Astragalus extract inhibits proliferation but enhances apoptosis in gastric cancer

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Abstract: We and others have shown that *Astragalus* extract (AE) regulates various cellular processes including inflammation and apoptosis. It remains elusive whether and how AE modulates apoptosis in gastric cancer cells *in vitro* and *in vivo*. The objective of this study is to determine the effects and mechanisms of AE on the proliferation and apoptosis of human gastric cancer SGC-7901 cells and on tumor growth in orthotopic transplantation gastric tumor model in nude mice. Human gastric adenocarcinoma SGC-7901 cells and nude mice implanted with gastric cancer cells were treated with different concentration of AE and 5-fluorouracil as control. Cellular proliferation, apoptosis and tumor growth as well as interleukin (IL)-6/signal transducer and activator of transcription (Stat) 3 signals pathway were determined. We found that AE inhibited proliferation but caused apoptosis in human gastric cancer cells. Furthermore, the tumor growth and volume were reduced by AE administration in nude mice implanted with gastric cancer cells. In addition, treatments with AE decreased the expression of Bcl-2 proteins, whereas the expression of Bax was increased after AE treatment in tumor tissues of nude mice transplanted with human gastric cancer cells. This was associated with AE-mediated reduction of IL-6, phosphorylated Stat3, survivin and vascular endothelial growth factor. Overall, AE enhances apoptosis in gastric cancer cells *in vitro* and *in vivo*, which is associated with decreased activation of IL-6/Stat3 signals.

Keywords: *Astragalus* extract, gastric cancer, apoptosis, STAT3, IL-6, vascular endothelial growth factor, surviving.

INTRODUCTION

Gastric cancer is the second leading cause of cancer-related deaths with high recurrence rate (Ezzati, Henley *et al*., 2005). There is no significant improvement for the overall survival rate of gastric cancer and few novel chemopreventive approaches have been developed for this disease (Delaunoit, 2011). This is associated with the incomplete understanding of signaling pathways for tumorigenesis and metastasis of gastric cancer. The apoptosis-inducing compounds have been shown to regulate cancer cell proliferation, which is considered a promising approach for treating cancer (Qiao and Wong, 2009, Wu, Nie *et al*., 2009). Hence, the agents bearing ability to cause apoptosis will be the promising therapeutic ways in inhibiting tumorigenesis and tumor recurrence. Signal transducer and activator of transcription 3 (Stat3) is a transcription factor and it can be activated by a variety of growth factors and cytokines (i.e., interleukin [IL]-6) through tyrosine phosphorylation. For instance, IL-6/IL-6R complex associates with glycoprotein 130, which can activate Stat1 and Stat3 through the signals mediated from the Janus-associated kinase during neoplastic growth (Aaronson and Horvath, 2002, Leu, Wong *et al*., 2003). Upon activation, Stat3 is recruited on the promoters of the targeted genes (e.g., cyclin D1, Bcl-2, Bcl-xL, matrix metalloproteinases and vascular endothelial growth factor [VEGF]), leading to increased transcription of these genes (Buettner, Mora *et al*., 2002, Niu, Wright *et al*., 2002, Gamero, Young *et al*., 2004). Therefore, targeting IL-6/Stat3 signal pathway may be beneficial to gastric cancer via apoptosis induction.

Astragalus is one of the Chinese tonic herbs, and its active components include astragalosides, flavonoids, and polysaccharides, which are extracted from the root of Astragalus membranceus (Fisch) Bge (Sinclair, 1998, Zheng, Liu *et al*., 1998). We and others have shown that astragalosides are able to regulate a variety of cell processes including aging, immune, inflammation, oxidative stress, host defense, and cell metabolism (Lei, Wang *et al*., 2003, Yin, Li *et al*., 2010, Ghafourian Boroujerdnia, Azemi *et al*., 2011, Qu, Yang *et al*., 2012, He, Du *et al*., 2013, Liu, Qin *et al*., 2013, Yang, Qu *et al*., 2013). Accumulating evidence has revealed that astragalus inhibits proliferation but induces apoptosis in cancer cells *in vitro* and *in vivo* (Chen, Xie *et al*., 2005, Cho and Leung, 2007). Sporadic reports have shown that astragalus affects proliferation, invasion and apoptosis in gastric cancer cells (Auyeung, Woo *et al*., 2012, Wang, Xuan *et al*., 2013). However, it remains elusive whether

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and how astragalus has any effect on gastric cancer through IL-6/Stat3 signals. We hypothesize that astragalus has an inhibitory effect on gastric cancer via regulation of IL-6/Stat3 signal pathway. To test this hypothesis, we employed *in vitro* in human gastric adenocarcinoma SGC-7901 cells and *in vivo* in nude mice implanted with gastric cancer cells, which were treated with astragalus extracts (AE). The molecular components of IL-6/Stat3 pathway were also determined in mice administered with AE.

