EFFECTS OF ELEMENE ON PROLIFERATION, APOPTOSIS AND AMPK/MTOR SIGNALING PATHWAY OF COLORECTAL CANCER DLD-1 CELLS

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ABSTRACT

Objective: *To analyze the effect of elemene on the proliferation, apoptosis, and AMPK/mTOR signaling pathway of colorectal cancer DLD-1 cells.*

Methods: *Colorectal cancer DLD-1 cells were cultured in vitro and treated with β-elemene concentrations of 50, 100, 200, and 300 μmol/L. A blank control group was established to detect the proliferation ability of DLD-1 cells by the MTS method. Flow cytometry was used to determine the cell cycle stage, Hoechst 33342 staining experiment was used to analyze apoptosis, and Western blots were used to detect p-AMPK, AMPK, p-mTOR, and mTO protein expression.*

Results: *Compared with the blank control group, the β-elemene concentrations of 50, 100, 200, and 300 μmol/L were used to treat DLD cells for 24, 48, and 72 h. As the concentration and duration of action increased, the cell proliferation capacity gradually decreased. Compared with the blank control group, with the gradual increase of β-elemene concentration, the cell content of DLD-1 cells in G0/G1 and S phases decreased significantly, and the cell content in G2/M phase increased significantly. After treatment of DLD cells with 50, 100, 200, and 300 μmol/L β-elemene for 24 hours, the nucleus of the blank control group was regular and uniform, and the nuclear membrane was relatively complete. With increasing β-elemene concentrations, the nuclear cells in each group were fragmented, and the chromatin was concentrated, appearing to be bright and dense. Compared with the blank control group, β-elemene at a concentration of 50, 100, 200, and 300 μmol/L significantly increased the expression level of p-AMPK protein and reduced the expression level of p-mTOR protein; these differences were statistically significant (P<0.05).*

Conclusion: *Elemene can significantly inhibit colorectal cancer DLD-1 cell proliferation and induce apoptosis. The associated mechanism may be related to AMPK/mTOR signaling pathway regulation.*

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Introduction

Colorectal cancer is a common digestive tract malignancy. In Western countries, it ranks third in incidence rate. Its occurrence is closely related to lifestyle, heredity, and colorectal adenoma. In recent years, with the change of diet structure and living environment, its incidence rate has been increasing yearly, threatening human life[(1)]. There is no significant symptom in the early stage of colorectal cancer, and the tumor is typically metastasized by the time the patients are treated. At present, the main treatment methods are surgical resection and adjuvant chemotherapy, but the low selectivity and toxic side effects of chemotherapy drugs mean that clinical treatment has certain limitations. Therefore, the search for safe, effective anti-colorectal cancer drugs has become a key focus for clinical scholars[(2)]. In recent years, the essential role of elemene for tumors has attracted increased clinical attention.

Elemene is one of the important anti-tumor monomers discovered by Chinese scholars. It is effectively extracted from the traditional Chinese medicine Zingiberaceae plant Curcuma aromatica. Its active monomer mainly causes "stagnation of Qi and blood, abdominal pain, dyspepsia and amenorrhea of women's blood stasis". β-elemene is its main component and can inhibit tumor cell proliferation, cell invasion, and metastasis, and has the advantages of a broad anti-tumor spectrum, high efficiency, safety, and non-toxicity.

In addition, β-elemene has high lipophilicity and low molecular weight and can be distributed in a variety of organs and tissues. Due to its unique advantages, it can easily pass the blood-brain barrier and has a significant inhibitory effect on various cancer cells[3-4]. Some scholars have found that β-elemene can inhibit the proliferation of human kidney cancer cells and induce apoptosis by inhibiting MAPK/ERK and PI3K/Akt/mTOR signaling pathways[5]. Therefore, this study aimed to analyze the effect of elemene on the proliferation, apoptosis, and AMPK/mTOR signaling pathway of colorectal cancer DLD-1 cells.

