

Metformin targets Axl and Tyro3 receptor tyrosine kinases to inhibit cell proliferation and overcome chemoresistance in ovarian cancer cells

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Abstract. Metformin, the most frequently prescribed anti-diabetic drug, has recently been paid attention as a chemotherapeutic agent. In this study, we demonstrated that metformin decreased the viability of parental as well as cisplatin/taxol-resistant ovarian cancer cells. Its anti-proliferative effect was further demonstrated by dose-dependent reduction of the clonogenic ability of the metformin-treated cells. We next observed the effect of metformin on expression of Axl and Tyro3 receptor tyrosine kinases (RTKs) which belong to the TAM subfamily of RTKs transducing pro-survival and anti-apoptotic signals. Metformin treatment of ovarian cancer cells decreased both mRNA and protein levels of Axl and Tyro3 in a dose-dependent manner. Axl promoter activity was also inhibited by metformin, indicating that metformin suppresses Axl and Tyro3 expression at the transcriptional level. Metformin treatment was also found to augment its anti-proliferative effect in SKOV3 and taxol-resistant SKOV3/TR cells transfected with Axl and Tyro3 specific siRNAs, siAxl and siTyro3, respectively, suggesting that metformin might target Axl and Tyro3 RTKs to restrain cell proliferation. In parallel, the level of X-linked inhibitor of apoptosis protein (XIAP), an anti-apoptotic molecule, was reduced in the metformin-treated cells. Collectively, our data showed that metformin caused

reduction of Axl and Tyro3 RTKs' expression, inactivation of downstream effectors, and decrease of anti-apoptotic protein level, forming a potent therapeutic strategy to facilitate its anticancer activity as well as to overcome chemoresistance in human ovarian cancer cells.

Introduction

More than 80% of ovarian cancer cases are discovered at the advanced stage. When the patients are first diagnosed, they already have metastasis (1). The combination of surgery and chemotherapy is common approach to treat ovarian cancer. Many chemotherapeutic agents including carboplatin, cisplatin and paclitaxel have been used as a single drug or in combination. The study conducted by Einzig *et al* in 1992, which reported the significant activity of paclitaxel in patients with advanced ovarian cancer (2), established the co-administration of paclitaxel and cisplatin/carboplatin as a standard chemotherapeutic regimen. Since 30% ovarian cancer patients in advanced stages are non-responders to the first-line chemotherapy and the acquired chemoresistance is also emerged from the initial responders (3), both intrinsic and acquired resistances have been the major causes of low survival rates and poor prognosis. Therefore, it is urgently required to understand the underlying molecular mechanisms involved in such chemoresistance, which can introduce novel attempts to overcome resistance and eventually success to treat ovarian cancer.

In human, there are >90 protein tyrosine kinases and 58 of them are receptor tyrosine kinases (RTKs) which are, in turn, divided into 20 families (4). TAM family, a subfamily of RTK, is comprised of three RTK members, which are Tyro3 (alternatively called Sky), Axl (alternatively called Ark and Ufo), and Mer (5). They share common extracellular structures, two immunoglobulin-like domains and two fibronectin type III repeats, and conserved kinase domain within cytoplasmic region (6). It has been reported that the typical ligands to interact with TAM RTKs are growth arrest-specific 6 (GAS6) and protein S which are vitamin K-dependent proteins and transduce many intracellular signals including survival, proliferation, inhibition of apoptosis, adhesion, morphology, invasion and motility (7-9). Indeed, overexpression of TAM

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Abbreviations: AMPK, AMP-activated protein kinase; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; GAS6, growth arrest-specific 6; IL-6, interleukin-6; mTORC, mammalian target of rapamycin complex; RTK, receptor tyrosine kinase; STAT, signal transducers and activators of transcription; XIAP, X-linked inhibitor of apoptosis protein

Key words: Axl, chemoresistance, Erk, metformin, ovarian cancer, STAT3, Tyro3

RTKs as well as GAS6 protein have been observed in the initiation and progression of many cancers (10) such as breast cancer (11), colon cancer (12), gastric cancer (13), leukemias (14), liver cancer (15), melanoma (16,17), ovarian cancer (18,19) and prostate cancer (20). Therefore, targeting TAM RTKs and their ligands might be a potent strategy to control these cancers.

