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Anti-cancer activity of Tonglian decoction against esophageal cancer cell proliferation through regulation of the cell cycle and PI3K/Akt signaling pathway

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Abstract *Objective:* The purpose of this study was to observe the anti-cancer activity of Tonglian decoction (TD) on esophageal cancer (EC) cells *in vitro*, and to elucidate the related molecular mechanisms in the cell cycle and PI3K/Akt signaling pathway.
Methods: EC9706 cells were cultured in RPMI 1640 medium supplemented with 10% calf serum at 37°C in a 5% CO_2 incubator. The cells were treated with rat serum containing TD or the serum of rats administered Xiaoaiping as a positive control drug. Cell proliferation was assessed by methylthiazolyldiphenyl-tetrazolium bromide assays. Cell morphology was observed under a microscope. The cell cycle was examined by flow cytometry. Protein expression in the PI3K/Akt signaling pathway was measured by western blotting.
Results: TD mainly inhibited cell proliferation. Concentrations of 50% cell inhibition by rat serum containing TD or Xiaoaiping were 73.6 and 153.8 μL/mL, respectively. TD also influenced cell morphology characterized by small shrunken cells. Cell colonies became small and the cell proliferation rate was slower. In cell cycle analysis, the percentage of cells in S phase was decreased significantly by TD and Xiaoaiping compared with the blank control group ($P < .05$). Western blotting showed that serum containing TD strongly down-regulated EGFR, PI3K, Akt, p-Akt, and mTOR expression compared with the blank control group ($P < .05$).

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Conclusion: TD could inhibit EC9706 carcinoma cell proliferation by blocking the cell cycle progression in S phase. The possible mechanism was inhibition of multiple targets in the PI3K/Akt signaling pathway by TD.

Introduction

Esophageal cancer (EC) was the fifth most commonly diagnosed cancer and the fourth leading cause of cancer-related death in China in 2009.[1] The average incidence of EC is 21.62/100,000 and mortality is 16.25/100,000 in 2011.[2] There is regional variation of EC incidence with the highest rates at the borders of Henan, Shanxi and Hebei provinces.[3] As an important complementary and alternative medicine, traditional Chinese medicine (TCM) has shown significant effects in treating this disease in recent years with few side effects.

A study has identified that the pathogenesis of blood stasis and stagnation of Qi have a close relationship with over-expression of proteins in the PI3K/Akt signaling pathway of EC9706 cells.[4] The Chinese medicinal formula Tonglian decoction (TD) has been approved as a National Invention Patent of China.[5] TD is applied to treat EC by activating the blood and promoting Qi.

This study focused on the anti-cancer activity of TD in EC. EC9706 cells were treated with rat serum containing TD and then cell proliferation, morphology and the cell cycle were examined. Protein expression of the PI3K/Akt signaling pathway was determined to elucidate the molecular mechanism of the anti-cancer effects.

Xiaoaiping tablet, which is widely used to treat EC in clinic, is a qualified drug approved by National Food and Drug Administration of China. The ingredients in Xiaoaiping are extracted from *Caulis marsdeniae tenacissimae*, a Chinese herbal plant growing in south-west of China. The plant contains anti-cancer substances which could eliminate cancer hormones and disrupt cancer cell proliferation or metastasis.[6] So Xiaoaiping was applied as a positive drug in this study to evaluate effect of TD on EC cells objectively.

Materials and methods

Experimental animals

Twenty-one male Sprague—Dawley (SD) rats (220 ± 20 g) were purchased from the National Institute for Food and Drug Control (Beijing, China). All animals were housed under specific pathogen-free conditions and had free access to water and standard rat feed. The experiment was approved by ethics committee of North China University of Science and Technology and the number of animal experimental ethical inspection form is 2014-023.

Cells and culture conditions

EC9706 human EC cells were a gift from the Tumor Institute, Beijing Union Medical College. EC9706 cells were cultured in RPMI 1640 medium supplemented with 10% calf serum (CS) at 37°C in a 5% CO_2 incubator.