Materials and methods

Experimental reagents and instruments

The colorectal cancer DLD-1 cell line was purchased from the cell bank of the Chinese Academy of Sciences; β-elemene was obtained from Dalian Jingang Pharmaceutical Co., Ltd. (purity ≥95%); DMEM medium was procured from Hyclon Corporation (US); fetal bovine serum was obtained from Hangzhou Four Seasons Qing Biological Engineering Co., Ltd.; Hoechst 33342 living cell staining solution and SDS-PAGE protein loading buffer were purchased from Biyuntian Biotechnology Research Institute; p-AMPK, AMPK, p-mTOR, and mTOR antibodies were purchased from Cell Signal Technology Corporation (US); the PVDF membrane was bought from Milipore (US); the horseradish enzyme-labeled goat anti-rabbit IgG was purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd. The ultra-clean workbench was purchased from Sujing Purification Equipment Co., Ltd.; the constant temperature incubator was obtained from Ningbo Jiangnan Instrument Factory; the high-speed refrigerated centrifuge was purchased from Suzhou Antai Biotechnology Co., Ltd.; the flow cytometer was obtained from Beckman Coulter (US); the enzyme-labeled instrument was purchased from MD Corporation (US); The electrophoresis tank and instrument were purchased from the Beijing Liuyi Instrument Factory; the vertical steam sterilizer was obtained from Shanghai Shenan Medical Instrument Factory; the gel imaging and analysis device were acquired from Shanghai Furi Technology Co., Ltd.; and the analytical balance was purchased from Sartorius (Germany).

Cell culture

The frozen human colorectal cancer DLD-1 cells were thawed in a 37 °C water bath. The cells were cultured in DMEM high-glucose medium containing 10% FBC, penicillin 20 u/ml, and streptomycin at 37 °C and 5% CO_2. The cells were digested and passaged after growing to approximately 80–90%. The cells were resuscitated and passaged to the third generation of stable growth, and then logarithmic growth cells were used for the experiment.

Detection method

The MTS method was used to detect the effect of β-elemene on the proliferation ability of DLD-1 cells: a logarithmic growth of DLD-1 cells was prepared as a cell suspension, and 5×10^3 200 μl cells per well were seeded in 96-well plates. When the cells adhered to grow to 50% to 60%, β-elemene concentrations of 50, 100, 200, and 300 μmol/L were added to each well in sequence. A blank control group was set up. Each well contained 6 duplicate wells. After 24, 48, and 72 hours of continuous culture, the cell morphology was observed, and the MTS and PMS mixtures were added to each well in turn for 1 hour. (OD). Cell proliferation capacity (%) = β-elemene group OD/blank control group OD × 100%. Flow cytometry was used to detect the effect of β-elemene on the cell cycle of DLD-1: the logarithmic DLD-1 cells were inoculated with 5 × 105 cells per well in the six-well plate. After the cells adhered to the wall, 50, 100, 200, and 300 μmol/L of β-elemene was added to each well in turn, and a blank control group was established. After 24 hours of continuous culture, digestion and counting were carried out, 1×10^6 cells were resuspended, 75% ethanol was added to disperse the cells evenly, and placed at approximately 2-8 °C overnight.

After 12 hours of centrifugation, the cells were resuspended. Twenty μl of Rnase solution was added to mix the cells. Four-hundred μl of PI staining solution was added to mix the cells and incubated in the dark for 30 minutes. Then, flow cytometry was used for detection.

Hoechst 33342 staining experiment to detect the effect of β-elemene on DLD-1 cell apoptosis: a logarithmic growth of DLD-1 cells was inoculated into 12-well plates with 1×10^5 cells per well. After the cells adhered to the wall, 50, 100, 200, and 300 μmol/L of β-elemene was added into each well in turn, and a blank control group was set up. After 24 hours, the drug solution was discarded and cleaned with PBS. Hoechst 33342 staining solution was added to each well in turn and incubated for 15 min. The staining solution was discarded and observed under a microscope after PBS cleaning. Protein expression detected by Western blot: after treating DLD-C cells with β-elemene concentrations of 50, 100, 200, 300μmol/L for 24h, protein lysate was added to extract the total protein of the cell. A BCA working solution was prepared, according to the BCA reagent Box instructions for protein concentration determination and quantification, separation and concentrated gels were prepared, and 10% SDS-PAGE was performed on 10 μL protein samples.

The appropriate PVDF membrane was cut off according to the size of the protein required by the experiment, transferred to the PVDF membrane after 1 h, placed in 5% skim milk, and shaken gently for 2 h in a shaker. The membrane was washed with PBST three times, and the appropriate primary antibody was diluted at a ratio of 1:1000 for p-AMPK, AMPK, p-mTOR, and mTOR, incubate at 4 °C for 12 h, and washed three times with PBST. The PVDF membrane was placed in the corresponding secondary antibody and shaken for 2 h. Finally, the membrane was washed three times with PBST, and electroluminescence was used to visualize the protein.

Statistical methods

Data were analyzed using the SPSS 20.0 software package, and all measurement data were expressed by ($\bar{x}\pm s$). An independent sample t-test was used to compare the means between two groups, and an analysis of variance was used to compare the means between multiple groups. Differences for which $P<0.05$ were considered statistically significant.

Results

Effect of β-elemene on DLD-1 cell proliferation ability

Compared with the blank control group, for DLD cells treated with β-elemene at concentrations of 50, 100, 200, and 300 μmol/L for 24, 48, and 72 hours, their proliferation ability gradually decreased with the increase of concentration and the extension of action time. See Figure 1.

