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Effect of Yiqi Jianpi plus anticancer herbs on spleen deficiency in colorectal cancer and its anti-tumor role

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ABSTRACT

Objective: To observe the effect of Yiqi Jianpi plus anticancer herbs on spleen deficiency in colorectal cancer and its anti-tumor role. **Methods:** Human intestinal cancer cell HT29 xenograft of nude mice model was established. The expression of EGF, VEGF, gastric cancer tumor growth in mice were observed. **Results:** Protein kinase C expression in the Yiqi Jianpi group and Yiqi Jianpi anti-tumor group was significantly better than the model group ($P < 0.01$, $P < 0.05$). There was significantly more apoptotic cells in Yiqi Jianpi anti-tumor group than Yiqi Jianpi group and model group ($P < 0.01$). Epidermal growth factor and vascular endothelial growth factor expression in Yiqi Jianpi group was significantly lower than Yiqi Jianpi group and model group ($P < 0.05$). **Conclusions:** Tumor can inhibit the expression of PKC inhibition. Yiqi Jianpi and anticancer treatment can reduce this inhibition. Besides this treatment can also inhibit expression of tumor related genes such as epidermal growth factor and vascular endothelial growth factor.

1. Introduction

Preliminary studies have found that, colorectal cancer is characterized by bloating, poor appetite, constipation, diarrhea and other spleen deficiency syndromes. It is proposed that the use of Yiqi Jianpi decoction combined with anticancer herbs is effective in treatment of colorectal cancer. Spleen deficiency related proteins and signaling pathways is helpful in further study on mechanism of Yiqi Jianpi decoction. In this study, we explored the molecular mechanisms about anticancer effects of Yiqi Jianpi decoction^[1].

2. Materials and methods

2.1. Cell lines and experimental animals

HT29, a human colon cancer cell line, was purchased from Cell Biology Laboratory of Zhengzhou University,

Zhengzhou, Henan Province, China. Forty nude mice and forage, of specific pathogen free grade, were purchased from Experimental Animal Center of Zhengzhou University, Zhengzhou, Henan Province, China. Sterile gauze pad was also prepared.

2.2. Drugs, reagents and instruments

Yiqi Jianpi decoction included radix pseudostellariae, poria, rhizoma atractylodis macrocephalae, radix glycythizae, rhizoma pinelliae, pericarpium citri reticulatae, radix Aucklandiae, and fructs amomi, total 400 g. Anticancer herbs contained pseudobulbus cremastrae seu pleiones, rhizoma smilacis glabrae, bulbus fritillariae thunbergii, and hedyotis diffusa, total 240 g. All were provided by the Bureau of Drugs, Department of Outpatient, Traditional Chinese Medicine Hospital of Henan Province, China. 200 mL drug solution was obtained through conventional boiling and then was concentrated to 85 mL, thus the concentration of the extracts was 2.2 g/mL. The extraction was packaged at 10 mL/bottle, deactivated at high pressure (0.1–0.15 KPa) for 15 min, and stored at 4°C.

Fetal bovine serum and phosphate buffer solution were purchased from Tianjin Haoyang Biological Manufacture Co., Ltd., Tianjin, China.

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Centrifuge machine, micro-medical imaging systems, microplate reader, plate washer, fluorescence microscopy, refrigerator, carbon dioxide incubator, inverted microscope, electric heat incubator, microscope, Beckman centrifuge tube, and low speed centrifuge machine were used in this study.

2.3. Methods

2.3.1. *In vitro* culture of HT29 cells

HT29 cell lines were cultured with RPMI 1640 medium containing 10% fetal calf serum in an incubator at 37 °C, 5% CO₂, and saturated humidity. The adherent cells at logarithmic growth phase were digested and prepared into 1×10⁴/mL suspension with fresh medium. Subsequently 100 μL cell suspension was added to each hole in the 96-well culture plate and incubated for 24 h at 37 °C, 5% CO₂. Afterwards the culture medium was replenished to serum-free medium, and the test serum was added to each hole (10 μL/hole), three holes at the same concentration. The control hole was added with 10 μL normal serum and the blank hole was added with 100 μL culture medium. All the cells were cultured for 72 h, and 100 μL MTT solution was added to each hole (5 mg/mL) and the cells were incubated for additional 4 h, each well was added with 100 μL lysate and stayed overnight. The absorbance value at 570 nm was measured with a automated microplate reader. T189 cells at logarithmic phase were obtained and prepared into cell suspension at 5.0 × 10⁴ cells/mL.

