Scutellarin suppresses growth and causes apoptosis of human colorectal cancer cells by regulating the p53 pathway

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Abstract. Scutellarin is a flavonoid isolated from a medicinal herb *Scutellaria barbata* D. Don and exerts therapeutic effects on cardiovascular diseases. However, it remains unclear whether Scutellarin exhibits anti-tumor actions on human colon cancer. The current study aimed to investigate whether Scutellarin produces antiproliferative and pro-apoptotic effects on HCT-116 human colon cancer cells and to elucidate the mechanisms involved. Human colon cancer cells were exposed to different concentrations of Scutellarin, and cellular growth and apoptosis were evaluated by MTT assay, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining, western blot analysis and other assays. A cell viability assay demonstrated that Scutellarin treatment reduced the viability of HCT-116 cells in a dose- and time-dependent manner. TUNEL staining demonstrated that Scutellarin also induced apoptotic changes in HCT-116 cells. The expression level of the anti-apoptotic protein, Bcl-2 apoptosis regulator (Bcl-2), was reduced by Scutellarin in HCT-116 cells, whereas the expression Bcl-2 associated X apoptosis regulator (Bax) and the activation of caspase-3 protein were increased by Scutellarin treatment. Further investigation revealed that Scutellarin significantly increased the phosphorylation of p53 protein in HCT-116 cells. Additionally, suppression of p53 using a specific inhibitor, pifithrin-α, abrogated the pro-apoptotic effects of Scutellarin in HCT-116 cells. Collectively, Scutellarin reduced the viability and induced apoptosis of human colon carcinoma cells, potentially by regulating p53 and Bcl-2/Bax expression. These data suggested that Scutellarin may be useful as a promising anti-tumor drug for treating colon cancer.

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Introduction

Colon cancer is a common cancer worldwide and remains the third most common cause of cancer-associated mortality in the United States. It accounts for >50,000 mortalities and ~137,000 cases are diagnosed each year (1). Currently, more patients with colon cancer are diagnosed at the earlier stage, however the chemotherapy is often ineffective in patients with colon cancer, because colon cancer has a high recurrence rate (2). Thus, it is important to develop novel, effective drugs to treat colon cancer.

Scutellaria barbata D. Don is a natural medicinal herb prevalent in Korea and southern China, and used to treat ischemia heart diseases, neurological disorders, hepatitis, inflammation and osteomyelitis (3,4). As a major type of active compound, Scutellarin has been reported to produce many biological activities, including anti-oxidative, anti-inflammatory, cardioprotective effects, and also effects against human immunodeficiency virus (5-7). It was previously reported that Scutellarin also exhibited a potent ability to inhibit the growth of colon cancer, tongue carcinoma and squamous cell carcinoma (8,9). However, whether Scutellarin produces therapeutic effects on colon cell carcinoma and the molecular mechanisms involved have not been elucidated. Thus, the current study aimed to elucidate these effects and the molecular mechanisms.

The present study reported that Scutellarin inhibits the growth of colon cancer cells and induces apoptosis. The molecular mechanisms may be associated with the activation of p53 and the regulation of Bcl-2 apoptosis regulator (Bcl-2) and Bcl-2 associated X apoptosis regulator (Bax). These findings suggest that Scutellarin may be useful as a therapeutic drug for colon cancer.

Materials and methods

Reagents. Scutellarin was purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany) and was dissolved in dimethyl sulfoxide (DMSO; less than 0.1%, v/v, without detectable effects) for all experiments of the current study. DMSO was used as the control treatment. The pifithrin-α was purchased from Sigma-Aldrich; Merck Millipore (Taufkirchen, Germany) and dissolved in DMSO to a final

concentration of 50 mM. All other reagents were purchased from Sigma-Aldrich; Merck Millipore unless specifically noted.

Cell culture. The HCT-116 human colon carcinoma cell line was purchased from American Type Culture Collection (Manassas, VA, USA), and cultured with Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck Millipore) supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck Millipore) and 1% penicillin-streptomycin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The HCT-116 cells were cultured as monolayer in an incubator at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The culture medium was changed every two days.

