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Original Research Article

# Anti-Cancer Activity of the Flower Bud of *Sophora japonica* L. through Upregulating Activating Transcription Factor 3 in Human Colorectal Cancer Cells

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**Abstract** - The flower buds of *Sophora japonica* L (SF), as a well-known traditional Chinese medicinal herb, have been used to treat bleeding-related disorders such as hematochezia, hemorrhoidal bleeding, dysfunctional uterine bleeding, and diarrhea. However, no specific anti-cancer effect and its molecular mechanism of SF have been described. Thus, we performed *in vitro* study to investigate if treatment of SF affects activating transcription factor 3 (ATF3) expression and ATF3-mediated apoptosis in human colorectal cancer cells. The effects of SF on cell viability and apoptosis were measured by MTT assay and Western blot analysis against cleaved poly (ADP-ribose) polymerase (PARP). ATF3 activation induced by SF was evaluated using Western blot analysis, RT-PCR and ATF3 promoter assay. SF treatment caused decrease of cell viability and increase of apoptosis in a dose-dependent manner in HCT116 and SW480 cells. Exposure of SF activated the levels of ATF3 protein and mRNA via transcriptional regulation in HCT116 and SW480 cells. Inhibition of extracellular signal-regulated kinases (ERK) 1/2 by PD98059 and p38 by SB203580 attenuated SF-induced ATF3 expression and transcriptional activation. Ectopic ATF3 overexpression accelerated SF-induced cleavage of PARP. These findings suggest that SF-mediated apoptosis may be the result of ATF3 expression through ERK1/2 and p38-mediated transcriptional activation.

**Key words** - Activating transcription factor 3, Cancer chemoprevention, Colorectal cancer, Flower bud of *Sophora japonica* L.

## 1. Introduction

Human colorectal cancer accounts for the second in the frequent malignancy and the leading cause of death from cancer worldwide (Walker *et al*., 2014). Although the most effective treatment of CRC is surgery and adjuvant chemotherapy, patients with late-stage of colorectal cancer still have poor prognosis and the overall mortality of the disease is around 40% (Luo *et al*., 2014). Thus, chemoprevention has received attention as an effective approach to reduce incidence and malignancy of colorectal cancer.

Approximately 60% of the current anti-cancer drugs have been derived from natural products (Gordaliza, 2007). Among the natural products, medicinal plants have shown the properties to reduce the risk of colorectal cancer and slow its progression through targeting various molecular aspects associated with colorectal cancer development (Kuppusamy *et al*., 2014).

Sophorae Flos (SF) as the flower buds of *Sophora japonica* L. has been well known to be traditional Chinese medicinal herb (Qi *et al*., 2007). In traditional Chinese medicine, SF has been used for treating bleeding related disorders such as hematochezia, hemorrhoidal bleeding, dysfunctional uterine bleeding, and diarrhea (Lo *et al*., 2009). However, the potential anti-cancer mechanisms of SF have not been elucidated and the effects of SF on human colorectal cancer have not been tested so far.

In light of the therapeutic potential of SF in colorectal

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cancer, this study was performed to elucidate the biological mechanism by which SF induces the reduction of cell viability and apoptosis in human colorectal cancer cells. Here, for the first time, we report that SF leads to ERK1/2- and p38-dependent activation of activating transcription factor 3 (ATF3) expression, which may result in apoptosis in colorectal cancer cells.

## Materials and Methods

### Chemicals

Cell culture media, Dulbecco's Modified Eagle medium (DMEM)/F-12 1:1 Modified medium (DMEM/F-12) was purchased from Lonza (Walkersville, MD, USA). 3-(4,5-dimethylthizaol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Aldrich (St. Louis, MO, USA). SB203580 and PD98059 were purchased from Calbiochem (San Diego, CA, USA). ATF3 antibody was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Antibodies against β-actin, Poly (ADP-ribose) polymerase (PARP), p38, p-p38, ERK1/2 and p-ERK1/2 were purchased from Cell Signaling (Bervely, MA, USA). ATF3 promoter constructs (-1420/+34, -718/+34, -514/+34, -318/+34, -147/+34 and -84/+34, pATF3-514 del Ftz and pATF3-514 del CRE) were kindly provided by Dr. S-H Lee (University of Maryland College Park, Maryland, USA). All chemicals were purchased from Fisher Scientific, unless otherwise specified.