MATERIALS AND METHODS

Reagents

Astragalus extract containing astragalosides>63% with brown powder was purchased from the Guizhou Hanfang Pharma Co., Ltd (batch number: 19993254, Guiyang, Guizhou, China) and was extracted with 95% ethanol solvents from the root of Astragalus membranaceus (Fisch) Bge. Quality control was performed using high-performance liquid chromatography coupled with diode array and evaporative light scattering detectors. 5-FU was purchased from the Tianjin Kingyork Group Co., Ltd (Tianjin, China), and CDDP was obtained from the Qilu Pharmaceutical Co., Ltd (Jinan, Shandong, China).

Cell culture and treatment

Human gastric adenocarcinoma SGC-7901 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Beijing), and cultured in DMEM Medium with FBS (10%) at 37^{o}C in a humidified atmosphere with 5% CO_2. Cells were split every 2-3 days via trypsinization, and fresh culture medium were added (Wang, Li *et al*., 2012). The SGC-7901 cells were treated with AE (100, 50, 25 μg/ml) and 5-FU (10 μg/ml) as a control for 3 days. The medium and reagents were changed daily until apoptosis analysis.

Morphological measurement of apoptosis

The morphological change of apoptosis was assayed under fluorescence microscope after Hoechst33258 staining. Briefly, cells were fixed in ethanol followed by Hoechst33258 (10μg/ml) staining for 30min at 37^{o}C, then visualized under the UV fluorescence microscope. Apoptotic cells were considered as cells showing nuclear and cytoplasmic shrinkage, chromatin condensation and apoptosis body. At least 400 cells were counted, and the percentage of apoptotic cells (Apoptotic Index) was calculated (Wu, Sun *et al*., 2001).

Cell proliferation by MTT assay

Sterile MTT dye (100μl of 0.5mg/ml, Amresco, Solon, OH, USA) was added to SGC-7901 cells cultured on 96-well plates for 4 h at 37°C. The culture medium was then removed followed by the addition of dimethyl sulphoxide (150 μl, Sigma, St. Louis, MO, USA). The absorbance was determined at 570 nm using a spectrophotometer. All experiments were performed for three times (Na, Liu *et al*., 2009, Lin, Dai *et al*., 2010). The inhibitory rate of cell proliferation was calculated using the formula ([OD of control-OD of treatment]/OD of control)×100%.

Mice and orthotopic implantation of gastric cancer cells

All BALB/c nude mice (male, 5-6 weeks old) were purchase from the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China), and underwent implantation of gastric cancer cells (SGC-7901) (Djokovic, Trindade *et al*., 2010). Briefly, The SGC-7901 cells (nearly 70-80% confluence) were harvested via trypsinization, and cell viability was assessed using the trypan blue exclusion test. Cells with >90% viability were subcutaneously injected in both rear flanks (3×10^6 cells/0.1ml/flank). Tumor nodules occurred ~8-10 days following cell injection, and these mice were treated with AE and 5-FU for 3 weeks once tumor volume reached 100-300mm^3. The tumor size and weight were measured. The tumor volume (V) (mm^3) was measured with the following formula $V=0.52 \times a \times b^2$ (a and b refer to the corresponding longer and shorter diameter of the tumor) (Djokovic, Trindade *et al*., 2010). No animal death was observed during tumor growth. All animals were treated under protocols approved by the Animal Research Committee of the Anhui Medical University at Hefei, China.

Drug administration

After tumor volume reached 100-300 mm^3, mice were then randomly grouped for the following four treatments: saline control, 5-FU (50 mg/kg, twice a week, i.p.), AE (120 mg/kg, daily, i.g.) and AE (60mg/kg, daily, i.g). These drugs were administered into mice for 3 weeks, and sacrificed for endpoint measurement as described below. The dose of 5-FU was used as per the previous report (Tao, Yang *et al*., 2015). According to previous reports (Krasteva, Nikolova *et al*., 2004, Ko, Lam *et al*., 2005) and our preliminary data (not shown), there are no toxicities found at the dose of 120mg/kg body weight of AE in mice and the doses used here are physiologically reasonable.

