Role of Natural Product Eriocalyxin B in Promoting Apoptosis of Colon Cancer Cells through ERK1/2 Pathway

Pingping TIAN[1], Wei SHI[1], Ying PAN[1], Xiaocong XIANG[2], Jin CHEN[1]*

1. Anhui Medical College, Hefei 230601, China; 2. North Sichuan Medical College, Nanchong 637000, China

Abstract [**Objectives**] To investigate the role of Eriocalyxin B (EriB) in promoting colon cancer cell apoptosis through ERK1/2 pathway in vitro, and to provide a natural candidate drug for colon cancer treatment. [**Methods**] Colon cancer cells treated with different concentrations of EriB were detected by CCK-8 assay; cell scratch assay and crystal violet staining were used to detect the invasion and migration of colon cancer cell; cell apoptosis was detected by Annexin V/PI double staining, and cell cycle was detected by PI staining; Western Blotting was used to detect epithelial-mesenchymal transition and apoptosis-related proteins in colon cancer cells treated with EriB. [**Results**] After EriB treatment, the proliferation, migration and apoptosis of colon cancer cells were significantly inhibited, and the ratio of P-ERK1/2 to ERK was significantly decreased. [**Conclusions**] EriB can effectively inhibit the proliferation of colon cancer cells and promote the apoptosis of colon cancer cells through ERK1/2 pathway.

Key words Eriocalyxin B (EriB), Colon cancer, Apoptosis, ERK1/2

1 Introduction

Colon cancer is one of the most important tumors in the world, and it is considered as the biggest tumor killer for human beings together with lung cancer, prostate cancer and breast cancer[1]. In recent years, with the improvement of living standards, the low-fiber and high-fat diet mode has greatly increased the incidence of colon cancer[2]. Although the research on colon cancer metastasis has been carried out for a long time, its complex mechanism is not completely clear[3]. Eriocalyxin B (EriB/ER-B) is a diterpene compound isolated and identified from *Rabdosia eriocalyx* (Dunn) Hara, and it is used for anti-inflammation and antisepsis, and can treat infectious diseases such as tonsillitis, pharyngolaryngitis and chronic bronchitis, as well as autoimmune diseases such as lupus erythematosus and rheumatoid arthritis[3]. Studies have shown that EriB has a very strong inhibitory effect on the growth of some tumor cell lines, and can prevent the occurrence and development of tumors[3-4]. However, little is known about the anti-tumor mechanism of EriB in human colon cancer. ERK1/2 is an important member of mitogen-activated protein family and plays an important role in the occurrence and development of cancer. Phosphorylated ERK (p-ERK) can enter the nucleus and act on many transcription factors such as c-myc and c-jun, regulate transcription and promote physiological activities such as cell growth, development and division, and play an important role in malignant transformation of cells[5]. However, little is known about the role of ERK1/2 and phosphorylated ERK1/2 in colon cancer. Therefore, in this study, we mainly explored whether EriB drugs inhibited the proliferation of colon cancer cells through ERK1/2 pathway, and promoted the apoptosis of cancer cells, so as to provide a new targeting site for the treatment of colon cancer.

2 Materials and methods

2.1 Cells and reagents Human colon cancer cell RKO cells were stored in the cell bank of Chinese Academy of Sciences and cultured in 10% standard fetal bovine serum, double antibodies (penicillin and streptomycin) and RPMI-1640 medium.

EriB reagent was purchased from Chengdu Herpurify Co., Ltd., and stored at -80 ℃ with DMSO (purchased from Shanghai Bioengineering Technology Co., Ltd.). 0.25% trypsin-EDTA digestive juice, RPMI-1640 culture medium, penicillin and streptomycin antibodies and fetal bovine serum were all purchased from Hyclone Company. CCK-8 kit was purchased from Wuhan Boster Bioengineering Co., Ltd. Annexin V-FITC apoptosis detection kit, RIPA lysate (strong), cell cycle and apoptosis detection kit were all purchased from Shanghai Biyuntian Biotechnology Co., Ltd. ERK1/2, p-ERK1/2, PARP, BCL2, Actin and GAPDH were all purchased from Anhui Dingxiu Biological Co., Ltd., and HRP-labeled second antibody was purchased from CST Company.