Drugs and reagents

TD is comprised of six Chinese herbs: oldenlandia (*Oldenlandia diffusa* (Willd.) Roxb.) safflower (*Carthamus tinctorius* L.), peach kernel (*Prunus persica* (L.) Batsch), bugbane rhizome (*Cimicifuga foetida* L.), betel nut (*Areca catechu* L.), and bearded scutellaria (*Scutellaria barbata* D.Don) (dosage proportion, 3:3:1:1:1:2). All herbs were purchased from Tongrentang (Beijing, China). The pharmaceutical preparation section of the Affiliated Hospital of North China University of Science and Technology was responsible for preparation and quality control of the formula. Xiaoaiping tablets (lot No.: 110706) were purchased from Sanhome (Nanjing, China). RPMI 1640 medium and CS were purchased from Gibco (Grand Island, NY, USA). Trypsin was purchased from Cusabio (Wuhan, China). Methylthiazolyldiphenyl-tetrazolium bromide (MTT) and dimethyl sulfoxide were purchased from Amresco (Solon, OH, USA). RNaseA and propidium iodide (PI) were purchased from Sigma—Aldrich (St. Louis, MO, USA). The following antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA): anti-EGFR monoclonal antibody, anti-PI3K polyclonal antibody, anti-Akt monoclonal antibody, anti-p-Akt monoclonal antibody, and anti-mTOR monoclonal antibody.

Scientific apparati

The following scientific instrumentation were used in this study: laminar airflow workstation (model 1100; Thermo Fisher Forma, Waltham, MA, USA); ultrasonic processor Sonics and Materials (Vibra-Cell 750; Sonics and Materials, Newton, CT, USA); CO_2 incubator (model 3336; Thermo Fisher Forma); The inverted microscope (model Diaphot TMD; Nikon Instruments, Melville, NY, USA); The fluorescence microscope (model EF-D, Nikon); FACSCalibur flow cytometry system (model FACSCalibur, BD Biosciences, San Jose, CA, USA). The mini-slab electrophoresis system (model AE-6531 mPAGE, ATTO, Tokyo, Japan); enzyme immunoassay (EIA) microplate reader (model Elx800, Bio-Tek, Winooski, VT, USA).

Preparation of animal serum containing drugs

Using a random number table, 21 male SD rats were divided into blank control (BC), positive control drug (PC, Xiaoaiping tablets), and TD groups (n = 7 each). The clinical dosages of TD (raw powder) and Xiaoaiping were 58 and 9 g/d, respectively, which are 828 and 129 mg/kg per day according to the 70 kg standard adult weight. The scaled rat dosages were 75 mg/kg per day (TD) and 11.5 mg/kg per day (Xiaoaiping). Rats received gastric lavage with the drugs for 5 days continuously. The same volume of normal saline was administered to rats in the BC group by lavage. Rats were maintained under nesteia for 12 h before the last lavage. After 12 h, pentobarbital sodium (1.5 mL/kg) was applied for intraperitoneal anesthesia, and blood was sampled through the aorta abdominalis. Serum was isolated and inactivated for 30 min at 56°C in a water bath. Serum was sterilized by passing through a 0.22 μm millipore filter and then stored at −80°C.

MTT assay

EC9706 cells were seeded on 96-well plates at a concentration of 1×10^4 cells/well in complete culture medium and allowed to adhere for 24 h. The adherent cells were incubated in the presence of various amounts of serum (0%, 2%, 4%, 6%, 8% and 10%) for 48 h and then subjected to MTT assays. The optical density (OD) of the treated samples in comparison with the BC group was measured with the EIA photometer. The wavelength for measuring the absorbance of the formazan product was 570 nm. The experiment was repeated three times. A fitting curve of dose-effect dependence was constructed and the median effective dose (IC_{50}) was calculated by SPSS 18.0 software (SPSS, Chicago, IL, USA) according to the following formula[7]:

Inhibition rate of EC9706 cells by serum = (1−OD of medicinal groups/OD of control group) × 100%.