Measurement of IL-6 and VEGF by ELISA

Mouse blood was harvested, and serum was isolated through the centrifuged at 1000g for 10min. The levels of IL-6 and VEGF in serum were measured using their corresponding ELISA kits from the R&D System (Minneapolis, MN) following the manufacture's instruction. In brief, ELISA plates were coated with a capture antibody overnight, and then the serum sample or the standards were added for 2 h at room temperature. Each well was added with the biotinylated antibodies for VEGF and IL-6 for 1 h incubation, and washed three times at room temperature. Streptavidin-horseradish peroxidase reagent was added to each well following by addition of substrate solution containing hydrogen

peroxide and tetramethylbenzidine. Finally, stop solution was added, and concentrations were determined at 450 nm based on a standard curve using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Flow cytometric analysis of apoptosis

After treatments, SGC-7901 cells were washed twice with cold PBS, and fixed by methanol (2mL) for 30min at 4^0C. After cells were fixed, the mixture was added with propid iumiodide solution (0.5mL, 100 μg/ml, Sigma) and RNase A (0.5 mL, 0.25 mg/ml, Sigma, St. Louis, MO, USA) for 30 min incubation at room temperature. Cells were resuspended in 1mL PBS and then assessed by flow cytometry with excitation 488 nm and emission >630 nm (Coulter, Brea, CA, USA) as per the manufacturer's instructions (Na, Liu *et al*., 2009, Lee, Lee *et al*., 2010). The cells in the subdiploid peak were considered apoptotic.

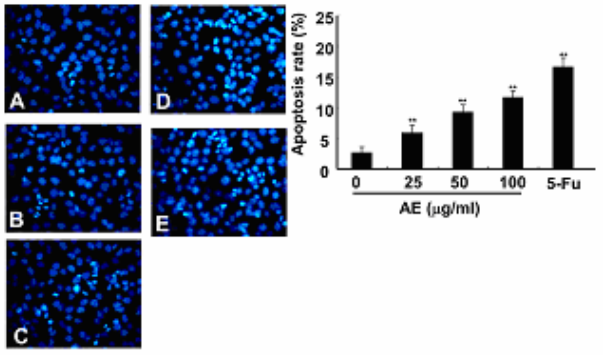

Fig. 1: Effect of AE and 5-Fu on the apoptosis of human gastric cancer cells detected by Hoechst33258 staining.
SGC-7901 cells were treated with AE (100, 50, 25 μg/ml) and 5-FU (10 μg/ml) for 3 days. (A-E) A representative image of morphological change of apoptosis was showed under fluorescence microscope following staining with Hoechst33258. Apoptotic cells were defined as cells showing nuclear and cytoplasmic shrinkage, chromatin condensation and apoptosis body. A: vehicle; B: 25 μg/ml of AE; C: 50 μg/ml of AE; D: 100 μg/ml of AE; E: 10 μg/ml of 5-FU. Original magnification ×200. (Bottom panel) At least 400 cells were counted and the percentage of apoptotic cells was calculated. Data are expressed as the mean ± SD. $^{**}P$<0.01, vs. control group.

Immunohistochemistry

The primary gastric specimens from gastrectomy were fixed with formalin, and paraffinized for the preparation of tumor xenograft sections (5 μm thick). For staining, tissue sections were deparaffinized with xylene, and incubated with the reduced concentrations of ethanol followed by the process of antigen unmasking using sodium citrate buffer (10mmol/L). Tissue sections were incubated with 3% hydrogen peroxide in methanol for 10 min to quench the endogenous peroxidase activity prior to blocking with 1% goat serum for 15 min. Tissue samples were then incubated with primary antibodies (anti-Bax, anti-Bcl-2, anti-IL-6, anti-VEGF, and anti-Survivin, Beijing Zhongshan Jinqiao Biotechnology Co., Ltd, Bejing, China) overnight at 4^0C. Negative controls were tissue sections immunostained with nonspecific IgG antibody. The slides were washed in PBS, incubated with biotinylated anti-mouse and anti-rabbit antibodies (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd, Beijing, China) and incubated with streptavidin-peroxidase according to the manufacturer's instructions. 3,3'-diaminobenzidine was used for development, and tissue sections were counterstained with haematoxilin. Finally, the sections were dehydrated in graded ethanol and embedded and the slides were observed under a bright-field microscope. The mean optical density (OD) value of all images was analyzed for the relative levels of protein expression (Wang, Si *et al*., 2013).

