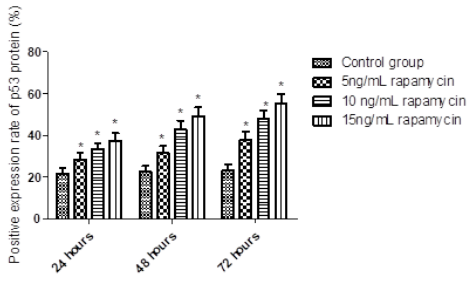

Figure 2. Effect of RAPA on p53 protein expression (n=3). (compared with the control group, *P< 0.05).

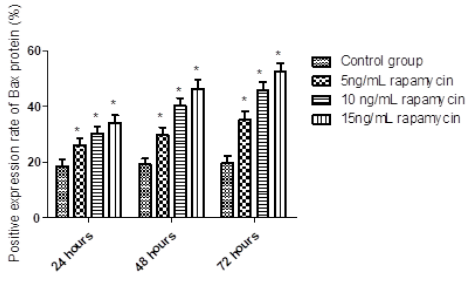

Figure 3. Effect of RAPA on Bax protein expression in cells (n=3). (Compared with the control group, *P<0.05).

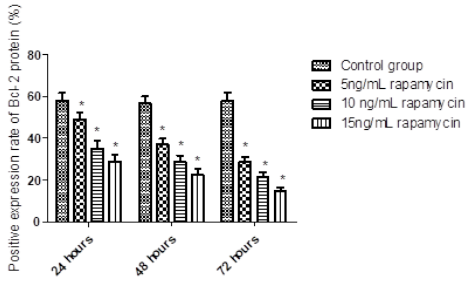

Figure 4. The effect of RAPA on the expression of Bcl-2 protein (n=3). (Compared with the control group, *P<0.05).

Table 1. Comparison of apoptosis in NCI-H446 cells (%, $\bar{x} \pm s$).

Group	n	24h	48h	72h
Control	6	1.91±0.75	2.23±0.64	2.61±0.83
5 ng/mL rapamycin	6	6.74±2.11*	14.58±3.26*	27.22±4.16*
10 ng/mL rapamycin	6	11.75±1.54*	26.84±2.03*	40.17±3.52*
15 ng/mL rapamycin	6	20.06±2.77*	40.13±3.95*	50.06±3.13*

Compared with the control group, *P<0.05.

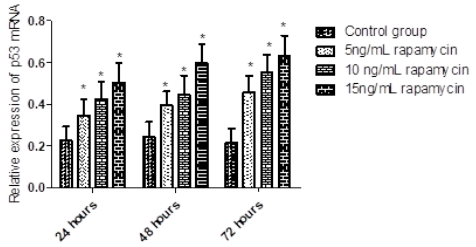

Figure 5. Effect of RAPA on p53 mRNA expression (n=3). (Compared with the control group, *P<0.05).

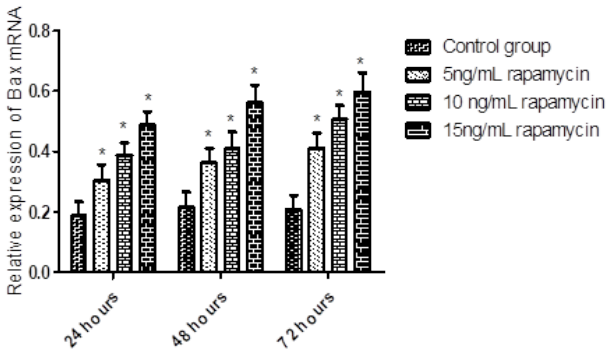

Figure 6. Effect of RAPA on Bax mRNA expression in cells (n=3). (Compared with the control group, *P<0.05).

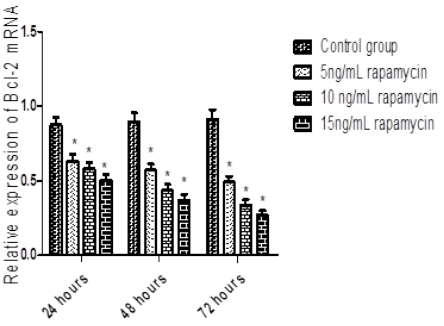

Figure 7. The effect of RAPA on the expression of Bcl-2 mRNA (n=3). (Compared with the control group, *P< 0.05).

with time; while in the 5ng / mL, 10ng / mL, and 15ng / mL RAPA group, the intracellular Ca^{2+} concentration in the RAPA group increased significantly after 12 h of administration (P <0.05); After that, with the prolonged action time of the medicine, the intracellular Ca^{2+} concentration in the 5ng / mL, 10ng / mL, and 15ng / mL RAPA group decreased, but at 72h, the effect was 5ng / mL, 10ng / mL, and 15ng / mL RAPA. The intracellular Ca^{2+} fluorescence intensity in the group was still significantly higher than that in the control group (P <0.05), as shown in Table 2.