Metformin is the most widely prescribed biguanide to treat type 2 diabetes (21). In addition to the primary effect on glucose metabolism, the antitumorigenic potential of metformin has come to the forefront, since the accumulating evidence suggests its anticancer effects on cell proliferation in various cancers and tumor growth in xenograft model (22-25). The anticancer mechanisms of metformin have been assessed by its ability to activate AMP-activated protein kinase (AMPK), resulting in the concomitant inhibition of mammalian target of rapamycin complex I (mTORC1) activity which plays an important role in protein synthesis and cell proliferation (26-29). Metformin has also been reported to directly inhibit mTORC1 independent of AMPK and to target epidermal growth factor receptor (EGFR) and Akt/mitogen-activated protein kinase (MAPK) which mediate pro-survival and anti-apoptotic signals (30-32).

In this study, we examined the effect of metformin on the expression of Ax1 and Tyro3 RTKs in both parental and chemoresistant ovarian cancer cells, which affects the viability of these cells. Metformin was found to suppress the expression of both Ax1 and Tyro3 RTKs at transcriptional level and deactivate downstream signaling molecules such as Erk and signal transducers and activators of transcription 3 (STAT3), which led to significant decrease of cell proliferation through downregulation of anti-apoptotic protein such as XIAP.

Materials and methods

Reagents and antibodies. Metformin was obtained from Sigma (St. Louis, MO, USA). For western blot analysis, specific antibodies against phospho-Akt, Akt, Ax1, phospho-Erk, Erk, phospho-STAT3, STAT3, Tyro3, XIAP and GAPDH and secondary antibodies were obtained from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Primers for PCR to amplify Ax1, Tyro3, and GAPDH were synthesized by Bioneer Inc. (Daejeon, Korea). TRI reagent was from Solgent (Daejeon, Korea). AmpliTaq DNA polymerase was obtained from Roche Inc (Indianapolis, IN, USA).

Cell culture. A2780 and SKOV3 cells were purchased from the American Type Culture Collection and A2780/Cis cells were from Sigma. SKOV3/TR cells, a variant of SKOV3 cells with resistance to taxol, were established as we previously reported. Briefly, SKOV3/TR cells had acquired the taxol resistance via long-term and stepwise exposure of the parental cells to the escalating concentrations of taxol. All of these 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% CO₂ in a water-saturated atmosphere.

RT-PCR. Cells (3x10⁵) were seeded in 60-mm culture dishes and grown overnight at 37°C and then treated with the indicated

concentrations of metformin for the 48 h. Total RNA was extracted using TRI reagent and subjected to cDNA synthesis and PCR. The specific primers were as follows: Ax1, sense 5'-AACCTTCAACTCCTGCCTTCTCG-3' and antisense 5'-CAGCTTCTCCTTCAGCTCTTAC-3'; Tyro3, sense 5'-GTGTGTGGCTGACTTCGGAC-3' and antisense 5'-CACGTCCTCCATACACTCCG-3'; GAPDH, sense 5'-GGAGCCAAAAGGGTCATCAT-3' and antisense 5'-GTGATGGCATGACTGTGGT-3'.

Western blot analysis. Cells were treated with the indicated concentrations of metformin for the 48 h. Whole 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 Na₃VO₄ and protease inhibitor cocktail]. Protein concentrations were determined using Bio-Rad protein assays. Proteins from whole 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 3x10-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™ ECL™ prime Western Blotting detection reagent; GE Healthcare, Piscataway, NJ, USA).

Colony-forming assay. Cells (1-2x10³ cells per dish) were seeded in 35-mm culture dishes and allowed to grow in the absence or presence of the metformin for 7-10 days to form colonies. Colonies of >50 cells were visualized by crystal violet (in 60% methanol, Junsei Chemical, Japan) staining and images were taken by RAS 3000 Image Analysis System (Fuji Film, Japan).

Ax1 promoter activity test. To measure the Ax1 promoter activity upon metformin treatment, the plasmid containing Ax1 promoter-luciferase reporter, pGL3-Ax1, was used. As we previously reported, pGL3-Ax1 plasmid is constructed by PCR amplification of Ax1 promoter region ranging from -887 to +7 bp of the transcriptional start site and the following subcloning of the PCR product into the pGL3-basic vector. The pGL3-Ax1 plasmid was co-transfected into cells (3x10⁴ cells in a 60-mm dish) with *Renilla* luciferase vectors, pRL-SV40, as an internal control. Luciferase activity was measured using a Dual-Glo luciferase assay system.

siRNA transfection. RNA interference silencing was performed to suppress Ax1 or Tyro3 protein levels. SKOV3 or SKVO3/TR cells (1x10⁶) were seeded in 100-mm culture dish and grown overnight and then transfected with 50 nM siRNA against Ax1 (sc-36438, Santa Cruz Biotech), Tyro3 (sc-36438, Santa Cruz Biotech), or control siRNA (sc-37007, Santa Cruz Biotech). For cell proliferation assay, cells were harvested 24 h after transfection and re-seeded into 35-mm culture dishes and allowed to grow for 48 h in the absence or presence of the metformin. Cells were then harvested and the number of

viable cells was counted. Whole cell lysates were prepared and used for western blot analysis to evaluate Axl and Tyro3 protein levels.