Observation of cell morphology by microscopy

EC9706 cells were seeded in 100 mm dishes at a concentration of 1×10^6 cells/dish in 10 mL culture medium/dish and incubated at 37°C in a 5% CO_2 incubator for 24 h. Serum containing drugs (IC_{50} concentration) was then added to medicinal groups, while the BC group was maintained in RPMI 1640 medium containing 10% normal rat serum for 48 h. Cell growth and morphological changes were observed under an inverted microscope.[8]

Cell cycle analysis by flow cytometry

Cells were seeded in 100 mm dishes at a concentration of 1×10^6 cells/dish in 10 mL culture medium/dish and incubated at 37°C in a 5% CO_2 incubator for 24 h. Serum containing drugs (IC50 concentration) was added to the culture medium, and the cells were cultured for another 24 h. The cells were collected and bathed in 70% ethanol/PBS. The cells were washed and then treated with 10 μL, 10 mg/mL RNaseA, 1% Triton-100, and then 1 mg/mL PI in turn for 5 min each. The cells were filtered through a 400 μm mesh prior to flow cytometry. Cell Quest Pro and Modifit LT software were used to analyze the cell cycle. The percentage was calculated in each cell cycle phase.[9]

Determination of protein expression in the PI3K/Akt signaling pathway by western blotting

Cells were seeded in 100 mm dishes at a concentration of 1×10^6 cells/dish in 10 mL culture medium/dish and cultured at 37°C in a 5% CO_2 incubator. Serum with or without drugs (IC_{50} concentration) was added to the culture medium, and the cells were cultured for 24 h. The cells were collected, lysed by ultrasound, and then centrifuged at 1676 g for 20 min. The supernatant was collected and the protein concentration was determined by the Bradford method.[10] Samples were stored at −20°C until analysis.

Proteins samples were subjected to 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Electrotransfer was conducted for 8 h. Ponceau was used for staining. Membranes were blocked and antigen−antibody reactions were conducted for 4 h. Then the membranes were developed by enhanced chemiluminescence and exposed to X-ray film.[11] Image J software (National Institutes of Health, USA) was used to analyze the grey values.

Statistical analysis

Continuous variables are expressed as the mean ± standard deviation. All samples were tested to determine whether they followed homogeneity of variance by one-way analysis of variance. Assuming the data had a normal distribution, least significant difference (LSD) was applied to compare differences between two groups. If data had an abnormal distribution, Dunnett's T3 was used. A value of $P < .05$ was regarded as statistically significant. SPSS 18.0 software was used for statistical analysis.

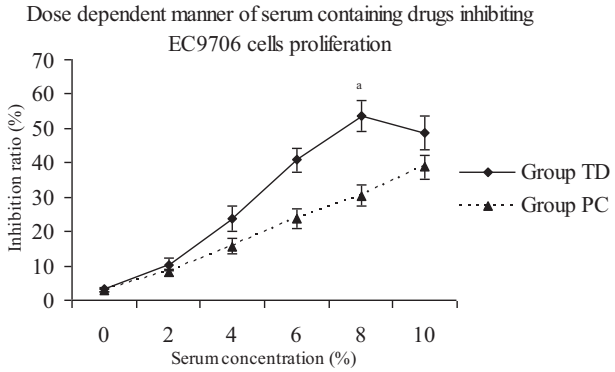

Fig. 1 Dose-dependent effect of rat serum containing drugs on inhibition of EC9706 cell proliferation. EC9706 human EC cells were seeded in 96-well plates and treated with various concentrations of TD and Xiaoaiping for 48 h. Three samples were tested for each treatment group. Data are expressed as the mean ± standard error of the mean and were analyzed by the paired *t*-test. The experiment was repeated three times. [a]$P < .05$ versus the PC group.