Cell viability assay. Evaluation of cellular viability of HCT-116 cells was performed using an MTT assay. The HCT-116 cells were seeded in 96-well plates, at a density of 1,500 cells/well, and incubated overnight. The cells were exposed to Scutellarin (10, 30 and 100 μM) for 48 h. Fresh DMEM containing 5 mg/ml MTT (Sigma-Aldrich; Merck Millipore) were introduced to the cells at 37°C for 4 h. DMSO (100 μM; Sigma-Aldrich; Merck Millipore) was then added to solubilize the MTT product. The absorbance was measured at 540 nm with a background subtraction at 650 nm using an EMax Endpoint Microplate Reader (Molecular Devices LLC, Sunnyvale, CA, USA). This assay was repeated five times.

Hoechst 33342 dye staining. Morphological evaluation of apoptosis of HCT-116 cells was performed using on Hoechst 33342 staining (Invitrogen; Thermo Fisher Scientific, Inc.). HCT-116 cells ($5x10^5$ cells/well) were incubated in the absence and presence of Scutellarin (10, 30 and 100 μM) for 48 h. The HCT-116 cells were then fixed in 4% paraformaldehyde at room temperature for 30 min and rinsed with phosphate-buffered saline (PBS). The fixed HCT-116 cells were exposed to Hoechst 33342 (20 μg/ml) at room temperature for 15 min. Apoptotic morphological changes of HCT-116 cells were observed using an inverted fluorescence microscope. This assay was repeated five times.

Acridine orange/ethidium bromide (AO/EB) double staining. The AO/EB (Sigma-Aldrich; Merck Millipore) stain was used to detect the apoptosis of cancer cells. HCT-116 cells ($5x10^5$ cells/well) were seeded in 6-well plates before they were incubated with 10 μl prepared AO/EB working solution (100 μg/ml AO and 100 μg/ml EB in PBS) for 5 min. The nuclear alterations and apoptotic body formation of HCT-116 cells were visualized immediately using an inverted fluorescence microscope (Eclipse TE300; Nikon Corporation, Tokyo, Japan). This assay was repeated three times.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Apoptosis of HCT-116 cells was determined using a *In Situ* Cell Death Detection kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's protocol. Following exposure to Scutellarin (10, 30 and 100 μM) for 24 h, HCT-116 cells ($1x10^6$) were fixed with 4% paraformaldehyde in PBS for 1 h at room temperature, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate

for 2 min on ice. Then the HCT-116 cells were treated with the prepared TUNEL reaction mixture for 1 h at 37°C in the dark, and TUNEL staining was visualized using a fluorescence microscope (Olympus Corporation, Tokyo, Japan). The TUNEL assay was performed three times.

Western blot analysis. Following treatment with Scutellarin for 24 h, HCT-116 cells ($1x10^6$) were homogenized on ice, and the cell lysates were prepared by centrifugation at 14,000 x g for 10 min at 4°C. The protein concentration was quantified using the bicinchoninic acid assay (Pierce; Thermo Fisher Scientific, Inc.). Equal quantities of protein (60 μg) were separated using SDS-PAGE (10-12%) and then transferred onto a nitrocellulose membrane. The blots were blocked with 5% non-fat milk for 1 h at room temperature and then washed three times with PBS (Sigma-Aldrich; Merck Millipore) supplemented with 0.1% Tween-20 (PBS-T; Sigma-Aldrich; Merck Millipore), before they were incubated with primary antibodies against phosphorylated p53, p53, Bcl-2, Bax, p21 and cleaved caspase-3 for 2 h at room temperature. After washing with three times with PBS-T, Membranes were subsequently incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody [cat. no. sc-2005; dilution, 1:5,000 in 5% bovine serum albumin (BSA); Santa Cruz Biotechnology, Inc., Dallas, TX, USA] and anti-mouse IgG (cat. no. sc-2030; dilution, 1:5,000 in 5% BSA; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature, before they were incubated with horseradish peroxidase conjugate (GE Healthcare Life Sciences, Chalfont, UK). The bands were detected by chemiluminescence using an ECL kit (GE Healthcare Life Sciences). ImageJ software (version, 1.50; National Institutes of Health, Bethesda, MD, USA) was used to quantify the expression of proteins based on the intensity of the bands. The experiments were repeated three times. The mouse monoclonal p53 (cat. no. sc-98; dilution, 1:500), mouse monoclonal phosphorylated p53 (cat. no. sc-99; dilution, 1:200), mouse monoclonal Bcl-2 (cat. no. sc-7382; dilution, 1:500), mouse monoclonal Bax (cat. no. sc-23959; dilution, 1:200), mouse monoclonal p21 (cat. no. sc-6246; dilution, 1:200), rabbit polyclonal cleaved caspase-3 (cat. no. sc-22171; dilution, 1:500) antibodies were obtained from Santa Cruz Biotechnology, Inc., and mouse monoclonal β-actin (cat. no. AC-15; dilution, 1:2,000) was purchased from Sigma-Aldrich; Merck Millipore.