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 treated with the indicated concentration of metformin for 48 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 \pm SD of triplicate samples or at least three independent experiments. Student's t-test was used to determine statistical significance with a threshold P-value of <0.05 .

Results

Metformin inhibits proliferation of both parental and chemoresistant ovarian cancer cells. To determine anti-proliferative effect of metformin on human ovarian cancer cells, A2780 and SKOV3 cells were incubated with 1, 3 and 10 mM of metformin for 48 h and then cell viability was measured. As shown in Fig. 1A, cell counting results showed that metformin treatment caused dose-dependent reduction of cell proliferation in both cell types. Of note, when the cells were exposed to 10 mM of metformin for 48 h, the number of viable A2780 and SKOV3 cells were diminished to 36 and 33% of that of untreated cells, respectively. The inhibitory effect of metformin on cell proliferation was further confirmed by colony-forming assay. Exposure of A2780 and SKOV3 cells upon 1, 3 and 10 mM metformin for 7 days were found to form less colonies in a dose-dependent manner (Fig. 1B). Especially, both cell types failed to grow into colonies in the presence of 10 mM metformin, indicating cytotoxic effect of metformin on human ovarian cancer cells.

We also found that metformin decreased proliferation of chemoresistant ovarian cancer cells. Cisplatin-resistant A2780/Cis cells and taxol-resistant SKOV3/TR, which are each of subline derived from the parental A2780 and SKOV3 cells, were treated with 1, 3, 10 mM of metformin for 48 h for cell counting or 7 days for colony-forming assay. As shown in Fig. 1C, the viability SKOV3/TR cells at 10 mM metformin was 37% of control, while that of A2780/Cis cells was 55% of control. The result from colony-forming assay also demonstrated that metformin induced a dose-dependent inhibition of cell proliferation (Fig. 1D). It seems to be evident that SKOV3/TR cells were more profoundly affected by metformin treatment than A2780/Cis cells, since both the cell viability and the number of colonies of SKOV3/TR cells incubated with metformin were less than each of those of A2780/Cis cells exposed to the same concentrations of metformin (Fig. 1C and D). Taken together, these results indicate that metformin decreases proliferative capacity of both parental and chemoresistant ovarian cancer cells, SKOV3, SKOV3/TR, A2780 and A2780/Cis cells.

Metformin suppresses the expression of Axl and Tyro3 RTKs at transcriptional level. Next, we explored if metformin affects the expression of receptor tyrosine kinases (RTKs), since TMA

