Anti-tumor activity of Sann-Joong-Kuey-Jian-Tang alone and in combination with 5-fluorouracil in a human colon cancer colo 205 cell xenograft model

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Abstract. Malignant tumors are the leading cause of death in Taiwan; among these, colon cancer ranks third as a cause of cancer-related death. Sann-Joong-Kuey-Jian-Tang (SJKJT), a traditional Chinese medicinal prescription, has been used to treat lymph node diseases and infectious lesions, and exhibits cytotoxic activity in many cancer cell lines. Our previous studies demonstrated that SJKJT inhibits the proliferation of human colon cancer colo 205 cells *in vitro*. The aim of this study was to evaluate the anti-tumor activity of SJKJT alone and in combination with 5-fluorouracil (5-FU) *in vivo*. SCID mice bearing human colon cancer colo 205 cell xenografts were administered SJKJT alone (30 mg/kg daily, p.o.), SJKJT (30 mg/kg daily, p.o.) in combination with 5-FU (30 mg/kg weekly, i.p.), or vehicle alone. At the end of the 4-week dosing schedule, the tumor and animal body weights were individually measured. The SCID mice were sacrificed with CO_2 inhalation, the xenograft tumors were dissected, and the protein expression of microtubule-associated protein light chain 3 (MAP-LC3-II) in colo 205 xenograft tumors was measured by Western blotting. In the control, SJKJT-, and SJKJT plus 5-FU-treated mice, the tumor weights were 6.37±2.57, 0.43±0.35 and 1.63±0.46 g, and the mice body weights were 29±0.55, 29±2.71 and 27±0.77 g, respectively. Treatment with SJKJT resulted in a reduction in tumor weight compared with the control group, indicating that SJKJT inhibits tumor growth in a colo 205 xenograft model. SJKJT also increased LC3-II protein expression as compared to the controls. The present study shows that SJKJT alone or in combination with 5-FU has a positive effect on the treatment of SCID mice bearing human colon cancer colo 205 cell xenografts. This suggests that SJKJT has therapeutic potential in the treatment of human colon cancer.

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Introduction

Traditional Chinese medicine, which is founded on over 5,000 years of tradition and employs an enormous variety of drugs of plant origin, is a well-known medical practice in Asia (1). Sann-Joong-Kuey-Jian-Tang (SJKJT) is a traditional Chinese medicine that has been prescribed to treat diseases with lymphadenopathy and inflammation or tumors with lymph node invasion. Colon cancer is the second leading cause of cancer-related death in Western societies (2). In Taiwan, it is ranked third as the principal cause of cancer-related death (3), and has been treated with SJKJT as a complementary medicine. Our previous studies showed that SJKJT inhibits the proliferation of colo 205 cells and up-regulates the protein expression of microtubule-associated protein light chain 3 (MAP-LC3-II) *in vitro* (4). However, the protein expression of MAP-LC3-II in human colon cancer treated with SJKJT *in vivo* is in need of further investigation. 5-Fluorouracil (5-FU) is one of the most commonly used chemotherapeutic agents in the treatment of colon cancer, but has limited efficacy (5). In the present study, we evaluated the anti-tumor efficacy of SJKJT in combination with 5-FU in a human colon cancer colo 205 cell xenograft model, and assayed the protein expression of MAP-LC3-II in these tumors.

Materials and methods

Chemicals and reagents. Fetal bovine serum (FBS), sodium pyruvate, HEPES, dimethyl sulfoxide (DMSO), RPMI-1640 and the antibodies to MAP-LC3-II and β-actin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Penicillin-streptomycin, trypsin-EDTA and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) running buffer (10X), Tris, Tween-20, SDS and 5X TBE buffer were obtained from Amresco (St. Louis, MO, USA). BioMax Film was obtained from Kodak.

