## Original Article
# Metformin inhibits the proliferation in EOC (epithelial ovarian cancer) mediated by AMPK/mTOR signaling pathway in vitro

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**Abstract:** Epithelial ovarian cancer (EOC) is the most lethal gynecological cancer and accounts for the fifth most common cause of cancer-related deaths in women. Initial treatment with surgery and chemotherapy has improved survival significantly. However, most patients relapse within 2 years and develop chemoresistance. Thus, it is urgent to develop more effective treatment strategies. Metformin is a first-line anti-diabetic drug, but also be found its promising anti-cancer effect in many solid tumors. This study was attempted to evaluate the effect and mechanism of metformin in epithelial ovarian cancer cells. Cells were treated with metformin at different doses and different time intervals. The proliferation ability of cells was analyzed by cell counting kit-8 (CCK-8). Glucose uptake and secretion of lactic acid were measured in the culture medium by ELISA. Glycolysis key enzymes: Hexokinase 2 (HK 2) and Lactate dehydrogenase-A (LDHA) were detected by RT-qPCR. The activation of AMPK/mTOR signaling pathway and the concentration of ATP were evaluated in cell lysates. Metformin inhibited cell proliferation on both dose-dependent manner and time-dependent manner in SKOV3 cells. Metformin increased glucose uptake, lactic acid secretion and the expression of HK 2 and LDHA, but decreased the production of ATP. Both AICAR (1 mM) and metformin (20 mM) enhanced the AMPK/mTOR signaling and glycolysis, but compound C (15 µM) could reverse these effects. These data suggest that metformin might inhibit the proliferation in ovarian cancer cells by modulating the AMPK/mTOR signaling pathway, which also indicate the chemotherapeutic potential of metformin in epithelial ovarian cancer.

**Keywords:** Metformin, ovarian cancer, glucose metabolism, glycolysis, AMPK/mTOR signaling pathway

## Introduction

Ovarian cancer is the first fatal gynecological malignance. Despite progress made in the treatment by improved debulking surgery and the introduction of platinum-taxane regimens, the 5-year survival rate of advanced-stage ovarian cancer is still very low [1]. Therefore, it is very important to find a new strategy to reverse chemoresistance and improve the survival rate for patients with ovarian cancer.

Metformin is the first-line drug for type-II diabetes mellitus [2]. Recently, metformin has drawn renewed interest as its potential effect in cancer therapy and chemotherapy adjuvant. In 2005, Evans et al. reported that metformin reduced the risk of cancer significantly in a large cohort of diabetic patients firstly [3]. Until now, metformine exhibited a strong and consis-tent anti-proliferative action on different cancer cell lines and xenografts or transgenic mice: such as breast [4-6], colon [7], pancreas [8], lung and prostate [9].

Alterations in cellular metabolism are an emerging hallmark of cancer. The Warburg effect is the best-characterized metabolic phenotype of cancer which shows up as the shift from oxidative phosphorylation to aerobic glycolysis [10]. According to recent study, cancer cells undergo metabolic reprogramming, which drives cancer cells' growth and progression [11].

Though there have considerable investigation on metformin's regulation of cancer cell proliferation and growth, mechanistic details regarding the effect of metformin on cancer cell metabolism is still unclear, such as how metformin affects glycolysis and ATP production. This

**Table 1.** Primers used in the present study

| Primer | Forward | Reverse |
|---|---|---|
| HK 2 | 5'-AAGGCTTCAAGGCATCTG-3' | 5'-CCACAGGTCATCATAGTTCC-3' |
| LDHA | 5'-ATCTTGACCTACGTGGCTTGGA-3' | 5'-CCATACAGGCACACTGGAATCTC-3' |
| β-actin | 5'-CATGTACGTTGCTATCCAGGC-3' | 5'-CTCCTTAATGTCACGCACGAT-3' |