Statistical analysis. Data used for statistical analysis are expressed as the mean ± standard error. Significant differences among groups was determined using Bonferroni-corrected analysis of variance. All statistical analysis was performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistical significant difference.

Results

Scutellarin inhibits the growth of HCT-116 cells. The effect of Scutellarin on the viability of HCT-116 cells was evaluated using an MTT assay. As demonstrated in Fig. 1A, 10, 30 and 100 μM Scutellarin resulted in a significant reduction in the viability of HCT-116 cells when compared with control cells (P=0.0578, P=0.0062 and P=0.0023, respectively). The reduction in
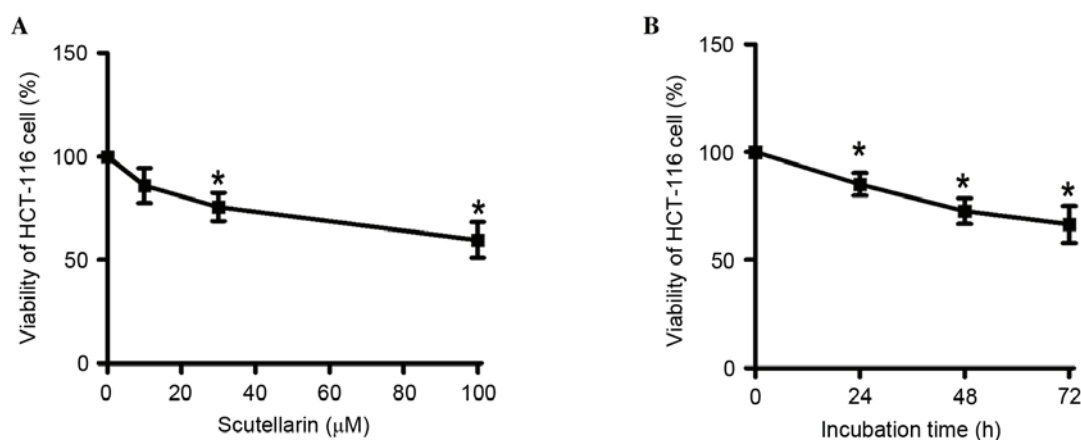
Figure 1. Scutellarin suppresses the growth of HCT-116 cells. (A) The cellular viability of HCT-116 cells was significantly reduced by Scutellarin (10, 30 and 100 μM) treatment for 24 h (*P<0.05 vs. 0 μM Scutellarin). (B) The growth of HCT-116 cells was decreased by treatment with 30 μM Scutellarin at 24, 48 and 72 h of incubation (*P<0.05 vs. Scutellarin 0 h).
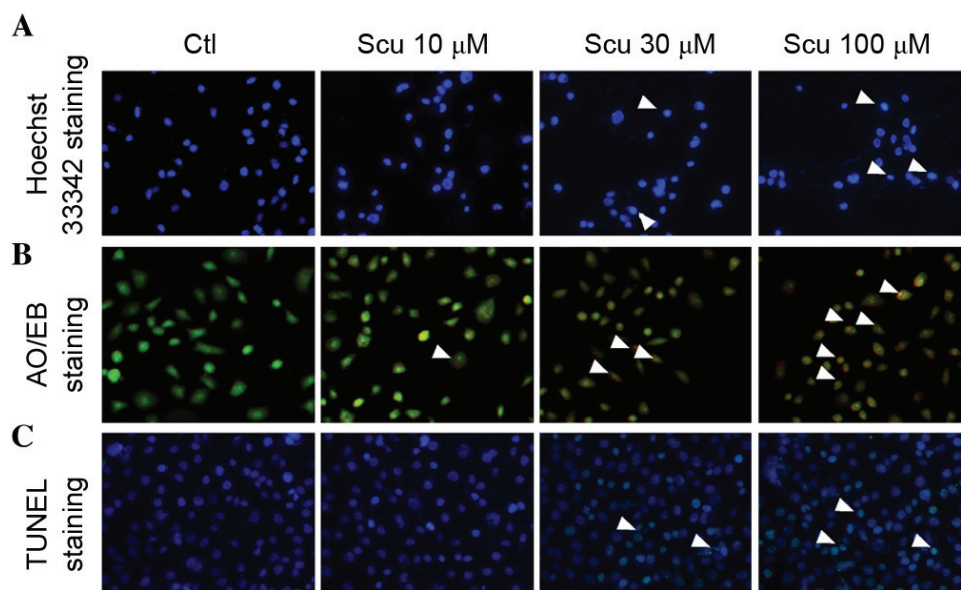