SJKJT consists of 17 species of medicinal herbs: *Coptis chinensis* Franch, *Cimicifuga heracleifolia* Komar, *Scutellaria baicalensis* Georgi, *Gentiana scabra* Bunge, *Trichosanthes cucumeroides* Maxim, *Phellodendron amurense* Rupr, *Anemarrhena asphodeloides* Bunge, *Platycodon grandiflorum*,

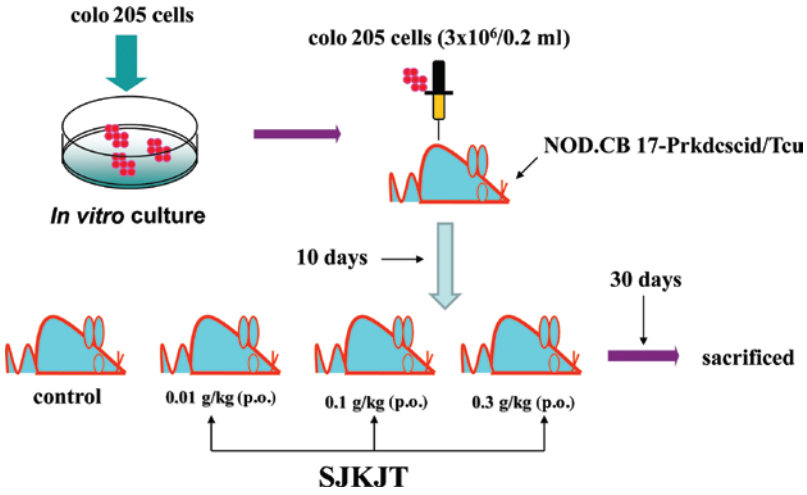

Figure 1. First experimental design. Three-week-old male nude SCID mice were xenografted with colon cancer colo 205 cells (3x10⁶/0.2 ml), divided randomly into four groups and maintained in a pathogen-free environment. From day 10, SJKJT (dissolved in normal saline) was administered orally at concentrations of 0.01, 0.1 and 0.3 mg/kg daily for 30 days. As a control, mice were treated with normal saline (0.1 ml/10 g body weight). Mice were sacrificed by CO_2 inhalation.

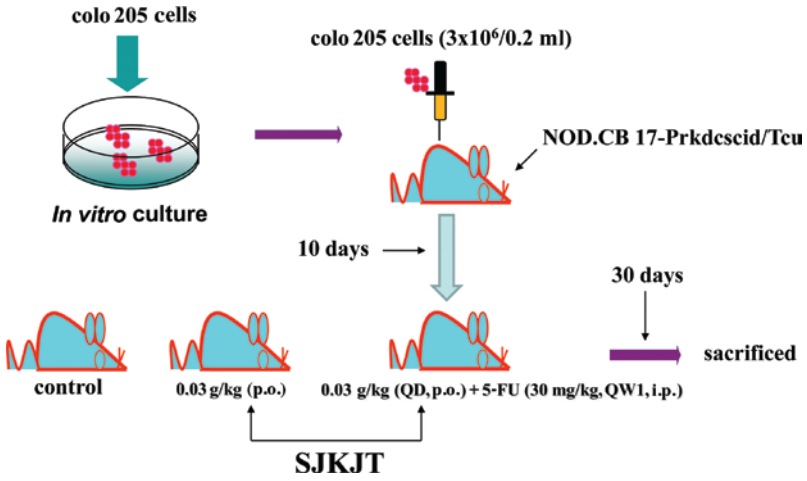

Figure 2. Second experimental design. Three-week-old male nude SCID mice were xenografted with colon cancer colo 205 cells (3x10⁶/0.2 ml), divided randomly into three groups and maintained in a pathogen-free environment. From day 10, the mice were administered SJKJT [30 mg/kg daily (QD), p.o.], a combination of SJKJT (30 mg/kg daily, p.o.) and 5-FU [30 mg/kg weekly (QW1), i.p.], or vehicle (normal saline) alone. At the end of the 4-week dosing schedule, mice were sacrificed by CO_2 inhalation.

Laminaria japonica Aresch, *Bupleurum scorzoneri folium* Willd (Bupleurum chinese DC), *Glycyrrhiza uralensis* Fisch, *Sparganium toloniferum* Buch, *Curcuma aeruginosa* Roxb, *Forsythia suspense* Vahl, *Pueraria lobata* Ohwi, *Paeonia lactiflora* Pall and *Angelica sinensis* Diels (6). Crude extract of SJKJT was obtained from Chuang Song Zong Pharmaceutical Co., Ltd. (Ligang Plant, Taiwan).