2.3.2. Establishment of xenograft model of HT29 colorectal cancer cells in nude mice

HT29 cells at logarithmic growth phase were adjusted to 1×10⁷/mL and then 0.1 mL suspension was transplanted into the armpit of mice, 1×10⁷ cells per mouse. The operations were performed under sterile conditions and tumor growth was observed daily. The tumor model was defined a success upon the appearance of 0.8 cm diameter of tumor nodule and hard tissue texture at 15 days after inoculation.

2.3.3. Grouping and treatment

The mice were divided into four groups: normal group, model group, Yiqi Jianpi treatment group, and Yiqi Jianpi plus anticancer treatment group. There were ten mice in each group. Normal group and model group were given intragastrical administration of saline for 14 days, while two treatment groups received Yiqi Jianpi decoction and Yiqi Jianpi decoction plus anticancer herbs respectively, for 14 days.

2.3.4. Index detection

The animal's diet, activity, fur, color, weight, and sweating were observed. The changes of animal's liver, kidney, and spleen after treatment were also detected. All mice were weighed.

At the end of the experiments, nude mice were sacrificed and the tumors were isolated, fixed in 10% formalin for 24 h, embedded in paraffin, and sliced. The obtained slices were stained with hematoxylin–eosin, and pathological changes

of colorectal cancer tissue were observed under light microscopy.

Protein kinase C (PKC) in spleen was detected using western blot analysis[2].

The colorectal cancer tissue in nude mice was incubated with protease K (20 mg/L) at 37 °C for 15–30 min after paraffin–embedding, dewaxing and hydration. Cell apoptosis was determined according to the instructions of TUNEL assay kit. In brief, the tissue was incubated with 50 μL reaction solution in a wet box at 37 °C in the dark, for 60 minutes, and then observed under a fluorescence microscope. Each slice was observed through more than five high–power fields, and the percentage of apoptotic cells was calculated. Percentage = number of positive cells / number of total cells × 100%[3]. The expression of epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) were detected according to the instructions of EGF and VEGF kits.

3. Results

3.1. PKC expression in the mouse spleen

There was significant difference in expression of PKC among different groups after treatment. As shown in Figure 1, the expression of PKC in spleen was the highest in normal group, then Yiqi Jianpi plus anticancer treatment group, Yiqi Jianpi treatment group and model group. Compared with the normal group, the expression of PKC was decreased in other three groups ($P < 0.01$). Compared with the model groups, the expression of PKC was significantly increased in normal group and Yiqi Jianpi plus anticancer treatment group ($P < 0.01$), and was slightly increased in Yiqi Jianpi treatment group, with significant difference ($P < 0.05$).

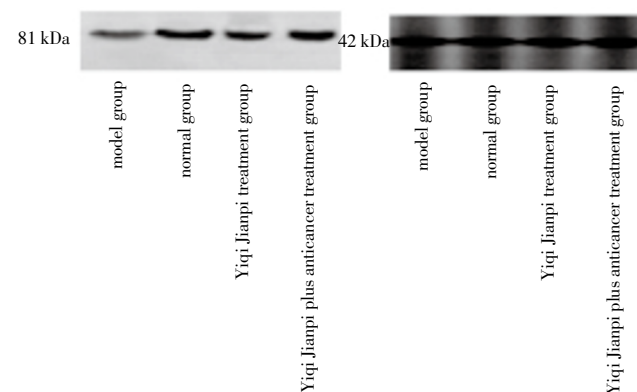


Figure 1. PKC expression in the spleen of rat from different groups. PKC molecular weight: 5.8 kDa; β-actin molecular weight: 42 kDa.

