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Original Article

Apatinib inhibits the proliferation of colon cancer cells by down-regulating the VEGFR2-PLC-ERK1/2 pathway

Xiaokun Ma[1*], Menghan Wang[1*], Ni Zheng[1], Guoyu Wang[1], Juhua Zhuang[1], Ying Ye[2], Wei Xia[1]

[1]Department of Nuclear Medicine, [2]Central Laboratory, The Seventh People's Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China. [*]Equal contributors and co-first authors.

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Abstract: Objective: To analyze the effect of apatinib on colon cancer cells via the VEGFR2-PLC-ERK1/2 pathway. Methods: Human colon cancer cell line LS174T in the logarithmic phase of growth were treated with apatinib solution at concentrations of 0, 25, 50, and 100 mol/L for 24, 48, and 72 hours, respectively. CCK-8 detected cell proliferation, flow cytometry evaluated the cell apoptosis and cell cycle. Western blot was used to detect the phosphorylation of key enzymes in VEGFR2-PLC-ERK1/2 pathway and expression of related apoptotic proteins. Results: Compared with 0 mol/L, the absorbance of LS174T cells at 25, 50, and 100 mol/L decreased significantly after 24, 48, and 72 h of cell culture ($P < 0.05$); and the inhibitory effect of apatinib on colon cancer cells was increased in a dose/time-dependent manner. The apoptosis rate of the control group was (6.55 ± 1.08)% which is significantly different from (12.58 ± 1.36)% (24 h), (18.85 ± 1.37)% (48 h) and (25.74 ± 1.43)% (72 h) in the 100 mol/L apatinib group ($P < 0.05$). Apatinib-induced apoptosis exhibited a time-dependent relationship. Compared with the control group, the 100 mol/L apatinib group showed a decrease in S-phase and G2/M-phase cells and an increase in G0/G1-phase cells ($P < 0.05$), while the expression of pPCL and pERK1/2 proteins in the 100 mol/L apatinib group decreased at 24, 48, and 72 h ($P < 0.05$). Conclusion: Apatinib can inhibit the proliferation of colon cancer cells and accelerate apoptosis, which was related to the inhibition of phosphorylation of key proteases in the VEGFR2-PLC-ERK1/2 pathway.

Keywords: Colon cancer, apatinib, apoptosis, VEGFR2-PLC-ERK1/2 pathway

Introduction

Colon cancer is a common digestive tract cancer from the colon or rectum (parts of the large intestine). Its incidence is closely related to a patients' lifestyle, genetics, and living environment [1, 2] Current treatment options for colon cancer include chemotherapy and surgery [3, 4], however, patients who undergo chemotherapy often experience serious toxic side effects, and tumors are prone to relapse or metastasis after surgical treatment. Studies have shown that the incidence and mortality of colon cancer in China are increasing annually, which has a serious impact on people's physical and mental health. Finding a safe and effective treatment for colon cancer has been the focus of clinical research.

Apatinib, a small-molecule tyrosine kinase inhibitor, exerts anti-tumor effects by inhibiting the neovascularization of tumors [5, 6]. A number of *in vitro* and *in vivo* studies have shown that apatinib has clear antitumor activity against various animal and human tumors, including gastric cancer, colon cancer and lung cancer. The VEGFR2-PLC-ERK1/2 pathway has been verified to be closely related to cell proliferation and cycle regulation. Phosphorylation of activated ERK1/2 activates the Jun oncogene, resulting in cell proliferation and DNA synthesis [7]. Therefore, this study analyzed the effect of apatinib on the proliferation of colon cancer cells by down-regulating the VEGFR2-PLC-ERK1/2 pathway, and provided some reference for clinical treatment.

Materials and methods

Human colon cancer cell line LS174T (Institute of Biology, Chinese Academy of Sciences); apatinib (Jiangsu Hengrui Corporation); trypsin,

DMEM-F12 medium, penicillin streptomycin double antibody and fetal bovine serum (Gibco Corporation, USA); Cell cycle, CCK-8 and apoptosis kits (Biyuntian Biological Company); Cleared caspase-3, Bcl-2 and Bax rabbit anti-polyclonal antibodies (Nanjing Enjing Biological Company); gel imaging System (Bio-Rad, USA), CO2 incubator, flow cytometer, and microplate reader (Sigma, USA).

Cell culture

DMEM-F12 medium (containing penicillin streptomycin double antibody and 10% fetal bovine serum) was used to culture LS174T cells at 95%, 5% CO_2, and 37°C. The cells were divided into control group and apatinib group. According to different culture time, the apatinib group was divided into apatinib 24 h group, apatinib 48 h group and apatinib 72 h group.

