Research Paper

Total Flavones of *Selaginella uncinata* (Desv.) Spring Inhibits Breast Cancer Cell Proliferation and Induces Apoptosis *via* Regulating microRNA-1269

LIDENG NI, XULIN WANG[1], JUN GU[1], SHUAI HAO[1] AND LIYANG SUN[2]*

Department of Oncology, Lanxi People's Hospital, [1]Research Institute of General Surgery, Jinling Hospital, Nanjing, Jiangsu 210002, [2]Department of Neurosurgery, Lanxi People's Hospital, Lanxi, Jinhua, Zhejiang 321100, People's Republic of China

Total flavones of *Selaginella uncinata* (Desv.) spring has inhibitory effects on cancer progression. The study aims to analyze the effects of total flavones of *Selaginella uncinata* on breast cancer cell tumor properties and the possible mechanism. MDA-MB-231 cells were treated with total flavones of *Selaginella uncinata* or transfected with anti-microRNA-negative control or anti-microRNA-1269. In addition, microRNA-negative control or microRNA-1269 mimics-transfected MDA-MB-231 cells were exposed to total flavones of *Selaginella uncinata*. Cell proliferation and apoptosis were investigated *via* cell counting kit-8, cell colony formation or flow cytometry analysis. MicroRNA-1269, cleaved caspase-3 or cleaved caspase-9 expression was determined through quantitative polymerase chain reaction or Western blotting. After total flavones of *Selaginella uncinata* treatment, the cell proliferation inhibition rate, cell apoptosis rate, cleaved caspase-3 and cleaved caspase-9 protein levels were increased, the number of cell colonies was decreased, and microRNA-1269 was downregulated in a dose-dependent way. Relative to MCF-10A cells, microRNA-1269 expression was upregulated in MDA-MB-231 cells. After transfection of anti-microRNA-1269, cell apoptosis rate, cleaved caspase-3 and cleaved caspase-9 protein levels and the cell proliferation inhibition rate were increased, but cell colony-forming ability was decreased. After treatment of microRNA-1269 mimics and total flavones of *Selaginella uncinata*, the cell proliferation inhibition rate, cell apoptosis rate, cleaved caspase-3 and cleaved caspase-9 protein levels were reduced and cell colony-forming ability was promoted. Total flavones of *Selaginella uncinata* can inhibit the proliferation and cell colony-forming ability and induce apoptosis of breast cancer cells *via* negatively modulating microRNA-1269.

Key words: Breast cancer, flavones, *Selaginella uncinata*, microRNA-1269, cell proliferation, apoptosis

Breast cancer is a heterogeneous tumor that is commonly diagnosed in women and its incidence is increasing in China. Estrogen level, gene mutation and other factors are closely related to the incidence[1]. Currently, surgery, radiotherapy, chemotherapy and other means are often used to treat breast cancer, but the toxic and side effects on breast cancer patients are relatively large and the prognosis of patients is poor[2,3]. Chinese traditional medicine has anti-inflammatory and anti-tumor effects and plays an anti-breast cancer role in many ways[4-6]. *Selaginella uncinata* (*S. uncinata*) (Desv.) spring belongs to Selaginellaceae family and has the effect of clearing heat and detoxifying[7]. Total Flavones of *S. uncinata* (Desv.) Spring (TFS) can inhibit lung cancer cell growth and cell cycle progression[8]. But, the effects of the extract of TFS on breast cancer cell biological behaviors are unknown. MicroRNA (miR-1269) is up-regulated in esophageal squamous cell carcinoma tissues, and promotes cancer cell proliferation, migration and invasion[9]. However, no study has shown whether miR-1269 is a potential target for TFS-mediated treatment for breast cancer. Therefore, this study is designed to demonstrate whether TFS

***Address for correspondence**
E-mail: lxsly9001@163.com

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can affect breast cancer cell tumor properties through miR-1269.

