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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 ($\Delta\psi_m$) 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 2 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. Activation of PI3K/AKT/mTOR pathway plays a vital role in ovarian cancer tumorigenesis, progression and chemotherapy resistance. The results showed that metformin significantly inhibited expression levels of key proteins of PI3K/Akt/mTOR signaling pathway.

CONCLUSIONS: We propose that metformin exhibits its 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. 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 [illegible] x 10^6 cells per well in 96-well plates for [illegible] riod of 12 h and then exposed to 0, 10, 20 [illegible] 40 mM metformin dose for 48 h. To each [illegible] MTT solution (20 µl) was added. Before the [illegible] dition of 500 µl of dimethyl s[illegible]/DMSO [illegible] the medium was completel[illegible] move[illegible] To solubilize MTT formazan c[illegible]ls, 500 [illegible] DMSO were added. ELISA [illegible] the determination [illegible] tical [illegible].

Colony Form[illegible] Assay

For clonogenic assay [illegible] arian cancer cell line SKOV3 [illegible] at the exp[illegible]tial growth phase were [illegible]ested and counte[illegible]ith a hemocytometer. Seeding [illegible]he cells was done at 200 cells per [illegible] in[illegible]ated for a period of 48 h to allow the ce[illegible]ttach, [illegible]then to the cell culture [illegible]rent [illegible] (0[illegible], 20 and 40 mM) of metformin [illegible]were a[illegible]fter the treatment, the cells w[illegible]again ke[illegible] for incubation for 6 days, the [illegible]done with phospate buffered saline (PBS), and methanol was used to fix colonies. [illegible]e cells were then stained with crystal violet [illegible]bout 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 Mitoc[illegible] Membrane Potential (MMP)

SKOV3 cells were seeded at a [illegible]y of 2 × 10^5 cells/well in a 6-well plat[illegible] and [illegible]for 24 h and treated with 0 mM t[illegible] mM m[illegible]min for 72 h at 37°C in 5% [illegible] and 95% air. [illegible]reafter cells from all [illegible]ples [illegible] collect[illegible], washed 2 times b[illegible] PI[illegible]nd[illegible] suspended in 500 µl of dichlo[illegible]hydr[illegible]rescei[illegible]acetate (DCFH-DA[illegible] µM) for [illegible]est[illegible]tion and 3,3'-dihexyl[illegible]cyanine i[illegible] ($DiOC_6$) (1 µmol/l) for [illegible]MP[illegible]7°C in dar[illegible] room for 30 min. Th[illegible]mples we[illegible]en examined instantly usi[illegible]cytometer as[illegible]ribed previously in li[illegible]ture[14].

E[illegible]nation of [illegible]l Cycle Distribution of [illegible]G2 Ce[illegible]

The [illegible]ed in 6 well plates (2 x 10^5 cells/well) and metformin was administrated to the cel[illegible]he doses of 0, 10, 20 and 40 mM followed [illegible]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

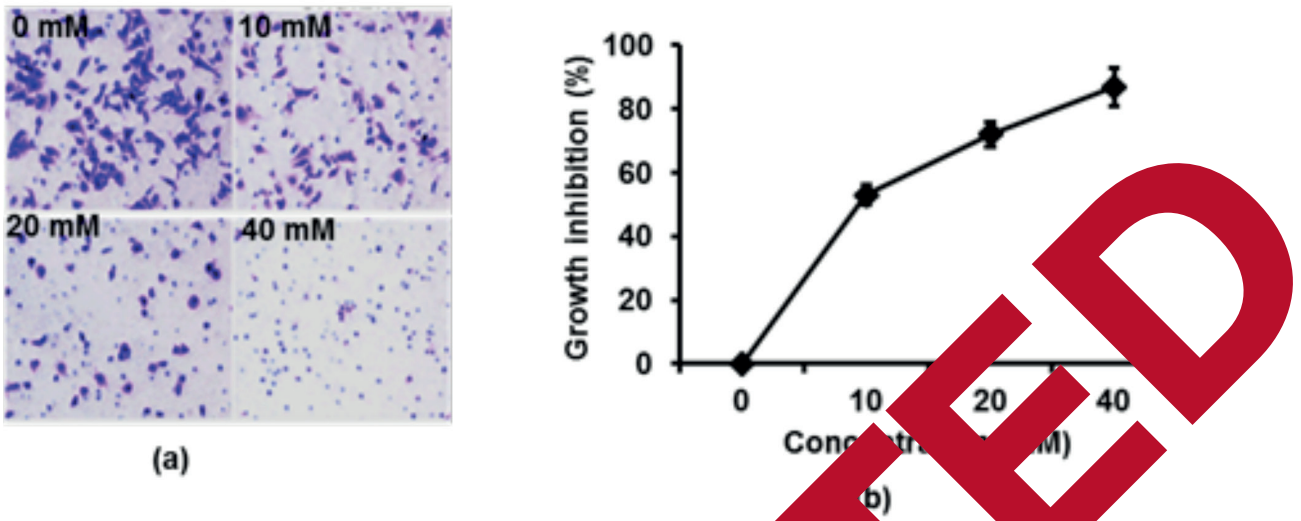

Figure 1. Effect of indicated doses metformin on ovarian cancer cell inhibition by Crystal violet assay (b) Growth inhibition curve. All values are mean of three independent replicates ± SD.

