RESEARCH ARTICLE

Tanshinone II-A Inhibits Angiogenesis through Down Regulation of COX-2 in Human Colorectal Cancer

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Abstract

Angiogenesis plays a significant role in colorectal cancer (CRC) and cyclooxygenase-2 (COX-2) appears to be involved with multiple aspects of CRC angiogenesis. Our aim was to investigate the inhibitory effects of Tan II-A (Tanshinone II-A, Tan II-A) on tumor growth in mice, as well as alteration of expression of COX-2 and VEGF in CRC. We established the mice xenograft model of C26 CRC cell line, and injected 0.5, 1, 2mg/kg of Tan II-A and 1mg/kg of 5-FU in respectively in vivo. Then, we assayed tumor weight and volume, and evaluated microvascular density and expression of VEGF. COX-2 promoter and COX-2 plasmids were transfected into HCT-116 cells, followed by detection of COX-2 promoter activity by chemiluminescence, and detection of COX-2 mRNA expression by fluorescence quantitative PCR. Taken together, the results showed Tan II-A could inhibit tumor growth and suppress the VEGF level in vivo. HCT-116 cell experiments showed marked inhibitory effects of Tan II-A on COX-2 and VEGF in a dose-dependent manner. The results indicate that Tan II-A can effectively inhibit tumor growth and angiogenesis of human colorectal cancer via inhibiting the expression level of COX-2 and VEGF.

Keywords: Tanshinone II-A - colorectal carcinoma - angiogenesis - cyclooxygenase 2 - vascular endothelial growth factor

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Introduction

Colorectal carcinoma (CRC) is the third most common and life-threatening malignant tumors in the world and has been rising over the years (Jemal et al., 2011). As one of the six hallmarks of carcinogenesis, micro-angiogenesis of tumor plays a significant role in carcinogenesis, development, invasion, as well as metastasis of the tumor (Arentz et al., 2011).

Cyclooxygenase-2 (COX-2) is involved with multiple aspects of tumorigenesis of CRC. Detection of COX-2 activity in tumor tissue or tumor cell can be used as an important method to monitor carcinogenesis and development of CRC; moreover, the expression of intracellular COX-2 level can serve as a significant marker for anti-tumor drug screening.

Tanshinone II-A (Tan II-A) is an effective constituent of Salvia Miltiorhizza – a traditional Chinese herb. Tan II-A is originally used for cardiovascular diseases, and recent studies have revealed a marked anti-tumor effect; the mechanism of which involves inhibition of cell proliferation (Chen et al., 2012), promotion of cell apoptosis (Dai et al., 2012), induction of cell differentiation (Wang et al., 2007; Zhang et al., 2010), and so forth. Further research is still required to determine whether the inhibitory effect of tumor angiogenesis is related to down-regulation of COX-2 expression.

Our study established the mouse xenograft model of C26 colorectal carcinoma cell line to evaluate the anti-angiogenesis effects of Tan II-A on CRC, as well as on the alteration of vascular endothelial growth factor (VEGF) expression and microvascular density. Using transfected luciferase reporter plasmid of COX-2 promoter in human CRC cells, we investigated the underlining mechanism.

Materials and Methods

Experimental animals

All animal protocols were approved by the Institutional Animal Use and Care Committee. A total of 48 BABL/c F1 mice, male, 18 ± 2 g, clean grade, were purchased from Shanghai Slac Laboratory Animal Co. Ltd (Shanghai, China, number of animal license SCXK (Shanghai) 2008-0016), and were fed in the experimental animal room in Putuo Hospital affiliated to Shanghai University of Traditional Chinese Medicine (Shanghai, China, number of animal laboratory license, SCHK (Shanghai) 2007-0005).

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Cell lines

Mouse C26 colorectal carcinoma cell line purchased from Bogoo Biotech Co., Ltd., Shanghai, China. human HCT-116 colorectal carcinoma cell line (purchased from Shanghai Institutes for Biological Sciences); the cells were cultured in RPMI 1640 culture media with 100 mL/L fetal bovine serum, 1×10^5 U/L penicillin and streptomycin. The culture media was placed into the incubator for routine subculture under 37 °C and 5% CO_2.