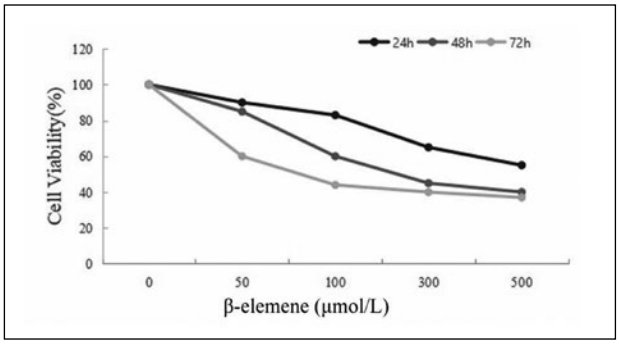

Figure 1: Effect of different concentrations of β-elemene on DLD-1 cell proliferation ability.

Effect of β-elemene on DLD-1 cell cycle

The results of flow cytometry showed that, compared with the blank control group, with the gradual increase of β-elemene concentration, the content of DLD-1 cells in G0/G1 and S phases was significantly reduced, and the content of G2/M phase cells was significantly increased. See Table 1.

β-elemene concentration	G0/G1 phase	S phase	G2/M phase
0 μmol/L	65.23±5.44	17.26±1.35	18.89±1.54
50 μmol/L	60.58±5.30	16.02±1.74	23.60±2.77
100 μmol/L	58.23±4.02	13.26±1.20	25.30±2.48
200 μmol/L	55.03±4.65	10.32±1.19	38.78±2.65
300 μmol/L	42.34±3.78	8.27±1.29	45.59±3.66

Table 1: Effect of β-elemene on DLD-1 cell cycle ($\bar{x}\pm s$).

Effect of β-elemene on DLD-1 cell apoptosis

After treating DLD cells with β-elemene concentrations of 50, 100, 200, and 300 μmol/L for 24 hours, the nucleus was regular and uniform in the blank control group, and the nuclear membrane was complete. With increasing β-elemene concentration, the nuclear cells became fragments, the chromatin was concentrated, and the cells were bright and dense in appearance. See Figure 2.

Effect of β-elemene on p-ampk, AMPK, p-mTOR, and mTOR protein expression in DLD-1 cells

Western blots showed that β-elemene at concentrations of 50, 100, 200, and 300 μmol/L significantly increased p-ampk protein expression and decreased p-mTOR protein expression compared to the control group ($P<0.05$). See Figure 3 and Table 2.

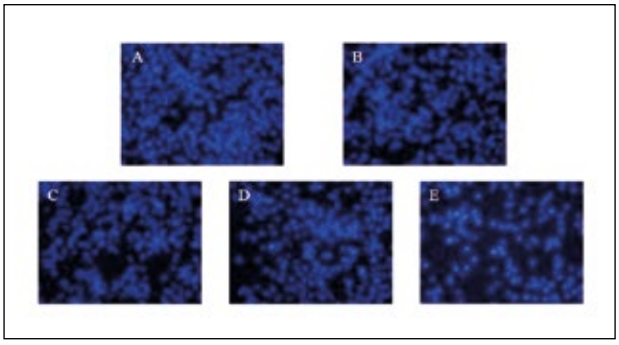

Figure 2: Effect of β-elemene at different concentrations on DLD-1 cell apoptosis.
A: Blank control group; B: 50μmol/L; C: 100μmol/L; D: 200μmol/L; E: 300μmol/L.

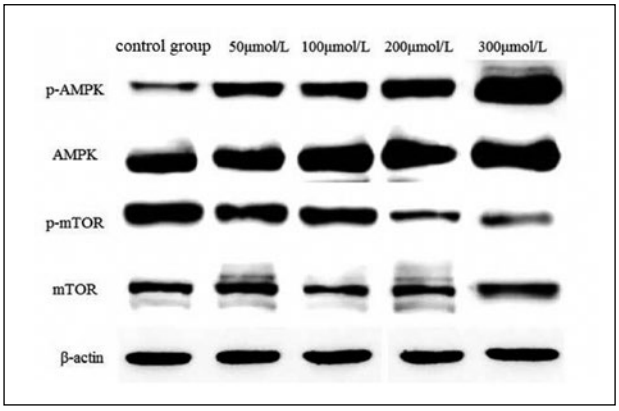

Figure 3: Effect of β-elemene on p-AMPK, AMPK, p-mTOR, and mTOR protein expression in DLD-1 cells.

