Abstract. *Patrinia scabiosaefolia* (PS) has long been used as an important component in traditional Chinese medicine formulas to treat gastrointestinal malignancies including colorectal cancer (CRC). We recently reported that PS can inhibit CRC growth through induction of apoptosis and inhibition of tumor angiogenesis. To further elucidate the mode of action of PS, in the present study, we used a CRC mouse xenograft model and a human CRC cell line HT-29 to evaluate the effect of the ethanol extract of PS (EEPS) on cancer cell proliferation and investigated the underlying molecular mechanisms. We found that EEPS inhibited CRC growth both in vivo and in vitro, which was associated with the inhibitory effects of EEPS on cancer cell proliferation. In addition, EEPS treatment significantly blocked G₁ to S phase cell cycle progression in HT-29 cells. Moreover, EEPS treatment decreased the expression of pro-proliferative CyclinD1 and CDK4, at both the mRNA and protein levels. Thus, inhibition of cell proliferation via G₁/S cell cycle arrest might be a potential mechanism whereby PS effectively treats cancers.

Introduction

Colorectal cancer (CRC) is one of the most common malignancies with high incidence in Western countries (1). At present, 5-fluorouracil-based regimens have been performed as international standard chemotherapy for CRC treatment (2). However, 5-FU-based regimens are often coupled with serious toxicity and side effects such as anemia, leucopenia, thrombocytopenia and peripheral neuropathy (3-5). Thus, the development of novel therapeutic drugs is necessary. Natural products, including traditional Chinese medicines (TCM), have been considered as alternative cancer remedies for many years. Numerous plants and their constituents have been shown to possess beneficial therapeutic effects for various diseases including cancer (6,7). *Patrinia scabiosaefolia* (PS), a perennial plant natively distributed in Eastern Asia, has long been used in China to clinically treat edema, appendicitis, endometritis and other inflammatory diseases (8-10). More importantly, PS has also been used as a major component in several TCM formulas for the treatment of gastrointestinal cancers (11,12). We recently reported that PS can inhibit CRC growth through induction of apoptosis and inhibition of tumor angiogenesis (12-14). To further elucidate the mode of action of PS, in the present study we used a CRC mouse xenograft model and a human CRC cell line HT-29 to evaluate the effect of the ethanol extract of PS (EEPS) on cancer cell proliferation and investigated the underlying molecular mechanisms.

Materials and methods

Materials and reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA, and TRIzol reagent were purchased from Life Technologies (Carlsbad, CA, USA). SuperScript II Reverse Transcriptase was obtained from Promega Corporation (Madison, WI, USA). PCNA assay kit was purchased from R&D Systems (Minneapolis, MN, USA). CDK4 and CyclinD1, β-actin antibodies, and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). A cell cycle kit was purchased from BD Biosciences (San Jose, CA, USA). All other chemicals, unless otherwise stated, were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of ethanol extract from PS. Ethanol extract from *Patrinia scabiosaefolia* (EEPS) was prepared as described previously (12). For animal experiments, EEPS powder was dissolved in saline to a working concentration of 250 mg/ml. In cell-based experiments, EEPS powder was dissolved in 50% DMSO to a stock concentration of 250 mg/ml, and the...
EEPS working concentrations were obtained by diluting the stock solution in the culture medium. The final concentration of DMSO in the medium for all cell experiments was <0.5%.

**Cell culture.** Human CRC HT-29 cells were obtained from the cell bank of the Chinese Academy of Science (Shanghai, China). The cells were grown in DMEM containing 10% (v/v) FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were cultured at 37°C in a humidified incubator with 5% CO₂.

**Animals.** Male BALB/c athymic (nude) mice (with an initial body weight of 20-22 g) were obtained from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and housed under pathogen-free conditions with controlled temperature (22°C), humidity, and a 12-h light/dark cycle. Food and water were given *ad libitum* throughout the experiment. All animal treatments were strictly in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. The experiments were approved by the Institutional Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine.

**In vivo tumor xenograft study.** HT-29 cells (1.5x10⁶) mixed with Matrigel (1:1) were subcutaneously injected into the right flank of the mice to initiate tumor growth. After 5 days of xenograft implantation, mice were randomly divided into 2 groups (n=6) and given intragastric administration with EEPS concentrations (1.93 g/kg/day of EEPS or saline daily, 5 days per week for 2 groups) were subcutaneously injected into the right flank of the mice to initiate tumor growth. After 5 days of treatment with Matrigel (1:1) were subcutaneously injected into the right flank of the mice to initiate tumor growth. After 5 days of treatment with EEPS and saline saline daily, 5 days per week for 3 weeks. Tumor size was determined by measuring the major (L) and minor (W) diameter with a caliper. The tumor volume was calculated according to the following formula: Tumor volume = π/6 x L x W².