Drugs

Tan II-A, purity >98%, purchased from Xi'an Guanyu Biotechnology Co., Ltd; serial number: 20070621; 5-Fluorouracil (5-FU), manufactured by Shanghai Xudong Haipu Pharmaceutical Co., Ltd; serial number: 20070802.

Establishment of mice xenograft model, grouping and drug administration

The mice were given subaxillary inoculation with exponential-phase C26 cells (5×10^6 per mouse); the tumor was developed in 7 to 10 days, and was dissected and shredded into 1 mm^3 pieces thereafter, followed by subaxillary inoculation; massive subaxillary inoculation was accomplished after three passages. After the tumor grew into 50 mm^3 approximately in size, the mice were randomized into 5 groups with 8 in each(another 8 mice without xenograft as blank group); the groups were labeled as model group, low, medium and high-dose Tan II-A group, and 5-FU group respectively; the Tan II-A groups were administered with 0.5, 1 and 2 mg (kg · d) of Tanshinone II-A respectively; the 5-FU group was administered with 1mg (kg · d) of 5-Flurouracil; the blank group and model group were administered with equal volume of saline. The injections were made through tail vein, and carried out consecutively for 1 week.

Calculation of tumor inhibition rate

After 7 days of treatment, blood sample was collected. The mice were then sacrificed by cervical dislocation, followed by dissection of the tumor and exclusion of connective tissue; the length, width and weight of the tumor were thereby evaluated. The tumor inhibition rate was calculated according to the change of volume and weight of the tumor between the model group and the administration groups. Tumor volume (TV) = $1/2 \cdot a \cdot b^2$ (9), in which a and b represented the length and width of the tumor respectively; tumor volume inhibition rate = (1 – mean tumor volume of experimental group / mean tumor volume of control group) · 100%; tumor weight inhibition rate = (1 – mean tumor weight of experimental group/mean tumor weight of control group) · 100%.

Immunohistochemical staining

Paraffin blocks of the aforementioned samples were collected to make 4 μm of consecutive slices, followed by de-waxing, gradient alcohol dehydration, removal of peroxidase, antigen retrieval, antigen blocking, antibody binding, DAB staining, rinsing in distilled water, followed by hematoxylin staining, hydrochloric acid/alcohol differentiation, bluing with dilute aqueous ammonia, dehydration with increasing gradient of alcohol, vitrification by dimethylbenzene, and regular resin sealing. Primary antibody: 1: 50 goat anti-mouse CD34 monoclonal antibody; secondary antibody was 1: 200 biotin-labeled, and 1: 200 HRP-labeled streptoavidin was added in coloring; the three reagents were diluted with phosphate buffer. Then, microvessels stained brown by CD34 were counted by microscope (200× magnification, unrepeated field); the mean value was applied as MVD.

Evaluation of VEGF expression with ELISA

Mice serum of normal, model and administration group extracted from orbital venous plexus, as well as HCT-116 cell surfactant transfected by pIRESI-COX-2 or processed for 48 hours, was evaluated following protocols of the ELISA kits, to determine the expression of VEGF.

Evaluation of drug sensitivity of cells with CCK-8

HCT-116 cells under routine culture were added to 200μL of culture media containing Tan II-A, the concentration of which were 2, 4, 8, 16, 32 and 64 μmol/L, respectively (10 wells for each concentration); 20 μL of CCK-8 was added in each well after 24, 48 and 72 hours of culture. After additional 4-hour cultivation, cells in different wells were placed on the enzyme-labeling instrument to evaluate the absorption value. The growth inhibition rate (GIR) of Tan II-A on HCT-116 cells was calculated according to the following formula: GIR = $[1- (OD_n - OD_0) / (OD_c - OD_0)] \cdot 100\%$, in which OD0 as absorption value of blank group, ODc as absorption value of control group, and ODn as absorption value of different administration groups; IC_{50} of Tan II-A was thereby determined.

