Inhibition of IL-6/STAT3 axis and targeting Axl and Tyro3 receptor tyrosine kinases by apigenin circumvent taxol resistance in ovarian cancer cells

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Abstract. Ovarian cancer is the number one cause of death from gynaecological malignancy. Platinum-based and taxol-based chemotherapy has been used as a standard therapy, but intrinsic and acquired resistance to chemotherapy is a major obstacle to treat the disease. In the present study, we found that in the chemoresistant ovarian cancer SKOV3/TR cells, interleukin-6 (IL-6), IL-6 receptor and signal transducers and activators of transcription 3 (STAT3) expression as well as STAT3 phosphorylation were upregulated compared to those in parental cells. Silencing of IL-6 using IL-6 siRNA was found to suppress IL-6 production, STAT3 and phospho-STAT3 levels, which eventually reduced proliferation and clonogenicity of taxol-resistant SKOV3/TR cells. In addition, stattic, a STAT3 inhibitor, was found to result in decrease of cell viability and clonogenicity of these cells, indicating that the elevated IL-6 and STAT3, phosphoSTAT3 levels are associated with the development of taxol resistance. Next, we found anti-proliferative effect of apigenin on both SKOV3 and SKOV3/TR cells. RT-PCR and western blot results showed that apigenin significantly reduced the expression of Axl and Tyro3 receptor tyrosine kinases (RTKs) at mRNA and protein level, which account for its cytotoxic activity. We further found that apigenin decreased Akt phosphorylation and the level of B-cell lymphoma-extra large (Bcl-xl or BCL2-like 1 isoform 1), an inhibitor of apoptosis. On the contrary to these results, apigenin had no effect on IL-6 production, STAT3 and phosphoSTAT3 protein levels, suggesting that apigenin exerts its anti-proliferative activity via downregulation of Axl and Tyro3 expression, Akt phosphorylation and Bcl-xl expression, but not modulation of IL-6/STAT3 axis. Taken together, our data suggest that inhibition of IL-6/STAT3 signaling pathway and downregulation of Axl and Tyro3 RTKs expression might be a therapeutic strategy to overcome taxol resistance in ovarian cancer cells.

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Abbreviations: Bcl-xl, B-cell lymphoma-extra large; ELISA, enzyme-linked immunosorbent assay; GAS 6, growth arrest-specific 6; IL-6, interleukin-6; RTK, receptor tyrosine kinase; STAT, signal transducers and activators of transcription

Key words: Axl, apigenin, IL-6, ovarian cancer, STAT3, taxol resistance, Tyro3

Introduction

Ovarian cancer is in the fifth place in respect of cancer-related death in woman (1). Worldwide the annual number of new cases and deaths of ovarian cancer is estimated to be around 0.22 and 0.14 million, respectively (2). Combination of surgery and chemotherapy has been used as a standard therapy for the treatment of ovarian cancer patients, but overall 5-year survival of the patients with stage III and IV still remains at only 20 to 40%. Such poor prognosis of advanced stage ovarian cancer is accounted for by the intrinsic and acquired chemoresistance, since 30% of patients with advanced stages have been reported not to respond to the first-line chemotherapy, paclitaxel and cisplatin/carboplatin, and ~80% of the initial responders eventually relapse and develop chemoresistance (3). However, the underlying molecular mechanisms of chemoresistance in ovarian cancer are not fully understood.

The signal transducers and activators of transcription (STAT) family proteins have been reported to be fairly upregulated and constitutively activated in many tumors (4) and known to be resulted from the upregulation of upstream signaling molecules such as interleukin-6 (IL-6) (5). Of note, in half of ovarian cancers, constitutive activation of STAT3 has been observed and considered to play an important role for growth, cell cycle progression and invasion of these cancer cells (4). Therefore, targeting IL-6/STAT3 signaling

axis via inhibition of the IL-6–IL-6R interaction or abrogation of STAT3 activation might be a clinically potential therapy to treat cancers.