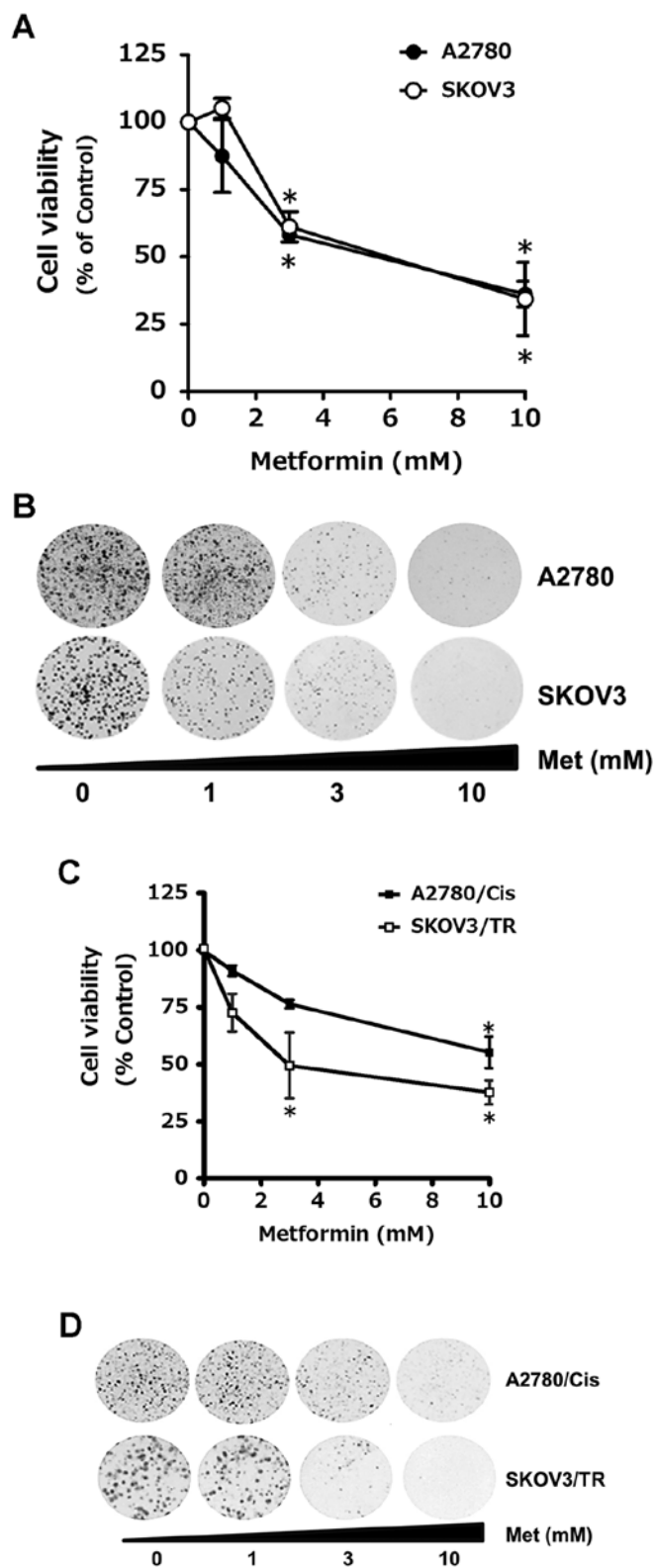


Figure 1. Metformin inhibits proliferation of both parental and chemoresistant ovarian cancer cells. (A and C) Cells (3×10^3 cells/dish) were seeded onto 60-mm dishes, grown overnight and treated with the indicated doses of metformin for 48 h. After treatment, cells were harvested, stained with trypan blue, and the number of viable cells was counted. Data are presented as mean \pm SD of at least three independent experiments. The asterisks indicate the significant difference compared to the control value ($*P < 0.05$ vs untreated group). (B and D) Cells (2×10^3 cells/dish) were seeded onto 35-mm dishes and allowed to grow under the indicated dose of metformin for 7-10 days. Colonies were visualized by crystal violet staining. The data shown is representative of three independent experiments.