Figure 2. Effects of Scu on the apoptosis of HCT-116 cells. (A) Evaluation of HCT-116 apoptosis by Hoechst 33342 staining following Scu (10, 30 and 100 μM) treatment (magnification, x200). White arrows indicate abnormal nuclei. (B) Apoptosis of HCT-116 cells caused by Scu (10, 30 and 100 μM) as demonstrated by AO/EB staining (magnification, x200). White arrows indicate AO/EB-positive cells. (C) TUNEL staining was used to assess the effects of Scu (10, 30 and 100 μM) on HCT-116 apoptosis (magnification, x200). White arrows indicate TUNEL-positive cells. Ctl, control; Scu, Scutellarin; AO/EB, acridine orange/ethidium bromide; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

HCT-116 cell viability was concentration-dependent. The viability of HCT-116 cells was then determined following treatment with 30 μM Scutellarin for 24, 48 and 72 h (Fig. 1B). It was demonstrated that 30 μM Scutellarin gradually decreased the viability of HCT-116 cells and suppressed cellular growth with increasing incubation time, when compared with the 0 h-time point (24 h, P=0.0131; 48 h, P=0.0025; 72 h, P=0.0044). These results suggest that Scutellarin demonstrated antiproliferative activities in HCT-116 cells.

Scutellarin induces apoptosis of HCT-116 cells. Additionally, the current study investigated whether the apoptosis HCT-116 cells was induced by Scutellarin treatment. The nucleolar changes of HCT-116 cells were detected using a fluorescent microscope following Hoechst 33342 (Fig. 2A) and AO/EB (Fig. 2B) staining. Scutellarin-treated HCT-116 cells exhibited condensation of chromatin and pyknosis of nuclei (Fig. 2A and B). By contrast, untreated HCT-116 cells exhibited intact nuclear architecture. That Scutellarin induced the apoptosis of HCT-116 cells was further confirmed using a TUNEL assay. Untreated HCT-116 cells were predominantly are negative for TUNEL fluorescence, however Scutellarin treatment markedly increased the number of TUNEL-positive HCT-116 cells compared with untreated cells (Fig. 2C). These results confirmed that Scutellarin increased apoptosis of human colon cancer cells.