Among more than 90 protein tyrosine kinases identified in the human genome, there are 58 receptor tyrosine kinases (RTK), which are classified into 20 families (6). One of the subfamily of RTK is the TAM family including three RTKs; Tyro3 (also called Sky), Axl (also called Ark and Ufo) and Mer (7). All the TAM RTKs have structural similarities, two immunoglobin-like domains and two fibronectin type III repeats in extracellular region and cytosolic kinase domain (8), and are recognized by growth arrest-specific 6 (Gas 6) and protein S in common, which are vitamin K-dependent proteins. In normal cells, intracellular signaling via TAM RTKs has been reported to be responsible for a various cellular functions such as survival, proliferation, blockage of apoptosis, adhesion, morphology and motility (9,10). However, in cancer cells, it plays a critical role in the initiation as well as progression of cancers, since Axl, Mer and/or Gas 6 have been demonstrated to be overexpressed in a variety of cancer cell lines and patient samples including breast (11), colon (12), gastric (13), leukemias (14), melanoma (15), multiple myeloma (16), ovarian (17) and prostate cancer (18). Therefore, expression level of TAM RTKs as well as their ligands and their changes seem to be good prognostic marker and targeting these RTKs and their signaling pathways might be a feasible strategy for the successful treatment of many cancers.

Apigenin (4',5,7,-tirhydoxyflavone), a dietary flavone, is found in many fruits, vegetables and seasonings (19-22) as a dimer. Anti-proliferative and anti-angiogenic property of apigenin has been demonstrated in various cancers including breast (23), cervical (23), lung (24), colon (25), hematologic (26), ovarian (27) and prostate cancer (28). Based on these unique effects of apigenin on various cancers along with its low intrinsic toxicity, apigenin has received great attention as a therapeutic as well as a chemopreventive agent.

In the present study, we demonstrated that inhibition of IL-6/STAT3 axis and targeting Axl and Tyro3 RTKs resulted in the reduced proliferation of both parental SKOV3 cells and taxol-resistant SKOV3/TR cells, suggesting a therapeutic potential of these approaches to improve the overall outcome of patients with chemoresistant ovarian cancer.

Materials and methods

Reagents and antibodies. Apigenin was from Sigma-Aldrich (St. Louis, MO, USA). Primers for Axl, IL-6, IL-6 receptor, Mer, STAT3, Tyro3 and GAPDH were synthesized by a domestic company, Bioneer Corp. (Daejoun, Korea). TRI reagent was from Solgent (Daejoun, Korea). AmpliTaq DNA polymerase was obtained from Roche Inc. (Indianapolis, IN, USA). Enzyme-linked immunosorbent assay (ELISA) kit for interleukin 6 (IL-6) was obtained from R&D Systems (Minneapolis, MN, USA). For immunoblotting, specific antibodies against Axl, STAT3, phosphoSTAT3, Tyro3 and GAPDH and secondary antibodies were obtained from Santa Cruz Biotechnology Inc. (Dallas, TX, USA).

Cell culture. SKOV3 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown in RPMI-1640 (Gibco-BRL, Grand Island, NY, USA) containing 10% FBS, 2 mM L-glutamine, 10 U/ml penicillin and 10 g/ml streptomycin at 37˚C in 5% CO_2 in a water-saturated atmosphere. The taxol-resistant SKOV3/TR cells were established by stepwise exposure of the parental SKOV3 cells to escalating concentrations of taxol, ranging from 1.5 to 24 nM for more than 6 months.

RT-PCR. Cells ($3x10^5$) were seeded in 60-mm culture dish and grown overnight at 37˚C and then treated with the indicated concentrations of apigenin for the 24 h. Total RNA was extracted using TRI reagent and subjected to the cDNA synthesis and PCR. The specific primers were as follows: Axl, sense 5'-AACCTTCAACTCCTGCCTTCTCG-3' and antisense 5'-CAGCTTCTCCTTCAGCTCTTCAC-3'; Tyro3, sense 5'-GTGTGTGGCTGACTTCGGAC-3' and antisense 5'-CACGTCCTCCATACACTCCG-3'; IL-6, sense 5'-ATGAACTCCTTCTCCACAAGCG-3' and antisense 5'-GAAGAGCCCTCAGGCTGGACT-3'; IL-6 receptor, sense 5'-CATTGCCATTGTTCTGAGGTTC-3' and antisense 5'-AGTAGTCTGTATTGCTGATGTC-3'; STAT3, sense 5'-TTCTCCTTCTGGGTCTGGCT-3' and antisense 5'-CCACCCAAGTGAAAGTGACG-3'; GAPDH, sense 5'-GGAGCCAAAAGGGTCATCAT-3' and antisense 5'-GTGATGGCATGGACTGTGGT-3'.