### Sample preparation

The buds of *Sophora japonica* L. were kindly provided by the Bonghwa Alpine Medicinal Plant Experiment Station, Korea. One kilogram of ginger leaf was extracted with 1000 ㎖ of 80% methanol with shaking for 24 h. After 24 h, the methanol-soluble fraction was filtered and concentrated to approximately 20 ㎖ volume using a vacuum evaporator and then fractionated with petroleum ether and ethyl acetate in a separating funnel. The ethyl acetate fraction was separated from the mixture, evaporated by a vacuum evaporator, and prepared aseptically and kept in a refrigerator.

### Cell culture and treatment

Human colorectal cancer cell lines, HCT116 and SW480 were purchased from Korean Cell Line Bank (Seoul, Korea) and grown in DMEM/F-12 supplemented with 10% fatal bovine serum (FBS), 100 U/㎖ penicillin and 100 ㎍/㎖ streptomycin. The cells were maintained at 37°C under a humidified atmosphere of 5% $CO_2$. The extracts from the buds of *Sophora japonica* L. (SF) were dissolved in dimethyl sulfoxide (DMSO) and treated to cells. DMSO was used as a vehicle and the final DMSO concentration did not exceed 0.1% (v/v).

### Cell viability

Cell viability was measured using MTT assay system. Briefly, cells were plated onto 96-well plated and grown overni ght. The cells were treated with 0, 12.5, 25 and 50 ㎍/㎖ of SF for 24 h. Then, the cells were incubated with 50 ㎕ of MTT solution (1 ㎎/㎖) for an additional 2 h. The resulting crystals were dissolved in DMSO. The formation of formazan was measured by reading absorbance at a wavelength of 570 ㎚.

### Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was prepared using a RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and total RNA (1 ㎍) was reverse-transcribed using a Verso cDNA Kit (Thermo Scientific, Pittsburgh, PA, USA) according to the manufacturer's protocol for cDNA synthesis. PCR was carried out using PCR Master Mix Kit (Promega, Madison, WI, USA) with primers for human ATF3 and human GAPDH as follows: human ATF3: 5′-gtttgaggattttgctaacctgac-3′, and reverse 5′-agctgcaatcttatttc tttctcgt-3′; huaman GAPDH: forward 5'-acccagaagactgtggatgg-3' and reverse 5'-ttctagacggcaggtcaggt-3'.

### Transient transfections and luciferase activity

Transient transfections were performed using the PolyJet DNA transfection reagent (SignaGen Laboratories, Ijamsville, MD, USA) according to the manufacturers' instruction. HCT116 and SW480 cells were plated in 12-well plates at a concentration of $2 \times 10^5$ cells/well. After growth overnight, plasmid mixtures containing 0.5 ㎍ of ATF3 promoter linked to luciferase and 0.05 ㎍ of pRL-null vector were transfected for 24 h. The transfected cells were cultured in the absence or presence of SF for the indicated times. The cells were then harvested in 1 × luciferase lysis buffer, and luciferase activity was normalized to the pRL-null luciferase activity using a

dual-luciferase assay kit (Promega, Madison, WI, USA).

**Expression vector**

ATF3 expression vector was provided from Addgene (Cambridge, MA, USA). Transient transfection of the vector was performed using the PolyJet DNA transfection reagent (SignaGen Laboratories, Ijamsville, MD, USA) according to the manufacturers' instruction.

