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Anticancer role of antidiabetic drug Metformin in ovarian cancer cells

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Original Article

Abstract

Purpose: Epithelial ovarian cancer is the most common ovarian cancer and has life threatening implications. Despite the progress in surgical and therapeutic strategies, resistance to chemotherapy is still a major concern. Chemotherapeutic agents cause cytotoxicity, primarily by the induction of apoptosis. The status of p53 is a key factor in determining the efficacy of apoptotic signaling. p53 is the most commonly mutated tumor suppressor gene in ovarian cancer. Metformin (an antidiabetic drug) has shown putative effects in many solid tumors. Hence we aimed to study the role of metformin in p53 mutated cancer cells. **Methods:** SKOV3 and OAW42 ovarian cancer cell line were used. The cancer cells were treated with metformin. MTT, Flow cytometry and Western blotting were used to characterize the effects of the different treatments. **Results:** Metformin treatment leads to cell cycle arrest in the G0/G1, S and G2/M phase of the cell cycle in SKOV3 and OAW42 respectively. Moreover, there was upregulation of Bax and downregulation of Bcl-2 protein and increased apoptosis in SKOV3 and OAW42 ovarian cancer cells. **Conclusion:** These findings support the potential of metformin to be used as chemoadjuvant and reflects its ability to sensitize cancer cells to apoptosis independent of p53 status.

Keywords: Metformin; Ovarian Cancer; Apoptosis; p53

1. Introduction

Epithelial Ovarian cancer is a lethal gynecological cancer being seventh most common cancer in world and fourth most common cancer in India (GLOBOCAN 2012). Most patients diagnosed in advanced stages undergo remission after optimal surgical cytoreductive and platinum/taxane based chemotherapy.[1] Despite the advent of new chemotherapies and molecular targeted therapies, prognosis still remains poor and most patients with metastatic cancer succumb to their disease.[2]

The major obstacle in the treatment of cancer is resistance to chemotherapy. Drug resistance either acquired or intrinsic often prevents tumor cells from undergoing sufficient levels of programmed cell death or apoptosis leading to survival of cancer cells and treatment failure.[2,3] Hence identifying novel therapeutic strategies or repositioning existing drugs that target key components of the apoptotic machinery is needed to improve patient survival. Metformin an antidiabetic drug has shown promising anticancer effects in an array of cancers like triple negative breast tumors[4] prostate[5], pancreas[6], colon[7] and gliomas.[8]

Metformin is a commonly prescribed oral hypoglycemic agent for type 2 diabetes. The anticancer effects of metformin are associated with both direct (insulin independent) and indirect (insulin dependent) actions. The indirect insulin dependent effects of metformin are mediated by reducing fasting blood glucose and insulin[9,10]. Metformin plays a major role in its anticancer activity by lowering insulin levels since insulin has mitogenic and prosurvival effects and tumor cells often express high levels of the insulin receptor.[11,12,13] Whereas it exerts its direct, insulin independent action through 5' adenosine monophosphate-activated protein kinase (AMPK) activation and decreased mammalian target of rapamycin (mTOR) signaling and protein synthesis in cancer cells. Moreoever, metformin is a cheap and widely available drug with minimal side effects like nausea, diarrhoea.[14,15]

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It has been reported that metformin treatment significantly inhibited proliferation of diverse chemoresponsive and resistant ovarian cancer cell lines, caused cell cycle arrest, decreased cyclin D1 and increased p21 protein expression.[16] Besides, metformin induced significant growth inhibition of OVCAR-3 and OVCAR-4 ovarian cancer cell lines in a time and dose dependent manner, increased cytotoxicity with cisplatin as compared to each agent alone[17], induced apoptosis by activating caspases 3/7, downregulating Bcl-2 and Bcl-xL expression, upregulating Bax and Bad expression.[18] Hence, we decided to study apoptotic potential of metformin in ovarian cancer.