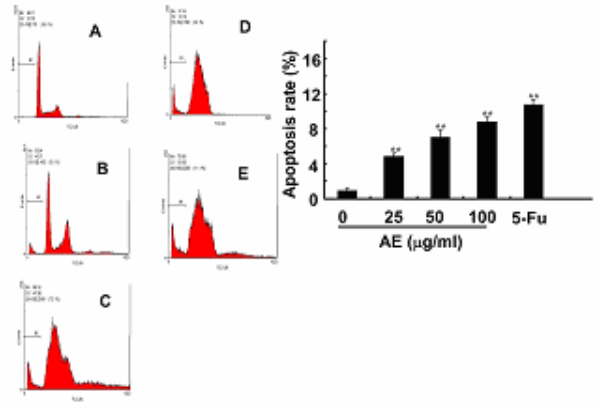

Fig. 2: Effect of AE and 5-Fu on the apoptosis of human gastric cancer cells detected by flow cytometry.
SGC-7901 cells were treated with AE (100, 50, 25 μg/ml) and 5-FU (10 μg/ml) for 3 days. (A-E) Apoptosis was determined by flow cytometry with excitation 488 nm and emission >630 nm using propid iumiodide staining. Finally, the cells were resuspended in 1 mL PBS and analyzed by flow cytometry with excitation 488 nm and emission >630 nm. The cells in the subdiploid peak were considered apoptotic. A: vehicle; B: 25 μg/ml of AE; C: 50 μg/ml of AE; D: 100 μg/ml of AE; E: 10 μg/ml of 5-FU. (Bottom panel) Apoptosis rate was shown in histogram in SGC-7901 cells treated with AE and 5-FU. Data are expressed as the mean ± SD. $^{**}P$<0.01, vs. control group.

Western blot

Cell lysate were prepared using lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 1 mg/L leupeptin, 1 mg/L aprotinin and 1 mM phenylmethylsulfonyl fluoride) on ice followed by the centrifugation at 3000 *g* for 30 min. Protein concentration in cell lysis was measured using the method of Lowry. Cell lysis were mixed with 4× SDS loading buffer, which was heated for 5min at 95^0C. Protein samples (20 μg) were resolved by SDS-PAGE gel, and then transferred onto nitrocellulose membranes (Bio-Rad). TBST buffer containing 5% skimmed milk for 2 h was added to block the membranes and the membranes were incubated with monoclonal antibodies against Bax, Bcl-2, IL-6, p-Stat3, Stat3, VEGF, and surviving (Beijing Zhongshan Jinqiao

Biotechnology Co., Ltd, Beijing, China) overnight. After three time of washing (15 min each), protein levels were assessed using the horseradish peroxidase-linked second antibodies (1:5,000 dilution in 2.5% BSA containing 0.1% Tween (v/v) 20) for 1 h. The bands were visualized using enhanced chemiluminescence method, and membranes were tested for β-actin to confirm equal loading.

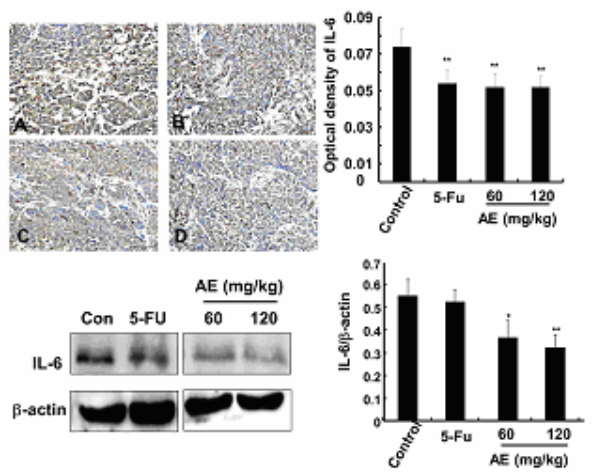

Fig. 3: Effect of AE and 5-Fu on the expression of IL-6 in tumor xenografts.

Nude mice implanted with SGC-7901 cells were administered with AE and 5-FU. (A-D) The expression of IL-6 in tumor xenografts was determined using immunohistochemical staining, and the representative images were shown from A to D. A: vehicle; B: 50 mg/kg of 5-FU; C: 60 mg/kg of AE; D: 120 mg/kg of AE. Original magnification ×200. Mean optical density (OD) value of all images was analyzed for relative protein expression levels. (Bottom panel) Protein levels of IL-6 in tumor xenografts were determined using Western blotting. β-actin was a loading control. Relative protein expression of IL-6 was normalized to that of β-actin. Positive immunoreactive bands were quantified densitometrically and expressed as IL-6 in optical density units, respectively. Data are expressed as the mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$, vs. control group.

STATISTICAL ANALYSIS

Results are shown as mean ± SE of three experiments. Statistical analysis of significance was calculated with one-way ANOVA followed by Tukey's post hoc test (two-sided) for multigroup comparisons.

RESULTS

Effect of AE on apoptosis and proliferation in gastric cancer cells

In order to determine whether AE treatment has any effect on apoptosis *in vitro*, Hoechst33258 staining and flow cytometry were performed in gastric cancer cells (SGC-7901) treated with AE (100, 50, 25 μg/ml) for 3 days. As expected, treatment with 5-FU (10 μg/ml) caused apoptosis in SGC-7901 cells. AE treatment induced apoptosis in a dose-dependent manner in SGC-7901 cells (figs. 1 and 2). Furthermore, AE treatment caused dose-dependent reduction in proliferation in SGC-7901 cells, which was also observed after 5-FU treatment (table 1). These data suggest that AE treatment causes imbalance of proliferation and apoptosis towards apoptosis in gastric cancer cells.