Western blot analysis. Cells were treated with the indicated concentration of apigenin or stattic for 24 h. Total cell lysates were prepared from those cells using lysis buffer [1% Triton X-100, 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM PMSF, 1 mM Na_3VO_4, and protease inhibitor cocktail]. Protein concentrations were determined using Bio-Rad protein assays. Proteins from cell lysates (20-40 μg) were separated on 12% SDS-PAGE, and electrotransferred to nitrocellulose membranes. Membranes were blocked for 30 min at room temperature in Tris-buffered saline-0.05% Tween-20 (TTBS) containing 5% non-fat dry milk, and then incubated with TTBS containing a primary antibody for 4 h at room temperature. After 3 x 10-min washes in TTBS, membranes were incubated with peroxidase-conjugated secondary antibody for 1 h. Following 3 additional 10-min washes with TTBS, protein bands of interest were visualized using an enhanced chemiluminescence detection system (Amersham).

siRNA transfection. RNA interference silencing was performed to inhibit IL-6 production. SKVO3/TR cells ($1x10^6$) were seeded in 100-mm culture dish and grown overnight and then transfected with 50 nM siRNA against IL-6 (sc-39627; Santa Cruz Biotechnology, Dallas, TX, USA), or control siRNA (sc-37007; Santa Cruz Biotechnology). At 48 h post-transfection, cells were harvested and the number of viable cells were counted and IL-6 level in conditioned media were determined by ELISA. STAT3 and phosphorylated STAT3 protein levels were determined by western blot analysis using whole cell lysates.

Clonogenic assay. Cells were seeded in 35-mm culture dishes ($2x10^3$ cells/dish) and allowed to grow for 7-10 days in the presence of and/or absence of apigenin or stattic to form colonies. Colonies of >50 cells were visualized by crystal violet (in 60% methanol; Junsei Chemical Co., Ltd., Tokyo Japan) staining

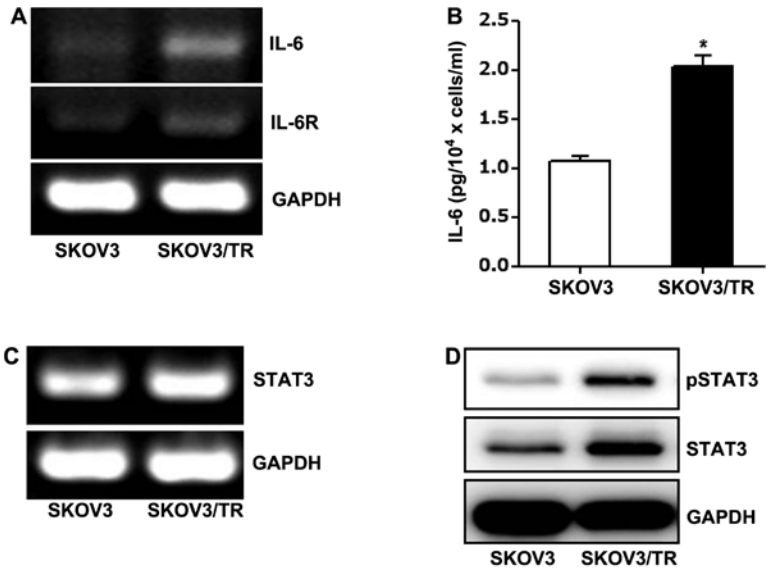

Figure 1. The levels of IL-6, IL-6 receptor, STAT3 and phosphorylated STAT3 were elevated in taxol-resistant ovarian cancer cells. (A and C) Total RNAs from SKOV3 and SKOV3/TR cells were isolated and used for RT-PCR to analyze *IL-6*, *IL-6* and *STAT3* mRNA expression. As an internal control, *GAPDH* mRNA was also amplified by RT-PCR. The data are representative of three independent experiments. (B) Culture media of both SKOV3 and SKOV3/TR cells were harvested and used for IL-6 ELISA. To assess IL-6 production per cell, the total amount of IL-6 was normalized by the number of viable cells. Results are from three independent experiments. Data are expressed as the mean ± SD of triplicate samples. The asterisks indicate the significant difference compared to the control value (*P<0.05 SKOV3 vs. SKOV3/TR). (D) Western blot analysis was also conducted to determine the levels of STAT3 protein and its phosphorylated form in SKOV3 and SKOV3/TR cells. GAPDH was used as a loading control. The data are representative of three independent experiments.

and images were taken by RAS 3000 image analysis system (Fuji Film, Tokyo, Japan).

Cell viability assay. The viability of cells was measured using Cell Counting Kit-8 assay kit (Dojindo Laboratories, Kumamoto, Japan). Cells (1-2x10^3 cells/well) were seeded in 96-well plates and grown overnight at 37˚C and then treated with the indicated concentrations of stattic for the 24 h. At the end of the treatment, 10 μl of CCK-8 solution was added and further incubated for 4 h. The absorbance at 450 nm was measured using a microplate reader (Model 680 microplate reader; Bio-Rad Laboratories). Values are the mean ± SD for triplicate wells and normalized to that of control group to determine the % of viability.

