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Antidiabetic drug metformin mitigates ovarian cancer SKOV3 cell growth by triggering G2/M cell cycle arrest and inhibition of m-TOR/PI3K/Akt signaling pathway

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Abstract. – OBJECTIVE: Metformin is one of most extensively prescribed oral hypoglycemic drug and has received increased attention in recent times for its antitumorigenic potential. Many possible mechanisms have been proposed for the ability of metformin to overturn cancer growth *in vitro* and *in vivo*. The objective of the present study was to evaluate the anticancer activity of metformin against ovarian SKOV3 cancer cells.

MATERIALS AND METHODS: Anticancer activity and IC50 value of metformin were determined by MTT assay. Reactive oxygen species (ROS), mitochondrial membrane potential (MMP) and effect on cycle were determined by flow cytometry. Protein expression was estimated by Western blotting.

RESULTS: Results indicated that metformin exhibited an IC50 of 20 mM against ovarian SKOV3 cancer cell line. Metformin also caused DNA damage in SKOV3 cells and also prompted ROS-mediated alterations in mitochondrial membrane potential. Nonetheless, it triggered cell cycle arrest of SKOV3 at G2/M checkpoint. The activation of the PI3K/AKT/mTOR pathway plays a vital role in ovarian cancer tumorigenesis, progression and chemotherapy resistance. The results showed that metformin significantly inhibited the expression levels of key proteins of PI3K/Akt/mTOR signaling pathway.

CONCLUSIONS: We propose that metformin exhibits anticancer activity in SKOV3 cells and may prove beneficial in the management of ovarian cancers.

Key Words:
Ovarian Cancer, mTOR, ROS, Metformin.

Introduction

Ovarian cancer is one of the most deadly causes of cancer-related deaths across the globe and chemotherapy remains the cornerstone for its management[1,2]. However, despite frequent preliminary responses to chemotherapy, the tumors often relapse. Moreover, there are limited chemotherapeutic agents available for the management of ovarian cancer[3,4]. So far bevacizumab is the only approved therapy for ovarian cancer for which consistent analytical markers are yet to be established. Furthermore, except for p53 signaling pathway, the PI3K/Akt/mTOR cascade is probably the most recurrently changed signaling pathway in cancer, such as ovarian cancer[1,5]. Consistent with this, first generation mTOR inhibitors exhibit significant anti-cancer properties and many of these inhibitors have even been approved for the management of different types of cancers, which include, but are not limited to, pancreatic, renal and breast cancers. Additionally, PI3K, Akt together with second-generation inhibitors of mTOR are undergoing clinical trials. Metformin is one of commonly prescribed oral hypoglycemic drugs across the globe[6]. Metformin has attained increased attention in recent times for its possible anticancer activity that is believed to be free of its hypoglycemic activity. Since metformin is already prescribed as hypoglycemic drug, there are limited toxicity-related issues, which are considered as an important aspect of anticancer drug development[7-12]. The current

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study was designed to determine the antitumor activity of metformin against ovarian cancer cells and to investigate its effects on TOR/PI3K/Akt signaling pathway. The present work is so far the only study that reports the anticancer activity of metformin via downregulating the TOR/PI3K/Akt signaling pathway in ovarian cancer cells.

Materials and Methods

Cell Line and Culture Conditions

Ovarian cancer cell line cell SKOV31 was procured from Cancer Research Institute of Beijing, China, and it was maintained in Dulbecco's Modified Eagle's Medium (DMEM) and was supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 µg/ml streptomycin and 100 U/ml penicillin G) in a incubator at 37°C (5% CO_2 and 95% air).

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) Assay

The anti-proliferation effect of metformin on ovarian cancer SKOV3 cells was demonstrated by MTT assay. SKOV31 cells were grown at 1 x 10^6 cells per well in 96-well plates for a period of 12 h and then exposed to 0, 10, 20, and 40 mM metformin dose for 48 h. To each well, MTT solution (20 µl) was added. Before the addition of 500 µl of dimethyl sulfoxide (DMSO), the medium was completely removed. To solubilize MTT formazan crystals, 500 µl DMSO were added. ELISA plate reader was used for the determination of optical density.

