Original Article
Metformin inhibits ovarian cancer growth and migration in vitro and in vivo by enhancing cisplatin cytotoxicity

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Abstract: The purpose of the current study was to investigate whether metformin can enhance the anti-cancer effect of cisplatin on epithelial ovarian cancer in vitro and in vivo. CCK-8 assays were performed to detect cell viability, and flow cytometry was performed to measure cell apoptosis rates. Transwell assays were used to detect the migration and invasion ability of ovarian cancer cells. Western blotting and qRT-PCR were performed to detect protein expression. Xenograft mouse models were constructed to clarify the treatment response in vivo. Metformin alone or cisplatin alone dose-dependently inhibited SKOV3 and Hey cell proliferation. The combination of these two drugs exerted a stronger inhibitory effect with a higher apoptosis rate than administration of either drug alone. Transwell assay results revealed that metformin promoted the inhibitory effect of cisplatin on ovarian cancer cell metastasis. Metformin and cisplatin co-treatment significantly inhibited N-cadherin and MMP-9 expression. The Western blotting results revealed that metformin and cisplatin co-treatment inhibited TGFβ1 expression and Smad2 and Smad3 phosphorylation. The in vivo study results were consistent with results from the in vitro study. Data from our study suggest that metformin enhanced the anti-tumour effect of cisplatin on epithelial ovarian cancer in vitro and in vivo, which provides more evidence supporting the use of metformin to treat epithelial ovarian cancer.

Keywords: TGFβ, metformin, epithelial ovarian cancer, cisplatin, metastasis

Introduction

As the most lethal gynaecological malignancy, an estimated 22440 new cases and 14080 deaths due to ovarian cancer were reported in America in 2016 [1]. Due to a lack of early symptoms, many patients are diagnosed at advanced stages. Currently, the standard treatment for ovarian cancer is radical surgery and platinum-based chemotherapy. Despite the development of novel chemotherapy drugs, the overall five-year survival rate of ovarian cancer patients remains approximately 40% [2]. Chemotherapy resistance is one of the leading causes of recurrence, and approximately 25% of patients experience recurrence within 6 months after the end of treatment [3]. Therefore, development of new, promising therapies for the treatment of ovarian cancer is an urgent need.

Metformin, a relatively safe, inexpensive and effective anti-diabetes mellitus drug, has been used clinically for several decades [4]. In 2005, Evans et al. reported that diabetic patients taking metformin had a lower risk of cancer than those who did not take metformin [5]. Since then, an increasing number of studies has reported the anti-cancer effect of metformin. Preclinically, metformin has been reported to inhibit the PI3K/AKT/mTOR signalling pathway, which plays a critical role in metabolism, proliferation and many other malignancy activities [6, 7]. Verifying the published research, our previous study concluded that metformin induced ovarian cancer cell apoptosis by activating the p38 MAPK/JNK pathway [8]. Interestingly, metformin exerted its anti-cancer effect by directly promoting cancer cell apoptosis [9, 10] and cancer cell cycle arrest at the G0/G1 stage [11, 12]. Additionally, a low dose of metformin inhibited cancer stem cell self-renewal in ovarian cancer, breast cancer, liver cancer and bladder cancer [13-16]. In addition, metformin attenuated epithelial-mesenchymal transition (EMT), which is critical for cancer metastasis in prostate cancer and lung cancer [17, 18]. In recent years, prospective clinical studies have
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indicated decreased Ki-67 expression in neoplasm tissue and reduced metastasis in patients diagnosed with breast cancer and endometrial cancer with metformin treatment [19-21]. In addition, combining metformin with chemotherapy has been attempted in various cancers. Lengyel E et al. treated ovarian cancer with metformin and paclitaxel in mouse models and found that metformin increased paclitaxel sensitivity [22]. In mouse xenografts generated with lung cancer and prostate cancer cell lines, metformin worked with a series of chemotherapeutic agents, including carboplatin, paclitaxel and doxorubicin, and had comparable effects on tumour regression and relapse prevention [23]. However, the effects of the metformin and cisplatin co-treatment on ovarian cancer proliferation, migration and invasion and the underlying mechanisms of these effects remain unclear.