CCK-8 detects cell proliferation

LS174T cells in a logarithmic phase of growth were seeded in a 96-well plate, and its concentration is adjusted to 1×10^5 cells/ml. After cell adhesion and apatinib treatment, the final cell concentrations are 0, 25, 50, and 100 mmol/L, and five parallel wells are set up at each concentration. After 24, 48 and 72 h of culture, 10 ml of CCK8 solution was added respectively and incubated for 2 hours, followed by dimethyl sulfoxide treatment. The absorbance value (A) was measured at 570 nm.

Flow cytometry detects apoptosis and cell cycle

A single cell suspension was prepared with LS174T cells in a logarithmic growth phase using 0.25% trypsin. Cells were seeded in a 6-well cell culture plate at 1×10^4 cells/ml. After the cells adhered, 0 and 100 mmol/L apatinib solutions were added. The apoptosis rate and cell cycle at 24, 48 and 72 h were detected using Annexin V FITC/PI apoptosis detection kit and cell cycle detection kit. Zero mmol/L was used as the control group, and each experiment was repeated 3 times.

Western blot detects apoptotic proteins and phosphorylation of key enzymes in VEGFR2-PLC-ERK 1/2 pathway

LS174T cells treated with 100 mmol/L apatinib for 24, 48 and 72 h, they were then ground, lysed and electrophoresis at 80 V. After the leading edge of bromophenol blue entered the upper edge of the separation gel, the voltage was increased to 100 V. The electrophoresis ended after the bromophenol blue moved out of the lower edge of the separation gel. Protein electrotransfer was performed within a PVDF membrane at 30 mA for 90 min using semi-dry electrotransfer instrument. After the PVDF membrane is taken out, it is sealed with 5% TBST skim milk powder and shaken for 60 minutes. Then, the membrane was washed 3 times with TNS-T solution, and transferred to the hybridization bag. Bcl-2 (1:100, Nanjing Enogene Biotech. Co., Ltd), Cleaved caspase-3 (1:100, Nanjing Enogene Biotech. Co., Ltd), and Bax (1:100, Nanjing Enogene Biotech. Co., Ltd) primary antibodies were added, and the membrane was incubated at 4°C overnight after sealing. After three times of washing the membrane with TBST, horseradish peroxidase-conjugated secondary antibody (1:1000, Nanjing Enogene Biotech. Co., Ltd) diluted in the rinse solution was added and shaken for 60 min. The PVDF film was placed in ECL developing solution and incubated for 5 minutes, exposed, developed, fixed, washed, and dried in a dark room. IPP software was used to analyze gray scale of the stripes.

Statistical analysis

SPSS 19.0 statistical software was used for data analysis. GraphPad Prism 6 software was used for illustrations. The measurement data ($\bar{x} \pm s$) was examined with single-factor variance and independent sample t test. $P < 0.05$ indicated statistical significance.

Results

Effects of apatinib on the proliferation of colon cancer cells

The 100 mol/L apatinib group and control group differ in apoptosis rate ($P < 0.05$), and the apatinib-induced apoptosis exhibited a time-dependent relationship. The concentration of 100 mol/L was used as the experimental concentration of the drug in subsequent experiments (**Table 1**).

The effect of apatinib on apoptosis

At 24, 48 and 72 h, the apoptosis rate of the apatinib group was (12.59 ± 1.18)%, (18.79 ±

Table 1. The effect of apatinib on the proliferation of colon cancer cells

Concentration	A		
	24 h	48 h	72 h
0 μmol/L	1.32 ± 0.12	1.55 ± 0.14	1.70 ± 0.13
25 μmol/L	0.99 ± 0.13*	1.24 ± 0.16*	1.45 ± 0.10*
50 μmol/L	0.56 ± 0.10*	0.70 ± 0.15*	0.82 ± 0.13*
100 μmol/L	0.42 ± 0.11*	0.52 ± 0.12*	0.64 ± 0.09*
F	16.834	19.073	15.861
P	< 0.05	< 0.05	< 0.05

Note: compared with 0 μmol/L, *$P < 0.05$.

1.40)%, (25.80 ± 1.51)%, which was significantly higher than that of the control group (6.56 ± 1.10)% ($P < 0.05$), suggesting apatinib induces LS174T cell apoptosis in a time-dependent manner (**Figure 1**).