Scutellarin regulates Bcl-2 and Bax expression in HCT-116 cells. The inactivation of Bcl-2 and the activation of Bax commit cancer cells to undergo apoptosis (10). Thus, the effect
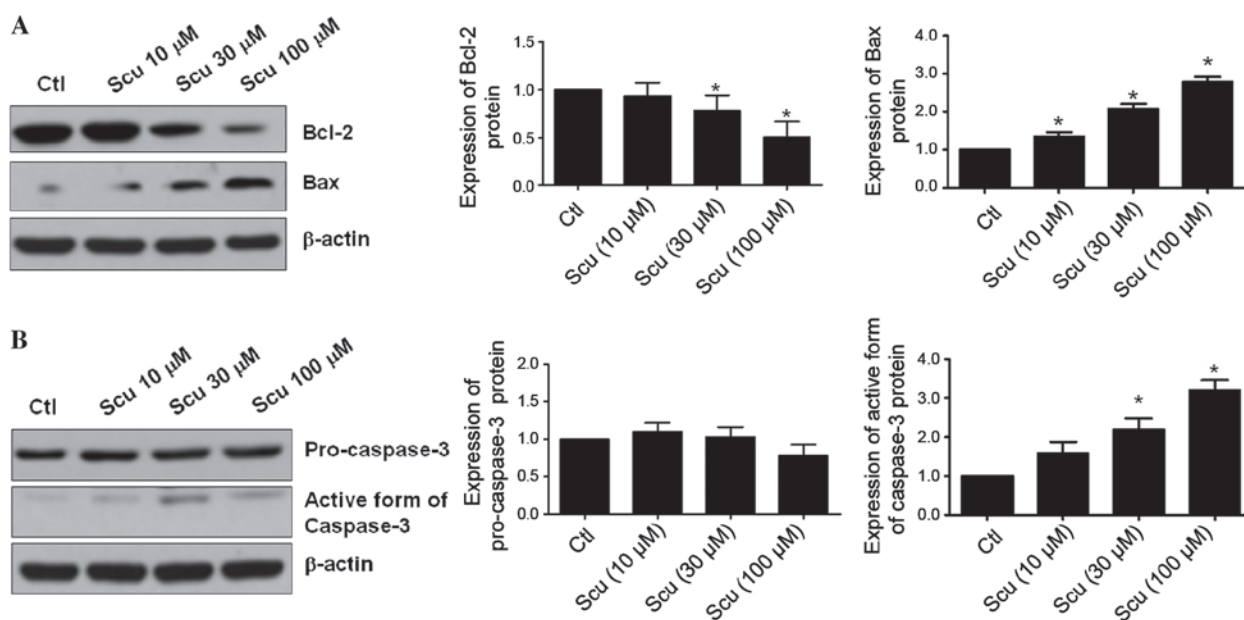
Figure 3. Scutellarin regulates the expression of Bcl-2/Bax proteins and the caspase-3 activity in HCT-116 cells. (A) The level of Bcl-2 protein was downregulated, and the expression of Bax protein was increased in Scu-treated HCT-116 cells. (B) The level of pro-caspase-3 and the active form of caspase-3 in HCT-116 cells in the absence and presence of Scu. β-actin was used as a loading control. *$P<0.05$ vs. Ctl. Ctl, control; Scu, Scutellarin; Bcl-2, Bcl-2 apoptosis regulator; Bax, Bcl-2 associated X apoptosis regulator.
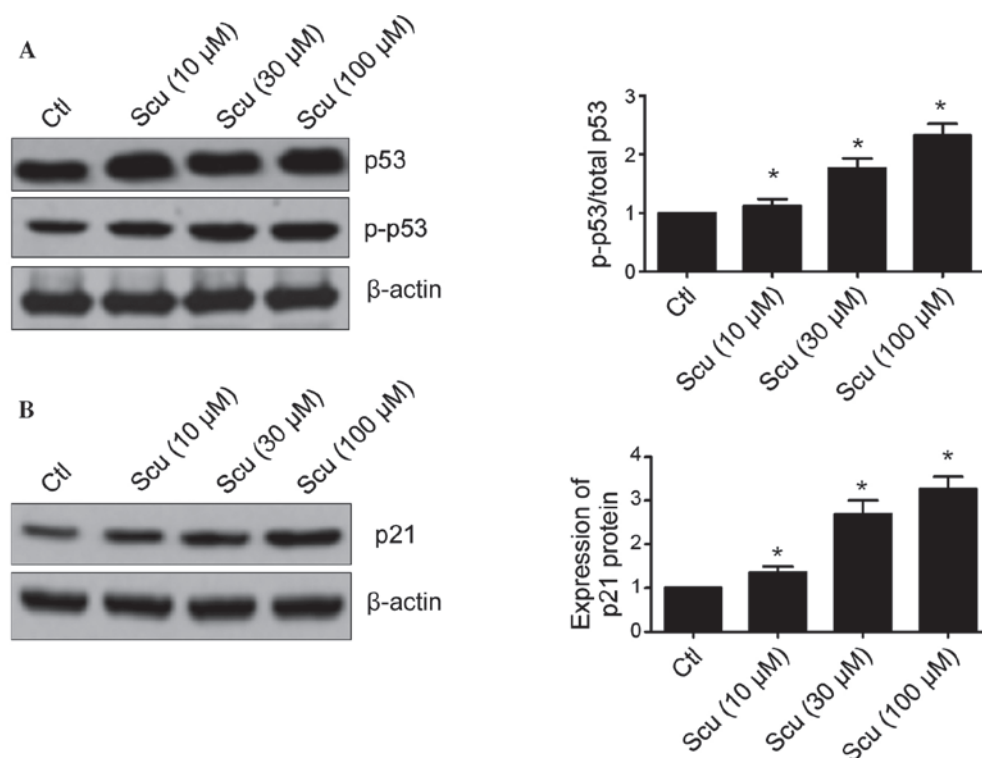