**Immunohistochemical staining (IHS).** After being fixed with 10% formaldehyde for 12 h, the tumor samples were processed conventionally for paraffin-embedded 4-µm-thick tumor slides. The slides were subjected to antigen retrieval, and the endogenous peroxidase activity was quenched with hydrogen peroxide. After blocking non-specific proteins with normal serum in phosphate-buffered saline (PBS) (0.1% Tween-20), the slides were incubated with rabbit polyclonal antibodies against PCNA (1:200 dilution) for 1 h. After washing with PBS, slides were incubated with a biotinylated secondary antibody followed by conjugated HRP-labeled streptavidin (Dako), and then washed with PBS. The slides were then incubated with diaminobenzidine (Sigma) as the chromogen, followed by counterstaining with diluted Harris’ hematoxylin (Sigma). After staining, five high-power fields (x400 magnification) were randomly selected in each slide, and the average proportion of positive cells in each field was counted using a true color multi-functional cell image analysis management system (Image-Pro Plus; Media Cybernetics, Rockville, MD, USA). To rule out any nonspecific staining, PBS was used to replace the primary antibody as a negative control.

**Evaluation of cell viability by MTT assay.** The HT-29 cell viability was assessed by MTT colorimetric assay. HT-29 cells were seeded into 96-well plates at a density of 1x10⁴ cells/well in 0.1-ml medium. The cells were treated with various EEPS concentrations for 24 h. At the end of the treatment, 100 µl MTT (0.5 mg/ml in PBS) was added to each well, and the samples were incubated for an additional 4 h at 37°C. The purple-blue MTT formazan precipitate was dissolved in 100 µl DMSO. The absorbance was measured at 570 nm using an ELISA reader (BioTek, Model ELX800, BioTek Instruments, Inc., Winooski, VT, USA).

**Colony formation assay.** HT-29 cells were seeded in 6-well plates at a density of 2x10⁵ cells/well in 2 ml medium. After treatment with various EEPS concentrations for 24 h, the cells were collected and diluted in fresh medium in the absence of EEPS and then reseeded in 6-well plates at a density of 1x10³ cells/well. Following incubation for 7 to 8 days in a 37°C humidified incubator with 5% CO₂, the formed colonies were fixed with 4% paraformaldehyde for 15 min, stained with 0.01% crystal violet, and counted. The numbers of colonies were observed, and the data were normalized to the number of control cells (100%).

**Cell cycle analysis.** Cell cycle was analyzed by flow cytometry using fluorescence-activated cell sorting (FACS Calibur; Becton-Dickinson, San Jose, CA, USA) and propidium iodide (PI) staining. Subsequent to treatment with various EEPS concentrations for 24 h, the cells were collected and adjusted to a concentration of 5x10⁵ cells/ml, and fixed in 70% ethanol at 4°C overnight. The fixed cells were washed twice with cold PBS and then incubated for 30 min with RNase (8 µg/ml) and PI (10 µg/ml). The fluorescent signal was detected through the FL2 channel, and the DNA proportion in different phases was analyzed using ModfitLT ver. 3.0 (Verity Software House, Topsham, ME, USA).

**RNA extraction and RT-PCR analysis.** Total RNA was isolated from the tumor tissues or HT-29 cells with TRizol reagent. Oligo(dT)-primed RNA (1 µg) was reverse-transcribed with SuperScript II reverse transcriptase according to the manufacturer’s instructions. The obtained cDNA was used to determine the mRNA amount of CDK4 and CyclinD1 by PCR. GAPDH was used as an internal control. The sequences of the primers used for amplification of CyclinD1 and CDK4 were as follows: Cyclin D1 forward, 5'-GGG TGG GTT GGA AAT GAA-3' and reverse, 5'-GGG TGG TGT GGT GGA AAT GAA-3' (Tm = 57°C, 394 bp); CDK 4 forward, 5'-GGG TGG GTT GGA AAT GAA-3' and reverse, 5'-GGG TGG TGT GGT GGA AAT GAA-3' (Tm = 57°C, 394 bp); CDK 4 forward, 5'-CTT CCC GTC AGC ACA GTTCC-3' and reverse, 5'-GGT CAG CAT TTC CAG TAGC-3' (Tm = 55°C, 687 bp); GAPDH forward, 5'-CGA CCA CTT TGT CAA GCTCA-3' and reverse, 5'-AGG GGT CTA CAT GGC AACGT-3' (Tm = 58°C, 240 bp). Samples were analyzed by gel electrophoresis (1.5% agarose). The DNA bands were examined using a Gel Documentation system (Model Gel Doc 2000; Bio-Rad, Hercules, CA, USA).