The effect of apatinib on LS174T cell cycle

Compared with the control group, the number of G2/M and S phase cells decreased and the number of G0/G1 cells increased at 24, 48, and 72 h in the apatinib group, indicating apatinib can inhibit the LS174T cell cycle (**Table 2**).

Apoptotic proteins in cells

Compared with the control group, at 24, 48 and 72 h, 100 mol/L apatinib group showed decreased expression of Bcl-2 protein and increased expression of Cleaved caspase-3 and Bax protein ($P < 0.05$), with statistically significant differences; suggesting that apatinib has an impact on the cell cycle-related proteins (**Figure 2**).

Phosphorylation of VEGFR2-PLC-ERK1/2 pathway-related proteins in cells

Compared with the control group, at 24, 48 and 72 h, 100 mol/L apatinib group showed decreased expression of p-ERK1/2 and p-PLC in a time-dose dependent manner, with statistically significant differences ($P < 0.05$). However, there was no significant difference in t-ERK1/2 and t-PLC protein expression between the control group and the apatinib group ($P > 0.05$). This suggested that apatinib can inhibit the phosphorylation of intracellular VEGFR2-PLC-ERK1/2 pathway-related proteins (**Figure 3**).

Discussion

Colon cancer is the third leading type of cancer worldwide. Patients with colon cancer die primarily from local recurrence and distant metastasis [7]. Studies have shown that although combined chemotherapy can improve the survival rate and disease remission rate, its toxic side effects negatively impact quality of life of patients [8, 9]. In recent years, targeted therapy such as angiogenesis inhibitors, anti-epidermal cell growth factor receptor 1, tyrosine kinase inhibitors, etc., have achieved satisfactory results for patients with colon cancer. Compared with cytotoxic drugs, targeted molecules drugs were characterized by low toxicity and less side effects, with high efficiency and high selectivity.

Apatinib is a compound derived from the anti-VEGFR small molecule tyrosine kinase inhibitor PTK787, which promotes anti-tumor growth through VEGFR inhibition. Studies have verified [10-13] that it has a significant anti-tumor effect on a variety of human tumors, such as lung cancer, breast cancer, colon cancer as well as gastric cancer of nude mice, and can also enhance the efficacy of adriamycin, oxaliplatin, and sitaxel [14-16]. In this study, LS174T cells were treated with different concentrations of apatinib. The results showed that compared with 0 mmol/L, the absorbance of LS174T cells decreased significantly at 25, 50, and 100 mmol/L in a time-dependent manner.

Apoptosis is a fundamental biological phenomenon, maintaining the stability of the intracellular environment. The caspase and Bcl-2 protein families are the most concerned proteins related to apoptosis. Bax gene and Bcl-2 gene are the main genes regulating cell apoptosis, and Caspase-3 is the main gene that triggers the execution of apoptosis. The above three genes exert important effects on tumor cell apoptosis [17, 18]. Quan et al. [19] showed that after 48 h of treatment with apatinib, the expression level of Bax was increased and the expression level of Bcl-2 was decreased in human multiple myeloma RPMI8226 cells, suggesting that apatinib significantly promoted apoptosis. This study shows that the expression of Bcl-2 protein was reduced and the expression of Cleaved caspase-3 and Bax protein was increased in the 100 mmol/L apatinib group in contrast to the control group, suggesting that apatinib can accelerate the apoptosis of colon cancer cells via reduced Bcl-2 protein expression and increased Cleared caspase-3