The flow cytometer was purchased from BD Company, the fluorescence inverted microscope (CKX41) was purchased from Olympus Company, and the Spectramax 190 microplate reader was purchased from Molecular Devices Company.

2.2 Methods

2.2.1 Cell culture. Human colon cancer cell line RKO was cultured in RPMI-1640 culture medium with 10% fetal bovine serum and double antibodies, and placed in a constant temperature incubator containing 5% CO_2 and sterile deionized water at the bottom

Received: May 13, 2023 Accepted: July 25, 2023
Supported by Natural Science Research Project of Colleges and Universities in Anhui Province (KJ2019A1110, KJ2020A0863); Innovation Team Research Fund Project of Anhui Medical College (WJH202007t, WJH202008t); 2021 Anhui Provincial Production-Education Integration Training Base Project for Quality Engineering (2021cjrh020).

* Corresponding author. E-mail: chenjin@ahyz.edu.cn

of the incubator at 37 ℃. All related cell operations were carried out in a bacteria-free environment.

2.2.2 CCK-8 colorimetry. RKO cells with good growth conditions were selected and inoculated in 96-well culture plate according to the density of 3×10^4/mL. After cell adhesion, the cells were treated with different concentrations of EriB (0.125, 0.25, 0.5, 1, 2, 4 μM); RKO cells without any drug treatment were used as blank control group (EriB 0 μM): EriB cells treated with 0.6% DMSO were used as control group, and three replications were set for each group. After finishing, the culture plate was put into the incubator for 48 h. After 48 h of drug treatment, the 96-well plate was taken out, and 10 μL of CCK-8 solution was added into each well, and then put into the incubator at 37 ℃ for continuous culture for 1 h. The well plate was taken out, and the absorbance *OD* value of each well was measured by microplate reader, and the wavelength was selected at 490 nm.

The survival rate of cell was calculated by relevant software as follows:

Cell survival rate (%) = (*OD* value of experimental group − *OD* value of blank group)/(*OD* value of control group − *OD* value of blank group) × 100%.

2.2.3 Detection of the effect of EriB on cell migration and invasion by crystal violet staining. The RKO cells were in good growth condition, about 5×10^5 cells were cultured overnight in a 6-well plate, fixed with 11% glutaraldehyde in the culture medium, and shaken on an oscillator for 20 min. After washing with deionized water twice, the cells were thoroughly dried in air or oven at 37 ℃, and then stained with 0.1% crystal violet solution, and shaken for 30 min. It was washed with distilled water until the excess crystal violet liquid was removed, then thoroughly dried in air or oven at 37 ℃, and photographed.

2.2.4 Detection of the effect of EriB on cell migration and invasion by cell scratch test. The RKO cells were in good growth condition, about 5×10^5 cells were cultured in a 6-well plate. After the cells grew overnight, they were scratched with pipette tip perpendicular to the horizontal line on the back, washed with PBS for 3 times, the scratched cells were removed, and serum-free culture medium was added. They were cultured in a 5% CO_2 incubator at 37 ℃. Samples were taken and photographed at 0, 24, 36 and 48 h.

2.2.5 Detection of the effect of EriB on apoptosis by flow cytometry. When the fusion degree of RKO cells in a 10 cm culture dish was 80%, the cells were collected and digested, and about 1×10^6 cells were inoculated into a 6-well plate. After inoculation for 24 h, different treatments were given. In the experimental group, EriB (0, 0.25 μM) with different concentrations was added, and DMSO was used as blank control. After treatment, the 6-well plate was placed in a constant temperature incubator at 37 ℃ for 48 h. After 48 h, the old culture medium was sucked out, the cells were digested by adding 0.25% trypsin, and then the cell suspension was collected in a centrifuge tube at 1 000 rpm for 5 min; the supernatant was removed, it was washed with PBS solution, centrifuged again, and the operation was repeated twice. The cells were re-suspended with PBS and counted. 50 000 − 100 000 suspended cells were taken, and centrifuged at 1 000 rpm for 5 min. The supernatant was discarded, and the cells were re-suspended with 195 μL of Annexin V-FITC binding solution. After 5 μL of Annexin V-FITC was added and gently mixed, 10 μL of propidium iodide staining solution was added, incubated at room temperature (20 − 25 ℃) without light for 10 − 20 min, and then placed in an ice bath for detection by BD FACScaliber.