Colony Formation Assay

For clonogenic assay, ovarian cancer cell line SKOV3 cells at the exponential growth phase were harvested and counted with a hemocytometer. Seeding of the cells was done at 200 cells per well, incubated for a period of 48 h to allow the cells to attach, and then to the cell culture different doses (0, 10, 20 and 40 mM) of metformin were added. After the treatment, the cells were again kept for incubation for 6 days, the washing was done with phospate buffered saline (PBS), and methanol was used to fix colonies. The cells were then stained with crystal violet for about 30 min before being counted under light microscope.

DAPI Staining

SKOV3 cells/well at a density of 2×10^5 cells/well were seeded in 6-well plates were administrated with 10 to 40 mM metformin for 48 h. The cells were then subjected to DAPI staining. Afterwards, the cell sample was studied and photographs taken under fluorescence microscopy as previously described[13].

Determination of ROS and Mitochondrial Membrane Potential (MMP)

SKOV3 cells were seeded at a density of 2×10^5 cells/well in a 6-well plate and kept for 24 h and treated with 0 mM to 40 mM metformin for 72 h at 37°C in 5% CO_2 and 95% air. Thereafter cells from all samples were collected, washed 2 times by PBS and re-suspended in 500 µl of dichloro-dihydro-fluorescein diacetate (DCFH-DA) (10 µM) for ROS estimation and 3,3'-dihexyloxacarbocyanine Iodide ($DiOC_6$) (1 µmol/l) for MMP at 37°C in dark room for 30 min. The samples were then examined instantly using flow cytometer as described previously in literature[14].

Estimation of Cell Cycle Distribution of HepG2 Cells

The cells seeded in 6 well plates (2 x 10^5 cells/well) and metformin was administrated to the cells at the doses of 0, 10, 20 and 40 mM followed by 24 h of incubation. DMSO was used as a control. For estimation DNA content, PBS was used to wash the cells and fixed in ethanol at -20°C. This was followed by re-suspension in phosphate buffered saline (PBS) holding 40 µg/ml propidium iodide (PI) and, RNase A (0.1 mg/ml) and Triton X-100 (0.1%) for 30 min in a dark room at 37°C. Afterwards, analysis was carried out by flow cytometry as reported previously[15].

Western Blotting Analysis

The metformin-administrated cells were harvested and lysed. The protein concentrations of the lysates were quantified by bicinchoninic acid assay (BCA) assay using specific antibodies. β-actin was used as a control. From each sample equal amounts of protein were loaded and separated by electrophoresis on a 12% denaturing SDS gel. Afterwards, the proteins were then electroblotted onto polyvinylidene difluoride membranes (0.45 m pore size).

Statistical Analysis

All experiments were carried out in at least three biological replicates and are expressed as mean ± standard deviation (SD). Statistical significance was determined using two-way ANOVA

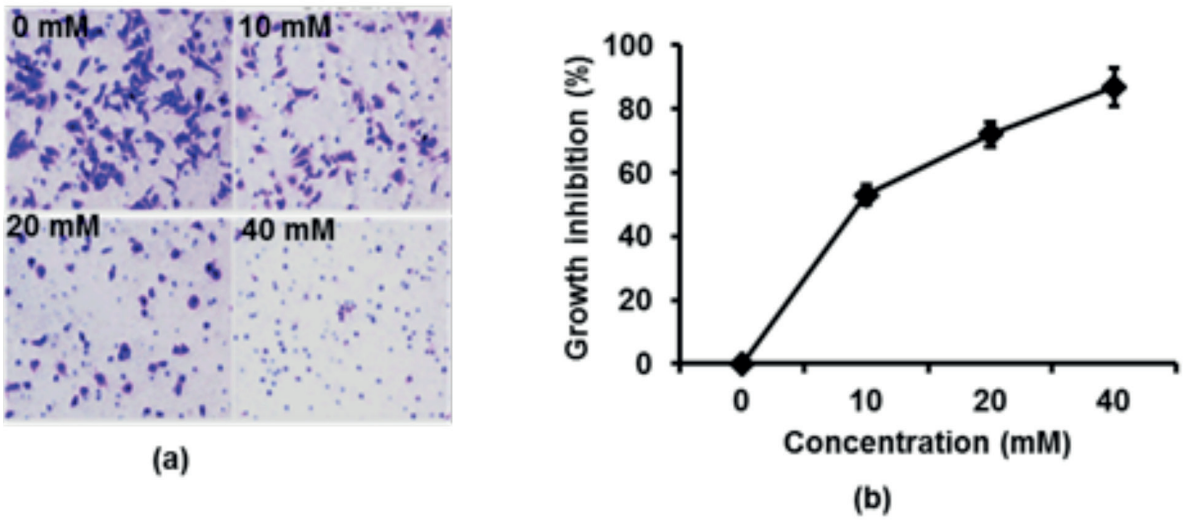

Figure 1. Effect of indicated doses metformin on ovarian cancer cell inhibition (a) Crystal violet assay (b) Growth inhibition curve. All values are mean of three independent replicates ± SD.