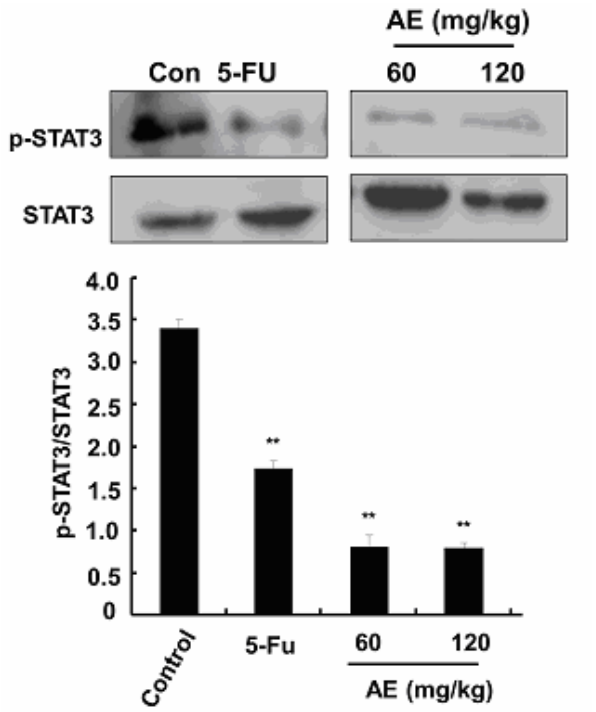

Fig. 4: Western blot analysis of protein level of p-Stat3 and Stat3 in tumor xenografts in nude mice treated with AE and 5-FU.

Nude mice implanted with SGC-7901 cells were administered with AE and 5-FU. Protein levels of p-Stat3 and Stat3 in tumor xenografts were determined using Western blotting. Relative protein expression of p-Stat3 was normalized to that of Stat3. Positive immunoreactive bands were quantified densitometrically and expressed as p-Stat3 in optical density units, respectively. Data are expressed as the mean ± SD. $^{**}P<0.01$, vs. control group.

Effect of AE on tumor volume and weight in nude mice with implantation of gastric cancer cells

To determine whether AE has an inhibitory effect on the growth of tumor, nude mice were implanted with SGC-7901 cells following by the administration of AE (60 and 120 mg/kg) and 5-FU (50 mg/kg). As expected, administration of 5-FU significantly reduced tumor volume from day 14 to day 21 after implantation of gastric cancer cells. Both doses of AE (60 and 120 mg/kg) treatment significantly reduced tumor volume and tumor weight in mice implanted with gastric cancer cells (tables 2 and 3). These data indicate that AE administration reduces tumor growth in nude mice with implantation of gastric cancer cells.

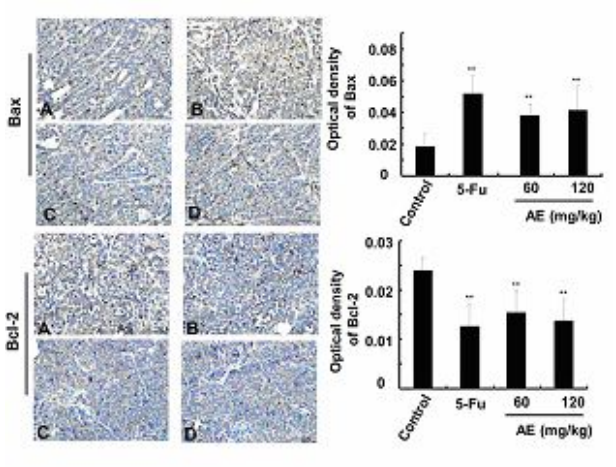

Fig. 5: Effect of AE and 5-Fu on the expression of Bax and Bcl-2 in tumor xenografts.

Nude mice implanted with SGC-7901 cells were administered with AE and 5-FU. The expression of Bax and Bcl-2 in tumor xenografts were determined using immunohistochemical staining, and the representative images were shown from A to D. A: vehicle; B: 50 mg/kg of 5-FU; C: 60 mg/kg of AE; D: 120 mg/kg of AE. Original magnification ×200. Mean optical density (OD) value of all images was analyzed for relative protein expression levels. Data are expressed as the mean ± SD. $^{**}P<0.01$, vs. control group.

