

Apigenin suppresses the growth of colorectal cancer xenografts via phosphorylation and up-regulated FADD expression

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Abstract. Apigenin is a flavonoid belonging to the flavone structural class. It has been implicated as a chemopreventive agent against prostate and breast cancers. However, to the best of our knowledge, no published data are available regarding apigenin in colorectal cancer (CRC). The effects and mechanisms of apigenin on CRC may vary significantly. This study aimed to analyze the effects of apigenin on the growth of CRC xenografts in nude mice derived from SW480, as well as to investigate the underlying mechanisms. Whole-body fluorescence imaging is an inexpensive optical system used to visualize gene expression in small mammals using reporter genes, such as eGFP as a reporter. In our study, the expression of eGFP may reflect the size of the tumor. A terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay showed that apigenin promoted the apoptosis of CRC cells. Furthermore, the expression of five genes related to the proliferation and apoptosis of CRC, i.e., cyclin D1, BAG-1, Bcl-2, yrdC and Fas-associated protein with death domain (FADD), was detected by real-time quantitative RT-PCR. Among these genes, the up-regulated expression of FADD was noted in CRC xenograft tumors treated with apigenin. Immunohistochemistry and Western blotting confirmed the results at the protein level. Furthermore, Western blot analysis

showed that apigenin induced the phosphorylation of FADD. Our findings suggest that apigenin enhances the expression of FADD and induces its phosphorylation, which may cause apoptosis of CRC cells and inhibition of tumor growth.

Introduction

Colorectal cancer (CRC) is one of the leading causes of morbidity and mortality both in the United States and worldwide. In 2009, the estimated new cases of colon and rectum cancers were 106,100 and an estimated 49,920 patients succumbed in the United States alone (1). Approximately 850,000 people develop CRC annually and 500,000 succumb to the disease (2). The prevention and treatment of CRC thus remains a global health issue.

Apigenin is a flavonoid belonging to the flavone structural class and is chemically known as 4',5,7-trihydroxyflavone (3). Apigenin has been shown to possess significant anti-inflammatory, antioxidant and anti-carcinogenic properties (4). Pre-treatment with apigenin induces the apoptosis of HCT-116 colon cancer cells (5) and inhibits the growth of colon carcinoma cell lines, including SW480, HT-29 and Caco-2 (6). A previous study showed that apigenin enhanced apoptosis in HT29 cells with wild-type adenomatous polyposis coli (7). *In vivo* studies showed that apigenin moderately reduces azoxymethane-induced colon carcinogenesis and inhibits the growth of colorectal cancer (8). However, to the best of our knowledge no published data are currently available regarding the *in vivo* study of apigenin on the growth of CRC in mice. Therefore, an *in vivo* study using fluorescence imaging may aid in a detailed analysis of the effectiveness of apigenin on xenograft tumors derived from SW480, a cell line established from a primary adenocarcinoma of the colon (9).

Materials and methods

Cell culture. SW480/enhanced green fluorescent protein (eGFP) was donated by Dr Zhao, Department of Pathology, Southern Medical University, China. Tumor cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/ml penicillin/streptomycin (Gibco)

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Table I. Primer sequence information.

Gene name	Primers	Product (bp)
BAG-1	Sense 5' - ACTGTCACCCACAGCAATGA-3' Antisense 5' - TGTGGAACCCCTATGACCTC-3'	116
Bcl-2	Sense 5' - ATGTGTGTGGAGAGCGTCAA-3' Antisense 5' - ACAGTTCCACAAAGGCATCC-3'	136
CCND1	Sense 5' - GATCAAGTGTGACCCGGACT-3' Antisense 5' - TCCTCCTCTTCCTCCTCCTC-3'	126
FADD	Sense 5' - CCTGTGTGCAGCATTTAACG-3' Antisense 5' - GTCCTCGATGCTGTCGATCT-3'	106
yrdC	Sense 5' - CGGATTCCTGATCATGCTTT-3' Antisense 5' - AGGACAACCTGAGGCCAGAGA-3'	139
GAPDH	Sense 5' - ACAGTCAGCCGCATCTTCTT -3' Antisense 5' - ACGACCAAATCCGTTGACTC -3'	94

and 500 $\mu\text{g/ml}$ G418, and incubated in a humidified chamber with 5% CO_2 at 37°C.