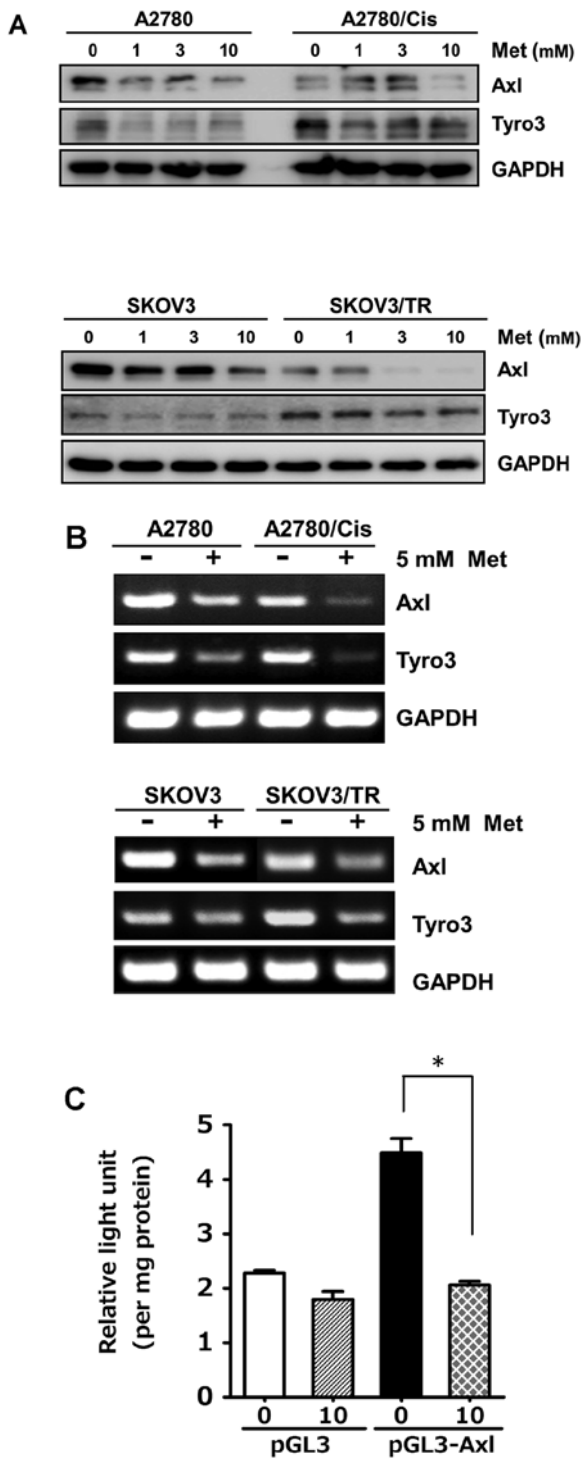


Figure 2. Metformin suppresses the expression of Axl and Tyro3 at transcriptional level. Cells (3×10^3 cells/dish) were seeded onto 60-mm dishes, grown overnight and treated with the indicated doses of metformin for 48 h. (A) The protein levels of Axl and Tyro3 in metformin-treated cells was determined by western blot analysis. GAPDH was used as a loading control. The result shown is a representative of three independent experiments. (B) For RT-PCR, total RNAs from the cells treated with 5 mM metformin for 48 h were isolated and used for analysis of Axl and Tyro3 mRNA expression. As an internal control, GAPDH mRNA was also amplified by RT-PCR. The data shown are representative of three independent experiments. (C) To examine the effect of metformin on Axl promoter activity, the SKOV3 cells (3×10^4 cells) were transfected with pGL3 or pGL3 Axl using Lipofectamine 2000. The cells were then incubated with 10 mM metformin for 24 h and total cell lysates were used to measure luciferase activity. Data are expressed as the means \pm SD of triplicate samples conducted in three independent experiments. The asterisks indicate the significant difference compared to the control value ($P < 0.05$, vs untreated group).

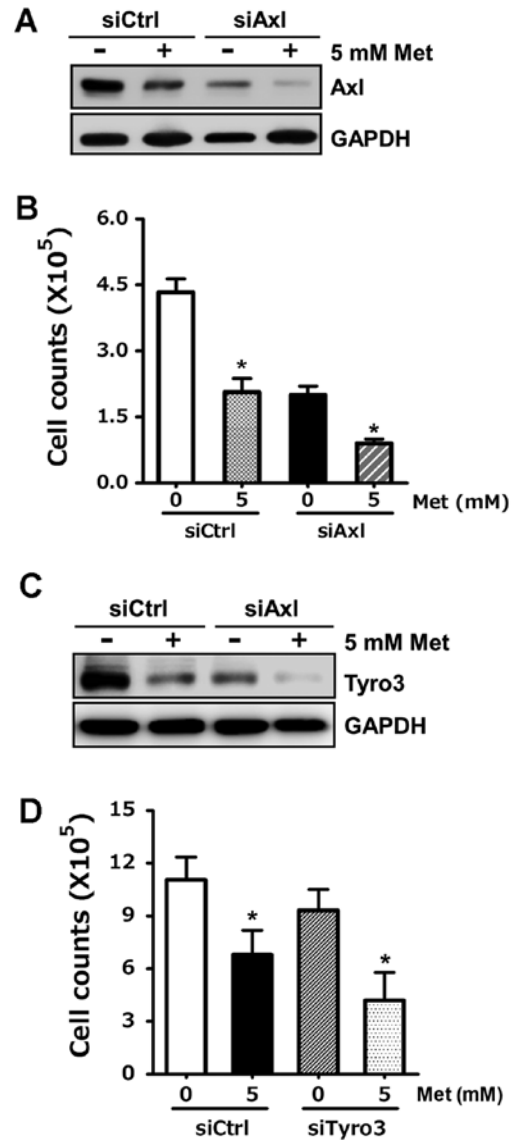


Figure 3. Silencing of Axl or Tyro3 augments the anti-proliferative effects of metformin in ovarian cancer cells. SKOV3 and SKOV3/TR cells (3×10^4 cells) were transfected with Axl- or Tyro3-specific siRNA, respectively. The control siRNA was also transfected to the same cells. (A and C) Cells were harvested 24 h post-transfection, split and treated with the indicated concentrations of metformin for 48 h. The total cell lysates were prepared and the Axl and Tyro3 protein levels were determined by western blot analysis. GAPDH was used as a loading control and results shown are representative of at least three independent experiments. (B and D) After treatment, cells were harvested and the number of viable cells was counted by trypan blue staining. Data are presented as mean \pm SD of at least three independent experiments. The asterisks indicate the significant difference compared to the control value ($P < 0.05$ vs untreated group).