On the basis of findings from previous preclinical and clinical studies, we hypothesized that metformin and cisplatin co-treatment exerts a powerful influence on ovarian cancer. In the present study, we aimed to explore whether metformin can enhance cisplatin cytotoxicity in epithelial ovarian cancer in vitro and in vivo. The results from our study revealed that metformin effectively boosted the inhibitory effects of cisplatin on tumour proliferation and metastasis.

Materials and methods

Cell lines and reagents

SKOV3 and Hey cell lines were purchased from the American Type Culture Collection (ATCC). The SKOV3 and Hey cells were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS) (Gibco, Life Technologies, USA), 1% penicillin, 1% streptomycin and 1% amphotericin B at 37°C with 5% CO₂. Cisplatin and metformin were purchased from Sigma-Aldrich (St. Louis, USA) and dissolved in phosphate-buffered saline (PBS). Compound C was purchased from MedChem Express (Shanghai, China).

Cck-8 assay

Cell viability was detected by a CCK-8 assay kit (Dojindo, Japan). Briefly, cells were seeded into 96-well plates at a density of 3000 cells/well and cultured for 24 hours. After starvation with FBS-free RPMI 1640 medium for 12 hours, the cells were treated with cisplatin or metformin for the indicated times. CCK-8 reagent (10 µl per well) and RPMI 1640 (100 µl per well) were added to the 96-well plates, and the cells were incubated for 30 min at 37°C. The absorbance values were measured at 450 nm, and cell viability was calculated. All experiments were repeated in triplicate.

Flow cytometry assay

The rate of cell apoptosis was measured by an Annexin V-FITC apoptosis detection kit (BD, USA). Briefly, cells were starved for 12 hours with FBS-free medium and treated with cisplatin or metformin after culturing in 6-well plates for 24 hours. The cells were resuspended in 1 x binding buffer after being washed twice with cold PBS. Then, 5 µl of PI and 5 µl of FITC were added and incubated for 15 min at room temperature in the dark. The apoptotic cells were detected by flow cytometry. FlowJo 7.6 (USA) was used to analyse the flow cytometry data. Each experiment was conducted in triplicate.

Transwell assay

Cell migration and invasion were detected with Transwell assays. For the migration assay, cells were resuspended in 200 µl of FBS-free medium after being starved for 12 hours and were plated into the upper chambers (BD, USA) of 24-well plates at a density of 1 x 10⁵ cells. RPMI 1640 (600 µl) supplemented with 20% FBS was added to the lower chambers. After being treated with the indicated drugs for 24 hours, the cells were fixed in 4% paraformaldehyde, stained with 1% crystal violet and rinsed in PBS three times. The migrated cells were counted under an inverted microscope. For the invasion assay, 50 µl of Matrigel (BD, USA) was pre-applied to the upper chambers, and the starved cells were plated. Similar to the migration assay, a total of 600 µl of medium containing 20% FBS was added into the lower chambers. The cells were incubated with the indicated drugs for 48 hours and then fixed and stained as described in the migration assay. Cells in the upper chambers were gently wiped with cotton swabs. An inverted microscope was used to detect the invading cells. Five visual fields were randomly selected for each chamber. All experiments were repeated in triplicate.
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**Quantitative real-time PCR**

Total RNA was extracted with TRIzol (Invitrogen, USA) and reverse transcribed into cDNA using PrimeScript RT Master Mix (Takara, USA). qRT-PCR was performed with the 7900HT Fast Real-Time PCR System (Thermo Fisher, USA). The primers for N-cadherin, MMP-9 and GAPDH were synthesized by BioTNT (Guantai, China). The primers were as follows: N-cadherin, forward 5'-CTATGAGTGGAA CAGGAAGC-3' and reverse 5'-GGTCTGAGTGGACTC-3'; MMP-9, forward 5'-CGAAGCTTGGACGACA-AG-3' and reverse 5'-TTCCAGGGCAGGACCATACG-3'; and GAPDH, forward 5'-TGACTTCAACA-GGACACCCA-3' and reverse 5'-CACCTGTGGCTAGCCAAA-3'. GAPDH was used as a control. The relative mRNA levels were calculated with the \(2^{-\Delta\Delta CT} \) method.