and $p<0.05$ was considered as significant using GraphPad Prism Ver. 5.01 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Anti-proliferative Potential of Metformin on SKOV3 Cell Line

To identify the anti-proliferative role of metformin on ovarian cancer SKOV3 cells, the cells were treated with metformin concentration range 0-40 mM for 48 h. Metformin displayed a potent anti-proliferative effect against SKOV3 cells with an IC_{50} of 20 mM (Figure 1a). In the colony formation assay, we observed that metformin treated cells reduced the number of colonies in a dose-dependent manner (Figure 1b).

Metformin Prompted DNA Damage in SKOV3 Ovarian Cancer Cells

The cells were separated from metformin and DNA damage was evaluated by DAPI staining. Our results indicated that metformin caused DNA damage in a dose-dependently as evident from evident from the greater density of white color nuclei (Figure 2).

Metformin Triggers ROS Production in Ovarian Cancer SKOV3 Cells

The potential of metformin to cause DNA damage observed through 4',6-diamidino-2-phenylindole (DAPI) staining suggested that metformin might induce generation of intracellular ROS. Therefore, we calculated the ROS level at varied concentrations of metformin for 48 h. The results showed that the intracellular ROS levels of treated cells increased up to 310% as compared to untreated cells (Figure 3a). Our result suggested that metformin is a potent molecule for activating ROS in SKOV3 cells.

Metformin Reduces the Mitochondrial Membrane Potential (MMP)

ROS generation is related to mitochondrial dysfunction. It disrupts the outer mitochondrial potential to release the death-promoting proteins[16]. Therefore, we examined whether metformin reduces the MMP in SKOV3 cells treated with metformin at varied concentrations (0-40 mM). Metformin treated SKOV3 cells showed a significant reduction in MMP in a dose-dependent manner. The MMP reduced up to 58 % at 40 mM of metformin as compared to untreated control (Figure 3b).

Metformin Caused Alterations in Cell Cycle Distribution of Ovarian Cancer SKOV3 Cancer Cell Line

It was observed that the percentage of SKOV3 cells was considerably increased in G2 at the concentrations of 0 to 40 mM concentrations of metformin causing cell arrest at G2/M checkpoint. After 48 h of treatment, cells in the G2/M population increased from 14.8% in control to 51.5% at 40 mM concentration (Figure 4). Additionally, the populations of SKOV3 cells in G2 phase