HK 2, Herokinase 2; LDHA, Lactate dehydrogenase A.
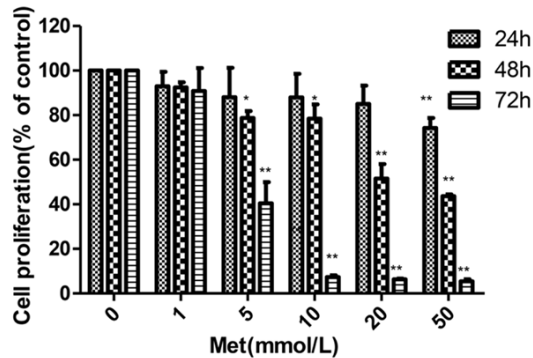


**Figure 1.** Metformin inhibited the cell proliferation in SKOV3 cells (*P<0.05 and **P<0.01).

study was to find out how metformin affected glycolysis in epithelial ovarian cancer in vitro and whether this process was related to AMPK activation.

## Materials and methods

### Cells and reagents

Epithelial ovarian cancer cell line, SKOV3 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 100 units/mL penicillin, 100 mg/mL streptomycin at 37°C and 5% $CO_2$. Metformin, AICAR (an AMPK agonist), and N,N-Dimethyl-1,3,5-triazine-2,4,6-triamine (an AMPK antagonist, namely Compound C) were obtained from Sigma Chemical Co. (St. Louis, MO). Metformin and AICAR were dissolved in culture media. Compound C was dissolved in DMSO, and the final concentration of DMSO was 0.075%.

### Cell viability assay

Cells ($1 \times 10^4$ per well) were incubated in medium containing 10% FBS. Twenty-four hours later, cells were starved in FBS-free medium for 12 hours. Then cells were treated with the indicated agents. After certain time, viable cells were counted using CCK-8 kit (Dojindo Japan).

### Glucose uptake and lactic assays

Cells ($4 \times 10^5$ per well) were seeded in 6-well plates with medium containing 10% FBS. Twenty-four hours later, cells were starved in FBS-free medium for 12 hours. Then cells were treated with the indicated agents. 48 hours later, the media was collected and measured for glucose and lactic acid using glucose kit and lactic acid kit (Jiancheng Nanjing China) according to manufacturer's protocol. Counts were normalized to protein concentration.

### ATP measurement

The level of ATP in SKOV3 cell lines was determined using the ATP Bioluminescence Assay Kit (Beyotime Technology Shanghai China) according to manufacturer's protocol. Briefly, Cells ($4 \times 10^5$ per well) were seeded in 6-well plates with medium containing 10% FBS. Twenty-four hours later, cells were starved in FBS-free medium for 12 hours. Then cells were treated with the indicated agents for 48 hours. Then cells were lysed with a lysis buffer, followed by centrifugation at 10,000 × g for 2 min at 4°C. Finally, the level of ATP was determined by mixing 50 μl of the supernatant with 50 μl of luciferase reagent, which catalyzed the light production from ATP and luciferin. The emitted light was linearly related to the ATP concentration and measured using a luminometer. Counts were normalized to protein concentration.