RTKs, Axl, Tyro3, and Mer, have been reported to mediate cell survival and proliferation signals (33). After exposure of cells with the indicated concentrations of metformin for 48 h, the expression of Axl and Tyro3 was examined at protein and mRNA levels. As shown in Fig. 2A, metformin treatment was found to induce dose-dependent downregulation of Axl and Tyro3 expression in both SKOV3 and taxol-resistant SKOV3/TR cells, while Axl and Tyro3 protein levels in A2780 cells and cisplatin-resistant A2780/Cis cells were significantly reduced upon 1 or 10 mM metformin treatment.

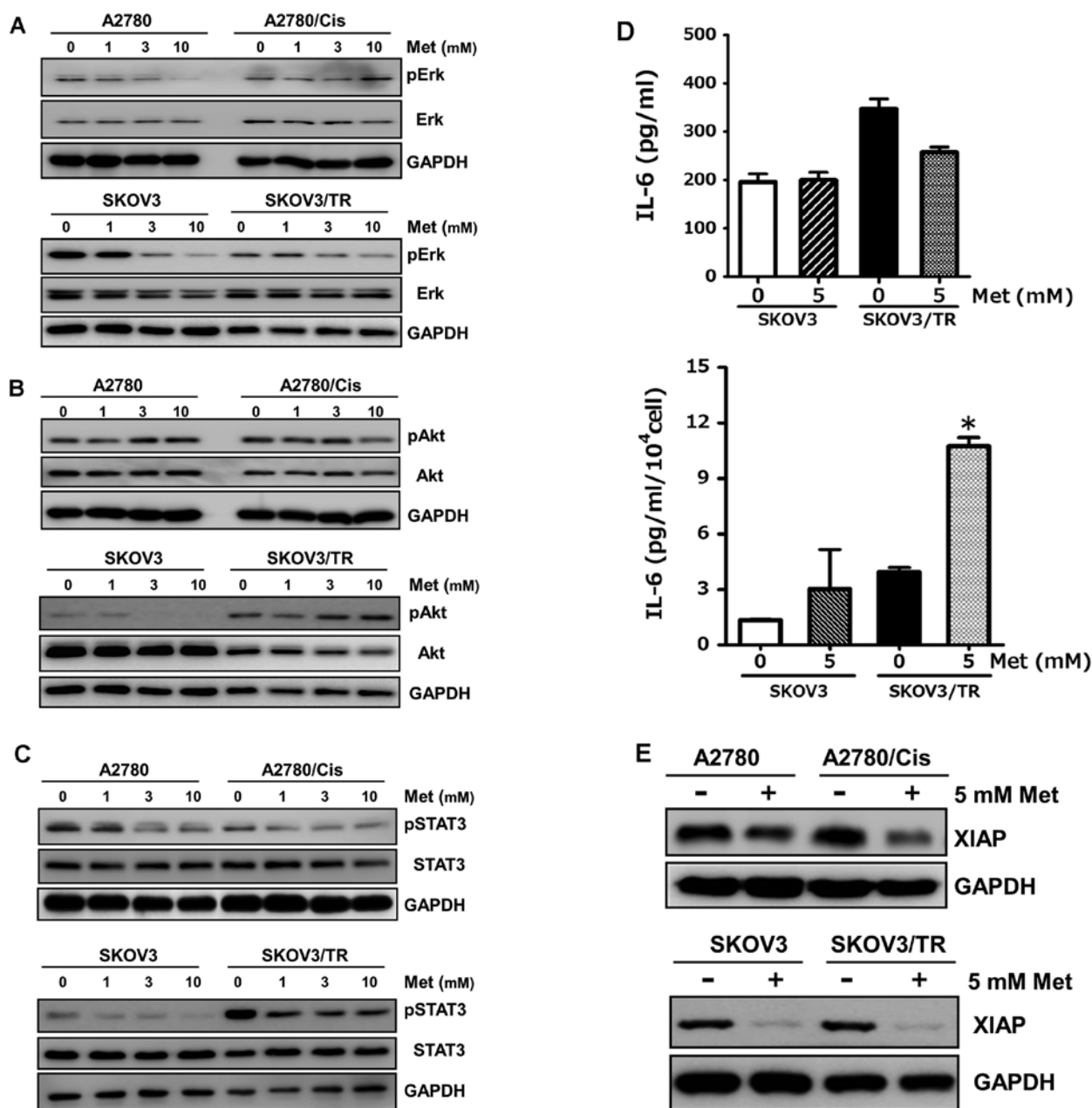


Figure 4. Metformin has inhibitory effect on Erk and STAT3 phosphorylation and XIAP protein level, but not IL-6 production, in ovarian cancer cells. Cells (3×10^3 cells/dish) were seeded onto 60-mm dishes, grown overnight and treated with the indicated doses of metformin for 48 h. (A-C) The total cell lysates were prepared and phospho-Erk, Akt and STAT3 levels were assessed by western blot analysis. Total protein levels of each protein were also analyzed by western blotting. GAPDH was used as a loading control. The result shown is a representative of three independent experiments. (D) In parallel, the conditioned media from metformin treated SKOV3 and SKOV3/TR cells were harvested and used to determine IL-6 protein levels via ELISA. The asterisks indicate the significant difference compared to the control value ($P < 0.05$ vs untreated control). The data are representative of three independent experiments. (E) XIAP protein levels of total cell lysates were also examined by western blot analysis. GAPDH was also detected as a loading control. The data shown are representative of three independent experiments.

The effect of metformin on Ax1 and Tyro3 expression was further confirmed by RT-PCR. Consistent with western blot results, the mRNA levels of Ax1 and Tyro3 were decreased by metformin treatment (Fig. 2B). It is notable that Ax1 mRNA expression was slightly reduced in chemoresistant A2780/Cis and SKOV3/TR cells, compared to that in their parental cells, while Tyro3 mRNA expression was increased in these two chemoresistant cells, indicating that the acquisition of cisplatin or taxol resistance resulted in the down/upregulation of Ax1 and Tyro3 expression, respectively.