**Western blotting analysis**

Cells were harvested and lysed with RIPA (Beyotime, China) supplemented with phenylmethyl sulfonyl fluoride (PMSF) (Beyotime, China) and phosphatase inhibitors. The protein concentration was quantified with a BCA kit (Beyotime, China), and 30 µg of protein was loaded onto SDS-PAGE gels for electrophoresis and transferred to PVDF membranes. The membranes were blocked in 5% fat-free milk for one hour at room temperature and incubated in primary antibodies at 4°C overnight. Then, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:4000) (Cell Signaling Technology, USA) for one hour at room temperature and detected with Immobilon Western substrate (Millipore, USA). The anti-N-cadherin, anti-MMP-9, anti-TGFβ1, anti-P-Smad2, anti-P-Smad3, anti-Smad2 and anti-Smad3 antibodies were purchased from Abcam (USA). The anti-Bcl-XL, anti-Bcl-2, anti-Bax, anti-cleaved caspase-3, anti-cleaved PARP, anti-cleaved caspase-9, anti-P-AMPKα, anti-AMPKα and anti-GAPDH antibodies were purchased from Cell Signaling Technology (USA). All primary antibodies were monoclonal and applied at a dilution of 1:1000. The relative protein expression levels were normalized to the GAPDH level. All experiments were repeated three times.

**Xenograft mouse experiment**

A total of twenty-four 5-week-old female nude BALB/c mice were purchased from Slac Laboratory Animal Corporation (China), and all animal experiments were approved by the Animal Care and Research Committee of Fudan University. SKOV3 cells were resuspended in 200 µl of RPMI 1640 at a concentration of 2 × 10⁷ cells/ml and injected subcutaneously into the flank of nude mice. The mice were randomly divided into four groups (n=6) when the tumour size increased to 50 mm³. The four groups were as follows: Group A (the control group), Group B (the metformin group), Group C (the cisplatin group) and Group D (the metformin and cisplatin co-treatment group). The mice in the control group were given PBS through intraperitoneal injection and oral gavage. Metformin (200 mg/kg) was given by oral gavage for 21 consecutive days. Cisplatin (4 mg/kg) was injected intraperitoneally every six days for three weeks. A Vernier caliper was used to measure the tumour size every three days. The tumour volume (mm³) was calculated as follows: 0.5 × length × width². All mice were sacrificed after 21 days. Tumours were gently resected and fixed in paraformaldehyde.

**Immunohistochemistry**

The fixed tumour tissue was embedded in paraffin and sliced into 5-µm-thick sections. After deparaffinization and dehydration, the slices underwent antigen retrieval at 95°C for 30 min and were blocked with goat serum for one hour. Then, the slices were incubated with primary antibodies at 4°C overnight and with HRP-conjugated secondary antibodies at 37°C for 45 min. DAB (Jiehao Biotechnology, China) was used for colour development. The slides were examined under the microscope, and the representative images were captured. For each slide, five visual fields were randomly selected at × 400. The anti-Ki-67 and anti-cleaved caspase-3 antibodies were purchased from Cell Signaling Technology (USA). The anti-N-cadherin, anti-MMP-9, anti-P-Smad2 and anti-P-Smad3 antibodies were purchased from Abcam (USA). Image-Pro Plus 6.0 (Media Cybernetics, USA) was used to analyse the captured images.