MATERIALS AND METHODS

Reagents:

S. uncinata (Desv.) spring was purchased from Sanyuan Tianyu Biological Products Co., Ltd. Shanghai Yubo Biotechnology Co., Ltd. provided human normal mammary epithelial cells MCF-10A and MDA-MB-231 cells. Thermo Fisher Biotech (Waltham, Massachusetts and United States of America (USA)) supplied RNA isolation reagents as well as Lipofectamine 2000. Fluorescence quantitative Polymerase Chain Reaction (PCR) reagents and complementary Deoxyribonucleic Acid (cDNA) synthesis reagents were provided by Tiangen Biotech (Beijing, China). Ribobio Co., Ltd. (Guangzhou, China) supplied anti-miR-NC, anti-miR-1269, miR-NC and miR-1269. Beyotime Biology (Shanghai, China) provided Cell Counting Kit-8 (CCK-8) reagent and cell apoptosis detection kit. The primary antibodies against caspase-3 and caspase-9 were provided by Amyjet (Wuhan, China). Abcam (Cambridge, Massachusetts, USA) supplied the primary antibody specific to Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) and the secondary antibody.

Method:

Preparation of TFS: Preparation of TFS was performed in accordance with previous methods[10]. 1000 g of *S. uncinata* (Desv.) spring was ground into powder, filtered through 60-mesh sieves and mixed with 60 % ethanol. After heating the mixture for 20 min, filtrate was collected and purified after decompression, evaporation and drying. The extracts were eluted with 70 % ethanol, concentrated and dried to obtain the extracts. After that, methanol was added to the extracts, and the peak wavelength of 150 nm was detected by enzyme-linked immune detector with rutin as the control substance. The absorption spectrum of the extracts was similar to that of rutin. Standard curve was made according to the value of rutin and then the content of TFS was calculated. Through calculation, we determined that the total content of TFS was 2.66 mg/g. TFS was dissolved in Dimethyl Sulfoxide (DMSO) and then added into medium to prepare 1 mg/ml TFS. According to the experimental requirements, the TFS was diluted to 5, 15 and 25 μg/ml.

Cell treatments: MDA-MB-231 cells were cultured for 24 h in medium containing TFS with different concentrations (5, 15 and 25 μg/ml), which were classified as the TFS-L group, TFS-M group and TFS-H group, respectively. At the same time, cells cultured in medium containing DMSO were regarded as the control group. To determine miR-1269 mediated influence on breast cancer cell proliferative and apoptotic abilities, we knocked down miR-1269 by transfecting miR-1269 inhibitors and its control into MDA-MB-231 cells, which were named as anti-miR-NC group and anti-miR-1269 group, respectively. To determine whether TFS affected breast cancer cell proliferative and apoptotic abilities by regulating miR-1269, the study performed transfection of miR-1269 mimics into MDA-MB-231 cells for 48 h, followed by culturing with medium containing 25 μg/ml TFS for 2 d, classified as the TFS+miR-NC group and the TFS+miR-1269 group, respectively.

Cell proliferation inhibition rate: Breast cancer cells from each group were collected and inoculated in 35 mm petri dishes (2×10^3 cells/well). Cells were cultured with CCK-8 solution in an incubator with 5 % Carbon dioxide (CO_2). Samples were detected by microplate reader. Cell proliferation inhibition rate=((Control group Optical Density (OD)–experimental group OD)/(control group OD–blank group OD)×100 %)

Cell colony formation assay: 0.25 % trypsin was used for digestion and the supernatant was discarded following centrifugation at 3000 r/min for 6 min. Phosphate buffer solution was used to wash the cell precipitation and 500 μl binding buffer was added. Annexin V-Fluorescein Isothiocyante (FITC) and prodium iodide were added to each well respectively. FACS Calibur flow cytometer was utilized to analyze cells.

MiR-1269 expression analysis by quantitative Reverse Transcription PCR (qRT-PCR):

The MCF-10A cells and MD-MB-231 cells were taken out and placed into Eppendorf (EP) tubes and 1 ml Trizol reagent was added to lyse samples. The cells were centrifuged at 12 000 r/min. Chloroform and isopropyl alcohol was added to each EP tube respectively and supernatant was discarded after centrifugation. The isolated Ribonucleic Acid (RNA) was then reversely transcribed to cDNA,

referring to the guidebooks. qRT-PCR samples were prepared according to the following reaction system including 10 µl SYBR Mixture, 0.8 µl positive as well as negative primers, 1 µl cDNA, and 7.4 µl double-distilled Water (ddH_2O). The samples were reacted on ABI Step One Plus qRT-PCR and miR-1269 expression was finally analyzed.