**SDS-PAGE and Western blot**

After SF treatment, cells were washed with 1×phosphate-buffered saline (PBS), and lysed in radioimmunoprecipitation assay (RIPA) buffer (Boston Bio Products, Ashland, MA, USA) supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MD. USA) and phosphatase inhibitor cocktail (Sigma-Aldrich), and centrifuged at 15,000 × g for 10 min at 4°C. Protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). The proteins were separated on SDS-PAGE and transferred to PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked for non-specific binding with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1h at room temperature and then incubated with specific primary antibodies in 5% non-fat dry milk at 4°C overnight. After three washes with TBS-T, the blots were incubated with horse radish peroxidase (HRP)-conjugated immunoglobulin G (IgG) for 1 h at room temperature and chemiluminescence was detected with ECL Western blotting substrate (Amersham Biosciences, Piscataway, NJ, USA) and visualized in Polaroid film.

**Statistical analysis**

Statistical analysis was performed with the Student's unpaired t-test, with statistical significance set at *, $P < 0.05$.

## Results

**Effect of SF on the cell viability and apoptosis in human colon cancer cells, HCT116 and SW480 cells**

To evaluate if SF attenuates the cell viability of human colon cancer cells, HCT116 and SW480 cells were treated with 0, 12.5, 25 and 50 ㎍/㎖ of SF for 24 h and then the cell viability was measured by MTT assay. As shown in Fig. 1A,

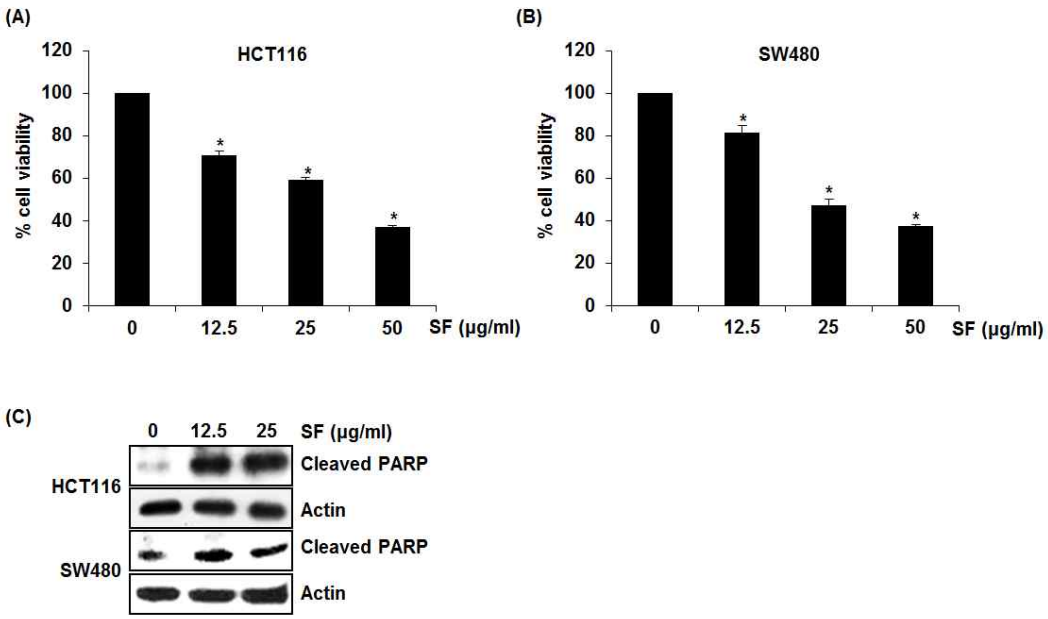


Fig. 1. Effect of SF on cell viability and apoptosis in HCT116 and SW480 cells. (A, B) HCT116 and SW480 cells were treated with the varying concentrations of SF for 24 h. Cell viability was measured using MTT assay system and expressed as % cell viability. *$P < 0.05$ compared to cells without SF. (C) HCT116 and SW480 cells were treated with the varying concentrations of SF for 24 h. Cell lysate was subjected to SDS-PAGE and Western blot was performed using PARP antibody. Actin was used as internal control.