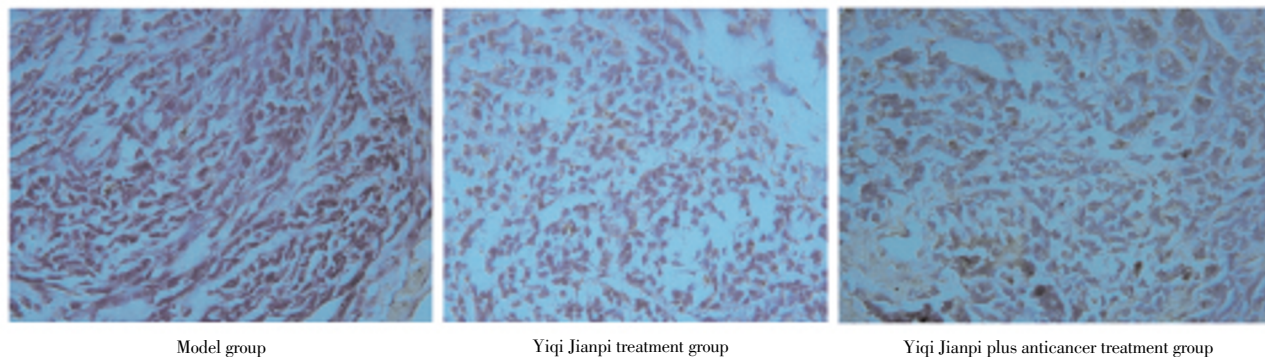
3.2. Detection of cell apoptosis

The cell apoptosis was detected with TUNEL assay after treatment. As shown in Table 2 and Figure 2, the number of apoptotic cells showed significant differences in Yiqi Jianpi plus anticancer treatment group and Yiqi Jianpi treatment group compared with the model group ($P < 0.01$). In addition,

Table 1

PKC expression among groups detected by western blot analysis.

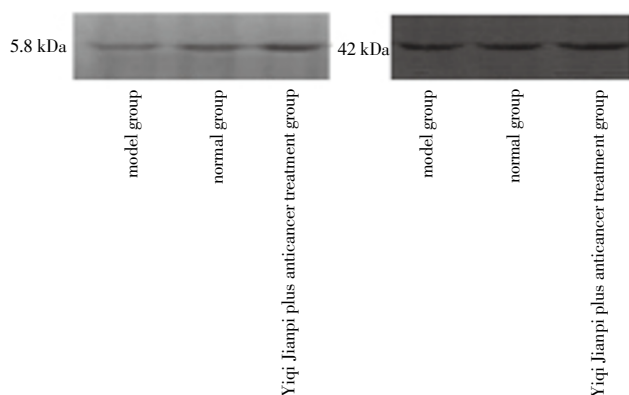
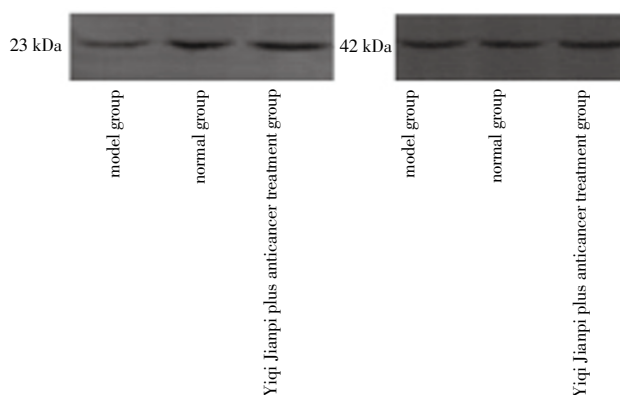
Group	PKC	HT29	EGF expression	VEGF expression
Normal group	0.567±0.011 [#]	–	–	–
Model group	0.422±0.008 [*]	0.60±0.70	0.574±0.020	0.596±0.014
Yiqi Jianpi treatment group	0.442±0.019 ^{##}	3.50±1.08 [*]	0.506±0.023 [*]	0.533±0.016 [*]
Yiqi Jianpi plus anticancer treatment group	0.480±0.014 ^{**}	8.70±1.34 ^{*#}	0.368±0.021 ^{*#}	0.419±0.012 ^{*#}

Note: [#] $P<0.01$, ^{##} $P<0.05$, vs. model group; ^{*} $P<0.01$, vs. control group.**Figure 2.** Apoptosis detected by TUNEL method (3,3'-diaminobenzidine chromogenic assay, × 400).

the number of apoptotic cells in Yiqi Jianpi plus anticancer treatment group was significantly higher than that in Yiqi Jianpi treatment group ($P<0.01$).

