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Apigenin inhibits proliferation of ovarian cancer A2780 cells through Id1

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ABSTRACT

Apigenin, a common dietary flavonoid, has been shown to possess anti-tumor properties. However, the mechanism by which apigenin inhibits cancer cells is not fully understood. Id1 (inhibitor of differentiation or DNA binding protein 1) contributes to tumorigenesis by stimulating cell proliferation, inhibiting cell differentiation and facilitating tumor neoangiogenesis. Elevated Id1 is found in ovarian cancers and its level correlates with the malignant potential of ovarian tumors. Therefore, Id1 is a potential target for ovarian cancer treatment. Here, we demonstrate that apigenin inhibits proliferation and tumorigenesis of human ovarian cancer A2780 cells through Id1. Apigenin suppressed the expression of Id1 through activating transcription factor 3 (ATF3). Our results may elucidate a new mechanism underlying the inhibitory effects of apigenin on cancer cells.

1. Introduction

Id (inhibitor of differentiation or DNA binding) proteins belong to the helix-loop-helix (HLH) family of transcription factors [1]. Id conventionally alludes to the ability of these proteins to inhibit DNA binding and differentiation. Id proteins, lacking the basic domain for DNA binding, associate with other members of the HLH family and prevent them from binding DNA or forming active heterodimers. There are four members of the Id family, Id1, Id2, Id3, and Id4. Recent studies suggest that Id proteins may function as oncogenes [2–4]. Expression of Id proteins has been demonstrated in a variety of human tumors [2,5–10]. Elevated Id1 expression has been seen in over 20 types of human cancers including prostate, breast, cervical, colon, liver, and ovarian cancers [11]. Overexpression of Id1 promotes cancer cell proliferation and resistance against apoptosis [12,13]. Id1 has also been suggested to take part in the malignant progression of human cancers [5,6,14].

Apigenin (4′,5,7,-trihydroxyflavone) is a common dietary flavonoid and is widely distributed in fruits and vegetables [15]. Apigenin is used as a healthy food supplement and has been shown to possess anti-tumor properties [16–19]. However, the mechanisms that apigenin inhibits cancer cells are not fully understood. In this manuscript, we have demonstrated that apigenin inhibits human ovarian cancer A2780 cells through Id1. Our results provide a new sight into the mechanisms that apigenin inhibits cancer cells.

Abbreviations: Id1, inhibitor of differentiation or DNA binding protein 1; ATF3, activating transcription factor 3

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2. Materials and methods

2.1. Reagents and cell culture

Apigenin, cycloheximide (CHX), and β-actin antibody were from Sigma (St. Louis, MO). The antibodies against Id1 and ATF3 (activating transcription factor 3) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human ovarian cancer A2780 and OVCAR3 cells were maintained as described [19]. The A2780 cell line was established from tumor tissue from an untreated patient. OVCAR3 cell line was established from the malignant ascites of a patient with progressive adenocarcinoma of the ovary.

2.2. Reverse-transcriptional PCR (RT-PCR)

Total cellular RNA was prepared and used for cDNA synthesis in a regular way. The primers used for Id1 are 5′-CCTGCCCTGCTGGACGAG-3′ (forward) and 5′-ATCTCGCCGTTGAGGGTGC-3′ (reverse). The primers used for ATF3 are 5′-GCTAAGCAGTCGTGGTATGGG-3′ (forward) and 5′-TCCTGGAGTTGAGGCAAAGAT-3′ (reverse). The primers used for GAPDH are 5′-CCACCCATGGCAAATTC CATGGCA-3′ (forward) and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ (reverse).

2.3. Plasmids

cDNA of Id1 were cloned into HindIII and Xho I sites of pcDNA™ 3.1/myc-His A vector. The primers used for cloning Id1 are: 5′-AAA AAAGCTTCCACCATGAAAGTC-3′ (forward) and 5′-AAAACTCGAGT-CAGCGACACAAG-3′ (reverse). For constructing ATF3, a 546 bp of

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ATF3 cDNA was subcloned into HindIII and BamHI sites of pcDNA 3.1. The primers for cloning ATF3 are: 5′-AAAAAGCTTCCACCATGAT GCTTCAACACCCA-3′ (forward) and 5′-AAAGGATCCTTAGCTCTGCA ATGTTCCT-3′ (reverse).