Western blotting analysis:

MDA-MB-231 cell samples of each group were added with 500 µl Radioimmunoprecipitation Assay (RIPA) buffer. 5×Sodium Dodecyl Sulfate (SDS) loading buffers were mixed with samples and boiled in hot water. Some protein samples were used for protein concentration detection *via* the Bicinchoninic Acid (BCA) method. After analysis of sample contents, 40 µg protein was used for SDS-Polyacrylamide Gel (SDS-PAGE) and protein bands were subjected to Polyvinylidene Difluoride (PVDF) membrane transfer and blocked at room temperature. The membranes were incubated with anti-cleaved caspase-3 (1:1000), anti-cleaved caspase-9 (1:1000), and anti-GAPDH (1:3000) for 24 h and secondary antibodies (1:5000) for 1 h. After Enhanced Chemiluminescence (ECL) was uniformly added to the membranes, the membranes were exposed and developed in the darkroom and the gray values of all bands were assessed by Image J software.

Statistical analysis:

Statistical Package for the Social Sciences (SPSS) 21.0 was used to analyze data and measurement data were expressed as ($\bar{x}\pm s$) t test and one-way analysis of variance were used for comparison $p<0.05$ indicated statistically significant.

RESULTS AND DISCUSSION

As shown in Table 1, cell proliferation inhibition rates in TFS-L, TFS-M and TFS-H groups were increased after TFS treatment in dose-dependent manners ($p<0.05$), while TFS treatment reduced the number of cell colonies ($p<0.05$).

As shown in fig. 1 and Table 2, the cell apoptosis rate and cleaved caspase-3 and cleaved caspase-9 protein levels in TFS-L, TFS-M and TFS-H groups were increased after TFS treatment in dose-dependent manner ($p<0.05$).

The data from Table 3 displayed miR-1269 expression was increased in MDA-MB-231 cells relative to MCF-10A cells ($p<0.05$).

As shown in Table 4, miR-1269 expression in TFS-L, TFS-M and TFS-H groups was decreased in dose-dependent manner after being compared with control group ($p<0.05$).

As shown in fig. 2 and Table 5, in comparison with anti-miR-NC group, the cell proliferation inhibition rate, cell apoptotic rates and cleaved caspase-3 and cleaved caspase-9 protein levels were increased in the anti-miR-1269 group ($p<0.05$), but the number of cell colonies was decreased ($p<0.05$).

As shown in fig. 3 and Table 6, compared with the TFS+miR-NC group, the cell proliferation inhibition rate, cell apoptosis rate and cleaved caspase-3 and cleaved caspase-9 protein levels were decreased in the TFS+miR-1269 group ($p<0.05$), whereas the number of cell colonies was increased ($p<0.05$).

TABLE 1: TFS MEDIATED EFFECT ON MDA-MB-231 CELL PROLIFERATION ($\bar{x}\pm s$, n=9)

Group	Cell proliferation inhibition rate (%)	Number of cell colonies
Control	0.00±0.00	111.72±9.50
TFS-L	25.31±2.11*	91.18±7.64*
TFS-M	43.61±4.29*#	73.49±6.72*#
TFS-H	64.23±5.52*#&	56.08±4.57*#&
F	503.094	95.414
p	0	0

Note: *$p<0.05$, #$p<0.05$ and &$p<0.05$ relative to control group, TFS-L group and TFS-M group, respectively