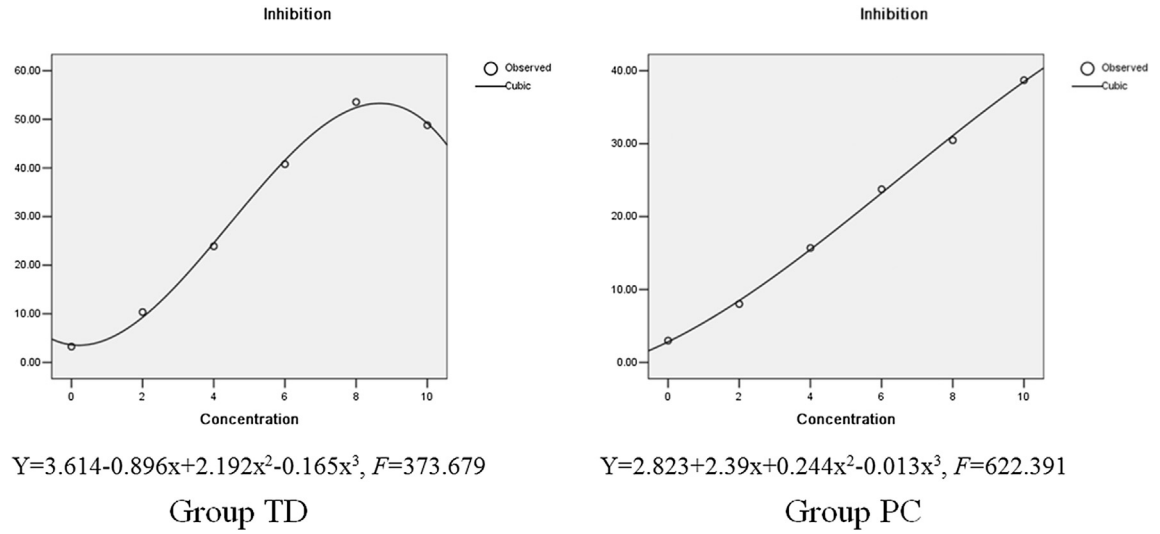

Fig. 2 Fitting curve of the dose-dependence of EC9706 cell inhibition by rat serum containing drugs. Fitting curves were constructed with SPSS 18.0 software, *F*-test. Dots indicate the actual values. The curves were fitted according to the actual values. A different formula was used in SPSS, to estimate the approximate IC_{50} values.

Results

Drugs sensitivity of EC9706 cells

Administration of serum containing drugs inhibited cell proliferation in a dose-dependent manner. The effect of TD was much stronger than that of Xiaoaiping. There was a significant difference between the two groups in terms of the inhibition ratio ($P < .05$). Within the test concentrations, serum containing TD had a peak value at 8%. Conversely, the IC_{50} of the PC group was outside of the test concentrations. The *F*-test fitting curve showed that the IC_{50} concentration of serum containing TD was 73.6 μL/mL, and the IC_{50} concentration of the PC group was 153.8 μL/mL (Figs. 1 and 2).

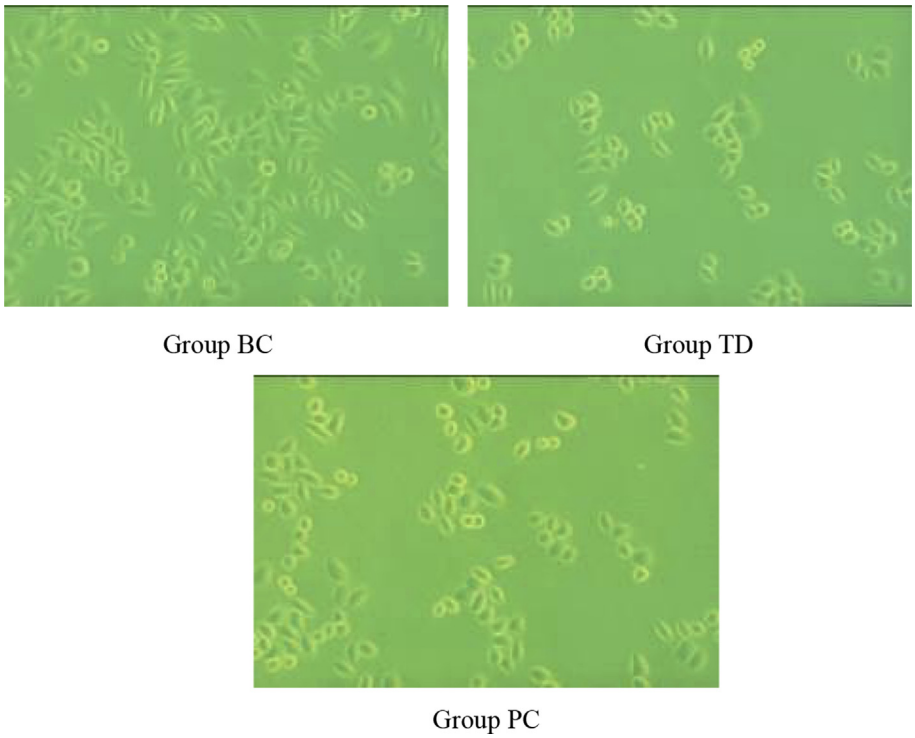

Fig. 3 Growth and morphology of cells treated with rat serum containing drugs. Samples were observed in five fields of vision at ×400 magnification. Drug dosages were as follows: normal serum concentration of 100 μL/mL in the BC group, IC_{50} serum concentration of 73.6 μL/mL in the TD group, and IC_{50} serum concentration of 153.8 μL/mL in the PC group.