Transfection of plasmid and evaluation of luciferase activity

HCT-116 cells were cultured on 24-well plate, within each well 750ng of pGL3-Basic-COX-2-promoter (or pGL3-Basic) and 50ng of pRL-TK plasmid DNA were transfected with Lipofectamine™ 2000 Transfection Reagent. The non-transfected cells were used as blank group; the control group was transfected with complete medium being added, as well as 4, 8, 12, 16 and 20 μmol/L of Tan II-A culture, respectively (3 wells for each concentration). After 48 hours, the activity of firefly luciferase and renilla luciferase was evaluated with the Dual Luciferase Assay System kit (Promega, United States).

RNA extraction and Real Time PCR

Cells were trypsinized, counted, and re-planted in 60mm Petri dishes at 1×10^6 per diffsh. The cells were then

Table 1. Tumor Weight, Volume and Tumor Inhibition Rate in Different Groups

Group	n	Tumor Weight(g)	Inhibition Rate of Weight (%)	Tumor Volume (cm^3)	Inhibition Rate of Size (%)
Model	8	0.73 ± 0.22	— —	1.00 ± 0.17	— —
Tan II-A-L	8	0.40 ± 0.22	45.8	0.50 ± 0.30	50.5
Tan II-A-M	8	0.29 ± 0.09	60.3	0.40 ± 0.12	60.7
Tan II-A-H	8	0.11 ± 0.06	84.5	0.16 ±0.09	84.2
5-FU	8	0.36 ± 0.17	50.3	0.47 ± 0.18	53.1

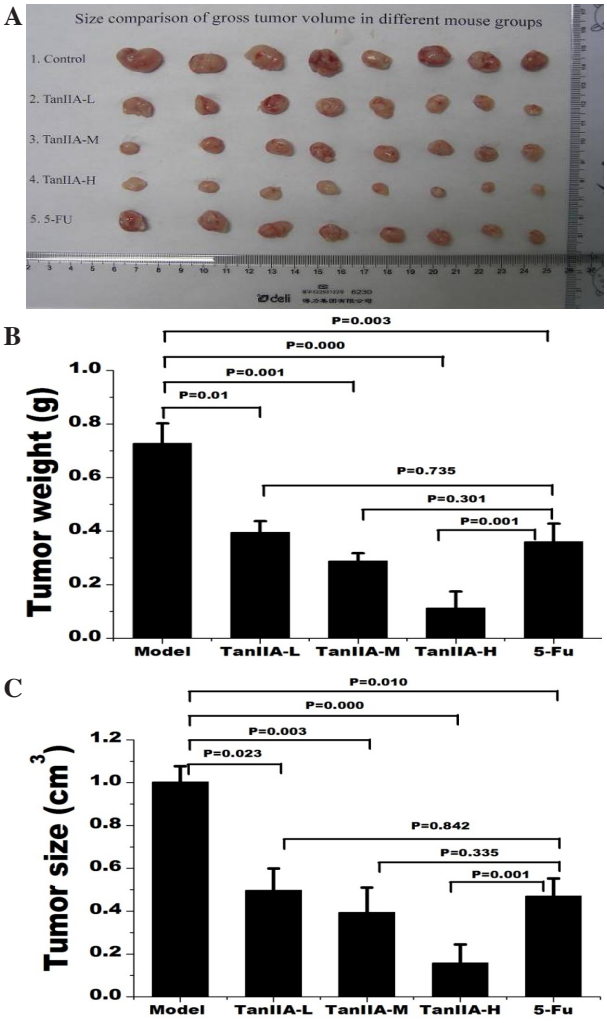

Figure 1. Inhibitory Effect of Tan II-A on Tumor Growth of Mice CRC. A. Comparison of tumor size in different groups of C26 CRC xenograft in mice after administration; tumor size of administration groups was lower than model group. B. Comparison of tumor weight in different groups; low, medium and high-dose Tan II-A groups were (0.40 ± 0.22) g, (0.29 ± 0.09) g, (0.11 ± 0.06) g, respectively. C. Comparison of tumor volume in different groups; low, medium and high-dose Tan II-A groups were (0.50 ± 0.30) cm3, (0.40 ± 0.12) cm3, (0.16 ± 0.09) cm3, respectively