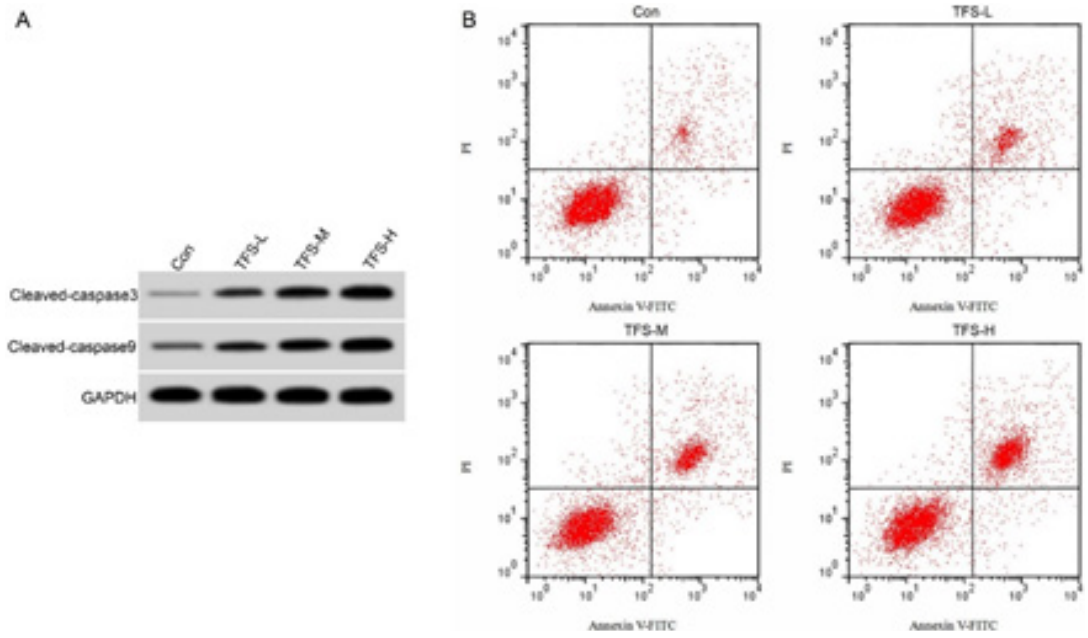

Fig. 1: TFS mediated effect on MDA-MB-231 cell apoptosis, (A): Cleaved caspase-3 and cleaved caspase-9 protein level detection and (B): Flow cytometry analysis of cell apoptosis

TABLE 2: TFS-MEDIATED EFFECT ON MDA-MB-231 CELL APOPTOSIS (x̄±s, n=9)

Group	Apoptotic rate (%)	Cleaved caspase-3 protein expression	Cleaved caspase-9 protein expression
Control	6.75±0.66	0.16±0.02	0.23±0.02
TFS-L	12.02±1.10*	0.29±0.02*	0.39±0.03*
TFS-M	18.32±1.21*#	0.41±0.03*#	0.53±0.04*#
TFS-H	27.65±2.09*#&	0.56±0.05*#&	0.68±0.05*#&
F	388.945	249.429	246.833
p	0	0	0

Note: *p<0.05, #p<0.05 and &p<0.05 relative to control group, TFS-L group and TFS-M group, respectively

TABLE 3: miR-1269 EXPRESSION IN BREAST CANCER CELLS (x̄±s, n=41)

Group	miR-1269
MCF-10A	1.00±0.13
MDA-MB-231	4.24±0.25*
t	73.625
p	0

Note: Compared with MCF-10A cells, *p<0.05

TABLE 4: TFS-MEDIATED EFFECT ON miR-1269 EXPRESSION IN MDA-MB-231 CELLS (x̄±s, n=9)

Group	miR-1269
Control	1.00±0.00
TFS-L	0.77±0.06*
TFS-M	0.61±0.05*#
TFS-H	0.44±0.04*#&
F	265.714
p	0

Note: *p<0.05, #p<0.05 and &p<0.05 relative to control group, TFS-L group and TFS-M group, respectively