Growth and morphology of EC9706 cells treated with rat serum containing drugs

Cell density in the BC group was much higher than that in the TD group. Cells proliferated rapidly with close connections in an adherent state and maintained their spindle-like shape. Cells in the TD group appeared to be small and shrunken. Cell colonies were small and the proliferation rate was low. Cells reduced sharply and vacuole material emerged intracellularly. In the PC group, cell morphology was similar to that in the TD group, but the cell number was much higher than that in the TD group, which was accompanied by close connections between cells (Fig. 3).

Cell cycle analysis

The percentages of cells in S phase of medicinal groups were 12.82% (TD group) and 15.26% (PC group), which was much lower than that in the BC group (Fig. 4). As shown in Fig. 5, there was a significant difference between medicinal groups and the BC group ($P < .05$). However, the percentages of cells showed no difference between medicinal groups (Fig. 5).

Expression of proteins in the PI3K/Akt signaling pathway

Western blotting was used to measure EGFR, PI3K, Akt, p-Akt, and mTOR levels. The TD group showed significant inhibition of the protein expression of the PI3K/Akt signaling pathway. Compared with the BC group, protein expression in medicinal groups was significantly different (Fig. 6). In semi-quantitative analysis with Image J software, grey values of the five proteins in the TD group were much lower than those in the BC group ($P < .05$) (Table 1).

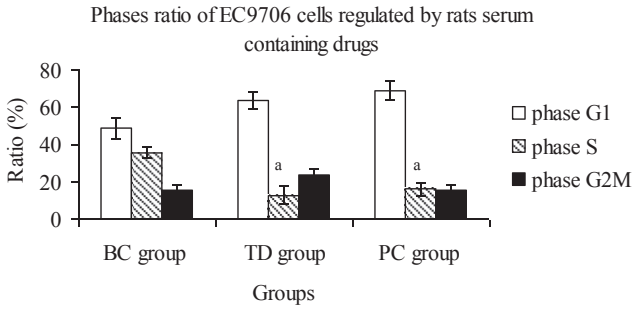

Fig. 5 Comparison of the ratio of EC9706 cells treated with rat serum containing drugs in cell cycle phases. Three samples were analyzed for each group. Drug dosages were as follows: normal serum concentration of 100 μL/mL in the BC group, IC50 serum concentration of 73.6 μL/mL in the TD group, and IC50 serum concentration of 153.8 μL/mL in the PC group. Data are expressed as mean ± standard error of mean and analyzed by the LSD test. $^{a}P < .05$ versus the BC group.

Discussion

TCM theory states that blood stagnation is important in the pathogenesis of cancers.[12] When cancers grow, blood stagnation worsens and blocks Qi circulation. In ancient China, many practitioners attached importance to blood stasis and Qi stagnation in terms of cancer. TD, which has been approved as a National Invention Patent of China,