### Analysis of gene expression using R-T qPCR

Cells ($4 \times 10^5$ per well) were seeded in 6-well plates with medium containing 10% FBS. Twenty-four hours later, cells were starved in FBS-free medium for 12 hours. Then cells were treated with the indicated agents. 48 hours later, total RNA was extracted using the Invitrogen™ TRIzol reagent (Thermo Fisher Scientific) following the manufacturer's instructions, and the quantity of RNA was analyzed by UV spectrophotometry. Then cDNA synthesis was performed using the reverse transcription kit (Tiangen). HK 2 and LDHA gene expression was evaluated by quantative R-T PCR using SYBR Premix Ex Taq (Takara). Briefly, 2 μL of aliquots containing cDNA was amplified in a total
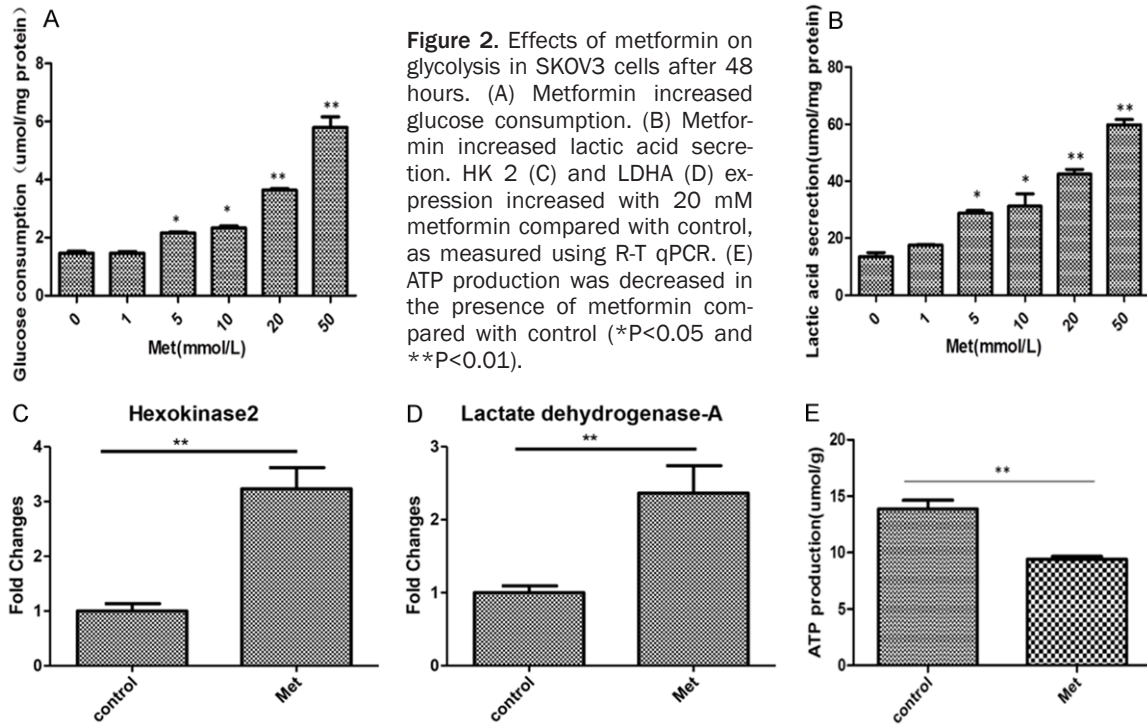
**Figure 2.** Effects of metformin on glycolysis in SKOV3 cells after 48 hours. (A) Metformin increased glucose consumption. (B) Metformin increased lactic acid secretion. HK 2 (C) and LDHA (D) expression increased with 20 mM metformin compared with control, as measured using R-T qPCR. (E) ATP production was decreased in the presence of metformin compared with control (*P<0.05 and **P<0.01).

volume of 20 μL containing 4 μL of a 5× SYBR Premix Ex Taq and 0.2 μM of primer. The oligonucleotide primers were synthesized by BIOTNT Corporation. Melting curves of the products were obtained after cycling by a stepwise increase of temperature from 55°C to 95°C. Expression values were normalized to the geometric mean of β-actin measurements. Primers used in the present study were presented in **Table 1**.

*Western blot analysis*

In brief, Cells ($4 \times 10^5$ per well) were seeded in 6-well plates with medium containing 10% FBS. Twenty-four hours later, cells were starved in FBS-free medium for 12 hours. Then cells were treated with the indicated agents. 48 hours later, cells were lysed in RIPA buffer containing protease inhibitor. Equal amounts of protein lysates were electrophoretically separated on 10% SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked with 5% nonfat dried milk for 1 hour at room temperature and then incubated with primary antibodies in PBS. After incubation with a horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature, the protein bands were detected using the ECL detection system (Pierce). The antibodies, anti-

p-AMPKα (Thr172), anti-AMPKα (23A3), anti-p-ACC, anti-p-mTOR and anti-p-4EBP1, were purchased from Cell signaling technology.

*Statistical analysis*

Each experiment was repeated at least three times. Results were presented as means ± SEM. Statistical analyses were carried out using Stata 12.0 software. Student's *t* test or one-way ANOVA was chosen to analyze statistical differences. *P*<0.05 was considered statistically significant.