Figure 4. Scu regulated the expression of p53 and p21 proteins in HCT-116 cells. (A) The expression of p-p53 protein was increased by Scu treatment. (B) The level of p21 protein expression was upregulated by Scu treatment. *$P<0.05$ vs. Ctl. Ctl, control; Scu, Scutellarin; p-p53, phosphorylated p53.

of Scutellarin on the expression levels of Bcl-2 and Bax in HCT-116 cells were investigated. HCT-116 cells were exposed to 10, 30 and 100 μM Scutellarin for 48 h, and the expression of Bcl-2 protein was significantly decreased when compared with untreated HCT-116 cells ($P=0.3036$, $P=0.0890$ and $P=0.0093$, respectively; Fig. 3A). Consistently, the expression

of Bax protein in HCT-116 cells was increased significantly in the presence of 10 ($P=0.011$), 30 ($P=0.0003$) and 100 μM ($P=0.0002$) Scutellarin when compared with the control. The changes in the levels of Bcl-2 and Bax protein expression in HCT-116 cells were associated with the concentration of Scutellarin used. Additionally, the active form of caspase-3
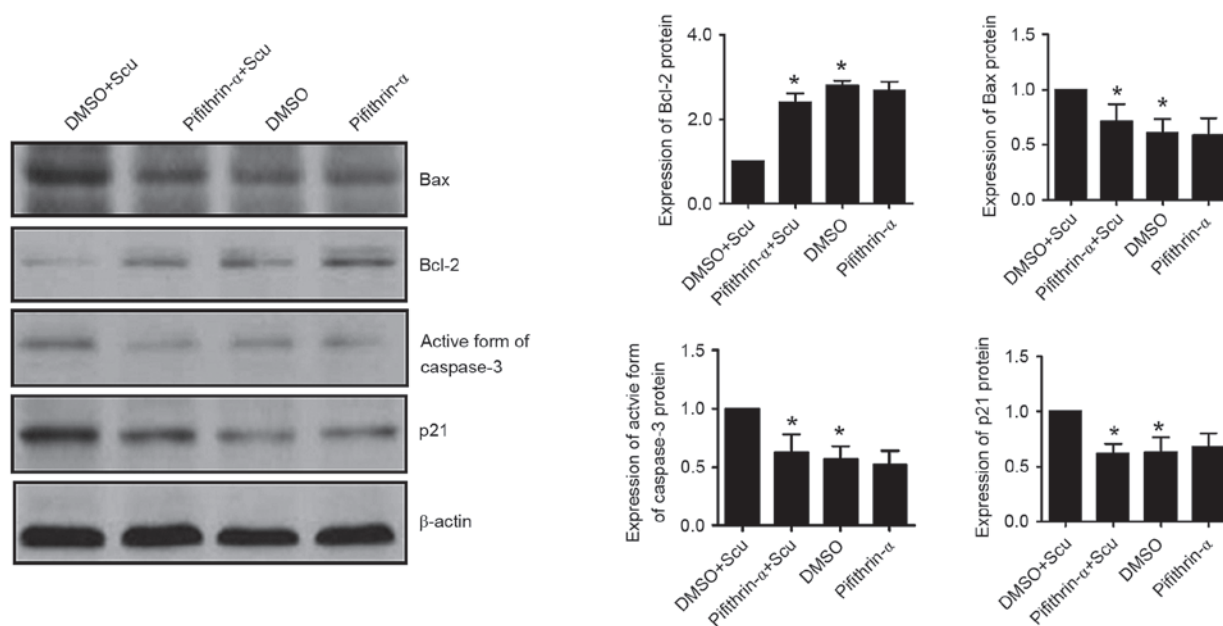
Figure 5. Inhibition of p53 abrogates Scu-induced apoptosis of HCT-116. The cultured cells were treated with DMSO, pifithrin-α (50 μM), DMSO+Scu (30 μM) and pifithrin-α (50 μM)+Scu (30 μM), respectively. The upregulation of the protein expression of Bax, active caspase-3 and p21 protein, and the reduction of Bcl-2 by Scu (30 μM) in HCT-116 cells was abrogated by pifithrin-α (50 μM) treatment. *P<0.05 vs. DMSO + Scu. DMSO, dimethyl sulfoxide; Scu, Scutellarin; Bax, Bcl-2 associated X apoptosis regulator; Bcl-2, Bcl-2 apoptosis regulator.