**Statistical analysis**

Quantitative data are expressed as the means ± SD. SPSS 16.0 (IBM, USA) was used to analyse the data. One-way ANOVA was used to compare the means among the four groups. \(P<0.05\) was considered statistically significant.
Metformin promotes the inhibitory effect of cisplatin on epithelial ovarian cancer cell proliferation by inducing apoptosis

To detect cell viability after metformin and/or cisplatin treatment, the CCK-8 assay was performed. The results revealed that both metformin and cisplatin dose- and time-dependently inhibited SKOV3 and Hey cell proliferation (Figure 1A-D). To clarify whether metformin can enhance the inhibitory effect exerted by cisplatin, low doses of metformin (5 mM) combined with cisplatin at different doses were added to the SKOV3 and Hey cells. Surprisingly, compared with cisplatin treatment alone, combination treatment with the two drugs significantly attenuated cell proliferation (P<0.001) (Figure 1E, 1F). To understand the underlying mechanisms by which metformin inhibited proliferation, flow cytometry was performed to examine the apoptosis rate. Data from our study indicated that compared with metformin or cisplatin alone, metformin and cisplatin co-treatment dramatically increased the apoptosis rate (Figure 2A-D). Western blot analysis was used to detect apoptosis-related proteins. The metformin and cisplatin co-treatment substantially increased the level of pro-apoptotic proteins cleaved caspase-3, cleaved PARP and cleaved caspase-9 and decreased the level of anti-apoptotic proteins Bcl-2, Bcl-XL and Bcl-XL/Bax (Figure 2E, 2F). In summary, we concluded that metformin promoted the inhibitory effect of cisplatin on epithelial ovarian cancer cell proliferation by inducing apoptosis.

Metformin promotes the inhibitory effect of cisplatin on ovarian cancer cell metastasis

To investigate the influence of metformin on ovarian cancer cell migration and invasion, a Transwell experiment was performed. Our results revealed that cisplatin slightly inhibited the migration and invasion of SKOV3 and Hey cells, which was enhanced by metformin treatment (Figure 3). EMT plays a critical role in ovarian cancer cell metastasis. qRT-PCR and Western blotting were performed to detect the
Figure 2. Metformin promotes cisplatin-induced cell apoptosis in vitro. A-D. SKOV3 and Hey cells were divided into four groups: the control group, metformin group (20 mM), cisplatin group (1.6 µg/ml) and metformin + cisplatin group (20 mM metformin + 1.6 µg/ml cisplatin). Flow cytometric analysis was used to detect the apoptosis rate in different groups after 48 hours of treatment. E, F. Western blotting was performed to detect apoptosis-related protein expression in different groups after 48 hours of treatment. GAPDH was used as a loading control. *, P<0.05, **, P<0.01, and ***, P<0.001.
Figure 3. Metformin enhances the inhibitory effect of cisplatin on cell migration and invasion. A, B. SKOV3 and Hey cells were divided into four groups: the control group, metformin group (20 mM), cisplatin group (1.6 µg/ml) and metformin + cisplatin group (20 mM metformin + 1.6 µg/ml cisplatin). Transwell assays were used to measure cell migration after 24 hours of treatment. C, D. SKOV3 and Hey cells were divided into four groups: the control group, metformin group (20 mM), cisplatin group (1.6 µg/ml) and metformin + cisplatin group (20 mM metformin + 1.6 µg/ml cisplatin). Transwell assays were used to measure cell migration after 48 hours of treatment. *, P<0.05, **, P<0.01, and ***, P<0.001.
Figure 4. Metformin inhibited EMT via the TGFβ signalling pathway. A. SKOV3 and Hey cells were divided into four groups: the control group, metformin group (20 mM), cisplatin group (1.6 µg/ml) and metformin + cisplatin group (20 mM metformin + 1.6 µg/ml cisplatin). qRT-PCR was performed to detect the mRNAs levels of N-cadherin and MMP-9 after 48 hours of treatment. B. SKOV3 and Hey cells were divided into four groups: the control group, metformin group (20 mM), cisplatin group (1.6 µg/ml) and metformin + cisplatin group (20 mM metformin + 1.6 µg/ml cisplatin). Western blotting was performed to detect the protein levels of N-cadherin and MMP-9 after 48 hours of treatment. C. D. SKOV3 and Hey cells were divided into four groups: the control group, metformin group (20 mM), cisplatin group (1.6 µg/ml) and metformin + cisplatin group (20 mM metformin + 1.6 µg/ml cisplatin). Western blotting was performed to detect the protein levels of TGFβ1, P-Smad2, P-Smad3, Smad2 and Smad3 after 48 hours of treatment. E, F. Compound C (10 µM) was added to SKOV3 and Hey cells that were pre-treated with metformin (20 mM) for two hours. Western blotting was performed to detect the protein levels of P-AMPKa, AMPKa, P-Smad2, P-Smad3, Smad2 and Smad3 after 48 hours of treatment. GAPDH was used as a loading control. *, P<0.05, **, P<0.01, and ***, P<0.001.
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Expression of EMT-related proteins. The results revealed that N-cadherin and MMP-9 expression levels were significantly inhibited transcriptionally and translationally by the drug combination in both SKOV3 and Hey cells (Figure 4A, 4B).