3.3. EGF and VEGF expression

After the apoptosis of tumor cells was detected, tumor tissue in model group, Yiqi Jianpi treatment group and Yiqi Jianpi plus anticancer treatment group was harvested to determine the EGF and VEGF expression. As shown in Table 1 and Figure 3, the EGF and VEGF expression reached a peak in the model group, which was significantly higher than that in Yiqi Jianpi treatment group and Yiqi Jianpi plus anticancer treatment group ($P<0.01$). In addition, Yiqi Jianpi treatment group showed significantly higher expression levels than Yiqi Jianpi plus anticancer treatment group ($P<0.05$).

**Figure 3.** EGF expression in HT29 tumor cells.EGF molecular weight: 5.8 kDa; β -actin molecular weight: 42 kDa.**Figure 4.** VEGF expression in HT29 tumor cells.VEGF factor molecular weight: 23 kDa; β -actin molecular weight: 42 kDa.

4. Discussion

Changes in PKC reflect the variation in the spleen and are positively correlated with splenic functions. Once the tumor forms, spleen PKC content sharply decreases, highlighting the impact of tumor on the function of the spleen^[4,5]. The present study found that the tumor had an apparent impact on the spleen and adverse effects on PKC, which underlines the tumor's damage on splenic function. After treatment of Yiqi Jianpi plus anticancer decoction, PKC expression began to increase. This indicates that the tumor-caused damage was decreased slightly. Although Yiqi Jianpi plus anticancer treatment showed an obvious clinical efficacy, the level was still lower than normal levels^[6–9].

The degree of cell apoptosis is an indicator of the efficacy of anti-tumor therapy. The present study found that there were a small number of apoptotic cells without drug intervention, while the number of apoptotic cells was increased after treatment. Yiqi Jianpi decoction alone could promote apoptosis, and Yiqi Jianpi plus anticancer decoction presents more pronounced effect^[10–12].

Both EGF and VEGF are the important factors that are conducive to the tumor growth. EGF is a stimulating factor of tumor growth, while VEGF functions to promote the growth of blood vessels and provide blood supply for tumor growth^[13–15]. In this study, the expression of EGF and VEGF was decreased after drug treatment, thus weakening tumor growth^[16]. Furthermore the inhibition effect was greatly improved after Yiqi Jianpi plus anticancer decoction, which was similar to the findings of cell apoptosis. Our experimental results indicate that Yiqi Jianpi plus anticancer herbs can promote the apoptosis of tumor cells and inhibit the contribution of EGF and VEGF, and its anti-tumor effect is more pronounced than Yiqi Jianpi decoction alone.

Tumor is prevalent to affect the functions of the spleen. Yiqi Jianpi treatment was shown to improve splenic function and Yiqi Jianpi plus anticancer treatment showed a stronger effect. Tumor also affects the growth of the spleen and may inhibit the expression of PKC in spleen, the inhibition effect could be reversed by Yiqi Jianpi plus anticancer treatment^[17,18]. Yiqi Jianpi plus anticancer treatment is superior to Yiqi Jianpi treatment alone due to it cannot only inhibit tumor growth, but also decrease the expression of EGF and VEGF. Therefore the combined treatment achieves a double therapeutic efficacy: nourishing the spleen and anti-cancer^[19]. In summary, we suggest the combination of Yiqi Jianpi plus anticancer herbs in the clinical practice, this remedy reflects the combination of “differential diagnosis of diseases” and “differential diagnosis of symptoms and signs” in traditional Chinese medicine treatment, and deserves further promotion.

Conflict of interest statement

We declare that we have no conflict of interest.

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