2.4. siRNA

The sequence of Id1 siRNA is 5′-AACGGCTGTTACTCACGCCTC-3′ (si-Id1) and that of control siRNA is 5′-ACTACCGTTGTTATAGGTGT-3′. The sequence of Id1 siRNA was submitted for BLAST search to ensure that only Id1 was targeted. pSilencer 2.0-U6 vector from Ambion (Austin, TX) was used to construct the siRNAs. For knocking down ATF3, two ATF3 siRNA oligoes (siATF3-1 and siATF3-2) and control siRNA oligo were purchased from Gene Pharma (Shanghai, China). The sequences of these oligoes are:

siATF3-1: CCUCUUUAUCCAACAGAUATT
siATF3-2: GGUUGUGCUUUCUAGCAAATT
Control: UUCUCCGAACGUGUCACGUTT

2.5. Transient transfection

The cells at 60–70% confluence were transfected with plasmids or siRNA oligoes using Lipofectamine and Plus reagents from Invitrogen as per the manufacture's instruction.

2.6. The orthotopic mouse ovarian cancer model

This experiment was performed as described previously [20]. In brief, 4-week-old female nude mice (Balb/c) were injected with A2780 cells subcutaneously at the rear flanks. Tumors were allowed to develop until they reach 15–20 mm^3 in size. These mice were sacrificed and 3-mm^3 pieces of the tumor were excised for orthotopic implantation. Additional 4-week-old female nude mice were anesthetized with nembutal. A right lateral incision was made and part of the right ovary was well exposed. One tissue block was implanted on the ovarian capsule. After transplantation, the skin was closed and the mice were divided randomly into two groups (each group containing 11 mice). In 2 days, apigenin (5 mg/kg body weight) was administered, IP, once a day. The apigenin dose was chosen according to previous work [21]. Primary tumors were allowed to develop for 30 days. The primary tumor size was measured using digital vernier caliper. Tumor volumes were calculated using the equation: volume = $(\pi \times a \times b^2)/6$, where, a is the longer dimension and b is the shorter dimension [22,23].

2.7. Statistics analysis

The data represent mean ± S.D. from three independent experiments except where indicated. Statistical analysis was performed by Student's t test at a significance level of $P < 0.05$.

3. Results

3.1. Apigenin inhibits proliferation and tumor growth of ovarian cancer A2780 cells

The effects of apigenin on proliferation of ovarian cancer cells were determined. As shown in Fig. 1, apigenin inhibited prolifera-