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 Metform on SKOV3 Cell Line

To identify the anti-proliferative of metformin on ovarian cancer SK cell he cells were treated with metfor concent n range 0-40 mM for 48 h. Me ori lis anti-proliferative eff gains V3 cells with an IC_{50} of 20 mM ure 1a). I colony formation assay, d that met n treated cells reduced the numb colonies in a dose-dependent er (Figure

Me min P pted DNA Damage in V3 arian Cancer Cells

The were se ted from metformin and da wa aluated by DAPI staining. Ou sults d that metformin caused DNA da e in a se-dependently as evident from the greater density of white color uclei (Figure 2).

formin 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 R level at v d concentrations of metformin for 48 h. T results showed that the intra lar RO evels of treated cells increased up as compared to untreated cells (Figure 3a). Our result suggested that metfor is a potent molecule for activating ROS in 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

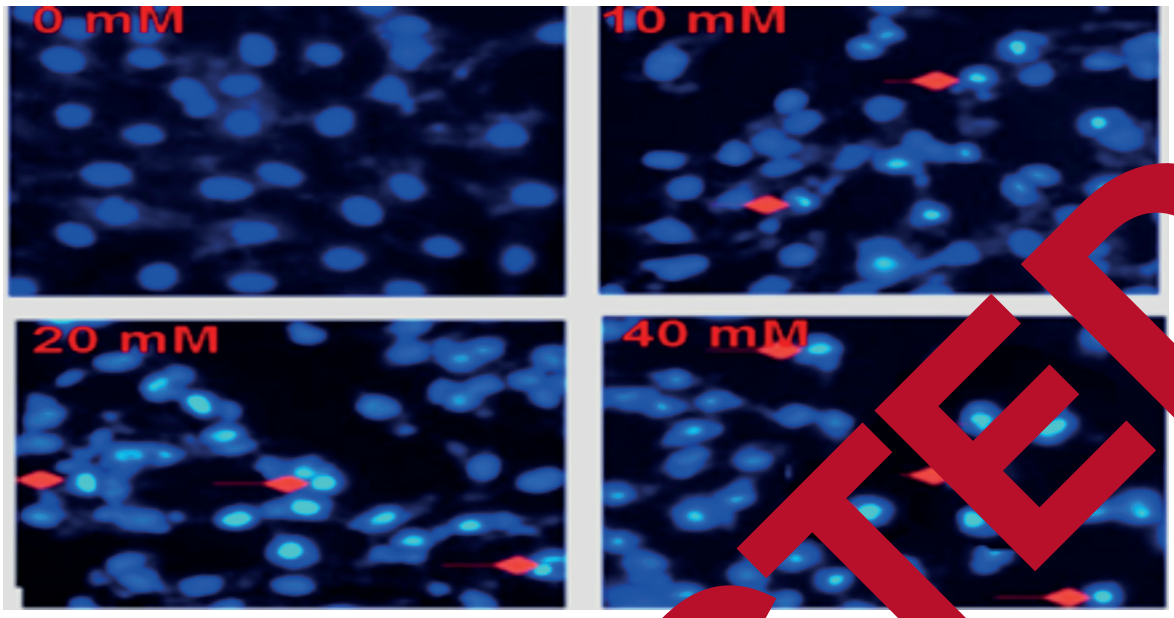

Figure 2. Effect of indicated doses of metformin on DNA damage as depicted by DAPI staining. The images are representatives of three biological replicates.

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 mTOR/PI3K/Akt Signaling Pathway

The m-TOR/PI3K/Akt signal is one of the main signally pathway activated in cancer cells. To confirm whether metformin could modulate 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 in the interesting outcome. Compared to the untreated control cells, metformin-treated cells showed a concentration-dependent down-regulation of mTOR and pm-TOR proteins. It also showed downregulation of PI3K/Akt proteins. Thus it may be concluded that metformin induces anticancer partly via mTOR/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-

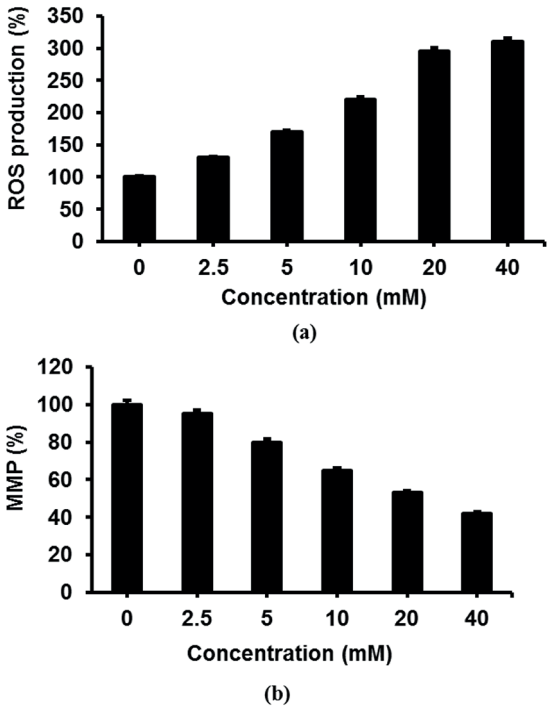

Figure 3. Effect of indicated doses metformin on (a) Mitochondrial membrane potential (b) ROS generation. All values are mean of three independent replicates ± SD.