randomly divided into 6 groups: control group (with blank culture medium), NS-398 group (with 10 μmol/L of NS-398), Tan II-A group (with 10 μmol/L of Tan II-A), COX-2 group (with 2 μg of pIRESI-COX-2 plasmid), COX-2 and NS-398 group (with 2 μg of pIRESI-COX-2 plasmid and 10 μmol/L of NS-398), and COX-2 / Tan II-A group (with 2 μg of pIRESI-COX-2 plasmid and 10 μmol/L of Tan II-A). Cells were harvested in the Trizol Reagent (TaKaRa Biotechnology (Dalian) Co., Ltd. China), and total RNA was isolated following the manufacturer's instructions. Reverse transcription was carried out using PrimeScriptTM RT-PCR Kit (TaKaRa Biotechnology (Dalian) Co., Ltd. China) reverse transcription reagents. The sequences of primers used for Real Time PCR amplification were as follows: human COX-2 forward: 5'-GAATCATTCACCAGGCAAATTG-3', and reverse: 5'-TCTGTACTGCGGGTGGAACA-3'; the TaqMan probe selected between the primers was fluorescent-labeled at the 5' end with 6-carboxyfluorescein (FAM) as the reporter

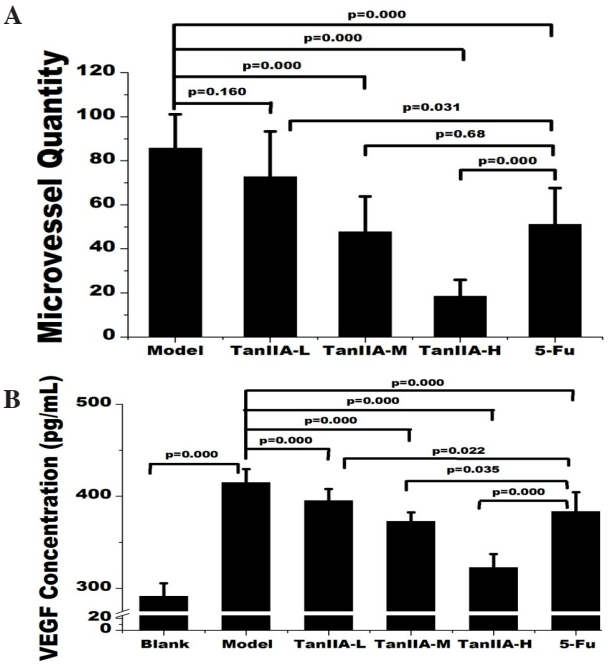

Figure 2. Inhibitory Effect of Tan II-A on Vasculogenesis of CRC. A. Mean microvascular count was calculated according to the result of different groups; microvascular density of model group was the highest, and low, medium and high-dose of Tan II-A decrease accordingly; a certain degree of dose-response relationship was observed; high-dose Tan II-A group had better effect than 5-FU group. B. VEGF expression in xenograft model groups were markly higher than blank group; serum VEGF expression in administration groups were lower than model group, in which high-dose Tan II-A group showed better result

dye and at the 3' end with 6-carboxytetramethylrhodamine (TAMRA) as the quencher; 5'-FAM-TGGCAGGGTTGCTGGTGGTAGGA-3-TAMRA-3' (Shine Gene, Shanghai, China; GenBank No. AY462100); GAPDH forward: 5'-CCACTCCTCCACCTTTGAC-3', and reverse: 5'-ACCCTGTTGCTGTAGCCA-3'; TaqMan probe: 5'-FAM-TTGCCCTCAACGACCACTTTGTC-3' (Shine Gene, Shanghai, China; GenBank No. AF261085). ABI PRISM 7300 SDS was applied to quantify mRNA level; data presented were representative of at least three independent experiments.

Statistics and analysis

Results were expressed as mean ± SD. Single factor analysis of variance was performed to compare multi-sample mean; paired comparison in different groups was performed with Student-Newman-KeμLs test; rank data were presented as rank data frequency; data analysis was performed with rank and test of multi-sample comparison; paired comparison in different groups was performed with Wilcoxon test. The P value was set at less than 0.05 for a statistical significance.