ELISA. The level of IL-6 in culture media was measured using ELISA kit from R&D Systems according to the manufacturer's protocol. Cells were transfected with siRNAs, siCtrl and siIL-6 or treated with apigenin for 24 h. Conditioned media were harvested and assayed for IL-6. The data are representative of at least three independent experiments.

Statistical analysis. Data are expressed as the mean ± SD of triplicate samples or at least three independent experiments. For statistical significance, Student's t-test was used with a threshold of P-values which is <0.05.

Results

IL-6, STAT3 and phosphorylated STAT3 levels are elevated in taxol-resistant ovarian cancer cells. To understand the molecular mechanisms underlying taxol resistance in ovarian cancer cells, we established a taxol-resistant subline, SKOV3/TR cells, by long-term and stepwise exposure of taxol to parental SKOV3 cells. Since elevated production of interleukin-6 (IL-6) and IL-6-mediated activation of signal transducers and the activators of transcription 3 (STAT3) have been reported to lead to chemoresistance to several chemotherapeutic drugs in various cancers (29), we examined IL-6, IL-6 receptor, STAT3, and phosphorylated STAT3 levels in both SKOV3 and SKOV3/TR cells. RT-PCR result showed that IL-6 and IL-6 receptor mRNA levels in SKOV3/TR cells were increased compared to those of SKOV3 cells, respectively (Fig. 1A). In addition, enzyme-linked immunosorbent assay (ELISA) result also showed that the level of IL-6 in culture media of SKOV3/TR cells was higher than that in culture media of parental cells, which is consistent with the transcriptional upregulation of IL-6 in SKOV3/TR cells (Fig. 1B).

Next, expression and phosphorylation status of STAT3 were examined. As shown in Fig. 1C, STAT3 mRNA level in SKOV3/TR cells was found to be increased compared to that in parental cells. Western blot results also showed that in SKOV3/TR cells, both STAT3 protein and phosphoSTAT3 level were significantly elevated (Fig. 1D), indicating the induction of STAT3 expression and its activation might be responsible for the development of resistance to chemotherapy.

Silencing of IL-6 and inhibition of STAT3 reduce proliferation of taxol-resistant cells. The biological relevance of the increase of IL-6 production, STAT3 protein level, and its phosphorylation status in SKOV3/TR cells was examined by silencing of IL-6 via siRNA and inhibition of STAT3 using stattic, a small molecule inhibitor of STAT3. SKOV3/TR cells were transfected with IL-6-specific siRNA, siIL-6 or control siRNA, siCtrl and assessed IL-6 level in culture media by ELISA. As shown in Fig. 2A, silencing of IL-6 via siIL-6

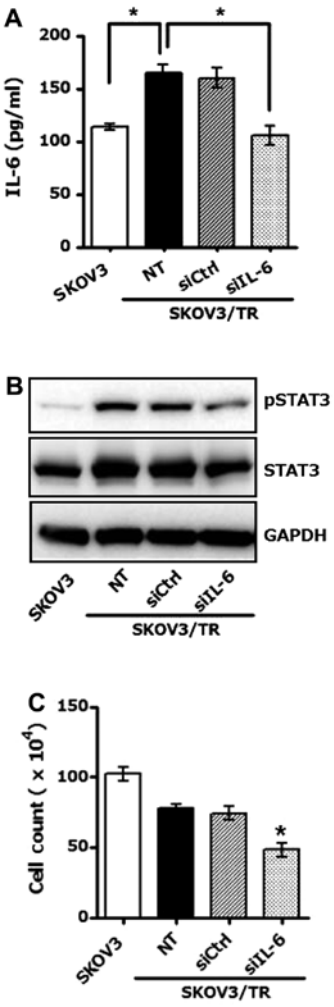

Figure 2. Silencing of IL-6 inhibits STAT3 expression and phosphorylation in taxol-resistant ovarian cancer cells, which eventually suppresses proliferation of these cells. (A) SKOV3/TR cells ($3x10^3$ cells) were transfected with IL-6-specific siRNA, siIL-6 and control siRNA, siCtrl, respectively. Cells were harvested 48 h after transfection and IL-6 protein level was determined by ELISA. The data are representative of three independent experiments. The asterisks indicate the significant difference compared to the control value (*P<0.05 SKOV3 vs. SKOV3/TR and SKOV3/TR vs. SKOV3/TR/siIL-6). (B) In parallel, the levels of STAT3 protein and its phosphorylated form were determined by western blot analysis. GAPDH was used as a loading control. The data are representative of three independent experiments. (C) To assess anti-proliferative effect of siIL-6, the number of viable cells was counted at 48 h post-transfection. Data are expressed as the mean ± SD of three independent experiments. The asterisk indicates a significant difference compared to the control value (*P<0.05 SKOV3/TR vs. SKOV3/TR/siIL-6). The results are representative of at least three independent experiments.