2.2.6 Detection of the expression level of proteins related to cell division cycle, apoptosis and molecular mechanism by Western blot. RKO cells were lysed in RIPA buffer, and the total proteins were collected after centrifugation. The total proteins were quantified by BCA method, and the samples for protein gel electrophoresis were prepared. Then the total protein was separated by SDS-PAGE gel electrophoresis and transferred to PVDF membrane. It was sealed with 5% skim milk for 1 h, the membrane was incubated overnight with primary antibody at 4 ℃, and then incubated with secondary antibody at room temperature for 1 h; chemiluminescence imaging was adopted.

3 Results and analysis

3.1 Inhibitory effect of EriB on the proliferation of colon cancer cells Colon cancer cell line RKO was treated with different concentrations of EriB (0.125, 0.25, 0.5, 1, 2, 4 μM) for 24, 48 and 72 h, and detected by CCK8 method. From the results, it can be seen that EriB inhibited the proliferation of colon cancer cells, and the cell survival rate decreased in a concentration-dependent manner with the increase of drug concentration, as shown in Fig. 1.

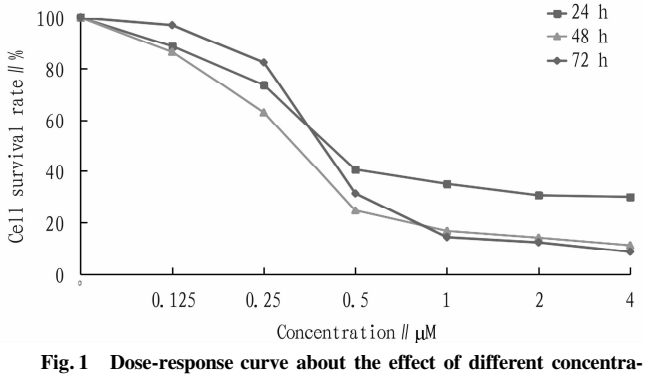

Fig. 1 Dose-response curve about the effect of different concentrations of EriB on proliferation of colon cancer cells

2.2 Inhibitory effect of EriB on the migration and invasion of colon cancer cells Cell scratch test was used to study the effect of drugs at different concentrations and different time (0, 1, 1.5 μM, 0, 24, 36, 48 h) on RKO cells. After EriB drug treatment, the migration speed of RKO cells in experimental group was significantly lower than that in 0 μM group, that is, EriB affected the migration of RKO cells in colon cancer with obvious time dependence (Fig. 2). At the same time, EriB (concentration 0.5 μM, time 24, 36, 48 h) acted on RKO cells. It can be seen from Fig. 3 that with the increase of drug concentration, the total num-

ber of cells passing through the membrane decreased significantly, indicating that EriB reduced the invasion of colon cancer cells.

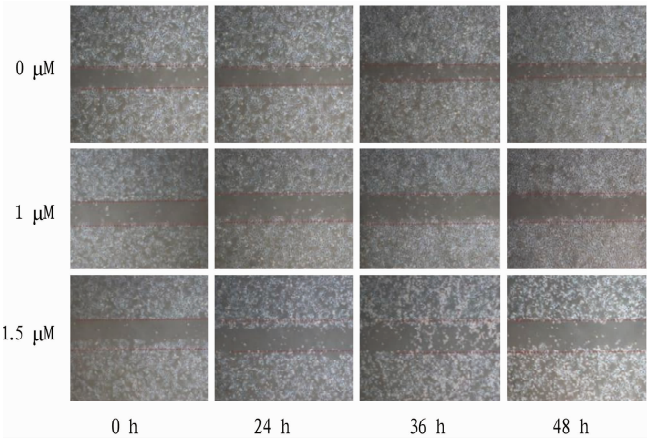

Fig. 2 Inhibitory effect of EriB on the migration of colon cancer cells at different concentrations and different time