SF attenuated the viability of HCT116 by 29% at 12.5 ㎍/㎖, 41% at 25 ㎍/㎖ and 63% at 50 ㎍/㎖, respectively. In addition, the viability of SW480 cells was inhibited by SF by 19% at 12.5 ㎍/㎖, 53% at 25 ㎍/㎖ and 62% at 50 ㎍/㎖, respectively. To test whether SF-mediated reduction of the cell viability results from apoptosis, we performed Western blot against cleaved PARP and observed that SF increased the cleavage of PARP (Fig. 1C).

**SF activates ATF3 expression through the increase of ATF3 transcriptional activity**

To evaluate whether SF activates ATF3 expression, Western blot was performed using HCT116 and SW480 cells treated with SF. As shown in Fig. 2A, SF dose-dependently increased ATF3 protein level in both HCT116 and SW480 cells. In time-course experiments, ATF3 expression began to increase at 10 h after SF treatment in both HCT116 and SW480 cells (Fig. 2B). To elucidate whether SF-induced activation of ATF3 expression results from its transcriptional regulation, mRNA level of ATF3 by RT-PCR and promoter activity by luciferase system were measured. As shown in Fig. 2C and 2D, SF activated both ATF3 mRNA level and promoter activity in HCT116 and SW480 cells. In addition, we found that ATF3 promoter activity began to increase at 10