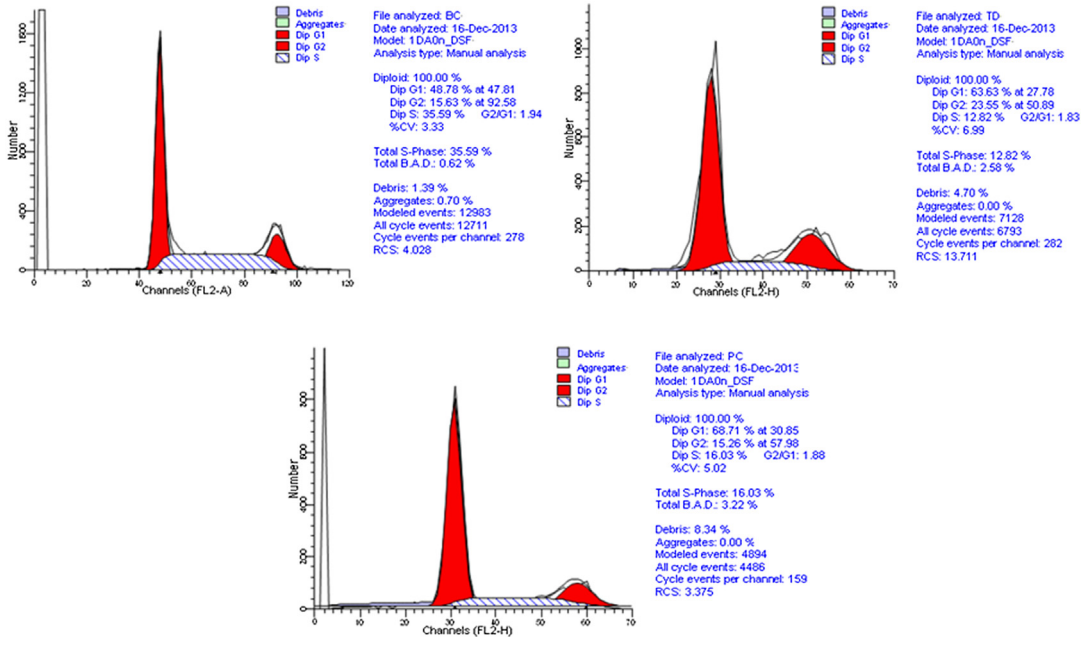

Fig. 4 Cell cycle distribution of EC9706 cells treated with rat serum containing drugs. A total of 50,000 events were recorded for each group. Three samples were observed for each group. Drug dosages were as follows: normal serum concentration of 100 μL/mL in the BC group, IC50 serum concentration of 73.6 μL/mL in the TD group, and IC50 serum concentration of 153.8 μL/mL in the PC group.

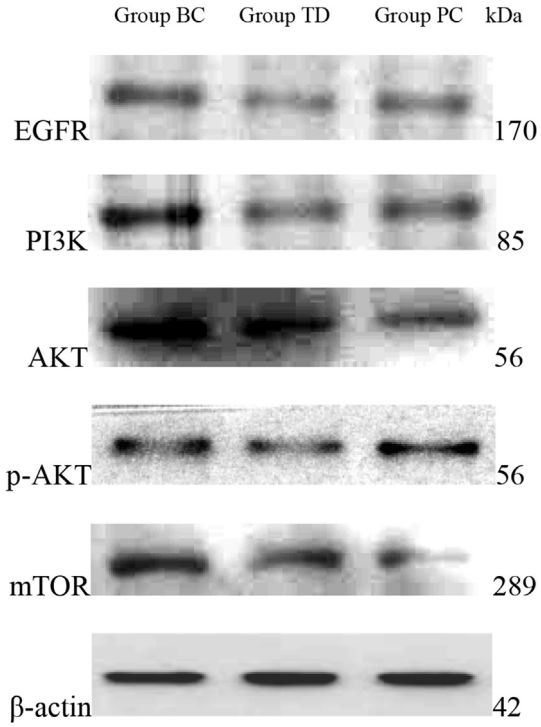

Fig. 6 Protein expression of the PI3K/Akt signaling pathway in cells treated with rat serum containing drugs. Drug dosages were as follows: normal serum concentration of 100 μL/mL in the BC group, IC50 serum concentration of 73.6 μL/mL in the TD group, and IC50 serum concentration of 153.8 μL/mL in the PC group.

originated from TCM theory in terms of the pathogenesis of EC influenced by blood and Qi stagnation.

There are six kinds of herbs in the TD formula. *Heterobathmia diffusa* and *S. barbata* are used to clear away heat and detoxification, removing stagnation and toxicity in the body. Areca nut tends to decease and move stagnation of Qi together with *Cimicifuga* that elevates nutrients and decreases turbidity in the body. Both facilitate Qi in the spleen and stomach. *C. tinctorius* and *Semen Persicae* act together. Each strengthens the other to activate blood and promote Qi. The formula works in two aspects: removal of pathogenic factors and cultivation of healthy Qi.