Concentration of β-elemene	p-AMPK	AMPK	p-mTOR	mTOR
0 μmol/L	0.60±0.05	0.88±0.12	1.38±0.32	1.00±0.11
50 μmol/L	0.67±0.08	0.91±0.11	1.13±0.12	1.05±0.12
100 μmol/L	0.78±0.05	1.05±0.10	1.00±0.08	1.10±0.12
200 μmol/L	0.86±0.11	1.01±0.09	0.45±0.06	0.98±0.07
300 μmol/L	1.20±0.18	0.97±0.13	0.23±0.05	1.03±0.10

Table 2: Comparison of p-AMPK, AMPK, p-mTOR, and mTOR protein content of DLD-1 cells in each group ($\bar{x}\pm s$).

Discussion

Tumors are caused by abnormal proliferation or differentiation of local tissue cells under the action of various oncogenic factors. The proliferation and diffusion speed is particularly rapid and causes serious damage and, eventually, death. Colorectal cancer is a common malignant tumor of the digestive tract. Its etiology is unclear. It can occur in any part of the colon or rectum. In recent years, its morbidity and mortality have increased yearly in China[6]. Many patients with this form of cancer were found to be in the middle and late stages. For advanced colorectal cancer, general systemic chemotherapy is used, and most chemotherapy drugs have specific side effects. Patients often have symptoms such as malignant vomiting, hair loss, and immune function decline[7]; hence, it is essential to develop high-efficiency, low-toxicity chemotherapy drugs for colorectal cancer.

Elemene is a non-cytotoxic, broad-spectrum anti-tumor drug developed in China. At present, β-elemene is the main component of the preparation. Pharmacological research[8] shows that it has a strong inhibition and killing effect on a variety of tumor cells and has prominent advantages, such as low toxic side effects and exact curative effect. At present, substantial progress has been made in analyzing the anti-tumor mechanism of β-elemene, which has significant potential for anti-tumor application. β-elemene can inhibit the proliferation of lung cancer A549 cells through the regulation of the tumor cell cycle[9], and its mechanism is closely related to the inhibition of tumor cell tubulin polymerization. Relevant data show that β-elemene can inhibit the proliferation of bladder cancer cells in a time- and dose-dependent manner and promote the expression of Smad4 to a certain extent[10]. Apoptosis is a gene-controlled process of cell death. The occurrence of many tumors is closely related to the disorder of cell apoptosis, which plays an important role in the embryonic development and internal environment stability of multicellular organisms[11]. A large number of studies have shown that the anti-tumor mechanism of β-elemene is different from that of traditional chemotherapy drugs. Apoptosis induction is one of the important mechanisms for β-elemene in terms of its anti-tumor role. β-elemene can significantly interfere with the metabolic pathway of cells, inhibit cell proliferation, and rapidly promote apoptosis by blocking tumor cells from S to G2/M phases. Although β-elemene has a wide spectrum of anticancer effects, due to the varying sensitivity of different tumor cells, the drug effect on these cells differs significantly[12]. In addition, β-elemene can improve the immune function of the body in many ways, promote auxiliary T cells, and kill T cells. Some scholars have found that β-elemene can enhance the phagocytosis of macrophages in tumor-bearing mice, improve the rDNA transcription activity of T lymphocytes, and activate the body's immune function[13].

In this work, the effect of β-elemene on colorectal cancer and its related molecular mechanism were analyzed at the cellular level. The

results showed that β-elemene could significantly inhibit the proliferation of DLD-C cells, and with the increase of β-elemene concentration, the cell contents of G0/G1 and S phase of DLD-1 cells decreased significantly. Further, the cell contents of G2/M phase increased significantly, which confirmed that β-elemene has a therapeutic effect on colorectal cancer. In addition, Hoechst 33342 staining showed that with increasing β-elemene concentrations, the nuclear cells in each group were fragmented, the chromatin was concentrated, and was bright and dense in appearance. These findings suggested that β-elemene can induce apoptosis of DLD-1 cells. AMPK, as an energy balancer in cells, plays a key role in energy metabolism and in the occurrence and development of cancer as a stress-response molecule[14]. MTOR, as the intersection of multiple signal pathways, determines the signal pathway of tumor growth or death mainly by affecting the autophagy process of tumor cells. Activated AMPK can activate downstream mTOR, affect the decrease of energy utilization in cells, and lead to tumor cell proliferation inhibition[15]. The results showed that β-elemene could increase the expression of p-AMPK and decrease the expression of p-mTOR, suggesting that β-elemene might regulate the proliferation and apoptosis of colorectal cancer cells through the AMPK/mTOR signaling pathway.

In conclusion, elemene can significantly inhibit the proliferation of DLD-1 cells and induce apoptosis, which may be related to the regulation of the AMPK/mTOR signaling pathway.

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