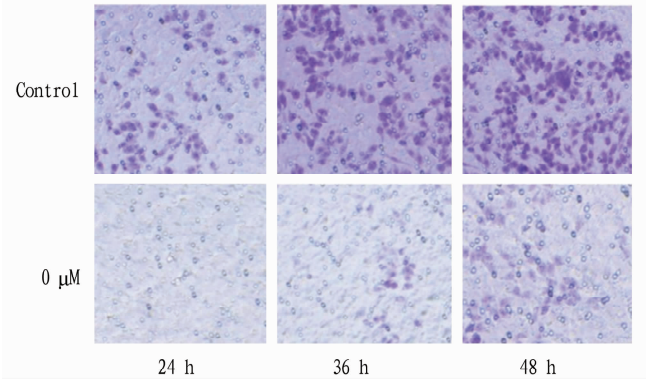

Fig. 3 Inhibitory effect of EriB on the invasion of colon cancer cells at different concentrations and different time

2.3 Role of EriB in promoting apoptosis of colon cancer cells through ERK1/2 pathway When colon cancer RKO cells were treated with different concentrations of EriB (0, 0.5, 1, 2 μM) for 24 h, it can be seen from Fig. 4 that all cells had different degrees of apoptosis, and the apoptosis rate increased with the drug concentration (from low to high), indicating that the apoptosis rate was positively correlated with the drug concentration (Fig. 4). At the same time, it was found that the content of ERK1/2, a key extracellular regulatory protein kinase that transmits signals from cell surface receptors to nucleus, decreased with the phosphorylation of p-ERK in RKO cells, and the apoptosis-related protein PARP was obviously up-regulated and gradually became obvious with the increase of EriB concentration (Fig. 5).

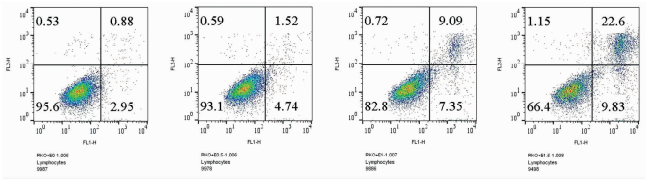

Fig. 4 Effect of different concentrations of EriB on apoptosis rate of RKO cells

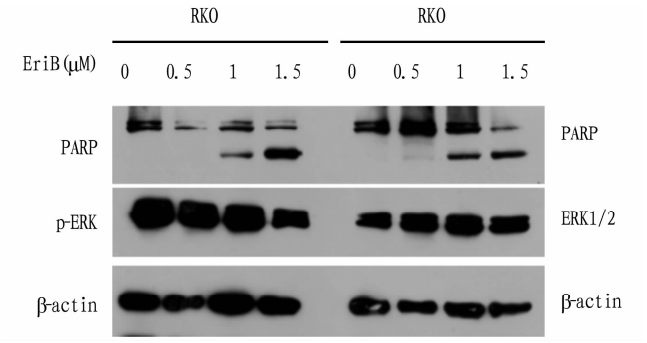

Fig. 5 Different concentrations of EriB regulating apoptosis through ERK pathway

4 Discussion

Colon cancer is a common malignant tumor in human life. In recent years, with the improvement of living standards, and especially due to uncontrolled diet among young people, the incidence of colon cancer is not only limited to old people, and more and more young people have developed colon cancer. The induction of colon cancer is related to many factors. Studies have shown that invasion and metastasis are the causes of high mortality caused by tumors, including colon cancer[6]. In this study, it was found that EriB promoted the apoptosis of colon cancer cells through ERK1/2 pathway, reduced the content of p-ERK and inhibited the expression of downstream genes. Normal cells in nature maintain the growth balance of the body through programmed death and cell proliferation. If this balance is broken, it will lead to imbalance of organisms and even the occurrence and development of many diseases, such as cancer. The purpose of human beings' research and development and application of anti-tumor drugs is to cause cell apoptosis and finally achieve the effect of treating tumors and improve human living standards[7-8]. This study demonstrated that EriB induced different degrees of apoptosis of cancer cells through ERK1/2 and there was a positive correlation between EriB and drug concentration.

This study updates the understanding of anti-cancer effect of natural drugs. Follow-up research can further explore the anti-cancer activity and mechanism of EriB *in vivo*, and it can be combined with clinical radiotherapy and chemotherapy technology to explore new therapies of integrated traditional Chinese and Western medicine, which will provide a new direction for the research and development of anti-cancer drugs from natural plants.

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ITS1 and ITS2 regions. The results of this experiment showed that the origily identified medicinal material was *P. capitatum* Buch. -Ham. ex D. Don in the Polygonaceae family.

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