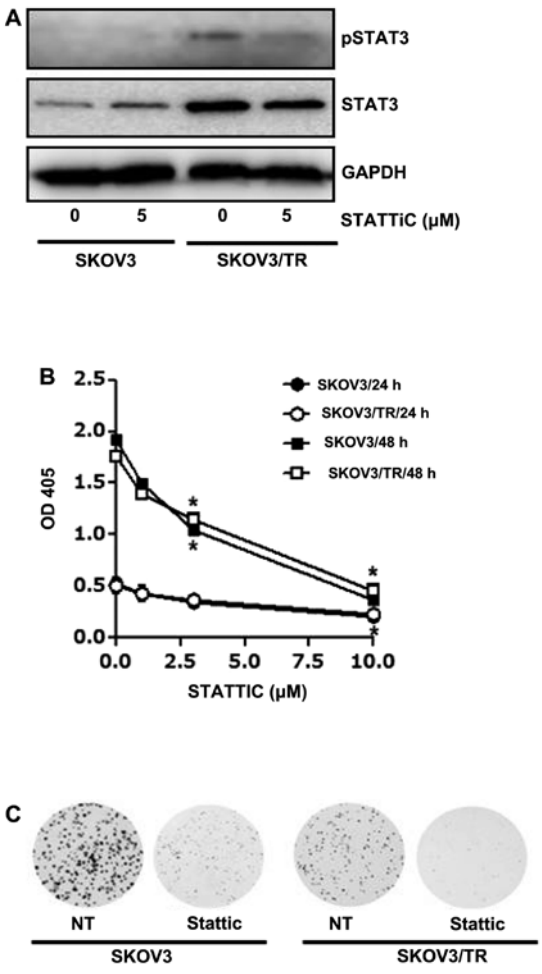

Figure 3. Stattic, a small molecule inhibitor, inhibits STAT3 phosphorylation and proliferation of taxol-resistant ovarian cancer cells. Cells ($3x10^3$ cells/dish) were seeded onto 100-mm dishes, grown overnight and treated with the indicated concentrations of stattic for 24 or 48 h. (A) After 24 h of treatment, cells were harvested. The levels of STAT3 protein and its phosphorylated form were determined by western blot analysis. GAPDH was used as a loading control. The data are representative of three independent experiments. (B) Cell viability was measured by CCK-8 assay. Each error bar represents mean ± SD of three independent experiments. The asterisk indicates a significant difference compared to the control value (*P<0.05 vs. untreated group). (C) Each cell ($2x10^3$ cells/dish) was seeded onto 35-mm dishes and treated with 5 μM stattic. After 48 h of treatment, cells were washed with PBS and allowed to grow for 7 to 10 days. The colonies were visualized by crystal violet staining. The results are representative of three independent experiments.

in SKOV3/TR cells resulted in significant decrease of IL-6 production. Western blot results further showed that phosphorylation and expression of STAT3 was also reduced in SKOV3/TR cells transfected with siIL-6 (Fig. 2B), confirming that STAT3 is a downstream effector of IL-6-mediated signaling pathway.

Next, we examined the effect of siIL-6 on cell proliferation. As shown in Fig. 2C, viability of SKOV3/TR cells transfected with siIL-6 was fairly reduced compared to that transfected with siCtrl.

The effect of stattic, a small molecule inhibitor of STAT3, on cell viability was also examined. We first found that both expression and phosphorylation of STAT3 were decreased by stattic treatment in SKOV3/TR cells (Fig. 3A). Then, both SKOV3 and SKOV3/TR cells were treated with 1, 3 and 10 μM stattic for 24 h and CCK assay results showed a dose-dependent decrease of the cell viability (Fig. 3B). Consistent with the CCK assay results, we also found that clonogenicity of stattic-treated cells was considerably reduced (Fig. 3C), confirming its anti-proliferative activity. Taken together, these results demonstrated that upregulation of IL-6 and STAT3 expression as well as the increased phosphorylation of STAT3 play a critical role in proliferation of SKOV3/TR cells and are associated with taxol resistance of SKOV3/TR cells.