Discussion

Cell proliferation, apoptosis and cancer cell death are closely related. Studies have found [14] that RAPA can induce apoptosis of liver cancer cells. Some studies have shown that (14,15). RAPA can effectively induce apoptosis of colon cancer cells. This study found that RAPA can inhibit cell proliferation and induce cell apoptosis after treating human small cell lung cancer (NCI-H446) cells in vitro, and its effect is dose-dependent. This conclusion is basically consistent with the previous research results that RAPA can inhibit the proliferation of lung cancer cells and induce their apoptosis (16).

Apoptosis is regulated by a variety of genes and mediated by apoptosis-related signaling systems. Therefore, whether cell apoptosis after receiving death signals is closely related to the expression of apoptosis-related genes. The proliferation and apoptosis of cancer cells are both positively regulated and negatively regulated by various genes, and their proliferation and apoptosis mechanisms are relatively complex. Tumor suppressor gene p53 belongs to a relatively important tumor suppressor gene, and its mutation is closely related to human tumors (17). The expression level of Bcl-2 is very important to the survival of cancer cells. Some studies have shown (18) that Bcl-2 transcription regulation is closely related to the proliferation and apoptosis of ovarian cancer cells. Bax is an important apoptosis-promoting gene. Previous studies have confirmed (19) that Bax can promote apoptosis of liver cancer cells. In this study, compared with the control group (RAPA, 0 ng/ml), the apoptosis rate of NCI-H446 cells treated with RAPA at 5ng/ml, 10 ng/ml and 15ng/ml increased ($p < 0.05$), and the levels of p53 and Bax proteins increased ($p < 0.05$), while the level of Bcl-2 protein decreased ($p < 0.05$). It is suggested that RAPA can inhibit the proliferation of human small cell lung cancer (NCI-H446) cells and promote their apoptosis, which may be related to the up-regulation of p53 and Bax protein expression and down-regulation of Bcl-2 protein expression by RAPA.

The induction and inhibition of apoptosis are also related to the apoptosis signal transduction pathway. Studies have shown that in most cell apoptosis processes, intracellular Ca^{2+} concentration increases (20). Ca^{2+}, as an important second messenger of intracellular signaling, can induce apoptosis through multiple pathways (21). The results of this study show that the intracellular Ca^{2+} concentration in the control group is constant and has no obvious change with time. However, the intracellular Ca^{2+} concentration in each RAPA group increased significantly 12 hours after administration and gradually increased with the increase of concentration,

Table 2. Changes of intracellular Ca^{2+} concentration after RAPA treatment.

Group	n	Intracellular Ca^{2+} concentration			
		12 hours	24 hours	48 hours	72 hours
Control	3	245.61±27.53	249.85±28.04	251.31±31.45	257.62±33.09
5 ng/mL rapamycin	3	733.42±67.37*	661.17±52.05*	645.77±49.32*	628.95±41.67*
10 ng/mL rapamycin	3	909.55±78.94*	890.63±81.48*	750.73±73.95*	717.32±69.14*
15 ng/mL rapamycin	3	1409.73±97.51*	1176.81±84.36*	985.34±91.36*	943.17±74.52*

Compared with the control group, *P< 0.05.

with dose-dependence. After that, the concentration of intracellular Ca^{2+} decreased with the prolongation of the action time, but the fluorescence intensity of intracellular Ca^{2+} in RAPA cells was still significantly higher than that in the control group after 72 hours of action. This is consistent with previous reports that Ca^{2+} increase is related to the initiation of early cell apoptosis (22). It is suggested that RAPA can induce apoptosis of lung cancer cells by increasing the concentration of Ca^{2+} in lung cancer cells, which may also be one of the molecular mechanisms of RAPA inducing apoptosis of lung cancer cells.

Examining and evaluating the expression of key genes, as well as controlling their expression, is one way to prevent unwanted traits and phenotypes. Genome editing can be effective in this regard (23-34). In the current research, the expression of p53, Bcl-2 and Bax proteins in cultured human small cell lung cancer was investigated.

In conclusion, RAPA can induce apoptosis of NCI-H446 cells by down-regulating Bcl-2 gene expression, up-regulating P53 and Bax gene expression, and increasing intracellular Ca^{2+} concentration and its apoptosis induction effect have timeliness and dose-effect. This apoptosis-inducing effect is one of the anti-lung cancer mechanisms of NCI-H446, and the regulation of apoptosis-related genes and intracellular Ca^{2+} concentration is the molecular mechanism of NCI-H446 inducing apoptosis of lung cancer cells. This study can provide a theoretical basis for the clinical application of RAPA.

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