Many tumor suppressor genes are implicated in the pathogenesis of ovarian cancer. These include the TP53 gene which plays a major role in chemotherapy resistance and is associated with disease metastasized beyond the ovary.[19,20] p53 is located on 17p13 which encodes a nuclear phosphoprotein and is altered in 50% of cases of ovarian cancer.[21,22] p53 expression is induced in response to oncogene activation, hypoxia and DNA damage and has multiple effects on gene expression. It causes transcriptional activation of p21, inhibitor of different cyclin/cyclin-dependent kinase complexes leading to cell cycle arrest. p53 protein also plays an integral role in apoptosis by downregulation of antiapoptotic genes and upregulation of proapoptotic genes**.** In the present scenario, there are no effective biological markers that can be used to assess patient response to chemotherapy. Several different oncomorphic mutations have been reported in literature each of which acts in a distinct manner and has a different effect on tumor progression and chemo resistance.[23] Numerous studies have seen metformin's anticancer potential but not enough light has been shed on p53 status of the cell. There is a paucity of literature demonstrating the role of metformin in p53 mutated ovarian cancer lines. Hence, it is essential to understand the altered pathways and design appropriate drug interventions to reduce morbidity and mortality of this lethal disease

In the OAW42 ovarian carcinoma cell line silent mutation (CGA-CCG substitution) is seen which codes for the same amino acid (Arginine) as is present in wild type (wt) p53 protein[24] whereas in SKOV3 cells p53 protein expression is absent due to single nucleotide deletion at position 267 (codon 90). Hence SKOV3 is p53(-/-) ovarian cancer cell line. [21] It has been seen that p53 inactivation and mutant p53 expression can endow the cells with additive growth and survival advantages such as increased proliferation, evasion of apoptosis and chemo resistance.[25,26] Thus we would like to see whether metformin could induce apoptosis in this p53 mutated (OAW42) and p53 null (SKOV3) ovarian cancer cells so that it could be used for chemo adjuvant therapy.

2. Methods and Materials

2.1 Cells lines and treatment

The ovarian cancer cell line SKOV3 and OAW42 was obtained from NCCS, Pune, grown in Dulbecco's modified Eagle's medium (DMEM) media supplemented with heat inactivated 10% fetal bovine serum (FBS), 2 mM glutamine, and 10µg/ml gentamicin. The cells were routinely passaged every 5–7 days. All cells were maintained at 37 °C in a 5% CO2, 95% air atmosphere incubator. Assays were performed in medium containing 1% FBS. Metformin was obtained from Sigma-Aldrich, USA (cat#D150959) and kept as a stock solution of 1 M in DMEM without serum.

2.2 Chemicals and antibodies

Cell culture material was obtained from Sigma Aldrich (cat#D150959), USA. Anti-Bax, anti-Bcl2, anti-p53 and anti-β-actin antibodies, Alkaline phosphates-conjugated anti-rabbit Ig G, anti-mouse Ig G, reagents were purchased from Santa Cruz, USA.

2.3 Cell viability Assay (MTT assay)

MTT assay was used to standardize the dose to be used in the study. About, $5x10^4$cells/well were plated in 96 well culture plates and were treated with varying concentrations of metformin for 24, 48, 72 hours after overnight incubation. It was followed by incubation of cells with 100 µl of 5 mg/ml MTT for 4hrs at 37^0C. Formazan crystals once formed were dissolved in Dimethyl sulfoxide (DMSO) and the absorbance was measured at 570nm using 620nm as the reference wavelength in an ELISA reader. The standardized doses of 15mM and 10mM metformin was used for treatment of SKOV3 cells and OAW 42 for future experiments.

2.4 Flow cytometry

SKOV3 and OAW42 ovarian cancer cells cells were treated with standardized doses of 15mM metformin and 10mM respectively for 48 hrs. The adherent cells were collected thereafter using trypsin Ethylenediaminetetraacetic acid (EDTA) while floating cells were collected by centrifugation. The cells were subsequently washed twice with ice cold phosphate buffered saline (PBS). After collection and washing, the cells were fixed in 70% ethanol. Subsequently for flow cytometric analysis the cells were then washed twice with ice cold PBS and resuspended in propidium iodide buffer (PBS, 0.1% Triton X-100, 0.1 mM EDTA, 0.05 mg/ml ribonuclease A, and 50 mM propidium iodide) for 30 minutes at room temperature. The cell cycle analysis was then done by flow cytometry (BD Facs, USA) using Win Mdi 2.9 software.[27]

2.5 Protein extraction and Western blot analysis

The ovarian cancer cells were lysed in Radioimmunoprecipitation assay buffer supplemented with protease inhibitor cocktail tablets (G biosciences, USA)**.** 60-100µg of protein lysates (estimated by