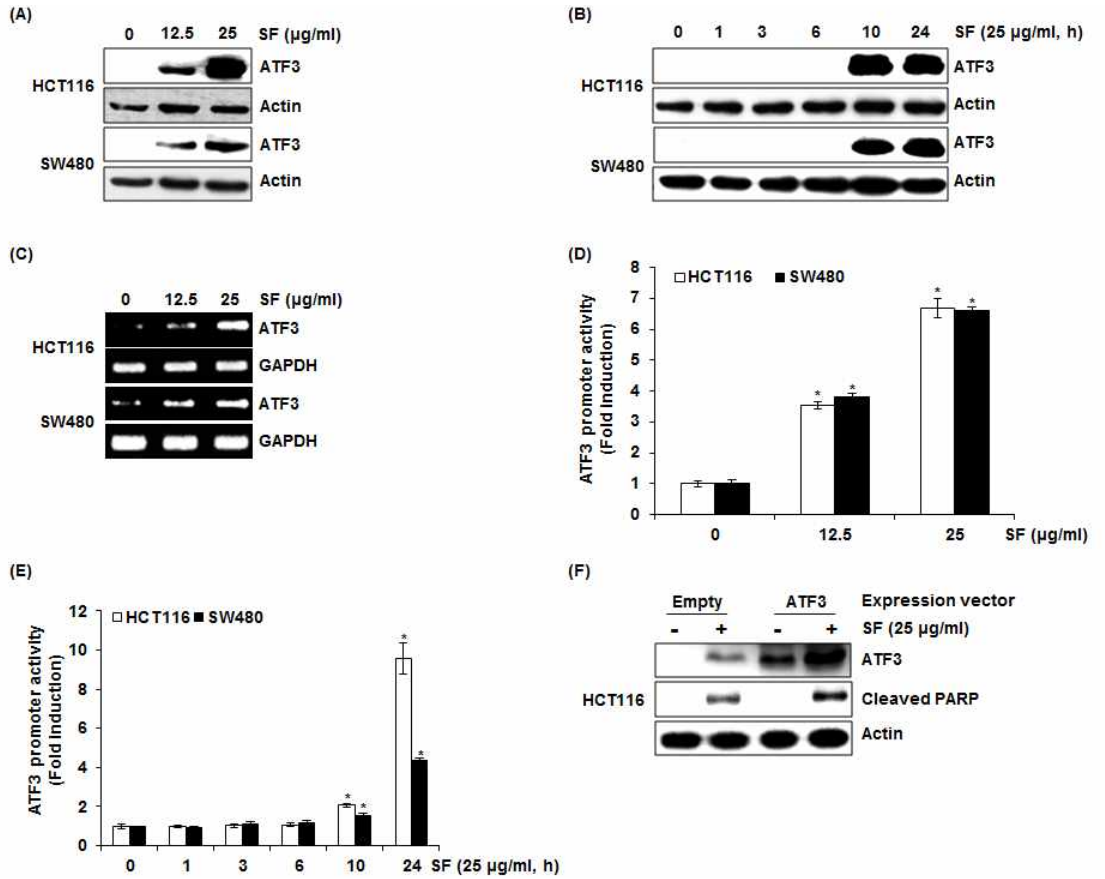


Fig. 2. Effect of SF on ATF3 activation in HCT116 and SW480 cells. (A, B) HCT116 and SW480 cells were treated with SF at the indicated concentrations for 24 h or 25 ㎍/㎖ of SF for the indicated times. Cell lysate was subjected to SDS-PAGE and Western blot was performed using ATF3 antibody. Actin was used as internal control. (C) RT-PCR analysis of ATF3 gene expression, total RNA was prepared after SF treatment for 24 h. GAPDH was used as internal control. (D, E) For ATF3 promoter activity, luciferase construct containing -1420 to +34 of human ATF3 promoter region was cotransfected with pRL-null vector and the cells were treated with SF and luciferase activity was measured. *P < 0.05 compared to cells without SF treatment. (F) HCT116 cells was transfected with empty- or ATF3 expression vector for 24 h and then treated with 25 ㎍/㎖ of SF for 24 h. Cell lysates were subjected to SDS-PAGE and Western blot was performed using ATF3 or PARP antibody. Actin was used as internal control.

h after SF treatment similar to SF-induced increase of ATF3 protein level (Fig. 2E). To see whether ATF3 expression affect SF-mediated apoptosis, cleaved PARP was measured by Western blot in SF-treated HCT116 cells after ATF3 overexpression. As shown in Fig. 2F, ATF3 overexpression increased PARP cleavage by SF, indicating that ATF3 may be one of important molecular targets in SF-mediated apoptosis.

**Identification of ATF3 promoter sites responsible for SF-induced ATF3 activation**

To elucidate a specific site of ATF3 promoter region associated with SF-mediated ATF3 promoter activation, ATF3 promoter constructs (pATF3-1420/+34, pATF3-718/+34, pATF3-514/+34, pATF3-318/+34, pATF3-147/+34 and pATF 3-84/+34) were transfected into SW480 cells and treated with 25 ㎍/㎖ of SF for 24 h. As shown in Fig. 3A, SF induced ATF3 promoter activation by 3.5, 3.4, 3.2, 3.2, 2.4 and 1.4 in pATF3-1420/+34, pATF3-718/+34, pATF3-514/+34, pATF3-318/+34, pATF3-147/+34 and pATF3-84/+34, respectively. SF increased ATF3 promoter activity by 2-fold using ATF3 promoter construct containing regions between -1420 and -147, while SF slightly increased ATF3 promoter activity using a construct containing the -84/+34 region, which indicates that ATF3 promoter region (-146/-85) may be important for ATF3 activation by SF. The Fushi tarazu (Ftz) and CREB have been reported to be cis-acting elements in ATF3 promoter containing -146 and -85 (Gene Regulation, TFSEARCH, and Transcription Element System). To identify the role of each cis-acting element, each site-deleted ATF3 promoter constructs were transfected into SW480 cells and treated with 25 ㎍/㎖ of SF for 24 h. As shown in Fig. 3B, SF-mediated ATF3 promoter activation was significantly decreased when the CREB site was deleted. However, the deletion of Ftz sites did not affect ATF3 promoter activity by SF. These data indicated that CREB is an important region in SF-induced ATF3 expression.