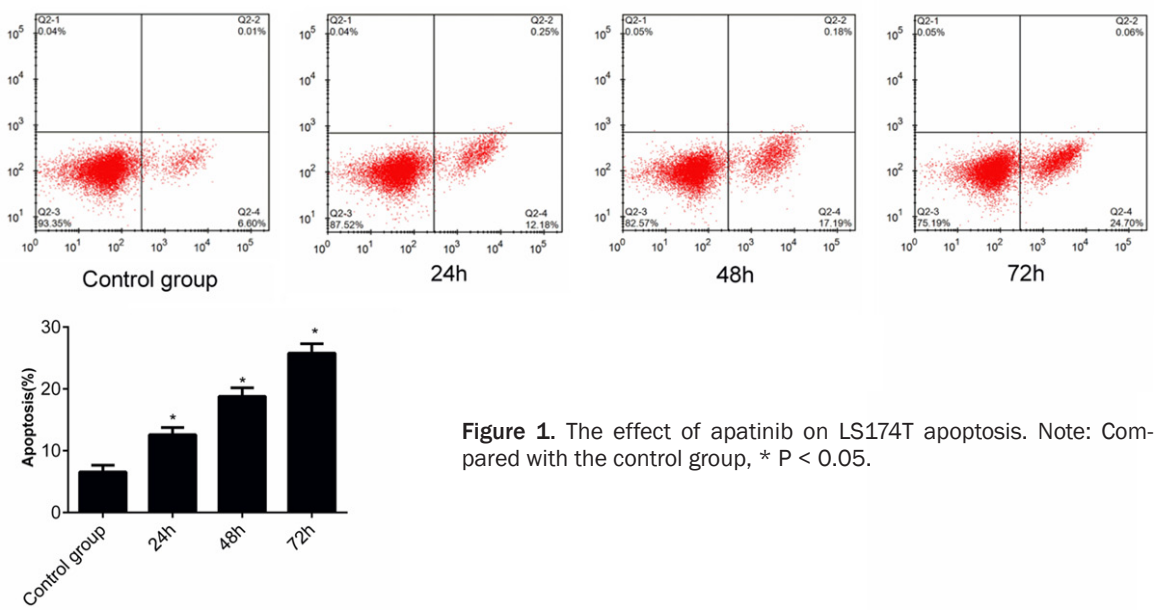

Figure 1. The effect of apatinib on LS174T apoptosis. Note: Compared with the control group, * P < 0.05.

Table 2. Effects of apatinib on the cell cycle of LS174T

Group	Time points	G_0/G_1	S	G_2/M
Control group	-	54.79 ± 3.10	32.89 ± 2.54	12.30 ± 1.38
Apatinib group	24 h	62.70 ± 3.52*	26.21 ± 2.38*	11.08 ± 1.25*
	48 h	63.48 ± 3.39*	28.69 ± 2.70*	7.78 ± 1.07*
	72 h	63.99 ± 3.27*	28.39 ± 2.41*	7.49 ± 1.15*
F		8.720	6.028	12.640
P		< 0.05	< 0.05	< 0.05

Note: Compared with the control group, *$P < 0.05$.

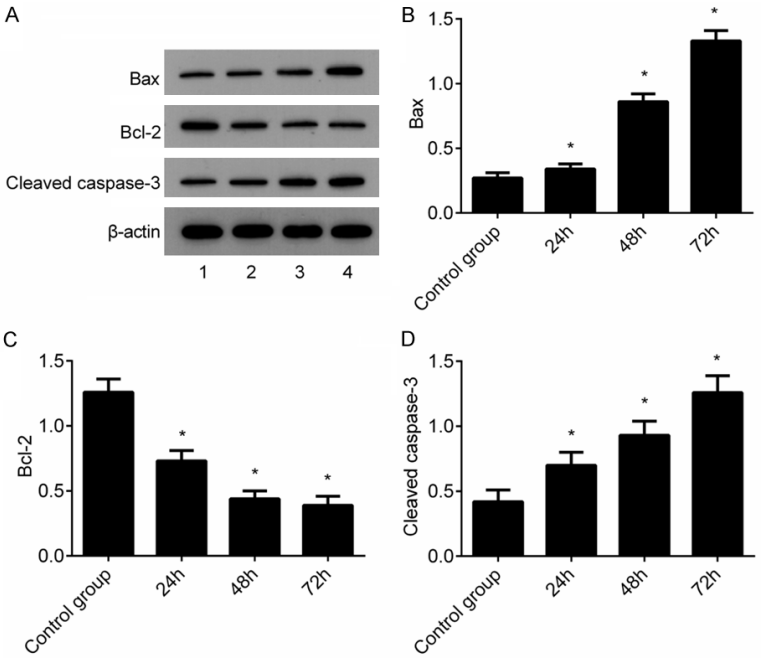

Figure 2. The effect of apatinib on apoptosis of related proteins. Note: Compared with the control group, * P < 0.05. 1. Control group; 2. Apatinib 24 h group; 3. Apatinib 48 h group; 4. Apatinib 72 h group.

and Bax protein expression, which is inconsistent with the above findings.