Bradford method) were resolved electrophoretically on 10%-15% denaturing SDS–polyacrylamide gels and transferred to nitrocellulose membranes. 5% non-fat milk was used for blocking following which membranes were probed with the primary antibodies specific to Bcl-2, Bax and β-actin. Immunoblotted proteins were visualized using Alkaline Phosphatase conjugated secondary antibodies. Final detection was performed with BCIP/NBT (5-bromo-4-chloro-3'-indolyphosphate/ nitro-blue tetrazolium chloride) substrate (Promega, USA). Appropriate positive and negative controls were run simultaneously. The bands were analyzed and quantitated using Alpha imager scanning densitometer (Alpha Innotech, USA) and its expression measured in Relative Units (RU). The density of the control was taken as 1 and the results of treatments were expressed in relation to the control. The methods were done as previously described.[27]

2.6 Statistical analysis

All results are expressed as mean±SEM. For multiple comparisons, data were analyzed by on-way ANOVA test followed by the post hoc Bonferroni test. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Metformin's effect on cell proliferation

The cells were treated with different doses of metformin 2.5, 5, 10, 15, 30, 50mM for duration of 24, 48 and 72 hrs. The IC 50 (dose at which 50% of cells were viable) doses were established by doing a dose response curve and MTT assay (Figure 1). Morphological changes characterized by membrane blebbing and formation of apoptotic bodies were also observed. Metformin inhibited growth of SKOV3. After 24 h, 30 mM of metformin was able to significantly reduce the number of viable cells whereas after 48 h, metformin at 15 mM showed similar antiproliferative effect. At 48 h, IC50 of metformin was found to be 15 mM. Based on these results and those in several published reports, 15 mM metformin was used in the following experiments. Similarly, in OAW42 cancer cells the IC50 at 48 hrs was found out to be 10mM.

3.2 Metformin affects cell cycle distribution

The effect of metformin on different phases of cell cycle was analyzed by flow cytometry after treatment with standardized doses of metformin. Metformin treatment in SKOV3 resulted in increase of cells in the S phase of the cell cycle (29.8%) as compared to control (14.7%) whereas no significant difference was seen in the G0/G1-phase and G2–M phase cells compared to control. Similarly, metformin treatment in OAW42 resulted in increase of cells in the G2–M phase of the cell cycle (36.8%) as compared to control (19.1%) whereas no significant difference was seen in the G1phase and the S-phase of cells compared to control (Figure 2a). To assess whether the induction of apoptosis also contributed to metformin mediated inhibition of ovarian cancer cell growth, the proportion of apoptotic cells was measured. The mean percentage apoptosis in untreated SKOV-3 and OAW42 control cells was 3.2 and 5.1% respectively. On treatment with standardized doses of metformin the mean percentage apoptosis was 38.06% and 32.2% (Figure 3) in SKOV- 3 and OAW42 cells respectively (Figure 2b). Our results demonstrate that the proportion of apoptotic cells was higher in metformin treated cultures compared with that in controls.