protein was also increased in HCT-116 treated with Scutellarin (Fig. 3B). These results suggest that Scutellarin induces apoptosis of HCT-116 cells via regulating Bcl-2/Bax expression and activating caspase-3.

Scutellarin affects the expression of p53 and p21 in HCT-116 cells. Lots of studies have demonstrated that p53 is an important tumor suppressor gene and its inactivation is involved in tumorigenesis and chemotherapy resistance (11-13). The effects of Scutellarin on the expression of p53 and p21 proteins were investigated. Fig. 4A demonstrated that Scutellarin significantly increased phosphorylation of p53 in HCT-116 cells when compared with the control (10 μM, P=0.0035; 30 μM, P=0.0005; and 100 μM, P=0.0002). p21 is a downstream target of the p53 pathway (12). Scutellarin-treated HCT-116 cells exhibited a significantly decreased level of p21 protein expression when compared with the control (10 μM, P=0.0096; 30 μM, P=0.0011; and 100 μM, P=0.0002; Fig. 4B). These results suggest that the p53 pathway may be involved in the Scutellarin-induced apoptosis of HCT-116 cells.

Scutellarin-induced apoptosis of HCT-116 was abrogated by suppressing p53. To further investigate the role of p53 in Scutellarin-induced apoptosis of HCT-116, the effects of a p53 inhibitor, pifithrin-α, on Scutellarin-induced apoptosis of HCT-116 was determined in the current study. Fig. 5 demonstrated that the p53 inhibitor, pifithrin-α (50 μM), abrogated the increase in Bax protein (P=0.0434) and decrease of Bcl-2 protein (P=0.0007) induced by 30 μM Scutellarin. In addition, the increase in the active form of caspase-3 induced by Scutellarin treatment was significantly abrogated by pifithrin-α in HCT-116 cells (P=0.0212). Furthermore, p21 protein expression was reduced by pifithrin-α treatment in HCT-116 cells

when compared with the levels in Scutellarin-treated cells (P=0.0032).

Discussion

Colon cancer is the third most common type of cancer, and >149,000 patients are diagnosed every year worldwide (14). In recent years, the occurrence of colon cancer has gradually increased. Currently, the therapeutics used to treat colon cancer are based on surgery combined with adjuvant chemotherapy (15). However, often these treatments fail to control the recurrence of colon cancer, as colon tumor cells develop resistance to the majority chemotherapy drugs (13). Thus, it is necessary to elucidate novel drugs to treat colon cancer.