Results

Tan II-A inhibits mouse CRC in vivo tumor growth

To investigate the biological effect of Tan II-A on CRC, we used the mouse xenograft model of colorectal carcinoma cell line C26. Tan II-A was administered in different concentration through tail vein with conventional, single

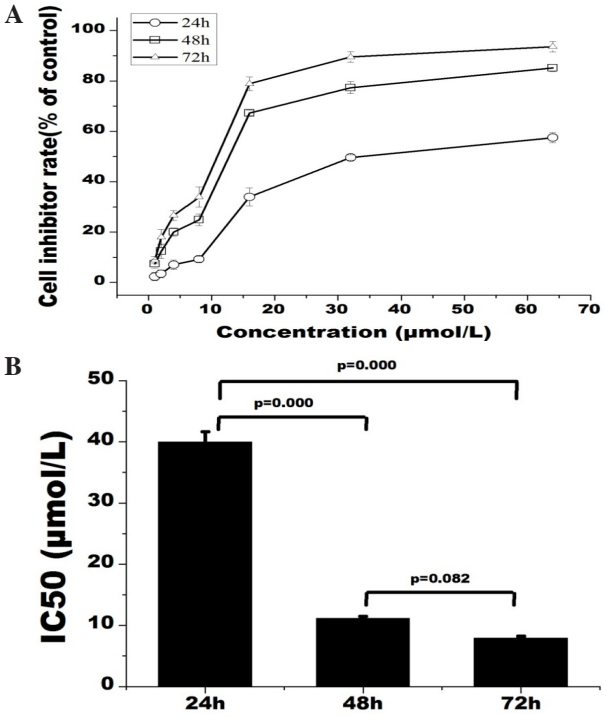

Figure 3. Inhibitory Effect of Tan II-A on HCT-116 Cell Growth. A. A marked inhibitory effect of Tan II-A on human HCT-116 CRC cells, with dose-response relationship. B. The inhibitory concentration 50% (IC50) of Tan II-A on human HCT-116 CRC cells in 24, 48 and 72-hour culture was (40.3 ± 5.22) μmol/L, (12.9 ± 3.24) μmol/L and (8.5 ± 1.47) μmol/L; the inhibitory effect in 48-hour showed significant difference compared with 24-hour culture (P = 0.000), but no significant difference was observed compared with 72-hour culture (P = 0.082)

chemotherapy reagent 5-FU as positive control on tumor reduction. Our data showed that, comparing to control group, the low, medium and high doses of Tan II-A induced significant 45.8%, 60.3%, 84.5% and 50.5%, 60.7%, 84.2% inhibition on tumor weight and tumor volume, respectively (P < 0.01, Figure 1A, 1B and 1C). Moreover, high dose of Tan II-A demonstrated a superior tumor inhibition potential over 5-FU (Figure 1B, 1C).

Tan II-A inhibits tumor angiogenesis and VEGF levels in CRC mice

To investigate effect of Tan II-A on micro-angiogenesis of mouse CRC tumors, we evaluated the protein levels of CD34, a well-used marker for angiogenesis, by immunohistochemistry. Microvascular density of the tumor was calculated under light microscope. Our results indicated that Tan II-A inhibited micro-angiogenesis of tumor tissue with a dose-dependent pattern (Figure 2A, 2B).

In the meantime, we evaluated the levels of VEGF, the soluble factor responsible for new blood vessel formation, in serum. ELISA data pointed out that, comparing with control, normal animals, VEGF protein in CRC tumor carrying mice was markedly elevated (P = 0.000, Figure 2C). Importantly, Tan II-A treatment significantly repressed the tumor-enhanced VEGF level by 15.8%, 34.2% and 74.8%, respectively, by low, medium and high doses of the drug (P = 0.001, Figure 2C).