**Results**

*Metformin inhibited the proliferation of SKOV3 cells*

The anticancer activity of metformin against SKOV3 cells was evaluated by CCK-8 analysis. SKOV3 cells were administrated with different concentrations of metformin (1, 5, 10, 20 and 50 mM) for different time intervals (24 hours, 48 hours and 72 hours). The result of CCK8 showed that the number of viable SKOV3 cells exposed to metformin treatment reduced considerably. Statistically, metformin inhibited the proliferation of SKOV3 cells in dose-dependent manner and time-dependent manner (**Figure 1**).
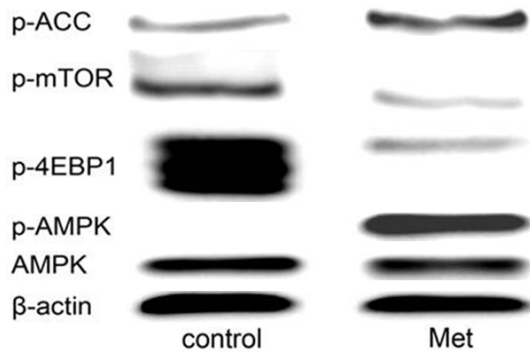
**Figure 3.** Western blot showed the activation of AMPK/mTOR signaling pathway. AMPK and ACC were activated, while mTOR/4EBP1 were inhibited by metformin in SKOV3 cells after 48 hours. β-actin was used as an internal loading control.

*Metformin promoted glycolysis but decreased ATP production in SKOV3 cells*

To investigate the effect of metformin on the metabolic reprogramming, glucose consumption and lactic acid secretion were detected in SKOV3 cell with the treatment of metformin. After 48 hours exposing to metformin, glucose consumption and lactic acid secretion were increased significantly in cell medium (**Figure 2A**, **2B**), which indicated that metformin promoted glycolysis. Hexokinase and lactic dehydrogenase are the key enzymes of glycolysis. HK is the first rate-limiting step in the glycolytic pathway, which catalyzes the conversion of glucose to glucose-6-phosphate [11]. Lactic dehydrogenase interconverts pyruvate and lactate at the end of the glycolytic pathway using NAD as a cofactor [12]. The data of q-PCR revealed that metformin increased HK 2 and lactic LDHA expression at mRNA level (**Figure 2C**, **2D**). Though inefficient, cells can generate ATP through glycolysis to meet energy needs. However, with the treatment of metformin, the production of ATP concentration decreased significantly in SKOV3 cells (**Figure 2E**).

*Metformin enhanced the activation of the AMPK/mTOR signaling pathway*

Emerging evidence suggests that activation of adenosine monophosphate-activated protein kinase (AMPK) may suppress cancer growth [13-15]. Identification of novel AMPK activators is therefore crucial to exploit potential targets for cancer prevention and treatment. Acetyl-CoA carboxylase (ACC) is directly phosphorylated by AMPK, which functions as an AMPK acti-

vation control. In this part, metformin treatment could enhance the activation of both AMPK and ACC in SKOV3 cells (**Figure 3**). mTOR, a main downstream molecule in AMPK signaling pathway. Phosphorylated AMPK can inhibit the activation of mTOR [16]. Western blot analysis showed that metformin inhibited the activity of mTOR as ACC in SKOV3 cells (**Figure 3**). Metformin was also proved to decrease the phosphorylation level of 4EBP1, the downstream of mTOR.