Apigenin suppresses proliferation of both parental and taxol-resistant cells. Since we previously reported that apigenin

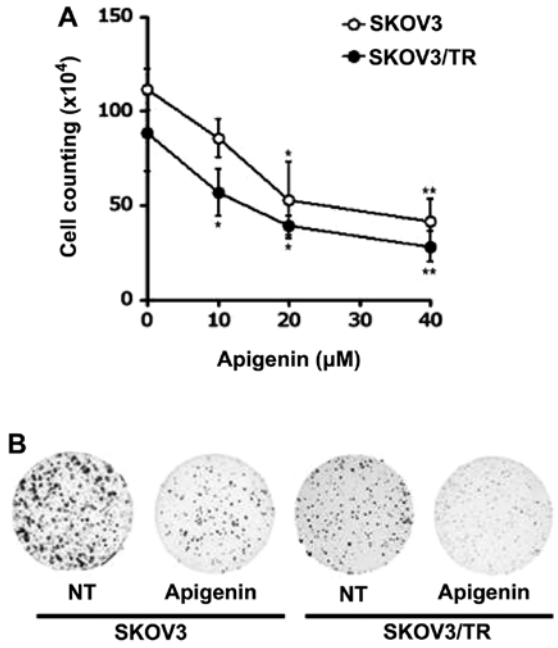

Figure 4. Apigenin inhibits proliferation of taxol-resistant ovarian cancer cells. (A) Cells ($3x10^3$ cells/dish) were seeded onto 100-mm dishes, grown overnight and treated with the indicated concentrations of apigenin for 24 h and harvested. Trypan blue exclusion assay was conducted to measure cell viability. Data are presented as mean ± SD of at least three independent experiments. The asterisks indicate a significant difference compared to the control value ($^{*}P<0.05$ vs. untreated group and $^{**}P<0.001$ vs. untreated group). (B) Cells ($2x10^3$ cells/dish) were seeded onto 35-mm dishes and allowed to grow in the absence or presence of 40 μM apigenin for 7 to 10 days. The colonies were visualized by crystal violet staining. The data are representative of at least three independent experiments.

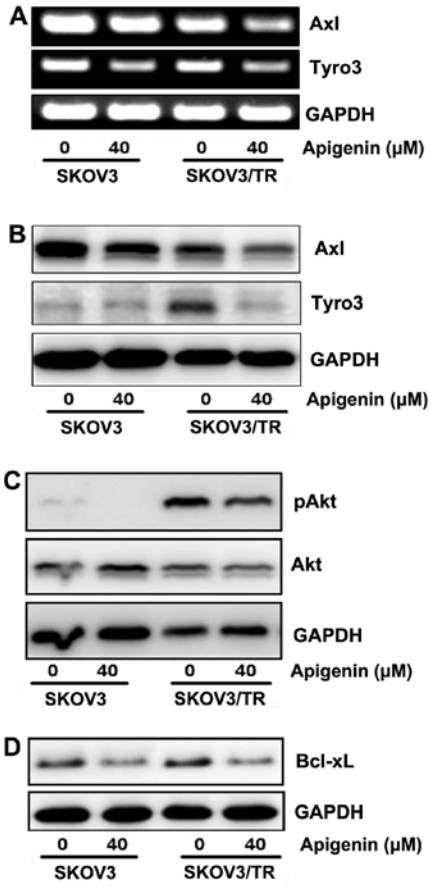

Figure 5. Apigenin suppresses the expression of TAM RTKs Axl and Tyro3 RTKs. Cells ($3x10^3$ cells/dish) were seeded onto 100-mm dishes, grown overnight and treated 40 μM apigenin for 24 h and harvested. (A) For RT-PCR, total RNAs from the cells were isolated and used for analysis of *Axl* and *Tyro3* mRNA expression. The data are representative of three independent experiments. As an internal control, *GAPDH* mRNA was also amplified. (B-D) The levels of Axl, Tyro 3, Akt, phosphorylated Akt and Bcl-xl protein were assessed by western blot analysis to determine the effect of apigenin on their expression. GAPDH was used as a loading control. The result are representative of at least three independent experiments.