Cell culture. Human colon adenocarcinoma colo 205 cells were obtained from the Food Industry Research and Development Institute (Hsin-chu, Taiwan). Cells were grown in 75-cm^3 tissue culture flasks at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air in RPMI-1640 medium containing 10% heat-inactivated FBS, 2% penicillin-streptomycin (10,000 U/ml penicillin; 10 mg/ml streptomycin), 1% HEPES, 1% sodium pyruvate and 1% glutamine.

Experimental design. In our first experiment, 3-week-old male nude SCID mice were xenografted with colon cancer colo 205 cells (3x10⁶/0.2 ml), divided randomly into four groups and maintained in a pathogen-free environment at the Laboratory Animal Center of Tzu Chi University, Hualien, Taiwan. As of day 10, SJKJT (dissolved in normal saline) was administered orally at concentrations of 0.01, 0.1 and 0.3 g/kg daily for 30 days. As a control, mice bearing xenograft tumors were treated separately with normal saline (0.1 ml/10 g body weight). Mice were sacrificed by CO_2 inhalation, then the xenograft tumors were dissected and individually weighed, and proteins were extracted for Western blot analysis (Fig. 1).

In the second experiment, 3-week-old male nude SCID mice were xenografted with colon cancer colo 205 cells (3x10⁶/0.2 ml), divided randomly into three groups and maintained in a pathogen-free environment (Laboratory Animal Center of Tzu Chi University). From day 10, the mice were administered SJKJT (30 mg/kg daily, p.o.), a combination of SJKJT (30 mg/kg daily, p.o.) and 5-FU (30 mg/kg weekly, i.p.), or vehicle alone (normal saline). At the end of the 4-week dosing schedule, the SCID mice were sacrificed by CO_2 inhalation, then the

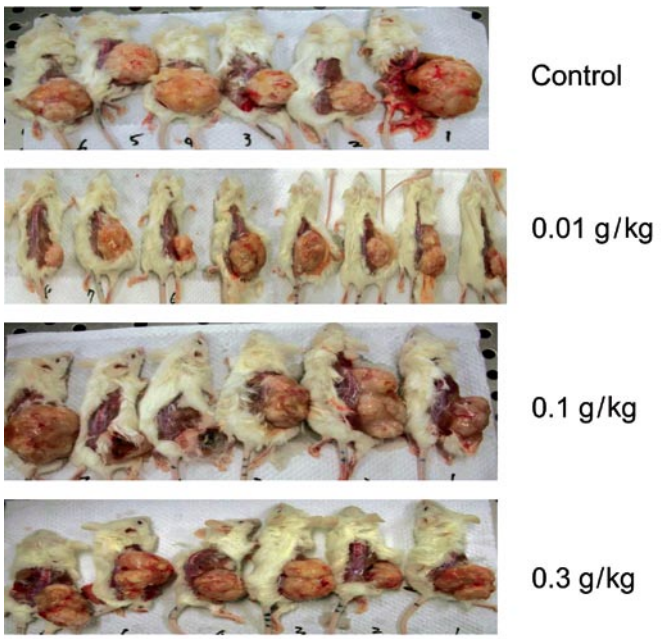

Figure 3. Dissected colo 205 cell xenograft tumors. Mice were treated for 30 days with normal saline only (control) or with SJKJT concentrations of 0.01, 0.1 and 0.3 g/kg daily, then sacrificed by CO_2 inhalation. The colo 205 cell xenograft tumors were then dissected.

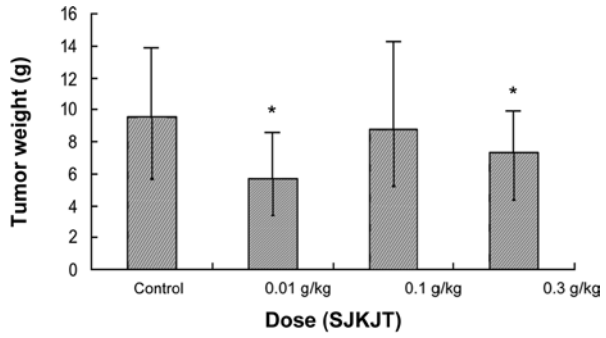

Figure 4. Colo 205 cell xenograft tumor weights. Mice were treated for 30 days with normal saline only (control) or with SJKJT concentrations of 0.01, 0.1 and 0.3 g/kg daily, and the colo 205 cell xenograft tumors were dissected and individually weighed.