Mice and xenograft tumors. Male BALB/c-nu mice (specific pathogen-free) were obtained from the Laboratory Animal Center of Southern Medical University. The animals were maintained at a constant room temperature with a 12-h light cycle in a conventional animal colony. The mice were between 6 and 8 weeks old at the beginning of the experiments, and weighed 15-20 g. Tumor xenografts were generated by subcutaneous injection of 5×10^6 cells on the right inguen of the nude mice. The procedures were performed using approved protocols, in accordance with the guidelines of the Animal Care and Use Committee of Southern Medical University. Three days following the injection, the fluorescence emitted by the tumor cells was imaged by a whole-body GFP imaging system (ImageStation 2000MM; Kodak, USA) to visualize the formation of the tumor. A total of 16 tumor-bearing mice were randomly divided into two groups, the apigenin and control groups. Apigenin (Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO) and diluted in phosphate-buffered saline (PBS). Each mouse in the apigenin group was injected intraperitoneally with 20 mg/kg of apigenin, while in the control group, each mouse was injected with the same amount of vehicle solution (DMSO-PBS). Images of tumors were obtained every 6 days and analyzed to calculate the tumor area and tumor growth using Molecular Imaging Software Version 4.0 provided by Kodak 2000MM System.

Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay. A TUNEL apoptosis assay kit for paraffin section was purchased from Nanjing KeyGen Biotech. Inc., China. The procedures were performed in accordance with the manufacturer's instructions. Through microscopic observation, cells were defined as apoptotic if the nuclei were positively stained. The positive cells were counted in three high-power fields. The apoptotic index (AI) was calculated as the percentage of positively-stained cells: $\text{AI} = (\text{number of positive cells} / \text{total number of nucleated cells}) \times 100\%$.

Real-time quantitative RT-PCR. The primer sequences are shown in Table I. cDNA was synthesized by oligo dT primed reverse transcription from 2 μg of total RNA using an access RT system (Takara). Real-time PCR was performed using the Mx3005P real-time PCR system (Stratagene) and Stratagene's Brilliant SYBR-Green QPCR Master Mix kit, following the manufacturer's instructions. Thermal cycling conditions were: 95°C for 5 min and 40 cycles at 95°C for 10 sec, followed by 60°C for 30 sec. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was amplified as an internal control. The target and GAPDH genes were amplified in the same reaction. The relative quantification was determined using the $2^{-\Delta\Delta C_t}$ method.

Immunohistochemistry assay. The tissues were embedded and cut into 4- μm sections. Immunohistochemistry was performed according to the protocol as previously described (10). The sections were incubated with the primary antibodies [rabbit anti-Fas-associated protein with death domain (FADD) antibody, Cell signaling technology (CST)] and biotinylated secondary antibody (Zymed Laboratories Inc., South San Francisco, CA, USA). Nuclear counterstaining was performed using haematoxylin. Brown stains in the immunostained regions was considered to be FADD-positive staining. The scoring approach in the assessment of immunohistochemically-stained tissue sections was based on a simple and reproducible protocol as previously described (10).

Western blotting. The protein lysates were loaded onto 10% SDS-polyacrylamide gel, electrotransferred to polyvinylidene fluoride (PVDF) (Immobilon P; Millipore, Bedford, MA, USA) membranes and blocked with 5% non-fat dry milk in Tris-buffered saline (TBST, 100 mM NaCl, 50 mM Tris, 0.1% Tween-20; pH 7.5). The membranes were incubated with FADD, anti-phospho-FADD (Ser 194) (CST) polyclonal antibodies or anti-GAPDH monoclonal antibody (CST) overnight at 4°C, respectively, followed by 1-h incubation with horseradish peroxidase (HRP)-conjugated secondary antibody. The signal was detected using an enhanced chemiluminescence system (ECL; CST) and documented with a charge-coupled