The inhibitory effect of metformin on the transcription of *Axl* gene was also demonstrated by promoter activity test. SKOV3 cells were transfected with pGL3-Axl construct, a luciferase expressing plasmid under the control of human Ax1 promoter and then incubated with 10 mM metformin for 48 h. As illustrated in Fig. 2C, metformin treatment was found to reduce Ax1 promoter activity to 46% compared with the untreated cells. The results from RT-PCR and promoter activity test indicate that metformin suppresses Ax1 expression at the transcriptional level.

Anti-proliferative effect of metformin is mediated by dysregulation of Axl and Tyro3 RTKs and downstream signaling molecules. We further examined whether the inhibitory effect of metformin on Axl and Tyro3 expression is responsible for its anti-proliferative property. Axl and Tyro3-specific siRNA, siAxl and siTyro3, was transfected into SKOV3 and SKOV3/TR cells, respectively, and then cells were treated with metformin to evaluate the synergistic effect on protein expression as well as cell viability. Western blot results showed that knock-down of Axl and Tyro3 protein level by specific siRNA augmented metformin-induced downregulation of each protein expression (Fig. 3A and C). The viability of the cells transfected with siAxl or siTyro3 was found to be 48% in SKOV3 cells and 45% in SKOV3/TR cells, thus being lower than each of the cells transfected with control siRNA, siCtrl (Fig. 3B and D). This indicates that decreased Axl and Tyro3 protein level by siRNA escalated the inhibitory effect of metformin on cell proliferation. Taken together, these data demonstrate that Axl and Tyro3 RTKs are novel targets of metformin for its anticancer activity in ovarian cancer cells.

We next examined the effects of metformin on several downstream effectors known as regulators of cell survival, proliferation and apoptosis. As shown in Fig. 4A, dose-dependent inhibition of Erk phosphorylation by metformin was found in A2780, SKOV3 and SKOV3/TR cells, but not in A2780/Cis cells. On the other hand, metformin was found to have no effect on phosphorylation of Akt which delivers proliferative signals and is elevated in SKOV3/TR cells, except in SKOV3 cells (Fig. 4B).

Western blot analysis further showed that metformin fairly reduced phosphorylation of signal transducers and activators of transcription 3 (STAT3), one of the key regulators driving cell proliferation in both parental and chemoresistant cells (Fig. 4C). Based on metformin-mediated suppression of STAT3 phosphorylation, we also examined IL-6 production upon metformin treatment, since activation of IL-6/STAT3 axis has been known to play an important role in cell viability (34,35). ELISA results showed that total IL-6 production was slightly reduced by metformin, whereas IL-6 production per cell was rather increased (Fig. 4D).