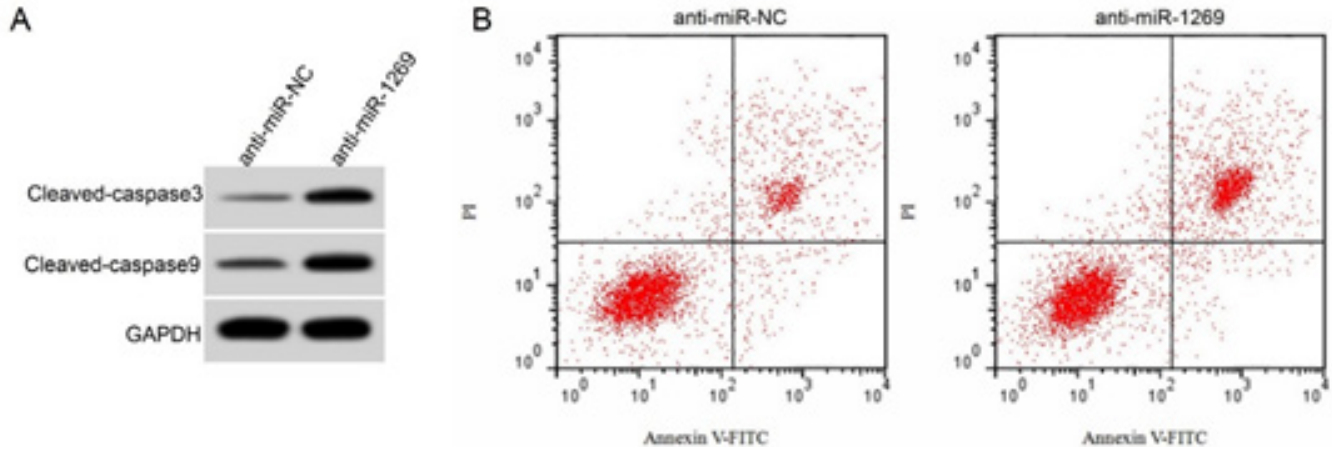

Fig. 2: miR-1269 inhibitors mediated effect on MDA-MB-231 cell proliferation and apoptosis, (A): Cleaved caspase-3 and cleaved caspase-9 protein level detection and (B): Flow cytometry analysis of cell apoptosis

TABLE 5: miR-1269 INHIBITORS MEDIATED EFFECT ON MDA-MB-231 CELL PROLIFERATION AND APOPTOSIS (x̄±s, n=9)

Group	miR-1269	Cell proliferation inhibition rate (%)	The number of cell colonies	Apoptotic rates (%)	Cleaved caspase-3 protein expression	Cleaved caspase-9 protein expression
Anti-miR-NC	1.00±0.00	6.27±0.49	113.52±12.49	6.37±0.56	0.15±0.02	0.22±0.02
Anti-miR-1269	0.33±0.03*	52.38±5.13*	63.46±5.13*	31.95±2.06*	0.51±0.04*	0.63±0.05*
t	67.000	26.843	11.122	35.948	24.150	22.841
p	0.000	0.000	0.000	0.000	0.000	0.000

Note: *p<0.05 relative to anti-miR-NC group

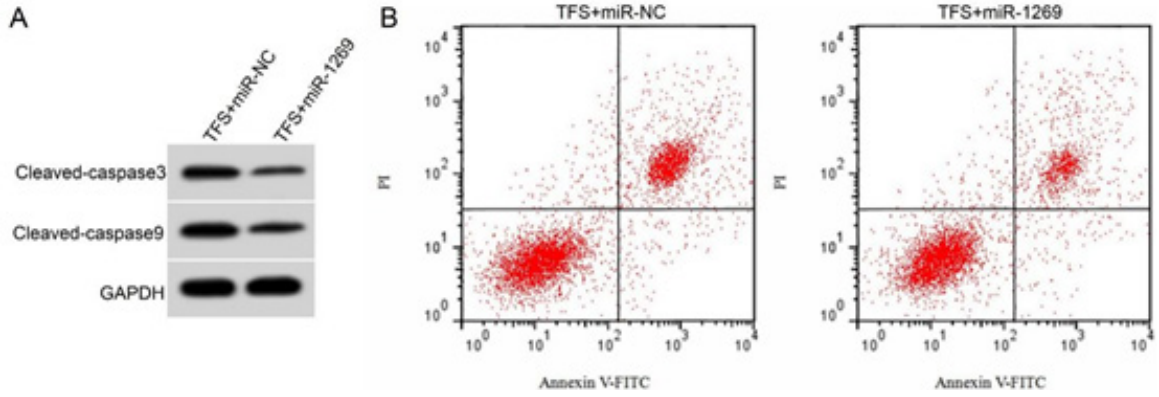

Fig. 3: Overexpression of miR-1269 reversed TFS-induced influence on breast cancer cell proliferation and apoptosis, (A): Cleaved caspase-3 and cleaved caspase-9 protein level detection and (B): Flow cytometry analysis of cell apoptosis