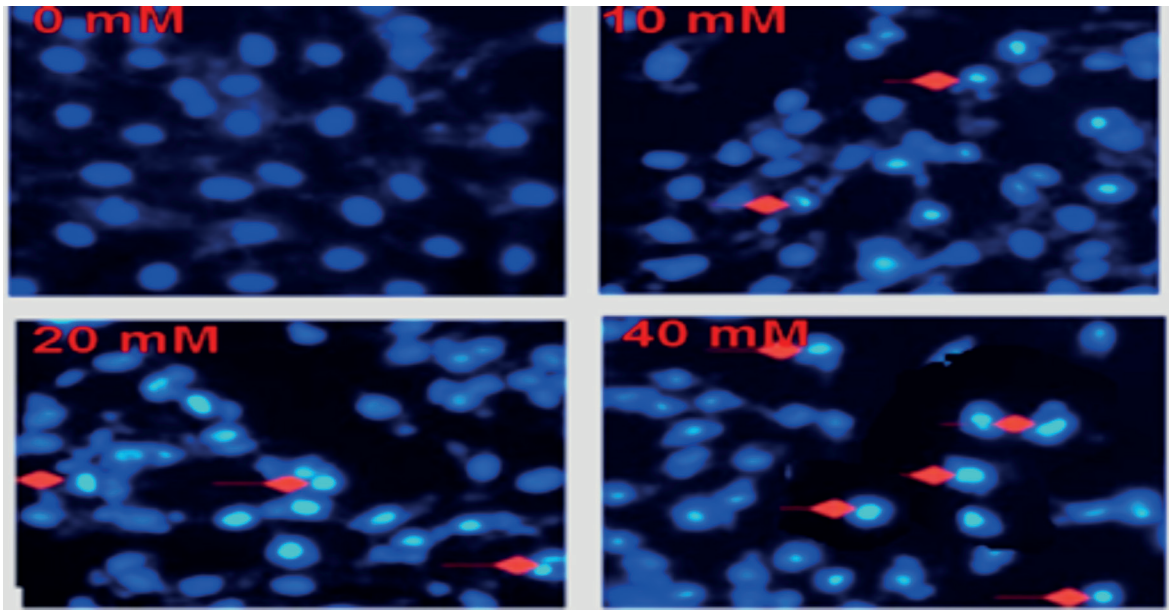

Figure 2. Effect of indicated doses of metformin on DNA damage as depicted by DAPI staining. The images are representatives of three biological replicates.

were marginally increased at a dose of 10 mM, reasonably increased at 20 mM, and dramatically increased at 40 mM. This metformin -induced G2 phase increase of SKOV3 cancer cells was observed to exhibit a dose-dependent pattern.

Metformin Acid Targets m-TOR/PI3K/Akt Signaling Pathway

The m-TOR/PI3K/Akt signaling pathway is one of the main signally pathway activated in cancer cells. To confirm whether metformin could modulate the protein expressions of m-TOR/PI3K/Akt signaling pathway Western blotting was carried to determine the expression of different proteins such as P13, AKT and mTOR. The findings are shown in Figure 5 indicate an interesting outcome. Compared to the untreated control cells, metformin-treated cells showed a concentration-dependent downregulation of m-TOR and pm-TOR proteins. It also showed downregulation of PI3K/Akt protein expressions. Thus it may be concluded that metformin induces anticancer partly via m-TOR/PI3K/Akt signaling pathway.

Discussion

Ovarian cancer is among the deadly reasons of gynecological cancer deaths around the globe. Despite preliminary responses to chemotherapy, the tumors consistently relapse. Metfor-

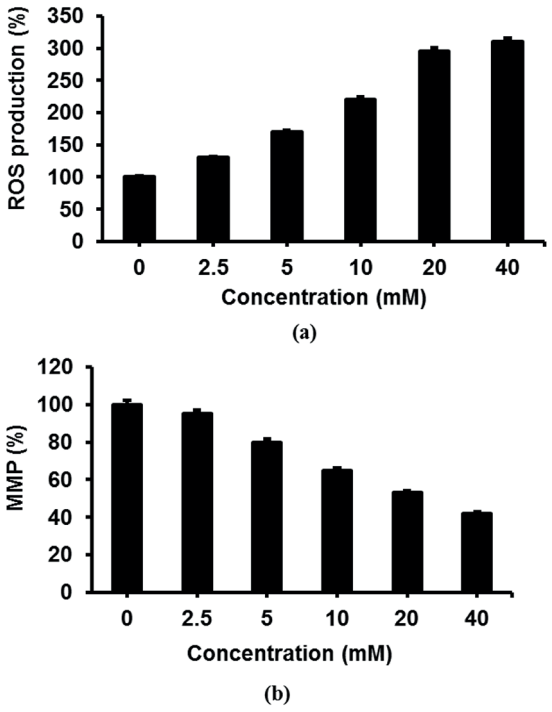

Figure 3. Effect of indicated doses metformin on (a) Mitochondrial membrane potential (b) ROS generation. All values are mean of three independent replicates ± SD.