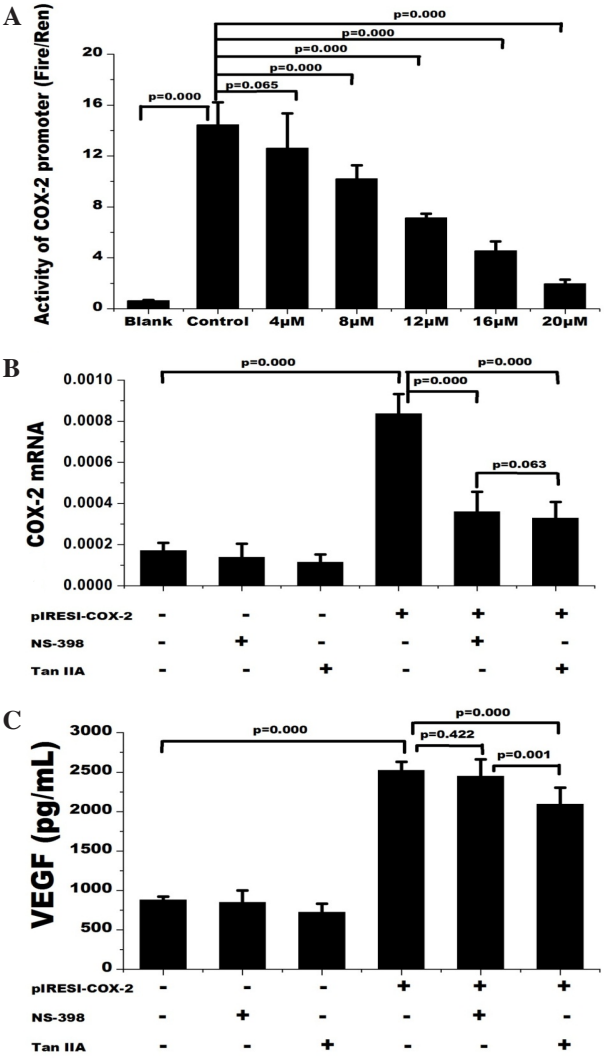

Figure 4. Regulatory Effect of Tan II-A on COX-2 in Inhibiting VEGF Expression of HCT-116 Cells. A. Cell luciferase activity upregulated by COX-2 promoter-transfected plasmid can be inhibited by Tan II-A, with dose-response relationship; relative activity decreased 7.25 fold from control group to highest-dose Tan II-A group, in which groups with Tan II-A concentration over 4μmol/L showed significant difference compared with control group (P = 0.000). B. COX-2 mRNA transcription activity was markedly elevated following transfection of pIRESI-COX-2 plasmid in HCT-116 cells; after processing with 10μmol/L of Tan II-A, COX-2 mRNA level was markedly inhibited, with no significant difference compared with positive control group (P = 0.063). C. Intracellular VEGF expression decreased as Tan II-A was being added; statistical significance was observed compared with control group (P = 0.000); the inhibitory effect of 10μmol/L Tan II-A on VEGF expression showed significant difference compared with NS-398 group (P = 0.001)

Tan II-A inhibits human CRC HCT-116 cell line in vitro growth

We next tested effect of Tan II-A on the in vitro growth of human HCT-116 CRC cells. Our data showed that the IC50 (The inhibitory concentration 50%) for Tan II-A were (40.3 ± 5.22) μmol/L (24-hour treatment), (12.9 ± 3.24) μmol/L (48-hour), (8.5 ± 1.47) μmol/L (72-hour), respectively (Figure 3A, 3B). This clear treatment-duration dependent pattern, i.e. longer drug incubation have more inhibitory effects on cancer cells than the shorter ones, corroborates well with numeral published observations of

other anti-tumor drugs. It may also suggests a tumoristatic, not tumoricidal, effect that TAN II-A has on cancer cell in vitro growth, since it needs longer time to reach its full potential.

Tan II-A down-regulates transcription of COX-2 gene and represses VEGF secretion in human CRC HCT-116 cells

To interrogate if Tan II-A inhibits angiogenesis of CRC tumor through modulating COX-2, we next tested the expression of COX-2 gene and the secreted VEGF levels in control and COX-2 over-expressed HCT-116 cells, with or without the presence of TAN II-A. Our results indicated that TAN II-A significantly repressed COX-2 mRNA levels as well as the amount of VEGF in conditioned culture media. The degree of inhibition by TAN II-A is similar to a COX-2 specific inhibitor NS-398 (Figure 4B). We also noted that TAN II-A had a more pronounced inhibitory effect in COX-2 over-expressed/over-activated cells. Lastly, we demonstrated that Tan II-A down-regulated COX-2 promoter activity using a luciferase reporter plasmid in HCT-116 cells, resulting in lowered COX-2 mRNA expression (Figure 4A).