**Metformin inhibited ovarian cancer metastasis via the TGFβ-1 pathway**

To investigate the mechanisms by which metformin inhibited metastasis, we focused on the TGFβ-1 pathway, which is a well-documented modulatory signalling pathway for ovarian cancer metastasis. The Western blotting results revealed that metformin alone significantly inhibited TGFβ-1, P-Smad2 and P-Smad3 expression, which was strengthened by the metformin and cisplatin co-treatment (Figure 4C, 4D). To investigate whether the impact of metformin on the TGFβ mediates tumour signalling pathway is AMPK dependent, compound C and/or metformin were added to the ovarian cancer cell lines. The results revealed that AMPK inhibition by compound C did not relieve the inhibitory effect of metformin on the TGFβ signalling pathway (Figure 4E, 4F). We thus concluded that metformin impacts the TGFβ pathway in an AMPK-independent manner in ovarian cancer.

**Discussion**

The TGFβ signalling pathway is a critical player in various biological functions, including stem cell renewal, immune system suppression, wound repair and neoplasm progression [24]. As a key modulator of cancer progression, TGFβ has been investigated in various kinds of tumours and is considered pleiotropic, coordinat-
Figure 6. Combination therapy with metformin and cisplatin inhibited ovarian cancer proliferation and EMT in vivo through the TGFβ signalling pathway. Representative (×400) staining showing N-cadherin, MMP-9, Ki-67, cleaved caspase-3, P-Smad2 and P-Smad3 expression in tumour tissues excised from mice at 3 weeks. *, P<0.05 and ***, P<0.001.
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Figure 7. Schematic diagram of the proposed mechanism by which metformin enhances the inhibitory effect of cisplatin on epithelial ovarian cancer metastasis. Metformin decreased the level of TGFβ-1, the subsequent Smad2/3 phosphorylation and EMT.

ed and contextual [25]. TGFβ enrichment in the tumour microenvironment has been verified in various tumours [26]. TGFβ binds to its membrane receptors, which phosphorylate Smad2 and Smad3. Phosphorylated Smad2 and Smad3 combine with Smad4 to form a complex and are shuttled to the nucleus to activate transcription factors, such as p21 [27]. EMT is a typical pathological feature in neoplasia, and TGFβ-induced EMT has been reported in several tumour cell lines, including breast cancer and skin carcinomas [28, 29]. In mesenchymal stem-like/claudin-low breast cancer, metformin attenuated the activation of Smad2 and Smad3 induced by TGFβ and effectively inhibited breast cancer cell proliferation, migration and motility [30]. Similar findings have been confirmed in prostate cancer, in which metformin inhibited the increase in N-cadherin and Vimentin expression and decrease in E-cadherin and β-catenin expression induced by TGFβ [18].