targets Axl receptor tyrosine kinase (RTK), one of TAM family members, which accounts for its anti-proliferative effects on non-small cell lung carcinoma (NSCLC) cell lines, we asked whether apigenin was cytotoxic in parental and taxol-resistant ovarian cancer cells, which might result from downregulation of TAM expression. As shown in Fig. 4A, apigenin treatment decreased the viability of both SKOV3 and SKOV3/TR cells in a dose-dependent manner. Of note, treatment with 40 μM apigenin for 24 h showed only 41.5% (SKOV3), and 28% (SKOV3/TR) survival of these cells, respectively (Fig. 4A), indicating a more profound anti-proliferative effect of apigenin on SKOV3/TR cells than parental SKOV3 cells. Colony-forming assay further demonstrated cytotoxic activity of apigenin on SKOV3 and SKOV3/TR cells. As shown in Fig. 4B, treatment of these cells with 40 μM apigenin was found to reduce not only the number of colonies but also the size of each colony.

Anti-proliferative effect of apigenin is mediated by the dysregulation of TAM RTKs and downstream effectors, but not IL-6/STAT3 axis. Since TMA family of RTKs, Axl, Tyro3 and Mer is known to be involved in cell survival, growth and proliferation, we then examined the effect of apigenin on TAM RTKs expression. Especially, in SKOV3/TR cells, Axl expression was found to be slightly reduced, while Tyro3 expression was increased, compared to those in parental SKOV3 cells, respectively. Both SKOV3 and SKOV3/TR cells were treated with 40 μM apigenin for 24 h and then expression of Axl and Tyro3 was examined at mRNA and protein level. RT-PCR results showed that apigenin treatment led to significant reduction of Axl and Tyro3 mRNA level in both parental and taxol-resistant cells (Fig. 5A). Downregulation of Axl and Tyro3 expression in apigenin-treated cells was further confirmed by western blot analysis. As shown in Fig. 5B, the protein levels of Axl and Tyro3 were decreased by apigenin treatment, which is consistent with RT-PCR results.

We next examined several downstream effectors which might be affected after apigenin-mediated inhibition of Axl and Tyro3 expression and subsequent reduction of cell proliferation. Western blot results showed that apigenin treatment decreased the level of phosphorylated Akt which transduces a strong signal for cell cycle progression and is fairly increased in SKOV3/TR cells (Fig. 5C). In addition, apigenin was also found to reduce the level of B-cell lymphoma-extra large (Bcl-xl, or BCL2-like 1 isoform 1) which is regulated by Akt and inhibits apoptosis (Fig. 5D). These data demonstrate that apigenin causes not only reduction of Axl and Tyro3 expression but also the decrease of Akt phosphorylation and Bcl-xl expression.

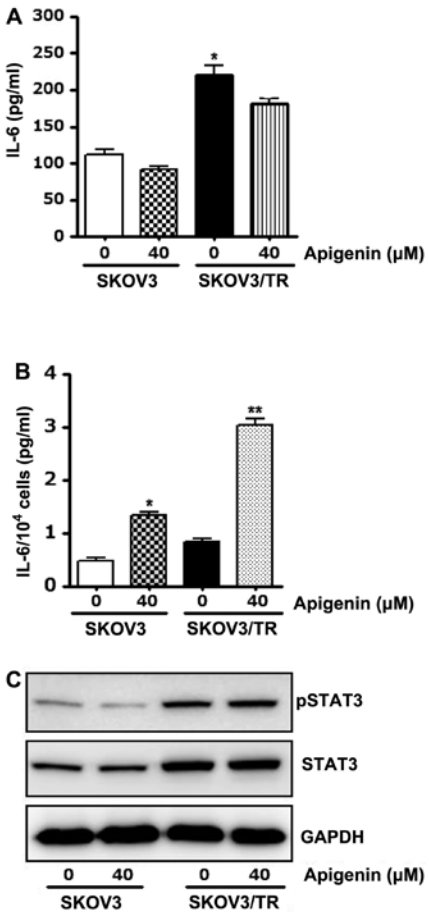

Figure 6. IL-6/STAT3 axis is not affected by apigenin. Cells ($3x10^3$ cells/dish) were seeded onto 100 mm dishes, grown overnight and treated 40 μM apigenin for 24 h. (A) Culture media were harvested and used for IL-6 ELISA. Data are expressed as the mean ± SD of triplicate samples. The asterisks indicate a significant difference compared to the control value ($^*P<0.05$ vs. untreated). The results are representative of at least three independent experiments. (B) To assess IL-6 production per cell, the total amount of IL-6 was normalized by the number of viable cells. Data are expressed as the mean ± SD of triplicate samples. The asterisks indicate a significant difference compared to the control value ($^*P<0.05$ vs. untreated SKOV3 cells and $^{**}P<0.001$ vs. untreated SKOV3/TR cells). (C) STAT3 and phosphorylated STAT3 protein levels were assessed by western blot analysis to determine the effect of apigenin on their expression. GAPDH was used as a loading control. The results are representative of at least three independent experiments.