*AMPK inhibitor partly abolished the effect of metformin on promoting glycolysis in SKOV3 cells*

To exploit the role of AMPK/mTOR signaling pathway in glycolysis by metformin, AICAR, an AMPK agonist, was used to activate the AMPK signaling pathway. AICAR could also promote cell glycolysis evidenced by increasing glucose uptake, lactic acid secretion and the expression of HK 2 and LDHA (**Figure 4A**-**D**). The data of western blot showed that metformin could activate AMPK/mTOR signaling pathway just like 1 mM AICAR (**Figure 4E**). In another way, compound C is a well-accepted AMPK inhibitor used in some studies [17, 18]. We found that 15 µM Compound C pretreated for 1 hour before 20 mM metformin or 1 mM AICAR could significantly inhibit AMPK/mTOR signaling pathway (**Figure 4F**) in SKOV3 cells. In addition, Compound C partly weakened the glycolysis promotion effect of both metformin and AICAR (**Figure 4A**, **4B**). Moreover, HK 2 and LDHA expression were decreased when cells were pretreated with Compound C for 1 hour before metformin compared to metformin only. This phenomenon also occurred in AICAR treated groups (**Figure 4C**, **4D**).

In conclusion, ovarian cancer is the most lethal gynecologic malignancy and the fifth leading cause for cancer death among women in the world. The majority of cases are diagnosed with advanced disease due to the lack of effective screening strategies, typical early signs and symptoms associated with this disease. Metastases are already present when diagnosed at advanced disease stages, leading to poor prognosis. Despite the encouraging developments in chemotherapy, surgery, and sometimes radiotherapy, the likelihood of long-term disease-free survival is still very low (15-20%) [19-22].
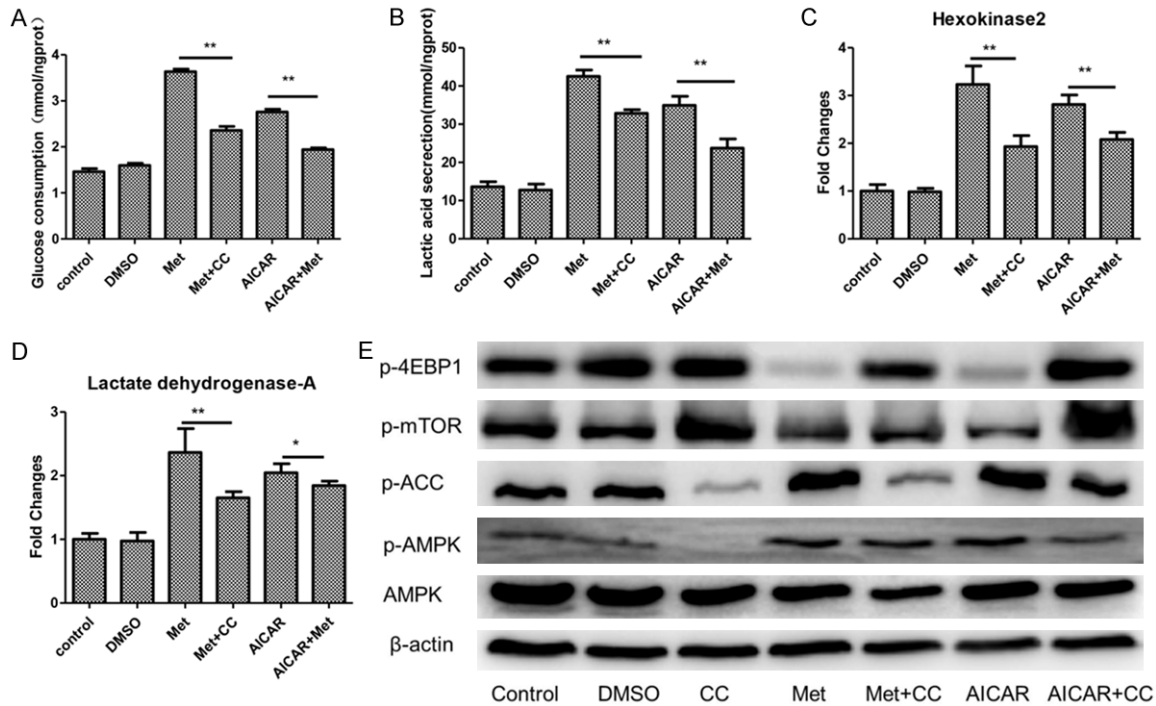
**Figure 4.** Compound C partly weakened the glycolysis promotion effect of metformin and AICAR. Compound C decreased glucose consumption (A) and lactic acid secretion (B) compared with metformin or AICAR alone. HK 2 (C) and LDHA (D) expression decreased with Compound C compared with metformin or AICAR alone, as measured using R-T qPCR. (E) Western blotting demonstrated the protein expression levels of AMPK/mTOR signaling pathway in the SKOV3 cell lines following pretreatment of Compound C (*P<0.05 and **P<0.01).