**Western blot analysis.** Tumor tissues were homogenized in non-denaturing lysis buffer and centrifuged at 14,000 x g for 15 min. Protein concentrations of the clarified supernatants were determined by BCA protein assay. HT-29 (1x10⁶) cells were seeded into culture flasks in 5 ml medium and treated with various concentrations of EEPS for 24 h. Treated cells were lysed in mammalian cell lysis buffer (M-PER, Thermo
**Results and Discussion**

**EEPS inhibits cancer cell proliferation in CRC xenograft mice.** Cancer cells are characterized by an uncontrolled increase in cell proliferation (15); therefore inhibiting excessive proliferation of tumor cells is one of the key approaches for development of anticancer drugs. We first evaluated the efficacy of EEPS against tumor growth in vivo in the CRC mouse xenograft model. As shown in Fig. 1A, EEPS treatment significantly reduced the tumor volume in CRC mice (P<0.05, vs. controls), demonstrating its in vivo anticancer activity. We next determined the cell proliferation in CRC tumor tissues using IHS to examine the expression of proliferating cell nuclear antigen (PCNA), a processivity factor for DNA polymerase that has been recognized as a histologic marker for cell proliferation (16). As shown in Fig. 1B, the percentages of PCNA-positive cells in the control and the EEPS-treated mouse group were 38.0 and 22.3%, respectively (P<0.05), suggesting that EEPS significantly suppressed CRC cell proliferation in vivo.

**EEPS inhibits cell proliferation in HT-29 cells.** The in vitro effect of EEPS on CRC cell proliferation was determined by MTT assay to compare the viability of HT-29 cells in EEPS-treated monolayers to untreated controls. As shown in Fig. 2A, treatment with 0.5 to 2 mg/ml of EEPS for 24 h dose-dependently reduced cell viability by 21-62% compared with the untreated control cells (P<0.05). These results were verified using a colony formation assay. EEPS treatment reduced the survival rate of HT-29 cells in a dose-dependent manner (P<0.05, Fig. 2B), suggesting that EEPS can inhibit CRC cell proliferation in vitro.

Scientific, Rockford, IL, USA) containing protease (EMD Biosciences) and phosphatase inhibitor (Sigma-Aldrich) cocktail and centrifuged at 14,000 x g for 15 min. Protein concentrations in the cell lysate supernatants were determined by BCA protein assay and resolved on 12% Tris-glycine gels. Equal amounts of protein from each tumor or cell lysate were separated by 10% SDS-PAGE gels and transferred onto PVDF membranes. Membranes were blocked for 2 h with blocking solution at room temperature, washed in TBS with 0.25% Tween-20 (TBS-T), and exposed to primary antibodies against CDK4, CyclinD1 and β-actin (all at 1:1,000 dilutions) overnight at 4˚C. After the membranes were washed in TBS-T, secondary HRP-conjugated antibodies (anti-rabbit or anti-mouse) were added at a 1:2,000 dilution for 1 h at room temperature, and the membranes were washed again in TBS-T followed by enhanced chemiluminescence detection.

**Statistical analysis.** Data are presented as means ± SD for the indicated number of independently performed experiments and analyzed using the SPSS package for Windows (ver. 18.0). Statistical analysis of the data was performed with the Student’s t-test and ANOVA. Differences with P<0.05 were considered statistically significant.
EEPS blocks cell cycle G<sub>i</sub>/S progression in HT-29 cells.

Eukaryotic cell proliferation is strongly regulated by the cell cycle, which consists of S, M, G<sub>i</sub>, and G<sub>j</sub> phases. G<sub>i</sub> to S transition is one of the two main checkpoints of the cell cycle that is responsible for initiation and completion of DNA replication (17). We, therefore, investigated the effect of EEPS on the G<sub>i</sub> to S phase progression. Using PI staining followed by FACS analysis we found that the percentage of HT-29 cells in the S-phase following treatment with 0, 0.5, 1 or 2 mg/ml of EEPS was 58.3, 32.6, 14.1 or 13.7%, respectively (P<0.05, vs. untreated control cells) (Fig. 3), indicating that the inhibitory effect of EEPS on CRC cell proliferation was mediated by G<sub>i</sub>/S cell cycle arrest.

EEPS downregulates the expression of CyclinD1 and CDK4 in vivo and in vitro. G<sub>i</sub>/S progression is highly mediated by CyclinD1, which forms an active complex with its CDK major catalytic partners (CDK4/6) (18-20). Overexpression of CyclinD1 and CDK4 is commonly found in many types of human cancer (21-24). To further explore the mechanism of the anti-proliferative activity of EEPS, we performed RT-PCR and western blot analyses to examine the mRNA and protein levels of CyclinD1 and CDK4. The results of the RT-PCR assay showed that EEPS treatment significantly reduced the mRNA expression of pro-proliferative CyclinD1 and CDK4 both in CRC tumor tissues and in HT-29 cells (Fig. 4A and C); and the pattern of protein expression of CyclinD1 and CDK4 was similar to their respective mRNA level (Fig. 4B and D).

In conclusion, in the present study, we report that *Patrinia scabiosaefolia* inhibits colorectal cancer cell proliferation through blockade of G<sub>i</sub>/S cell cycle and the modulation of cell cycle-regulatory gene expression, which may be one of the mechanisms through which *Patrinia scabiosaefolia* exerts its antitumor function.

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**References**