**SF-mediated ATF3 expression is dependent on ERK1/2 and p38 activation**

To investigate the upstream kinases associated with the

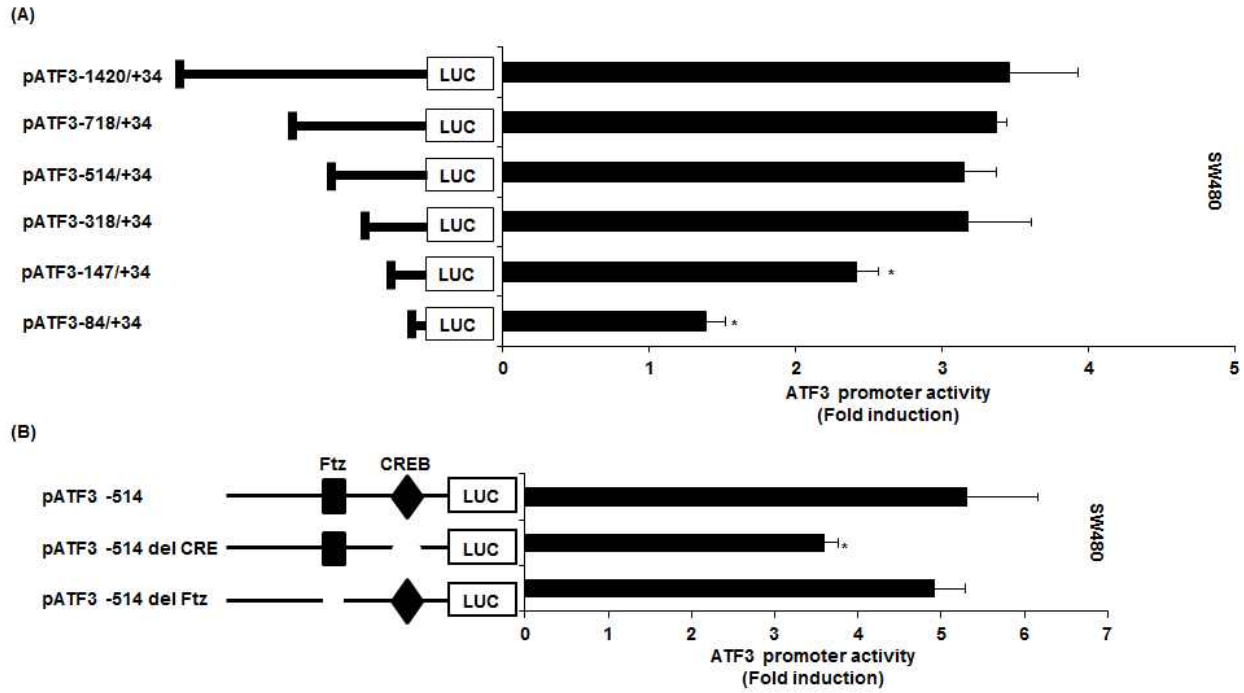


Fig. 3. Identification of ATF3 promoter sites responsible for SF-induced ATF3 activation. (A, B) Each indicated construct of the ATF3 promoter (0.5 ㎍) was co-transfected with 0.05 ㎍ of pRL-null vector into SW480 cells, and cells were treated with 25 ㎍/㎖ of SF. Luciferase activity was measured. *P < 0.05 compared to cells transfected with luciferase construct containing -1420 to +34 of human ATF3 promoter region.

activation of ATF3 expression by SF, HCT116 and SW480 cells were pretreated with PD98059 for ERK1/2 inhibition or SB203580 for p38 inhibition, and then co-treated with SF. In this experiment, we found that inhibitions of both ERK1/2 and p38 attenuated SF-mediated increase of ATF3 protein level (Fig. 4A). In addition, ATF3 promoter activity by SF was suppressed by inhibitions of both ERK1/2 and p38 (Fig. 4B). These two findings indicate that SF-mediated ATF3 expression may be associated with ERK1/2 and p38 activation. Thus, we performed Western blot against the phosphorylation of ERK1/2 and p38 to evaluate if SF activates ERK1/2 and p38. As shown in Fig. 4C, SF induced the hyperphosphorylation of ERK1/2 and p38.