Effect of AE on IL-6-Stat3 signals in tumor xenografts

We and others have shown that IL-6-Stat3 signal plays an important role in the progression and invasion of gastric cancer (Zhu, Chen *et al*., 2011, Wang, Si *et al*., 2013, Zhu, Zhang *et al*., 2014). Hence, we determined the levels and expression of IL-6/Stat3 signals in nude mice implanted with gastric cancer cells in response to AE and chemotherapeutic agents using ELISA, immunohistochemical staining and Western blot. AE administration (60 and 120 mg/kg) reduced the levels of IL-6 in serum of mice implanted with gastric cancer cells (table 4). In tumor xenografts, the expression and level of IL-6 were reduced by the administration of AE and 5-FU (fig. 3). In addition, the phosphorylation of Stat3 was attenuated by AE and 5-FU treatments (fig. 4). These data suggest that the inhibitory effect of AE on tumor growth is associated with decreased activation of IL-6/Stat3 signal.

Effect of AE on apoptosis regulatory proteins in tumor xenografts

Induction of apoptosis by Stat3 signals is one of the major mechanisms for chemotherapeutic agents (Real, Sierra *et al*., 2002, Wiita, Ziv *et al*., 2013). Therefore, we determined the expression and levels of Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic) in tumor xenografts of mice treated with 5-FU and AE. As expected, 5-FU treatment increased the expression of Bax in tumor xenografts by immunohistochemical staining (fig. 5). Administration of AE (60 and 120 mg/kg) also induced the expression of Bax in tumor xenografts (fig. 5). In contrast, the expression of anti-apoptotic proteins Bcl-2 and survivin was reduced by the treatment of 5-FU and AE in tumor xenografts (figs. 6 and 7). Western blots also showed that treatment of 5-FU and AE increased Bax level, whereas Bcl-2 and survivin levels were reduced by these treatments in tumor xenografts of nude mice (figs. 6 and 7). Altogether, AE induces apoptotic response in tumor xenografts of nude mice.

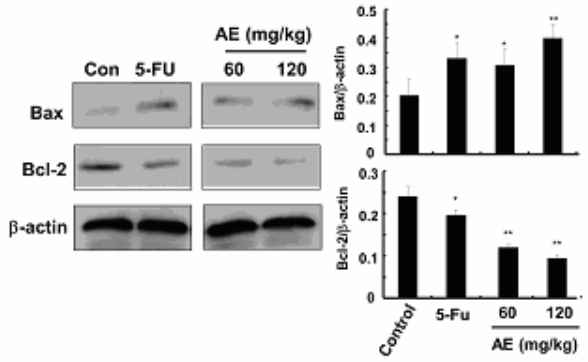

Fig. 6: Effect of AE and 5-Fu on the levels of Bax and Bcl-2 in tumor xenografts.

Nude mice implanted with SGC-7901 cells were administered with AE and 5-FU. Protein levels of Bax and Bcl-2 in tumor xenografts were determined using Western blotting. Relative protein expression of Bax and Bcl-2 was normalized to β-actin. Positive immunoreactive bands were quantified densitometrically and expressed as Bax and Bcl-2 in optical density units, respectively. Data are expressed as the mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$, vs. control group.

Effect of AE on VEGF in tumor xenografts

Angiogenesis is regulated by pro-angiogenesis factors including VEGF, which is an important hallmark of cancer development (Park, Thomas *et al*., 2014). Thus, we further determined the expression of VEGF after AE and 5-FU treatments. It was found that the expression and levels of VEGF were reduced by AE and 5-FU treatments (table 4, fig. 8). These data suggest that the inhibitory effect of AE on tumor growth is associated with decreased expression of VEGF.

DISCUSSION

In this study, we demonstrated that the chemo preventive potential of AE against gastric cancer cells by its ability to induce apoptosis *in vitro* in the SGC-7901 cells and *in vivo* in nude mice transplanted with gastric cancer cells. This is in agreement with the previous findings that astragalus inhibits proliferation and induces apoptosis in other cancer cells (Chen, Xie *et al*., 2005, Cho and Leung, 2007, Tin, Cho *et al*., 2007, Liu, Chen *et al*., 2011, Auyeung, Woo *et al*., 2012, Wang, Xuan *et al*., 2013, Auyeung, Law *et al*., 2014). Nevertheless, further study using 5-FU-resistant gastric cancer cells will reveal the chemo sensitizing effects of AE on gastric cancer, despite AE enhanced the chemotherapeutic response to 5-FU in SGC-7901 cells (Wang Z, *et al*. unpublished data).