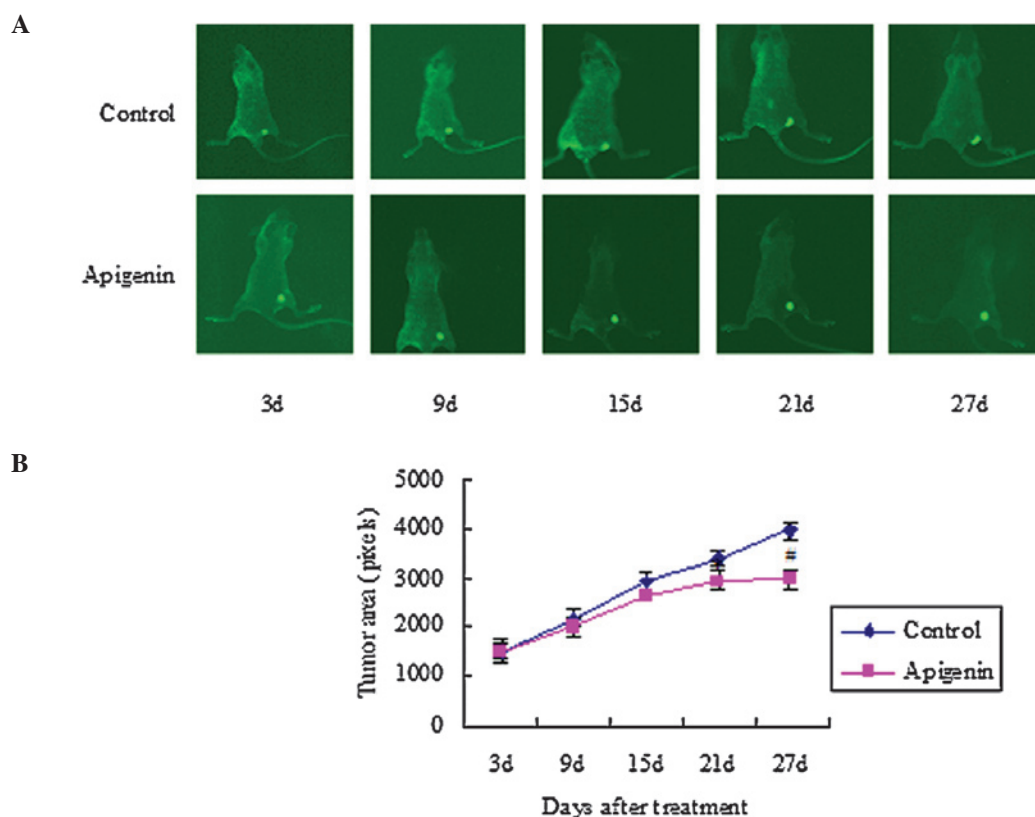


Figure 1. Effects of apigenin on the growth of xenograft colorectal tumors. (A) Representative images *in vivo* of xenograft tumors with stably-expressed GFP. (B) Effects of apigenin on tumor. Apigenin significantly decreased the growth of tumors compared to the control group. * $P < 0.01$, * $P < 0.05$ vs. control group.

device system (ImageStation 2000MM; Kodak). Data analysis was performed using the Molecular Imaging Software Version 4.0. The protein level in each sample was normalized to the level of GAPDH for the corresponding sample.

Statistical analysis. The experiments were repeated a minimum of three times. The data were expressed as means \pm SD. The data were analyzed with the Statistical Package (version 13.0; SPSS). Differences between the two groups were analyzed using Student's t-test. $P < 0.05$ was considered to be statistically significant.

Results

Effects of apigenin on the growth of tumor xenografts. The tumor areas increased rapidly from 1525.88 to 3991.38 pixels in the control group, while the tumor areas increased from 1496.13 to 2963.38 pixels in the apigenin group after 27 days. Apigenin treatment inhibited the growth of colorectal cancer xenografts after 21 ($P < 0.05$) and 27 days ($P < 0.01$) compared to the control group (Fig. 1).

Effects of apigenin on the apoptosis of tumor and expression of FADD. Based on TUNEL staining, a higher number of brown-stained cells were found in the apigenin group compared to those of the control group ($P < 0.01$) (Fig. 2A-C). Five genes that related to the proliferation and apoptosis of CRC, i.e., cyclin D1 (CCND1), BAG-1, Bcl-2, yrdC and FADD were screened, and it was found that FADD was up-regulated by apigenin (Fig. 2D).