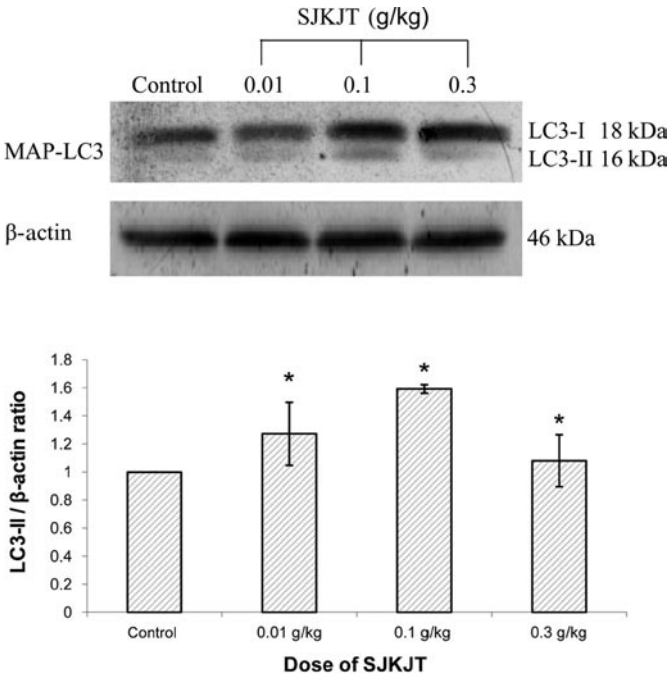

Figure 5. Protein expression of MAP-LC3-II in SCID mice with colo 205 cell xenograft tumors treated with SJKJT concentrations of 0.01, 0.1, 0.3 g/kg daily. The tumors were dissected, and the proteins were extracted for Western blot analysis. SJKJT up-regulated the protein expression of MAP-LC3-II as compared to the controls. Values are expressed as the mean ± SD. *P<0.05, significant difference compared to the controls.

xenograft tumors were dissected and individually weighed, and proteins were extracted for Western blot analysis (Fig. 2).

Determination of protein expression by Western blot analysis. MAP-LC3-II as well as β-actin proteins were extracted as previously described (7). Briefly, the xenograft tumors were dissected, and the thick liquid was resuspended in modified Pro-prep™ buffer (Intron Biotechnology) for 40 min at 4˚C. Lysates were immediately centrifuged at 13,000 x g for 20 min at 4˚C, and the supernatant was collected, aliquoted (20 µl/tube) and stored at -80˚C until assay. The extracted protein concentrations were determined using the Bradford method (8). Protein samples were separated by 8% (β-actin) or 15% (MAP-LC3-II) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (9). The SDS-separated proteins were equilibrated in transfer buffer (25 mM Tris, pH 8.5, 0.2 M glycine and 20% methanol) and transferred to PVDF membranes (Millipore Corp., Bedford, MA, USA). The membranes were incubated with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h, then washed and incubated with appropriate dilutions of specific antibodies, including anti-AP-LC3-II (1:1000) and anti-β-actin (1:15000) (Sigma-Aldrich), at 4˚C overnight. Following incubation with anti-mouse peroxidase-conjugated antibody (1:15000) (Sigma-Aldrich), the immunoreactive bands were visualized with an enhanced chemiluminescence (ECL) detection kit (Millipore Corp.). β-actin levels were used as an internal control for Western blotting. Immunoreactive bands were scanned (GS-800; Bio-Rad Life Science Products, Hercules, CA, USA) and analyzed using a digital scanning densitometer (Quantity One, v4.4.0, Bio-Rad Life Science Products).