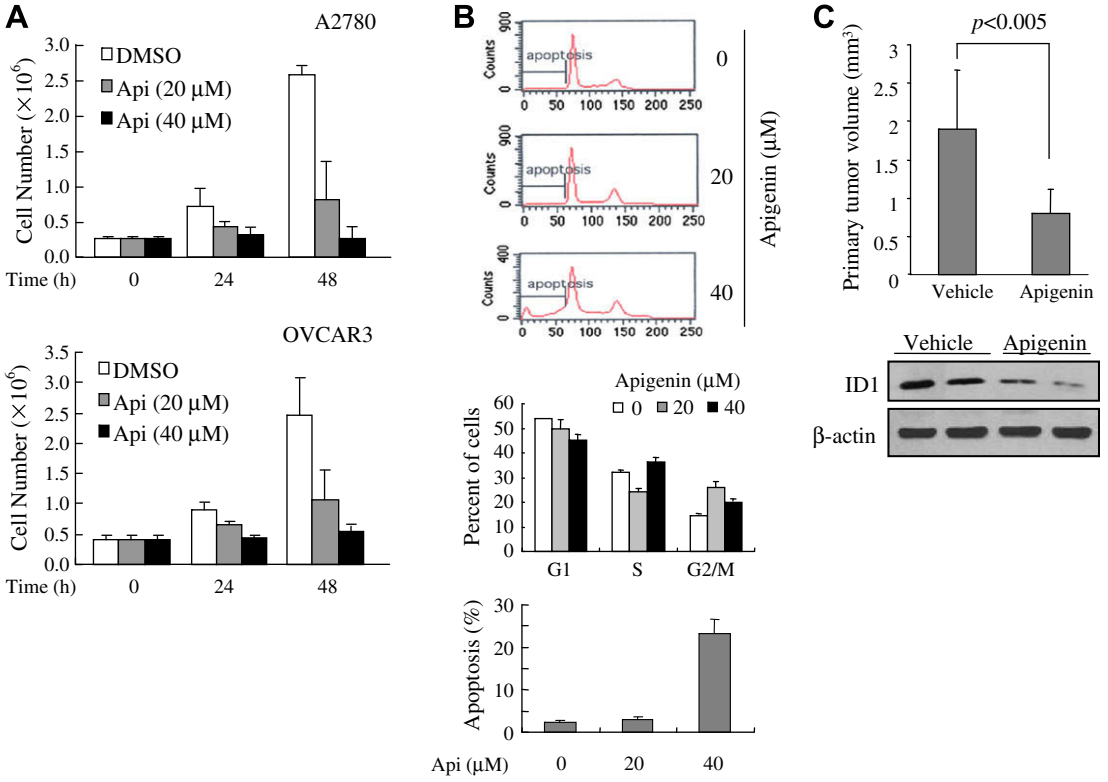

Fig. 1. Apigenin inhibits proliferation of ovarian cancer cells. (A) A2780 and OVCAR3 cells were seeded in a six-well plate and incubated overnight. The next day, apigenin was added and cells were treated for 24 and 48 h, respectively. Cell proliferation was determined by counting the cell numbers by using haemocytometer under a microscope. Cells treated with solvent alone were used as control. (B) Apigenin arrests A2780 cells at G2/M stage and induces apoptosis of the cells. Flow cytometry was performed as described previously [44]. Briefly, A2780 cells were seeded onto six-well plates and incubated overnight. The next day, apigenin was added and the cells were treated for 24 h. The cells were trypsinized, fixed with ice-cold ethanol (70%), stained with propidium iodide, and subjected to FACS analysis. (C) Apigenin inhibited tumor growth of A2780 cells. Orthotopic mice ovarian tumor implantation and treatment of mice with apigenin was as described in Methods. Four primary tumors from vehicle and apigenin groups were randomly selected for immunoblotting analysis.

tion of A2780 and OVCAR3 cells at a dose- and time-dependent manner. We next determined the effects of apigenin on A2780 cells by means of Flow cytometry. Apigenin treatment induced cell cycle arrest of A2780 cells at G2/M stage (Fig. 1B), eliciting an anti-proliferative effect of apigenin via blocking the cell cycle. This is consistent with previous reports that apigenin arrests cells at G2/M phase [17,18,24]. Apigenin at 20 μM did not induce apoptosis of the cells. However, at higher concentration (40 μM), apigenin treatment caused apoptosis of the cells (Fig. 1B).

Finally, we determined the effects of apigenin on tumor growth of A2780 cells in nude mice. The in vivo animal experiment indicates that administration of mice with apigenin repressed the growth of A2780 xenografts (Fig. 1C). Immunoblotting analysis of the primary tumor extracts showed that apigenin treatment attenuated expression of ID1 (Fig. 1C).

3.2. Apigenin inhibits expression of Id1 at a transcriptional level

Apigenin inhibited expression of Id1 at a dose- and time-dependent manner (Fig. 2A and B). Treatment of A2780 cells with CHX, an inhibitor of protein synthesis, led to a decrease of Id1 protein to the same extent as treatment with CHX plus apigenin did (data not shown). The data suggest that apigenin does not influence stability of Id1 protein. We found that apigenin treatment decreased mRNA levels of Id1 (Fig. 2), suggesting that apigenin regulates Id1 expression at mRNA level. It has been reported that ATF3 regulates expression of Id1 transcriptionally [25]. Therefore, we next determined whether ATF3 was involved in Id1 regulation by apigenin. Treatment of A2780 cells with apigenin induced expression of ATF3 and inhibited that of Id1 (Fig. 3A and B). We found that overexpression of ATF3 suppressed expression of Id1 (Fig. 3C). These data suggest that apigenin may inhibit Id1 through ATF3. To confirm this, we determined whether knockdown of ATF3 could restore Id1 expression after apigenin treatment. We found that knockdown of ATF3 indeed rescued expression of Id1 (Fig. 3D). Altogether, these results suggest that apigenin inhibits Id1 expression through ATF3.

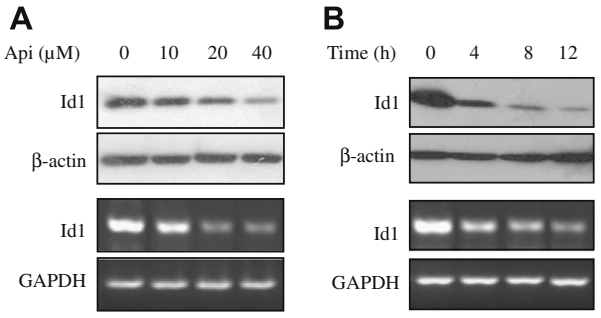

Fig. 2. Apigenin inhibits Id1 expression. (A) Apigenin inhibited expression of Id1 at a dose-dependent manner. A2780 cells were treated with different concentrations of apigenin for 8 h. Immunoblotting and RT-PCR were performed as described under Methods. (B) Apigenin inhibited expression of Id1 at a time-dependent manner. A2780 cells were treated with apigenin (40 μM) for different hours as indicated.