Immunological staining showed that the expression of FADD was mainly present in the cytoplasm of CRC cells in the control group, while the overexpression of FADD was observed in both the cytoplasm and nucleus (Fig. 2E-F) in the apigenin pre-treated mice. Western blotting showed that the phosphorylation ($P < 0.05$) and expression ($P < 0.05$) of FADD increased significantly in the apigenin group compared to the control group (Fig. 2G-H).

Discussion

The real-time *in vivo* imaging system utilizes light emitted by a bioluminescent or fluorescent reporter gene (or fluorescent molecule, such as a dye or quantum dot) expressed in a living organism and analyzes the source and strength of that bioluminescent or fluorescent signal non-invasively, allowing for extensive longitudinal modeling in the same live animal (11). In our study, a CRC cell line stably expressing eGFP was selected since cancer growth is observed directly in live animals by epifluorescence. A high correlation was found between the fluorescence intensity of the cancer and the mass of cancer cells. Regarding CRC tumors, an imaging system would be more reliable for sizing, due to the difficulty experienced in compressing the CRC tumor with the caliper (12). Our study using *in vivo* imaging shows that apigenin decreases the region of colon cancer derived from SW480 by increasing AI. Apigenin inhibits the proliferation of the human anaplastic thyroid carcinoma cell line (ARO). The inhibitory effect is associated with the increase of programmed cell