Statistical analysis. Values are presented as the mean ± SD. The Student's t-test was used to analyze statistical significance. A p-value <0.05 was considered statistically significant.

Results

Effect of SJKJT on colo 205 cell xenograft tumors. In the first experiment, the growth of colo 205 cell xenograft tumors was found to be inhibited in SCID mice treated daily for 30 days with normal saline or SJKJT concentrations of 0.01, 0.1, 0.3 g/kg (Fig. 3). SJKJT at a low dose (0.01 g/kg daily) inhibited the growth of colo 205 cell xenograft tumors significantly compared with the higher doses (0.1 and 0.3 g/kg daily) (Fig. 4).

According to the results of Western blotting, the protein expression of MAC-LC3-II in the SJKJT-treated xenograft tumors was up-regulated as compared to the controls (Fig. 5).

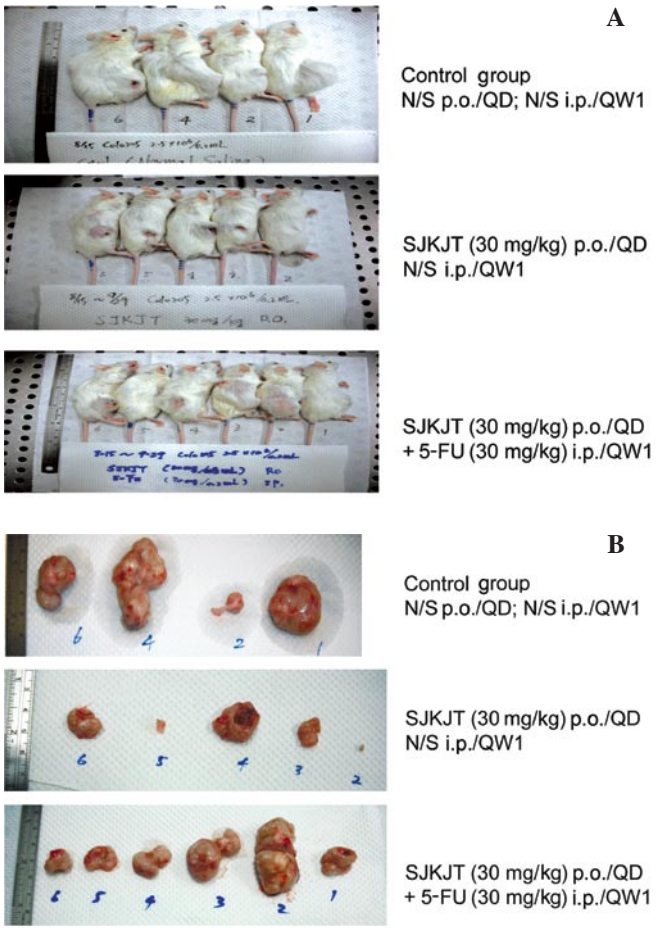

Figure 6. Dissected colo 205 cell xenograft tumors. Mice were treated for 30 days with normal saline (N/S) only (control), SJKJT [30 mg/kg daily (QD), p.o.], or SJKJT (30 mg/kg daily, p.o.) plus 5-FU [30 mg/kg weekly (QW1), i.p.], sacrificed by CO_2 inhalation (A) and the colo 205 cell xenograft tumors were dissected (B).

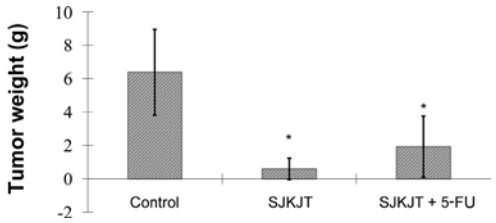

Figure 7. Colo 205 cell xenograft tumor weights. SCID mice with colo 205 cell xenograft tumors treated with normal saline only (control), SJKJT (30 mg/kg daily, p.o.), or SJKJT (30 mg/kg daily, p.o.) plus 5-FU (30 mg/kg weekly, i.p.). The tumors were dissected and weighed.