Apart from the aforementioned studies, several other preclinical and clinical studies have demonstrated that metformin attenuates the growth and progression of multiple tumours and is beneficial to patients. Under glucose-deprivation conditions, metformin synergistically enhanced cisplatin cytotoxicity in oesophageal squamous cancer cells by deregulating AKT and AMPK [31]. In human nasopharyngeal cancer, the combination of metformin and cisplatin significantly downregulated MMP-9 expression and upregulated E-cadherin expression; therefore, the combination therapy inhibited nasopharyngeal cancer cell metastasis [32]. Similar tumour-inhibitory effects have also been found in lung cancer, gastric cancer, colon cancer and endometrial cancer [33-36]. In ovarian cancers, a number of studies has reported that metformin enhanced the anti-cancer activity of cisplatin or paclitaxel in vitro and in vivo. In 2011, Ramandeep Rattan et al. constructed intraperitoneal ovarian cancer models and found that metformin effectively inhibited the proliferation and migration of ovarian cancer [37]. Additionally, the researchers demonstrated that metformin exerted anti-tumour effects through both AMPK-dependent and AMPK-independent pathways [38]. Another recent study indicated that metformin combined with cisplatin inhibited cell viability and induced apoptosis in human ovarian cancer cells by inactivating ERK 1/2 [39]. In addition, several retrospective and prospective studies have demonstrated the benefits of metformin on cancer patient survival. A total of 102 patients with newly diagnosed endometrial cancer were divided into the control group and metformin group. Metformin substantially decreased the level of insulin and insulin-like growth factor-1 (IGF-1) and the number of metastatic patients [19]. A retrospective study revealed that obese patients with type I endometrial cancer who received metformin treatment had a recurrence rate of 7.8% versus 15.3% in the control patients [40]. A case-control study consisting of 215 cases indicated that metformin intake improved ovarian cancer patient survival [41].

Recently, a series of novel anti-cancer metformin mechanisms have been described. It was reported that metformin can inhibit tumours by epigenetically modulating gene expression. SUV39H1 (a histone methyltransferase of H3
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Lys9) overexpression promotes prostate cancer cell migration. However, SUV39H1 expression was reduced by metformin in prostate cancer cells in a time-dependent manner by disturbing the integrin-FAK (focal adhesion kinase) signalling pathway [42]. Zhong T et al. reported that metformin induced genome-wide alterations in DNA methylation by regulating S-adenosylhomocysteine hydrolase (SAHH) activity [43]. These researchers conducted the study with an endometrial cancer cell line and a breast cancer cell line and found that metformin treatment induced the hypermethylation of tumour-promoting genes and inhibited tumour proliferation. In summary, the comprehensive anti-cancer mechanisms of metformin remain elusive.

Herein, we developed in vitro and in vivo studies. Consistent with previous studies, our results revealed that the combination of metformin and cisplatin inhibited the proliferation and promoted the apoptosis of epithelial ovarian cancer cells. In addition, we found that the combination therapy significantly decreased N-cadherin and MMP-9 expression in vitro and in vivo, which indicated that metformin can inhibit EMT in epithelial ovarian cancer. To our knowledge, this was the first study to demonstrate that metformin inhibits epithelial ovarian cancer metastasis via the TGFβ signalling pathway (Figure 7). By using xenograft mouse models with epithelial ovarian cancer cells, we indicated that metformin enhanced cisplatin cytotoxicity in vivo. However, our study still has some limitations. For example, we constructed ovarian cancer cell xenograft mouse models subcutaneously rather than intraperitoneally. Therefore, further studies are needed to investigate whether the metformin and cisplatin co-treatment can inhibit metastasis and ascites formation in intraperitoneal models. Additionally, platinum-resistant ovarian cancer cell lines should also be investigated to better determine if metformin can restrict the growth of platinum-resistant ovarian cancer. Furthermore, additional studies are needed to explore the novel mechanisms underlying the anti-tumour effect of metformin.

Collectively, our results revealed that metformin alone or combined with cisplatin attenuated epithelial ovarian cancer growth and metastasis in vitro and in vivo. We propose that metformin could be used as adjunct therapy in the treatment of epithelial ovarian cancer.

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Disclosure of conflict of interest

None.

Abbreviations

qRT-PCR, quantitative reverse transcription polymerase chain reaction; MMP-9, matrix metalloproteinase-9; TGFβ1, transforming growth factor beta 1; PI3K, phosphatidylinositol-3-kinases; mTOR, mammalian target of rapamycin; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinases; AMPK, AMP-activated protein kinase.

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