## Discussion

Natural products have received attention as an effective approach for chemoprevention of colorectal cancer. Here, we demonstrate direct evidence that Sophorae Flos (SF) as the flower buds of *Sophora japonica* L. enhances apoptosis of human colorectal cancer cells through activation of ATF3 gene transcription via activation of ERK1/2 and p38 in human colorectal cancer cells.

Activating transcription factor 3 (ATF3) as a member of the ATF/CREB family of transcription factors has been known to be an adaptive-response gene (Hai *et al*., 2010). In the cellular processes to adapt to extra- and/or intracellular

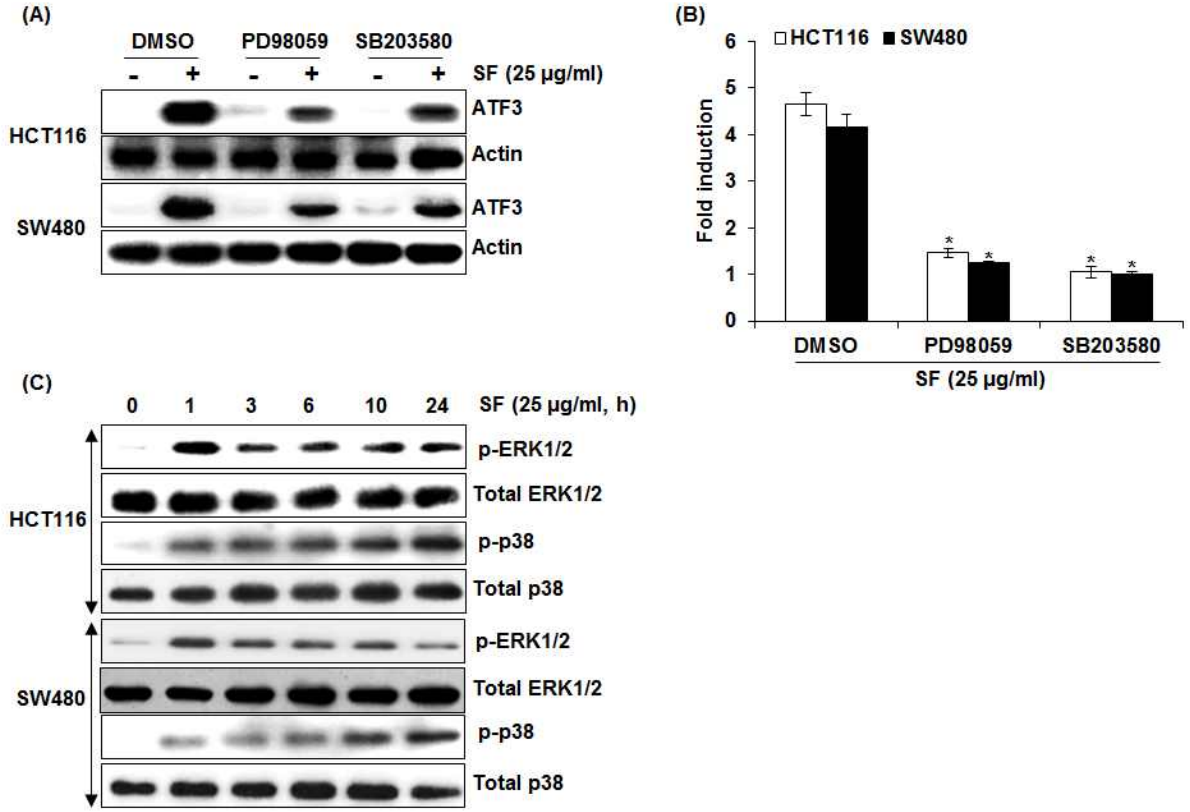


Fig. 4. SF-mediated ATF3 activation is dependent on ERK1/2 and p38 MAPK. (A) HCT116 and SW480 cells were pretreated with 20 μM of PD98059 and SB203580 for 2 h and then co-treated with 25 ㎍/㎖ of SF for 24 h. Cell lysates were subjected to SDS-PAGE and Western blot was performed using ATF3 antibody. Actin was used as internal control. (B) HCT116 and SW480 cells were transfected with luciferase construct containing -1420 to +34 of human ATF3 promoter region and pRL-null vector. The cells were pretreated with 20 μM of PD98059 and SB203580 for 2 h and then co-treated with 25 ㎍/㎖ of SF for 24 h. Luciferase activity was measured. *P < 0.05 compared to cells without treatments of PD98059 or SB203580. (C) HCT116 and SW480 cells were treated with 25 ㎍/㎖ of SF for the indicated times. Cell lysates were subjected to SDS-PAGE and Western blot was performed using antibodies for p-ERK1/2, ERK1/2, p-p38 and p38. Actin was used as internal control.

change, ATF3 plays a role to activate or repress gene expression (Hai *et al*., 2010). In cancer development, ATF3 have dichotomous roles including pro- or anti-apoptotic mechanisms (Miyazaki *et al*., 2009; Yin *et al*., 2008). Recently, there is a growing evidence indicating that ATF3 plays a pro-apoptotic role in human colorectal cancer cells (Baek *et al*., 2004; Cho *et al*., 2007; Fan *et al*., 2002; Lee *et al*., 2005; Lee *et al*., 2006; Piyanuch *et al*., 2007; Yamaguchi *et al*., 2006), which indicates that ATF3 may be an important molecular target in prevention of colorectal cancer.