Effect of SJKJT versus SJKJT plus 5-FU on colo 205 cell xenograft tumors. In the second experiment, the growth of colo 205 cell xenograft tumors treated with SJKJT (30 mg/kg daily, p.o.) or a combination of SJKJT (30 mg/kg daily, p.o.) and 5-FU (30 mg/kg weekly, i.p.) was inhibited by 93.25 and 74.41%, respectively, as compared to the vehicle (normal saline) only controls. In the control, SJKJT-, and SJKJT plus 5-FU-treated SCID mice, the weights of the colo 205 cell xenograft tumors were 6.37±2.57, 0.43±0.35 and 1.63±0.46 g (Fig. 6), and the mice body weights were 29±0.55, 29±2.71

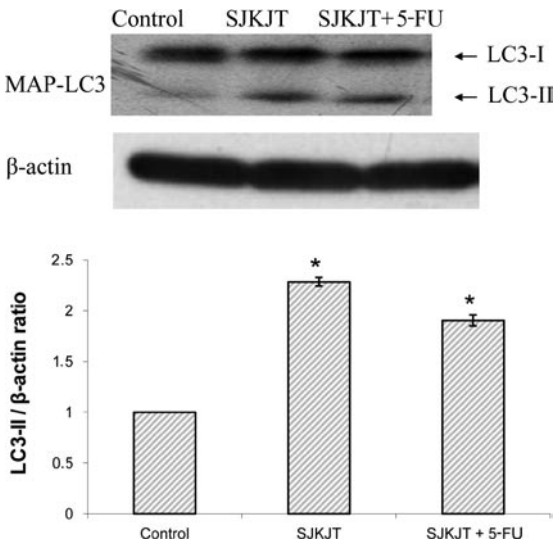

Figure 8. Protein expression of MAP-LC3 II in SCID mice with colo 205 cell xenograft tumors treated with normal saline only (control), SJKJT (30 mg/kg daily, p.o.) or SJKJT (30 mg/kg daily, p.o.) plus 5-FU (30 mg/kg weekly, i.p.). Tumors were dissected, and the proteins extracted for Western blot analysis. SJKJT and SJKJT plus 5-FU up-regulated the protein expression of MAP-LC3 II as compared to the controls. Values are expressed as the mean ± SD. *P<0.05, significant difference compared to the controls.

and 27±0.77 g, respectively (Fig. 7). These results indicate that SJKJT alone or in combination with 5-FU has a positive effect on the human colon cancer colo 205 cell xenografts, although SJKJT alone is more effective than SJKJT in combination with 5-FU.

Additionally, the results of Western blotting showed that SJKJT, as well as SJKJT plus 5-FU, up-regulated the protein expression of MAP-LC3-II compared to the controls (Fig. 8).

Discussion

It has been demonstrated that the examination of plant extracts used in traditional Chinese medicine may lead to the identification of novel active constituents (10), and the combination of traditional Chinese medicine and modern molecular medicine approaches has the potential to contribute to the development of the future practice of cancer medicine (11).

It is well documented that autophagy is one means of programmed cell death, and that targeting the autophagic pathway may be utilized to improve cancer therapy (12,13). MAP-LC3-II protein expression has been used as a marker of autophagy, and can be detected using Western blot analysis (14,15).

In a previous study, we demonstrated that SJKJT inhibited proliferation in human colon cancer colo 205 cells in a dose-dependent manner *in vitro*. (4). The results of the present study confirm that SJKJT (30 mg/kg daily, p.o.), as well as SJKJT (30 mg/kg daily, p.o.) plus 5-FU (30 mg/kg weekly, i.p.), inhibit colo 205 cell xenograft tumor growth *in vivo*, although SJKJT alone was more effective than SJKJT plus 5-FU. In both the experiments conducted, an increase in the protein expression of MAP-LC3-II as compared to the control was observed. Therefore, one of the molecular mechanisms behind

the inhibition of proliferation in colo 205 cell xenografts may be the up-regulation of MAP-LC3-II protein expression.

In a previous study, we showed that SJKJT induced apoptosis in colon cancer cells by up-regulating Fas, TNF-α, Caspase-8 and Caspase-3 protein expression *in vitro* and *in vivo* (16). Together, these findings suggest that SJKJT has therapeutic potential in the treatment of human colon cancer.

Acknowledgements

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