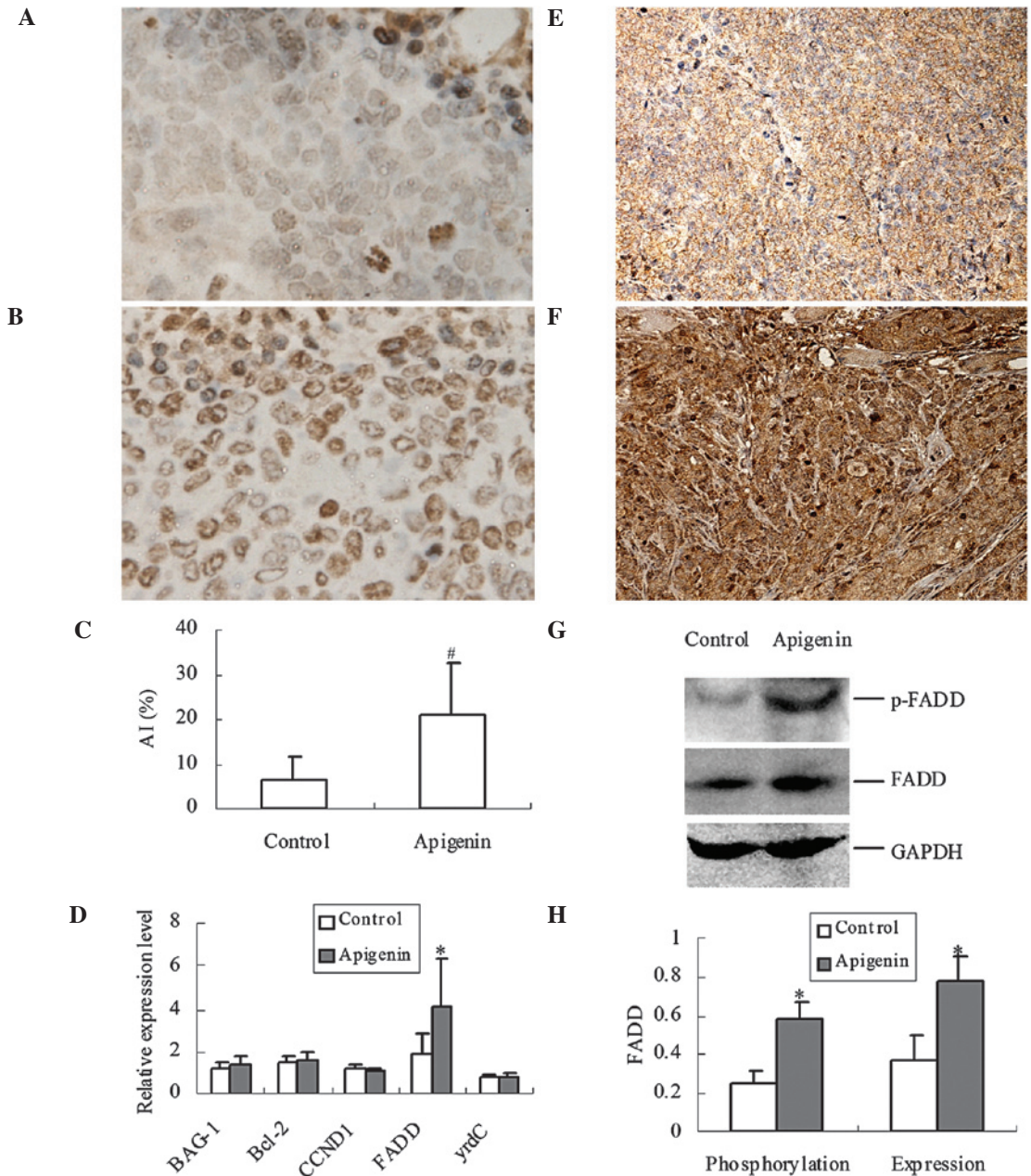


Figure 2. Effects of apigenin on the apoptosis of tumor and expression of FADD. (A-C) TUNEL analysis of apoptotic cells in colorectal tumor. Apoptosis index (C) shows that apigenin (B) increased the number of apoptotic cells compared to the control group (A). (D) Real-time RT-PCR evaluates the expression of CCND1, BAG-1, Bcl-2, yrdC and FADD. Apigenin significantly up-regulated the expression of FADD compared to the control group. (E-F) Immunohistochemical assay detected FADD expression in the tumor. Apigenin significantly increased the expression of FADD (F) compared to the control group (E). (G-H) Western blotting detected the expression and phosphorylation of FADD in the tumor (G). A semi-quantitative analysis showed that apigenin increases the expression and phosphorylation of FADD compared to the control group. [#]P<0.01, ^{*}P<0.05 vs. control group.

death (13). Treatment with apigenin significantly inhibited the cell proliferation in MDA-MB-453 cells that were resistant to 5-fluorouracil. This inhibition resulted in apoptosis, as evidenced by the increased number of apoptotic cells and the activation of caspase-3 (14). Taken together, the results suggest that apigenin inhibits the proliferation of cancer cells by up-regulating the cell apoptosis rate.

To establish why apigenin inhibits the growth of CRC, five genes were selected to determine the effect of apigenin on the proliferation and apoptosis of CRC. CCND1 (15) and BAG-1 (16) overexpression in colorectal tumor cells contribute to

abnormal growth and tumorigenicity, and the expression of yrdC promotes the proliferation of SW-480 (17). A decreased expression of Bcl-2 in tumor cells significantly relates to increased tumor size (18). Transfection of a dominant negative FADD prevents the induction of apoptosis (19). Therefore, the five genes were screened by real-time quantitative RT-PCR and only FADD was found to be up-regulated by apigenin.

Fas is a death receptor, transducing cell death signaling upon stimulation by Fas ligand. During Fas-initiated cell death signaling, the formation of Fas-death-inducing signaling complex (Fas-DISC) is the initial step. Previous observations

showed that FADD is an essential component of DISC, which contains a death domain and induces apoptosis when overexpressed in multiple cell types (20-23). Our results show that FADD expression is up-regulated in CRC cells with a high AI, which is consistent with the observation that transfection of a dominant-negative mutant of FADD decreases the apoptotic response of SW480 cells to resveratrol (24). These results suggest that cells reprogrammed to increase the abundance of FADD may promote apoptosis in cells doomed to die. Since quantitatively phosphorylated FADD plays an essential role in Burkitt lymphoma BJAB cell cycle arrest at the G2/M phase (25), the up-regulated phosphorylation of FADD induced by apigenin may be involved in the regulation of proliferation of CRC cells. Thus, we propose that apigenin suppresses the growth of CRC by enhancing the expression of and inducing the phosphorylation of FADD, which mediates the apoptosis of tumor cells and inhibits cell proliferation. However, further studies should be conducted as to which mechanisms of phosphorylation and translocation of FADD to nucleus are involved in order to elucidate the preventive effects of apigenin on the growth of CRC.

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