Metformin, an anti-hyperglycemic agent, has for decades been the first-line treatment for type II diabetes mellitus. Since the first report of its potential anti-cancer effect in mammals in 2001, many studies have been reported that metformin may have the potential as an antitumor drug and could combine with other chemotherapy drugs in some solid cancers. The anti-neoplastic activity of metformin has been reported in epidemiological and animal studies in many cancers including ovarian cancer, as it could reduce the risk of ovarian cancer and improve the survival rate [22, 23]. In addition, many studies showed that metformin played a crucial role in tumor growth and progression in many other solid cancers [4-6]. However, the comprehensive role and mechanism of metformin in ovarian cancer, especially how metformin affects glycolysis and finally ATP production, is still out of understanding.

In this study, CCK8 analysis showed that metformin inhibited the proliferation of ovarian cancer cells in a dosage and time-dependent manner. It implied that metformin indeed has the potential anti-cancer effect.

As we know, cells have two ways to generate ATP: glycolysis and oxidative phosphorylation. Though insufficient, ATP generated by glycolysis is important in maintaining energy balance of cells. In this study, metformin was confirmed to promote high glycolytic rate in SKOV3 cells, but decreased the production of ATP. This unexpected result could be explained by the role of metformin on mitochondrial respiration [24-26]. Metformin is identified as a new class of complex I and ATP synthase inhibitor [25], and it acts directly on mitochondria to limit respiration, which causes energy inefficient and reduces glucose metabolism through the citric acid cycle [24]. In addition, biguanide-based molecules act more effectively in cellular models with defects in glucose utilization and mitochondrial function, such as mitochondrial DNA mutation in complex I genes [27].

AMPK, a serine/threonine kinase, regulates cellular homeostasis in response to energy stress and energy unbalance, as the primary target of metformin [28]. Decreasing intracellular ATP concentration leads to phosphorylation AMPK in Thr172. Metformin increases

AMP/ATP ratio, therefore it activates AMPK switching cells from anabolic to a catabolic state. In order to investigate the mechanism involved in the glycolysis promotion effect of metformin, the AMPK/mTOR signaling pathway was analyzed. In this study, western blot showed that both metformin and AICAR could active AMPK/mTOR signaling pathway. In addition, they both increased glucose uptake, lactic acid secretion and the expression of HK 2 and LDHA. However, these promotion effects could be reversed by an AMPK inhibitor. These results confirmed that the activating of AMPK/mTOR signaling pathway might play a key role in promoting glycolysis procedure.

AMPK senses cellular energy and coordinates metabolic activities in many tissues. During periods of intracellular metabolic stress, AMPK activation suppresses cell growth and proliferation, leading to hypothesis that AMPK may function as part of a tumor suppressor pathway [29]. Faubert firstly provided genetic evidence that AMPKα displays tumor suppressor activity in vivo [30]. In this study, results revealed that metformin promoted glycolysis via the AMPK signaling pathway, which may be helpful to exploit more and newer mechanism for its anti-cancer effect.

In summary, the present study presented that metformin inhibited the proliferation in ovarian cancer cells and promoted glycolysis rates. This effect might be due to metformin modulate the activation of the AMPK/mTOR signaling pathway. According to previous studies, metformin may act directly on mitochondria to limit respiration and ATP production. As a result of energy insufficient, metformin maintained high glycolysis rates to produce ATP, but this compensation was still not enough to support cell proliferation. From this perspective, it provides a new possibility that metformin may be combined with glycolysis inhibitors, such as 2-deoxyglucose [31] or 3-bromopyruvate [32], to achieve a better proliferation inhibition effect on ovarian cancer cells. We will continue to devote to further research related to the metabolic interference of metformin in the future.

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### Disclosure of conflict of interest

None.

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