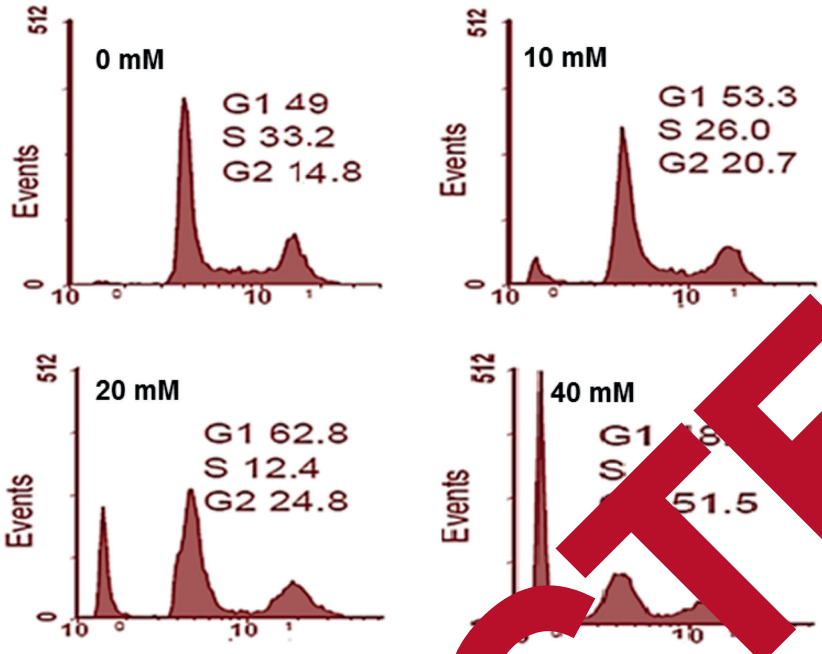

Figure 4. Effect of indicated doses metformin on Cell cycle arres[illegible] results are r[illegible]entatives of three independent biological replicates.

min is extensively prescribed oral hypogly[illegible] drug and has recently received attention i[illegible]s antitumorigenic activity. Metformin sho[illegible] potential growth inhibition activity agai[illegible] SKOV3 cells as evident from[illegible]T assa[illegible] As reported previously, [illegible]y dr[illegible] exhibi[illegible] antiproliferative effects[illegible]nducti[illegible]f apoptosis. For instance, [illegible]ve[illegible]he[illegible] drugs, such as cisp[illegible][17-23] [illegible]en reported to alter explicit[illegible]totic path[illegible] and cause DNA damage[illegible]ess wheth[illegible]etformin induces DN[illegible]damag[illegible] SKOV3, we carried out the D[illegible] staining o[illegible]reated cells. It was obser[illegible]hat metformin i[illegible]s DNA damage in a[illegible]centr[illegible] dependent manner. Further, it w[illegible]bs[illegible]d that metformin treated cells disp[illegible]ROS-n[illegible]ated MMP reduction[22]. [illegible]efor[illegible] re[illegible]s suggest that metformin m[illegible]nduce[illegible] damage through increasing in[illegible]ellular [illegible]S and reduction in MMP. Our [illegible] agreement with studies wherein [illegible]everal anti-cancer drugs have been reported to [illegible]get cancer cells partly by accretion of high [illegible]s 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 [illegible]n cell cycle progression. Metformin induced [illegible] cell cycle arrest and led to a significant [illegible] 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

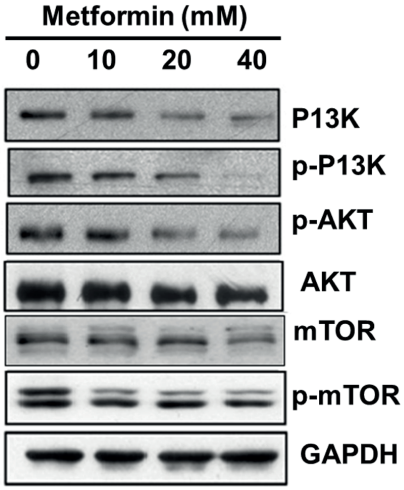

Figure 5. Western blots showing effect of indicated doses of metformin on protein expression of m-TOR/PI3K/Akt signaling pathway proteins. The images are representatives of three biological replicates.

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.

Referen

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