3.3. Apigenin inhibits A2780 cell proliferation through Id1

Knockdown of Id1 inhibited proliferation of A2780 cells (Fig. 4A), indicating that Id1 plays a role on A2780 cells proliferation. To know whether apigenin inhibited proliferation of A2780 cells via Id1, A2780 cells were transfected with Id1 and then treated with apigenin. Overexpression of Id1 partially restored proliferation of A2780 cells inhibited by apigenin (Fig. 4B). We next determined whether the ATF3 knockdown could restore proliferative capacity of A2780 cells in the presence of apigenin. As shown in Fig. 4C, knockdown of ATF3 partially restored proliferation of A2780 cells as well.

4. Discussion

Apigenin has been shown to be capable of inhibiting growth of a few types of human cancer cells including leukemia and carcinomas of breast, colon, lungs, skin, thyroid, and prostate. A number of molecular mechanisms for anti-carcinogenic activity of apigenin

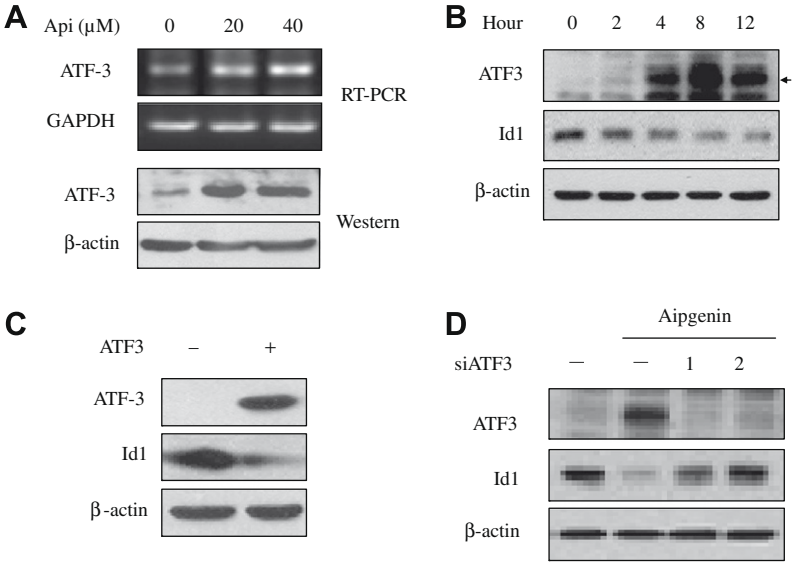

Fig. 3. Apigenin inhibits Id1 expression through ATF3. (A) Apigenin induced expression of ATF3. A2780 cells were treated with apigenin for 8 h. The cells were harvested and cellular proteins and total RNA were prepared. Immunoblotting and RT-PCR were performed. (B) Kinetics of ATF3 induction by apigenin. A2780 cells were treated with apigenin (40 μM) for different hours. The cells were harvested and cellular proteins were prepared for immunoblotting analysis of ATF3. (C) Overexpression of ATF3 suppressed expression of Id1. A2780 cells were transfected with ATF3 or control vector. In 24 h post-transfection, the cells were harvested and expression of Id1 was determined. (D) Knockdown of ATF3 restored Id1 expression inhibited by apigenin. A2780 cells were transfected with siATF3 or control siRNA oligoes. In 20 h post-transfection, the cells were treated with 40 μM of apigenin for 12 h. The cells were then harvested and cellular proteins were prepared for immunoblotting with Id1 and ATF3 antibodies. Api, apigenin.