In addition, we observed that the metformin reduced the level of X-linked inhibitor of apoptosis protein (XIAP) which inhibits apoptosis (Fig. 4E). Collectively, these data indicate that metformin induces the downregulation of Axl and Tyro3 expression and the subsequent decrease of Erk and STAT3 phosphorylation as well as XIAP expression by which it exerts its anticancer activity in ovarian cancer cells.

Discussion

Cisplatin has primarily been approved as an anticancer agent to treat testicular and ovarian cancer in 1978 (36,37), and is the first-line chemotherapy drug used in many cancers such as bladder, cervical, ovarian cancers, lymphomas and sarcomas, as a single agent or in combination with other anticancer agents, including paclitaxel (38-40). Taxol, a microtubule-stabilizing drug, which in turn prevents depolymerization of microtubule, has been frequently used to treat breast, lung, stomach or ovarian cancers (41). Although the combination of platinum-based agent and taxol is still standard chemotherapy

regimen in ovarian cancers, most patients eventually develop tolerance against cisplatin and taxol, leading to a low 5-year survival rate (3,42). However, the molecular mechanisms of this chemoresistance are not yet fully understood.

We found that metformin suppressed the viability of ovarian cancer cells (A2780 and SKOV3) and their cisplatin/taxol-resistant cells (A2780/Cis and SKOV3/TR) (Fig. 1). Consistent with our data, the anticancer and chemopreventive effects of metformin have been suggested by many epidemiological and preclinical studies which demonstrated that metformin treatment is associated with the reduction of cancer risk and better prognosis in patients with type II diabetes (43-46). Of note, however, there are also several studies which failed to demonstrate any beneficial effect of metformin (47-49).

We then observed that metformin downregulates the expression of Axl and Tyro3 receptor tyrosine kinases (RTKs) at transcriptional level (Fig. 2). TAM family of RTKs, Tyro3, Axl and Mer, have been reported to play critical roles in cell survival, proliferation, apoptosis, and adhesion (33). Dysregulation of these RTKs and their ligands including GAS6 has been shown in many human cancers. In ovarian adenocarcinoma tissues, the overexpression and activation of Axl, Mer and ligands have been observed and found to be correlated with disease stages and metastasis (19). In acute lymphoblastic leukemia (ALL) and non-small cell lung cancer (NSCLC) cell lines, the Mer expression was found to be elevated to promote cancer cell survival, tumorigenesis and chemoresistance. In addition, Axl or Mer inhibition using RNA interference, monoclonal antibodies and shRNA have been demonstrated to abrogate proliferation, metastasis and tumor progression in xenograft model, while it enhanced chemosensitivity in ALL and NSCLC (9,50,51). Consistent with these reports, we also found that the silencing of Axl and Tyro3 RTKs by specific siRNAs, siAxl and siTyro3, augmented the anti-proliferative effect of metformin in SKOV3 and SKOV3/TR cells, respectively (Fig. 3B and D). Our data suggest that Axl and Tyro3 RTKs might be novel targets of metformin to inhibit cell proliferation as well as to overcome chemoresistance.

A recent study by Feng *et al* demonstrated that metformin-induced inactivation of STAT3 signaling promotes autophagy and apoptosis as a mechanism to inhibit cell growth in esophageal squamous cells (52). In our setting, metformin was found to decrease STAT3 phosphorylation (Fig. 4C). Since STAT3 is a transcription factor to induce IL-6 production and IL-6 phosphorylates STAT3, we examined the effect of metformin on IL-6 expression. Interestingly, metformin was found to have almost no effect of on IL-6 production and IL-6 production per cell was even shown to be increased upon metformin treatment (Fig. 4D). These results indicate that metformin directly inhibits STAT3 phosphorylation, while cells must somehow have a strategy to compensate the IL-6 production which might provide residual cell viability upon metformin exposure.

In conclusion, our data demonstrate that metformin has inhibitory effects on Axl and Tyro3 expression and subsequent activation of Erk and STAT3, resulting in its anti-proliferative activity in both parental and chemoresistant ovarian cancer cells. Thus, Axl and Tyro3 and/or their downstream signaling pathway seem to be novel therapeutic targets of metformin to

control proliferation and chemoresistance of ovarian cancer cells.

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