We further examined if apigenin affects IL-6/STAT3 axis, which is associated with cell viability. ELISA results showed that total amount of IL-6 in culture media was slightly decreased by apigenin treatment (Fig. 6A), whereas IL-6 production per cell was increased, especially in taxol-resistant SKOV3/TR cells (Fig. 6B). We also found that apigenin treatment had no effect on STAT3 phosphorylation in these cells (Fig. 6C). Taken together, the results indicate that apigenin has no effect on IL-6 production and concomitant STAT3 phosphorylation and its anti-proliferative effect does not result from suppression of IL-6/STAT3 axis.

Discussion

Cisplatin- and taxol-based chemotherapy is still the first-line therapeutic choice for ovarian cancer. However, the intrinsic and acquired resistance to these drugs have major limitations leading to the failure of treatment (30-33). Therefore, it is urgent to elucidate characteristics and underlying molecular mechanisms of the resistance to improve final outcomes.

We found that in taxol-resistant SKOV3/TR cells, the levels of IL-6, IL-6 receptor, STAT3 and its phosphorylated form were significantly increased compared to those in parental SKOV3 cells. Moreover, intervention of this IL-6/STAT3 signaling via silencing of IL-6 and a STAT3 inhibitor, stattic, were found to exert anti-proliferative effect on SKOV3/TR cells. These results indicate that activation of IL-6/STAT3 axis resulted from the long-term exposure of cells to taxol and need a strategy or a compensation to survive under the pressure of taxol. Because of dual function of STAT3 as a downstream effector of IL-6 and a transcription factor to induce IL-6 expression, a positive feedback loop between STAT3 and IL-6 is established, which results in autocrine production of IL-6 and constitutive activation of STAT3. Consistent with our data, the anti-apoptotic effect of IL-6 and the involvement in drug resistance have been reported in various cancers including myeloma (34), prostate (35) and breast cancers (36), which supports the idea that combination of IL-6/STAT3 pathway inhibitor with chemotherapeutic agents could be effective, in patients with acquired chemoresistance.

A clinical significance of TAM receptor tyrosine kinases (RTKs), Tyro3, Axl and Mer, as well as their ligands has been demonstrated. For example, in 48.3% of ovarian adenocarcinoma tissues, Axl protein level was elevated and reflected in disease stage and lymph node metastasis. In lung cancer cases, overexpression of Axl, Mer, and their ligands was also found in more than half of non-small cell lung cancer (NSCLC) cell lines (37,38) and RNA interference or monoclonal antibodies against Axl have been reported to reduce NSCLC proliferation, metastasis and xenograft tumor growth (39). In accordance with these studies, we recently demonstrated that anti-proliferative effect on apigenin, a dietary phytochemical derived from various fruits and vegetables resulted from downregulation of Axl expression in NSCLC cells, suggesting that Axl is a novel target of apigenin. Since the initial report showed the inhibitory effect of apigenin on mutagenesis and tumor promotion (40), many follow-up studies further demonstrated its anti-oxidant, anti-inflammatory, anti-angiogenic and anti-proliferative activities. Based on the above evidence, apigenin has received considerable attention as a chemotherapeutic and chemopreventive agent. In the present study, apigenin was further found to suppress the expression of Axl and Tyro3, incurring decreased proliferation of both parental and taxol-resistant SKOV3 cancer cells. Of note, Tyro3 induction contrary to downregulation of Axl in SKOV3/TR cells seems to be a compensation or another strategy for survival, which resulted from long-term treatment of taxol. However, IL-6 and STAT3 expression and STAT3 phosphorylation were not affected by apigenin, IL-6 production per cell was increased, suggesting that IL-6/STAT3 signaling pathway is not involved in the anti-proliferative effect of apigenin.

In summary, our data demonstrated that silencing of IL-6 and STAT3 inhibition intervened IL-6/STAT3 signaling pathway and apigenin caused downregulation of expression

in all TAM RTKs, which eventually restricted in proliferation of taxol-resistant ovarian cancer cells, suggesting that inhibition of IL-6/STAT3 axis and targeting TAM RTKs might be feasible approaches to overcome taxol resistance in ovarian cancer cells.

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