This study focused on the influence TD on EC9706 cell behavior including cell proliferation, morphology, the cell cycle, and PI3K/Akt signaling pathway to determine whether TD might affect EC onset and development. Xiaoaiping, which has been approved as a National qualified pharmaceutic, served as positive control drug. It is an extract from *Marsdenia tenacissima*, an herb that grows in South West China.

Different concentrations of rat serum containing drugs led to different degrees of EC9706 cell inhibition. As shown in Fig. 1, with increasing concentrations of serum, the inhibition rates increased correspondingly in a direct manner. Serum containing TD had a much stronger effect than serum containing Xiaoaiping with a IC_{50} value of only 73.6 μL/mL. Morphological observations supported the results of MTT assays, revealing fewer cells in the TD group, which were small and shrunken. In contrast, the cell density in the BC group was much higher at 70% confluence. Cells maintained their spindle-like appearance with close connections. These observations indicated that rat serum containing TD provided an inhibitive environment for cell growth.

The cell cycle refers to the process of mitosis.[13] A typical cell cycle has four phases, G1, S, G2, and M, which are controlled by signal transduction and a feedback loop. Abnormal modulation of the cell cycle is a major mechanism of carcinogenesis.[14] A malignant tumor cell cycle is uncontrolled and will hinder cell differentiation while driving excessive proliferation.[15] The proportion of cells in S, G2 and M phases reflects active proliferation, which, to some extent, indicates the condition of cell proliferation.[16] Cells in G1 phase indicate suppression of cell proliferation. After application of TD, the distribution of cells in G1, S, G2 and M phases changed compared with the control group. As shown in Figs. 4 and 5, there were fewer cells in S phase after application of TD and Xiaoaiping compared with the BC group, indicating that TD inhibited EC9706 cell proliferation by blocking carcinoma cells from entering S, G2 and M phases.

PI3K/Akt signaling pathway can regulate cellular processes including cell cycle progression, cell survival and migration, and protein synthesis. Owing to kinds of extracellular growth factors up-regulating, PI3K/Akt signaling pathway will be activated, which is one of reasons leading to occurrence and development of human cancer.[17,18] Activation of the PI3K/Akt pathway has been reported to be conducive to EC cells survival *in vitro* as well as *in vivo*.[19,20] What's more, it has been proved that cell cycle-related proteins (such as cyclin D1 and p27) were regulated by the PI3K/Akt pathway in human EC cells.[21] Protein expression of PI3K/Akt pathway has close relation to cell cycle, which is a possible mechanism to elucidate change of cell cycle in EC cancer.

Western blotting showed that rat serum containing TD down-regulated protein expression in PI3K/Akt signaling pathway of EC9706 cells, including EGFR, PI3K, Akt, p-Akt,

Table 1 Relative grey values of proteins ($\bar{X} \pm s$).

	EGFR/β-actin	PI3K/β-actin	Akt/β-actin	p-Akt/β-actin	mTOR/β-actin
Group BC	1.534 ± 0.336	2.340 ± 0.409	2.252 ± 0.587	0.939 ± 0.245	1.233 ± 0.465
Group TD	0.785 ± 0.216[a]	1.102 ± 0.350[a]	1.166 ± 0.471[a]	0.433 ± 0.134[a]	0.944 ± 0.238
Group PC	0.859 ± 0.302[a]	1.327 ± 0.571[a]	0.597 ± 0.189[a]	1.007 ± 0.346	0.436 ± 0.178[a]

Grey values were estimated by Image J software. Three samples were analyzed for each group. Data are expressed as mean ± standard error of mean and analyzed by the LSD test.

[a] $P < .05$ versus the BC.

and mTOR. This indicated that the specific mechanism of the anti-EC effects of TD involved multiple targets. Semi-quantitative analysis indicated that TD and Xiaoaiping had significant inhibitory effects on protein expression compared with the BC group, ($P < .05$), which inhibited EC cell growth.

Conclusion

Based on the results, TD inhibits EC cell proliferation *in vitro*. The mechanism is associated with regulation of the cell cycle through the PI3K/Akt signaling pathway. However, these are only *in vitro* results and TD should be tested *in vivo* and in a clinical trial.

Funding

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Conflict of interest

No conflict of interests to declare.

Acknowledgments

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