Discussion

Angiogenesis is a very crucial factor in the growth of colorectal carcinoma; it serves not only as the nutrition source for the proliferation of CRC cells, but also provides material basis for the invasion and metastasis of CRC to other organs or tissues. In 1970s, Folkman proposed an intimate relation of tumor growth and angiogenesis (Folkman, 1974). New vessels provide nutrition and carry away metabolic wastes for the tumor, increase vascular density inside the tumor tissues, as well as increase the opportunity for metastasis of tumor cells by entering the vessels. In the meantime, VEGFs secreted by tumor cells can enhance neovascularization and proliferation of vascular endothelial cells to accelerate differentiation; newly-formed microvessels surround the tumor tissues, thus enhancing invasion and metastasis of the tumor (Casimiro et al., 2012). Hence, neovascularization is believed to be a crucial factor in the failure of tumor treatment, and it is also a key issue in the comprehensive treatment of CRC demanding prompt solution. Thus, effective inhibition of VEGF expression serves as a premise against micro-angiogenesis of CRC (Amini et al., 2012; Waldner et al., 2012).

Tan II-A is an effective constituent of Salvia Miltiorhizza, and is believed to have antitumor effects. Recent studies have discovered the inhibitory effect on proliferation and promotive effect on apoptosis of Tan II-A on many types of tumor cells such as hepatocellular carcinoma, stomach carcinoma, colorectal carcinoma and so forth; thus, it serves as an antitumor monomer of traditional Chinese medicine with promising investigation potential (Chien et al., 2012; Zhang et al., 2012). Our preliminary studies discovered that Tan II-A can markedly inhibit proliferation of hepatocellular carcinoma and promote apoptosis; it can also regulate COX-2 expression through p38MAPK signaling.

Our study established mice xenograft model of C26 colorectal carcinoma cell line to evaluate the micro-angiogenesis effect of Tan II-A as well as its inhibitory effect on tumor growth in terms of tumor weight, volume, microvascular density, and serum VEGF. The results indicated a good inhibitory effect on tumor growth and anti-neovascularization with a dose-response relationship.

COX-2 is a key rate-limiting enzyme in the transformation of arachidonic acid to prostaglandin; it is also a crucial promoting factor in carcinogenesis and development of tumor (Karahan et al., 2007; Itatsu et al., 2009; Chien et al., 2012), which promotes proliferation of tumor cells, inhibits tumor cell apoptosis, promotes neovascularization, and so forth (Franchi et al., 2004; Dohadwala et al., 2006). Recent studies have discovered the co-expression of VEGF and COX-2 around newly-formed vessels (Lazar et al., 2008). In the meantime, overexpression of COX-2 leads to massive formation of VEGF, thus providing a marked promoting effect on angiogenesis of CRC tissues.

By separately transfecting the pGL3-Basic-COX-2 promoter recombinant plasmid and pIRESI-COX-2 recombinant plasmid into HCT-116 human CRC cells, the activity of COX-2 promoter and COX-2 mRNA was markedly elevated, and VEGF expression was indirectly upregulated (Figure 4A, 4B, 4C). Tsujii discovered that the CaCo-2 CRC cell line transfected with COX-2 produces 4 to 8 fold of VEGF compared with non-transfected group, and this effect is reversed to normal with COX-2 inhibitor (Tsujii et al., 1998). In the meantime, we processed the transfected cells aforementioned with Tan II-A of different concentrations, and intracellular COX-2 and VEGF expression was markedly downregulated (Figure 4A, 4B, 4C), which indicates that Tan II-A can inhibit angiogenesis by regulating COX-2 and VEGF expression; detailed mechanism of which requires further approach.

Our data showed Tan II-A significantly repressed COX-2 mRNA expression, gene promoter activity as well as VEGF mRNA levels. Therefore, Tan II-A can effectively inhibit tumor growth and angiogenesis in CRC of mice, through inhibiting the expression level of COX-2 and VEGF.

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