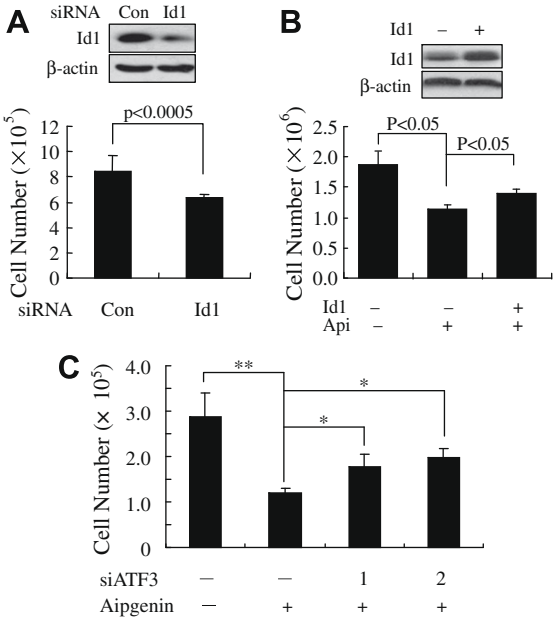

Fig. 4. Apigenin inhibits A2780 proliferation through Id1. (A) Knockdown of Id1 inhibited proliferation of A2780 cells. A2780 cells were transfected with si-Id1 or control siRNA and split to a 24-well plate. In 24 h post-transfection, cell proliferation was determined by cell counting as described in Fig. 1A. (B) Overexpression of Id1 restored proliferation of A2780 cells inhibited by apigenin. A2780 cells were transfected with Id1 or control vector and split to six-well plates. In 24 h post-transfection, apigenin (20 μM) was added and incubation continued for another 24 h. Cell proliferation was determined as described above. (C) Knockdown of ATF3 restored cell proliferation by apigenin. A2780 cells were transfected with siATF3 or control siRNA oligoes and split to six-well plates. In 24 h post-transfection, the cells were incubated with apigenin (20 μM) for another 24 h. Cell proliferation was determined as described. $^{**}P < 0.01$; $^{*}P < 0.05$.

have been proposed [26]. For example, apigenin has been shown to modulate the PI3K/AKT signaling [19,27], the insulin-like growth factor growth axis [28], and beta-catenin signaling [29]. Although several pathways have been proposed, the mechanisms that apigenin inhibits cancer cells are not fully understood. Here, we have demonstrated that apigenin inhibits proliferation of ovarian cancer cells through Id1.

The level of Id1 expression correlates with the malignant potential of ovarian tumors [30]. In cancer samples, high Id1 expression is associated with poor differentiation and more aggressive behavior of tumor cells. It was found in vitro that expression of Id1 promote proliferation of ovarian cancer cells [31]. These results suggest that Id1 is a potential target for the development of novel strategies in the treatment of ovarian cancer.

Knockdown of Id1 inhibits proliferation of A2780 cells (Fig. 4A), and overexpression of Id1 partly restores cell proliferation inhibited by apigenin (Fig. 4B). Moreover, knockdown of ATF3, which restores expression of Id1 in apigenin-treated cells (Fig. 3D), also partially restores proliferative capacity of A2780 cells (Fig. 4C). These results suggest that apigenin inhibits proliferation of ovarian cancer cells, at least partially, through Id1. In addition to Id1, apigenin may have other targets. So, we cannot exclude the possibility that apigenin inhibits proliferation of A2780 cells through other pathways. We found that apigenin decreased mRNA levels of Id1 (Fig. 2) but had little effects on its protein stability (data not shown), suggesting that apigenin inhibits Id1 expression at a transcriptional level. Our results are consistent with the recent report that expression of Id1 was regulated at a transcriptional level [32]. ATF3 is a member of the ATF/CREB family of transcription factors. ATF3 is expressed at low levels in normal and quiescent cells, but can be rapidly induced in response to diverse stress signals [33–36]. In most cases ATF3 was found to repress transcription of target genes [37,38]. We found that apigenin induced ATF3 (Fig. 3A) and overexpression of ATF3 suppressed expression of Id1 (Fig. 3C). Moreover, knockdown of ATF3 could restore Id1 expression inhibited by apigenin (Fig. 3D). Taken together, these results suggest that apigenin inhibits expression of Id1 via ATF3.

Apigenin inhibited proliferation of many types of cancer cells and a number of molecular mechanisms have been proposed [26]. For example, apigenin can inhibit growth of breast cancer cells as both an antiestrogen and a protein kinase inhibitor [39]. A recent study indicates that apigenin inhibited proliferation of prostate cancer cells via IGF-1/IGF-1R axis [40]. These results suggest that apigenin may have multiple targets. We have demonstrated herein that apigenin inhibits proliferation of ovarian cancer cells through, at least partially, ATF3/Id1 pathway. There is growing evidences that higher intake of plant flavonoids reduces the risk of certain chronic diseases including cancer [41–43]. Our results provide more evidence that the dietary flavonoid apigenin might be a potential agent for chemoprevention and/or treatment of cancers such as ovarian cancer.

Acknowledgements

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