TABLE 6: OVEREXPRESSION OF miR-1269 REVERSED TFS-INDUCED INFLUENCE ON BREAST CANCER CELL PROLIFERATION AND APOPTOSIS (x̄±s, n=9)

Group	miR-1269	Cell proliferation inhibition rate (%)	The number of cell colonies	Apoptotic rate (%)	Cleaved caspase-3 protein expression	Cleaved caspase-9 protein expression
TFS+miR-NC	1.00±0.00	65.13±5.66	54.97±4.81	29.13±2.01	0.58±0.05	0.69±0.04
TFS+miR-1269	2.66±0.23*	22.77±2.21*	94.22±7.16*	11.09±1.09*	0.28±0.03*	0.31±0.03*
t	21.652	20.915	13.651	23.669	15.435	22.800
p	0.000	0.000	0.000	0.000	0.000	0.000

Note: *p<0.05 relative to TFS+miR-NC group

Traditional Chinese medicine contains a variety of active ingredients and can play an important regulatory role in many diseases. Considerable evidence has revealed that extracts of traditional Chinese medicine can play an anti-breast cancer role by regulating non-coding RNA[11,12]. As endogenous non-coding small RNA molecules, miRNAs are able to modulate the biological behavior of breast cancer cells *via* binding to target genes and are potential targets for breast cancer treatment[13,14]. However, whether miRNA is a potential target for breast cancer therapy by Chinese herbal extracts has not been clarified.

The extract of *S. uncinata* (Desv.) spring has anti-tumor effects and its main active component is bioflavonoids, which is able to repress tumor cell growth and metastasis[15,16]. Currently, there is no work on the association between TFS and breast cancer. Our study showed that the increased TFS concentration led to the increased proliferation inhibition rate and the decreased number of colony formation of breast cancer cell lines, suggesting that TFS is able to repress cell proliferation. The activation of the caspase cascade can promote cell apoptosis and the main executive genes of the cascade are caspase-9 and caspase-3, which form cleaved caspase-9 and cleaved caspase-3 respectively after activation and then induce cell apoptosis[17]. Our work revealed TFS promoted the apoptosis rate of breast cancer cells and cleaved caspase-3 and cleaved caspase-9 protein levels in concentration-dependent manner, demonstrating that TFS can promote breast cancer cell apoptosis.

MiR-1269 was up-regulated in gastric cancer tissues and its ectopic expression increased cell proliferation and decreased cell apoptosis[18]. MiR-1269 was up-regulated in liver cancer tissues and cells and its downregulation led to reduced proliferation of liver cancer cells[19]. Our study displayed MDA-MB-231 cells had high miR-1269 expression. MiR-1269 expression was inhibited by TFS stimulation in breast cancer cells in a concentration-dependent manner, suggesting that TFS may play an anti-breast cancer role by inhibiting miR-1269. Also, our results showed that miR-1269 absence could weaken the proliferation and colony formation of breast cancer cell lines and increase cell apoptotic rates, while miR-1269 overexpression could antagonize the effects of TFS on cell proliferation and apoptosis. The above data suggested that TFS can inhibit breast cancer cell tumor properties by down-regulating miR-1269.

In conclusion, TFS can inhibit breast cancer cell proliferation and promote apoptosis and the regulatory of TFS in breast cancer cell phenotypes involved its inhibition in miR-1269 expression. The present results indicate that miR-1269 has the potential to be a potential target for breast cancer therapy by TFS. Additionally, our results not only lay an experimental foundation in exploring the underlying mechanisms of TFS in breast cancer therapy but also provide a new direction for the research and development of therapeutic drugs for breast cancer. However, it remains to be further explored whether TFS can play an anti-breast cancer role by regulating other non-coding RNAs.

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Conflict of interests:

The authors declared no conflict of interests.

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