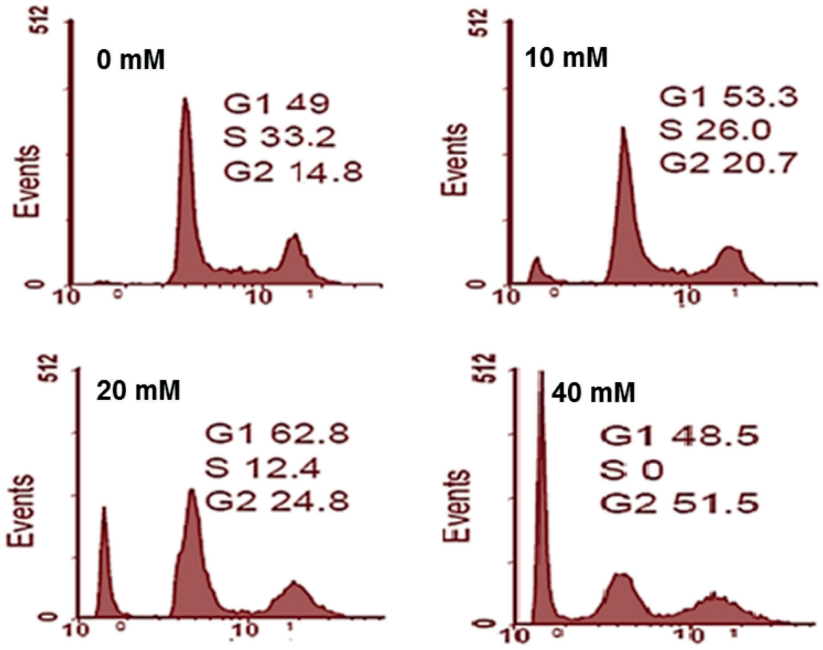

Figure 4. Effect of indicated doses metformin on Cell cycle arrest. The results are representatives of three independent biological replicates.

min is extensively prescribed oral hypoglycemic drug and has recently received attention for its antitumorigenic activity. Metformin showed potential growth inhibition activity against SKOV3 cells as evident from the MMT assay. As reported previously, many drugs exhibit antiproliferative effects via induction of apoptosis. For instance, several chemotherapeutic drugs, such as cisplatin[17-23] have been reported to alter explicit apoptotic pathways and cause DNA damage[24]. To assess whether metformin induces DNA damage in SKOV3, we carried out the DAPI staining of the treated cells. It was observed that metformin induces DNA damage in a concentration dependent manner. Further, it was observed that metformin treated cells displayed ROS-mediated MMP reduction[22]. Therefore the results suggest that metformin may induce DNA damage through increasing intracellular ROS and reduction in MMP. Our results are in agreement with studies wherein several anti-cancer drugs have been reported to target cancer cells partly by accretion of high levels of ROS[24]. Moreover, mitochondria play a key role in ROS[25]. For example, capsaicin disrupts MMP and mediates oxidative stress resulting in apoptosis in pancreatic cancer cells[26]. Flow cytometry using propidium iodide as a probe was used to study effects of metformin on cell cycle progression. Metformin induced G2/M cell cycle arrest and led to a significant increase of cells in G2 phase dose dependently. These findings are promising since it is well established that ovarian cancer is one of the most lethal cancers and metformin could inhibit this behavior[27]. Finally, effects of metformin

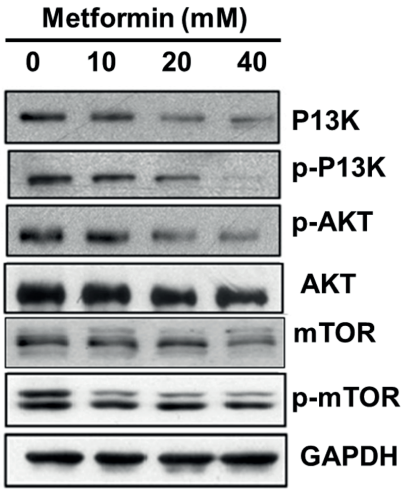

Figure 5. Western blots showing effect of indicated doses of metformin on protein expression of m-TOR/PI3K/Akt signaling pathway proteins. The images are representatives of three biological replicates.

on the expression levels of various proteins including m-TOR, pm-TOR, PI3K, p-PI3K and Akt were studied using Western blot assay. Results showed metformin-treated cells revealed a concentration-dependent downregulation of m-TOR and pm-TOR proteins. It also caused downregulation of PI3K/Akt protein expressions. It has been reported that activation of the PI3K/AKT/mTOR pathway plays a vital role in ovarian cancer tumorigenesis, progression and chemotherapy resistance[1]. Therefore, inhibitory effect of metformin on this pathway may prove crucial in the treatment of ovarian cancers.

Conclusions

Metformin may prove a potential candidate for the treatment of ovarian cancer by controlling m-TOR/PI3K/Akt signaling pathway. Since limited drug options available for ovarian cancer and metformin have limited toxicity, it seems a strong option for treatment of ovarian cancer and deserves further research endeavors.

Conflict of interest

The authors declare no conflicts of interest.

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