Cell cycle disorders resulted in uncontrolled and unlimited cell growth. Chemotherapeutic drugs can block tumor cells at the G0/G1 phase, inhibit the synthesis of related proteins and DNA, and play a role in fighting tumors [20, 21]. This study shows that apatinib has also exhibited similar effects. The VEGFR2-PLC-ERK1/2 pathway has been confirmed to be closely related to cell proliferation and cycle regulation. After activation of ERK1/2, after activation of ERK1/2, phosphorylation activates the Jun oncogene, resulting in cell proliferation and DNA synthesis. This study used Western blot to detect the phosphory-

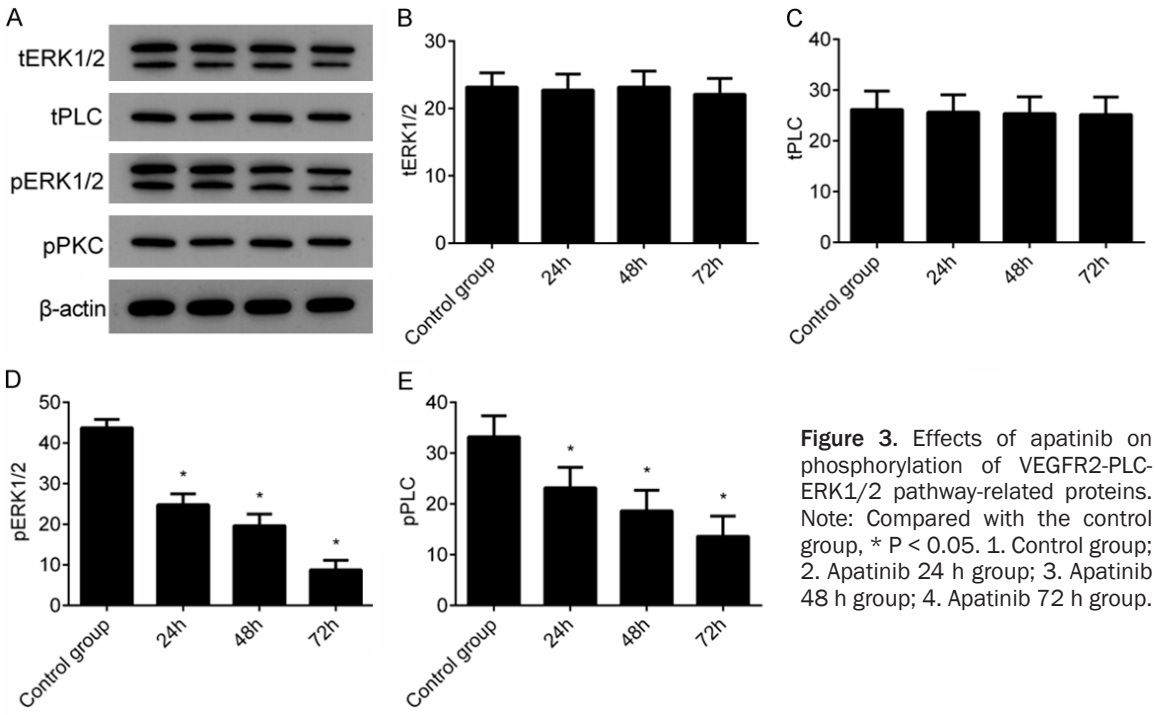

Figure 3. Effects of apatinib on phosphorylation of VEGFR2-PLC-ERK1/2 pathway-related proteins. Note: Compared with the control group, * $P < 0.05$. 1. Control group; 2. Apatinib 24 h group; 3. Apatinib 48 h group; 4. Apatinib 72 h group.

lation of key protease in the VEGFR2-PLC-ERK1/2 pathway. The results showed that compared with the control group, expression of pPCL and pERK1 proteins in the 100 mmol/L apatinib group decreased, indicating that apatinib can inhibit the phosphorylation of key proteases in the VEGFR2-PLC-ERK1/2 pathway, thereby preventing DNA synthesis of colon cancer cell and inhibiting tumor cell growth.

In summary, apatinib can inhibit the proliferation and accelerate apoptosis of colon cancer cells, which may be related to the inhibition of phosphorylation of key proteases in the VEGFR2-PLC-ERK1/2 pathway.

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Disclosure of conflict of interest

None.

Address correspondence to: Wei Xia, Department of Nuclear Medicine, The Seventh People's Hospital, Shanghai University of Traditional Chinese Medicine, No.358, Datong Road, Gaoqiao, Pudong New Area, Shanghai 200120, China. Tel: +86-021-58670561; E-mail: wxeiia00@163.com; Ying Ye, Central Laboratory, The Seventh People's Hospital, Shanghai University of Traditional Chinese Medicine, No.358, Datong Road, Gaoqiao, Pudong New Area, Shanghai 200120, China. Tel: +86-021-58611047; E-mail: yy49453324@163.com

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