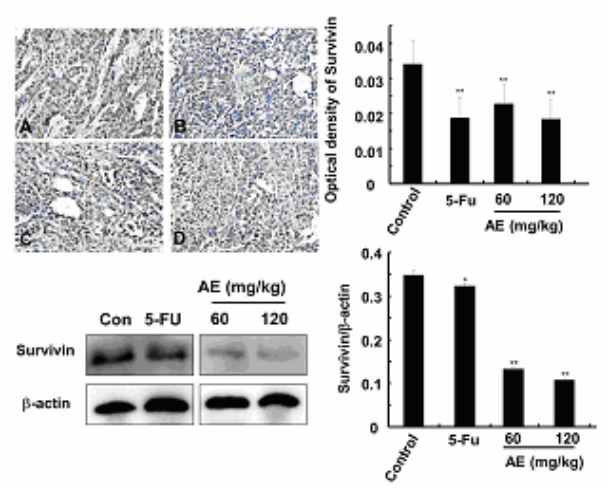

Fig 7: Effect of AE and 5-Fu on the expression of survivin in tumor xenografts.

Nude mice implanted with SGC-7901 cells were administered with AE and 5-FU. (A-D) The expression of survivin in tumor xenografts were determined using immunohistochemical staining, and the representative images were shown from A to D. A: vehicle; B: 50 mg/kg of 5-FU; C: 60 mg/kg of AE; D: 120 mg/kg of AE. Original magnification ×200. Mean optical density (OD) value of all images was analyzed for relative protein expression levels. (Bottom panel) Protein levels of survivin in tumor xenografts were determined using Western blotting. β-actin was a loading control. Relative protein expression of survivin was normalized to that of β-actin. Positive immunoreactive bands were quantified densitometrically and expressed as survivin in optical density units, respectively. Data are expressed as the mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$, vs. control group.

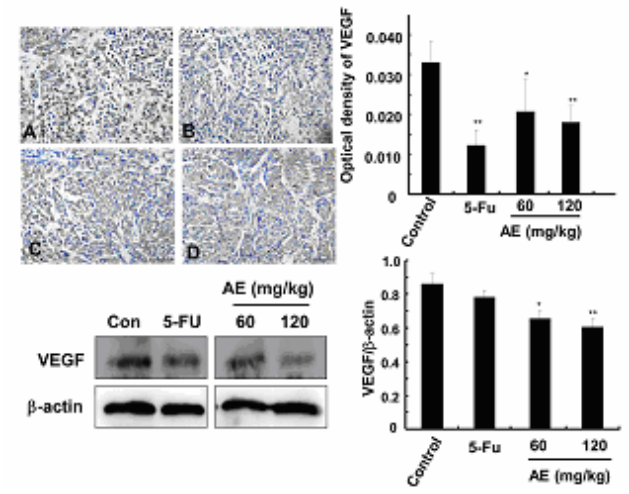

Fig. 8: Effect of AE and 5-Fu on the expression of VEGF in tumor xenografts.

Nude mice implanted with SGC-7901 cells were administered with AE and 5-FU. (A-D) The expression of VEGF in tumor xenografts was determined using immunohistochemical staining, and the representative images were shown from A to D. A: vehicle; B: 50 mg/kg of 5-FU; C: 60 mg/kg of AE; D: 120 mg/kg of AE. Original magnification ×200. Mean optical density (OD) value of all images was analyzed for relative protein expression levels. (Bottom panel) Protein levels of VEGF in tumor xenografts were determined using Western blotting. β-actin was a loading control. Relative protein expression of survivin was normalized to that of β-actin. Positive immunoreactive bands were quantified densitometrically and expressed as survivin in optical density units, respectively. Data are expressed as the mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$, vs. control group.

IL-6 causes the sustained activation of STAT3, which plays an important role in enhancing invasion of gastric cancer cells through (Lin, Lin *et al*., 2007, Kinoshita, Hirata *et al*., 2013). Furthermore, STAT3 activation also leads to growth stimulation, anti-apoptosis, and angiogenesis, and all these processes are liked to inflammation, immunity, and oncogenesis (Aaronson and Horvath, 2002, Bromberg, 2002, Yu, Pardoll *et al*., 2009). It has been shown that blockade of the JAK/STAT3 signal reduces the growth of human cancers (Toyonaga, Nakano *et al*., 2003). Recent studies have shown that STAT3 maintains the survival of gastric cancer cells (Kanda, Seno *et al*., 2004, Sekikawa, Fukui *et al*., 2008, Jackson and Giraud, 2009, Giraud, Menheniott *et al*., 2012, Hsu, Hsieh *et al*., 2012). Furthermore, STAT3 activation is considered as a predictive marker for poor prognosis in human gastric cancer (Yakata, Nakayama *et al*., 2007, Kim, Cha *et al*., 2009, Xiong, Du *et al*., 2012). We found that AE treatment reduced the expression and level of IL-6 and Stat3 phosphorylation in tumor xenografts from nude mice. These findings suggest that the chemo preventive potential of AE is associated with deactivation of IL-6/Stat3 signal pathway.