SF induced the decrease of the cell viability and the increase of the apoptosis in HCT116 and SW480 cells. In addition, SF activated the expression of the activating transcription factor 3 (ATF3) in both protein and mRNA level, which resulted from the transcriptional regulation. In the experiment to evaluate the relationship between ATF3 and SF-mediated apoptosis, ectopic expression of ATF3 using an ATF3 over-expression vector accelerated the apoptosis by SF in HCT116 cells. Therefore, our data indicate that ATF3 may be one of the molecular targets in the anti-cancer properties of SF in human colorectal cancer.

Various response elements such as activating protein-1, ATF3/CRE, NF-κB, E2F and Myc/Max are contained in ATF3 promoter region (Liang *et al*., 1996). In this study, we found that the CRE binding site in ATF3 promoter region (-146/-85) may be important for the transcriptional activation of ATF by SF. CRE binding site in the ATF3 promoter region has been reported to be important for the activation of ATF3 transcription (Fu and Kilberg, 2013; Lee *et al*., 2014; Park *et al*., 2014).

This study also indicated that SF treatment caused activation of ERK1/2 and p38. It has been shown that ERK1/2 and p38 are the upstream kinases associated with ATF3 activation (Baek *et al*., 2004; Lee *et al*., 2010). So, we examined whether SF-mediated ATF3 activation is associated with the activations of ERK1/2 and p38 and found that inhibitions of both ERK1/2 and p38 attenuated ATF3 promoter activation and protein expression by SF in HCT116 and SW480 cells. These data indicates that ERK1/2 and p38 may be important upstream kinases for SF's effect on ATF3 activation.

Taken together, the current study provides information on the molecular mechanism of anti-cancer activity by SF. ERK1/2 and p38 influence SF-induced ATF3 expression via the transcriptional regulation. The resulting ATF3 activation induces apoptosis in human colorectal cancer cells.

## Acknowledgment

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