Scutellarin is a major active compound extracted from *Scutellaria barbata* D. Don, an herbal plant that has been used for centuries to treat various aliments (4,8). Scutellarin has various biological activates, including anti-oxidant, anti-inflammatory and antibacterial effects, and thus, has been used in the clinical treatment of coronary heart diseases, cerebral thrombosis, cerebral infarction and hypertension (6-9). It was previously reported that Scutellarin induced the apoptosis of colon cancer, lymphoma and tongue cancer cells, with promising potential application in the clinic (8,9). For instance, Scutellarin suppresses the growth and induces apoptosis of human U937 leukemia cells via the mitochondrial apoptosis pathway (16). In addition, Scutellarin was demonstrated to effectively sensitize resveratrol and 5-fluorouracil-stimulated colon cancer cell apoptosis via enhancing caspase-6 activation (9). *In vivo* studies also confirmed that Scutellarin combined with ultrasound significantly delayed tumor growth, inhibited tumor angiogenesis and caused cancer-cell apoptosis of human tongue carcinoma xenografts via decreasing the expression of matrix metalloproteinase 2 and 9 (17).

To the best of our knowledge, the current study was the first to demonstrated the antiproliferative and pro-apoptotic effects of Scutellarin on human colon cancer cells. The present study demonstrated that Scutellarin significantly reduced the viability of HCT-116 cells in a time- and dose-dependent manner. Reduced cell viability was observed even at the lowest concentration of Scutellarin used (10 μM). Tumor cells with inhibited growth are usually prone to undergo apoptosis (18). Thus, the current study determined whether Scutellarin treatment induced apoptosis in HCT-116 cells, which may explain the observed loss of viability. Hoechst 33342 staining and AO/EB staining demonstrated nuclear changes and apoptotic body formation in HCT-116 cells. Treatment with Scutellarin also increased the number of apoptotic cells, as assessed by TUNEL staining. These findings confirmed that Scutellarin was able to cause apoptosis of human colon cancer cells.

Subsequently, the further analsyis was performed to understand the molecular mechanism underlying the anti-tumor effect of Scutellarin on human colon cancer. Carcinogenesis or tumorigenesis is closely associated to the uncontrolled growth of tumor cells and the loss of tumor cells apoptosis (19). Upregulated expression of the anti-apoptotic protein, Bcl-2, and the downregulation of pro-apoptotic protein, Bax, are involved in the initiation and aggression of tumors (10,20). Conversely, inhibiting Bcl-2 or increasing Bax expression was suggested as an important approach for treating cancer (10). The current study demonstrated that Scutellarin treatment reduced the expression of Bcl-2 protein and increased the expression of Bax protein in colon cancer cells. Additionally, the protein level of the active form of caspase-3, a downstream target of Bcl-2 and Bax the apoptotic pathway was also demonstrated to be increased by Scutellarin treatment (21). These results suggested that the regulation of Bcl-2/Bax expression and the activation of caspase-3 protein were a molecular mechanism underlying the Scutellarin-induced apoptosis of human colon cancer cells.

Various studies have demonstrated that p53 is an important tumor suppressor gene, and regulates cell cycle, apoptosis, metastasis and senescence (11,12). Inactivation or mutations of p53 have been well documented in human tumors (12). By contrast, overexpression or activation of p53 can induce cell apoptosis, and attenuate cancer cell migration and invasion through regulating numerous targets, including Bcl-2 and Bax (22). Also, p53 induces the apoptosis of tumor cells via regulating the transcription of p21 (19). The current study demonstrated that Scutellarin induced an increase in the ratio of phosphorylated p53 to total p53, which was accompanied by an upregulation in p21 protein expression. Further investigation demonstrated that inhibition of p53 led to a reduction in the expression of pro-apoptotic proteins (Bax and caspase-3) and an increase in the expression of anti-apoptotic proteins (Bcl-2) in HCT-116 cells. This indicated that Scutellarin induced apoptotic cell death in colon cancer and inhibited cell growth *in vitro* via regulation of the p53/p21 pathway. This result is consistent with a previous report (9).

In conclusion, Scutellarin, a bioactive flavonoid extracted from *Scutellaria barbata* D. Don induces apoptosis in HCT-116 human colon carcinoma cells via activating p53 and regulating Bcl-2/Bax expression. The results of the current study study suggested that Scutellarin may be useful as a novel therapeutic agent against colon cancer.

Acknowledgements

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