It has been shown Stat3 is able to bind to recognition sequence in the promoter of target genes including Bcl-2, surviving and VEGF, thereby increasing their transcription (Buettner, Mora *et al*., 2002, Niu, Wright *et al*., 2002, Gamero, Young *et al*., 2004). Furthermore, angiogenesis blockade is a promising mean to inhibit tumor growth, invasion and metastasis. In the current study, the expression and level of Bcl-2, Bax, and survivin was abnormally altered in gastric cancer, which is redressed by AE administration. Moreover, AE treatment reduced the expression of VEGF in tumor xenografts in mice. These findings implicate that the chemo preventive potential of AE is associated with increased apoptosis and reduced angiogenesis. However, future study is needed to determine whether AE treated alters the recruitment of Stat3 on the promoters of genes including Bcl-2, Bax, VEGF and surviving in gastric cancer.

Radix astragalus membranaceus is commonly used to reduce the side-effects of cytotoxic antineoplastic drugs. This is in agreement with the findings that polysaccharides significant ameliorates the degree of myelosuppression caused by chemotherapeutic drugs in cancer patients (Ma, Shi *et al*., 2002). Our unpublished

Table 1: Effect of AE on the proliferation of human SGC-7901 cells

Group	Concentration (μg/ml)	OD value	Inhibition rate (%)
Control	-	0.549±0.070	-
AE	25	0.330±0.033**	39.4±7.3
	50	0.306±0.041**	44.1±6.5
	100	0.276±0.031**	49.0±8.2
5-Fu	10	0.228±0.012**	58.1±5.1

Statistical analysis of significance was calculated with one-way ANOVA followed by Tukey's post hoc test (two-sided). $^{**}P<0.01$ ' compared with control group.

Table 2: Effect of AE on tumor volume in nude mice xenografts with SGC-7901 cells

Group	Dose	Tumor volume (mm^3)						
		0d	3d	7d	10d	14d	17d	21d
Control	—	289.6 ±79.6	411.4 ±106.1	767.8 ±375.0	866.0 ±408.8	1026.6 ±328.3	1248.8 ±416.0	1524.9 ±465.0
5-Fu (mg/kg)	50	276.5 ±65.9	331.2 ±100.4	382.7 ±115.0	438.3 ±137.2†	572.9 ±113.3*†	702.8 ±98.1*††	752.2 ±254.2*††
AE (mg/kg)	120	276.8 ±87.2	364.8 ±65.2	465.3 ±74.1	572.6 ±87.7†	683.2 ±58.1†	811.6 ±50.9*†††	998.1 ±155.2*†††
	60	297.0 ±36.2	377.7 ±67.6	471.2 ±108.2	586.4 ±175.2†	691.5 ±153.6†	948.4 ±195.2†††	1134.4 ±285.2†††

Statistical analysis of significance was calculated with one-way ANOVA followed by Tukey's post hoc test (two-sided) for multigroup comparisons. $^{*}p<0.05$, compared with control group; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$; $^{\dagger\dagger\dagger}p<0.001$ compared with day 0.

Table 3: Effect of AE on tumor growth in nude mice xenografts with SGC-7901 cells

Group	Dose	Tumor weight (g)	Inhibitory rate (%)
Control	—	2.41±0.84	-
5-Fu (mg/kg)	50	1.11±0.54*	53.94
AE (mg/kg)	120	1.41±0.38*	41.49
	60	1.67±0.97	30.7

Table 4: Effect of AE on the levels of IL-6 and VEGF in the serum of nude mice with SGC-7901 xenograft tumor

Group	Dose	IL-6 (pg/ml)	VEGF (pg/ml)
Control	—	48.7±5.3	123.5±31.7
5-Fu (mg/kg)	50	41.1±8.4	75.6±10.6*
AE (mg/kg)	120	34.5±5.6**	90.1±22.9*
	60	36.5±3.3**	108.0±20.6

Statistical analysis of significance was calculated with one-way ANOVA followed by Tukey's post hoc test (two-sided). $^{*}P<0.05$, compared with control group

findings showed that AE enhanced the immune function including $CD4^+$, $CD8^+$ and NK cells in patient with gastric cancer. This is corroborated by the study that astragalus attenuates chemotherapy-induced impairment of the immune function in cancer patients (Duan and Wang, 2002). The limitation of the study is to use 5-FU-sensitive SGC-7901 gastric cells, which cannot extrapolate into 5-FU-resistant gastric cancer. Overall, AE is a promising chemo sensitizing agent by enhancing the chemo sensitization in gastric cancer.

In conclusion, the present study provides molecular evidence both *in vitro* and *in vivo* that AE inhibit IL-6/Stat3 signal pathway and thus result in the chemo sensitization of gastric cancer cells to 5-FU-induced tumor growth reduction.

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