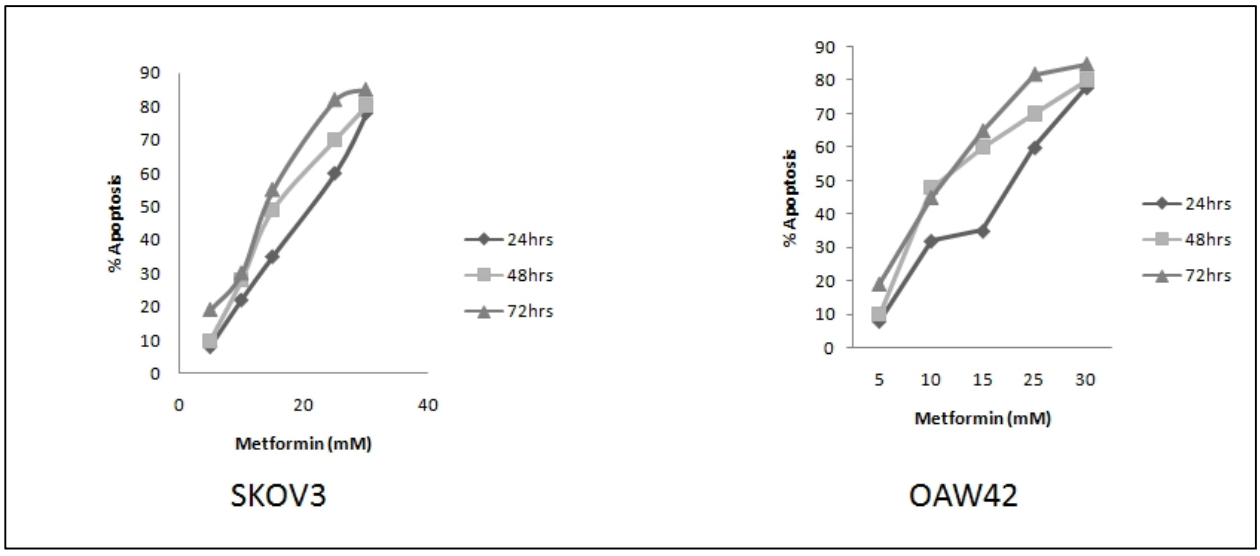

Figure 1: (left) Cell viability of SKOV3 and (right) OAW42 ovarian cancer cells treated with increasing doses of metformin for 48 hours measured by MTT assay.

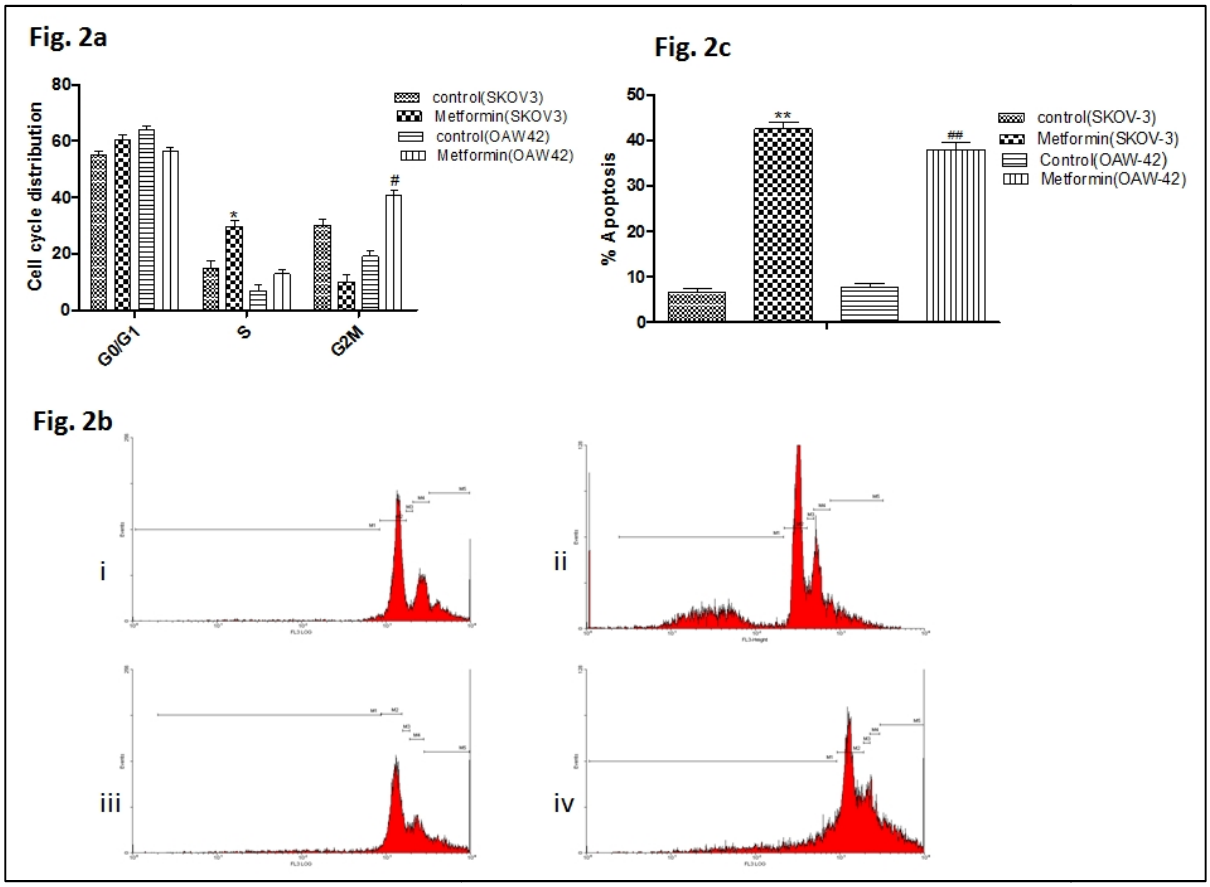

Figure 2: (a) Bar diagram showing cell cycle distribution in control and treated SKOV-3(metformin 15mM) and OAW42 (metformin 10mM) ovarian cancer cells for duration of 48 hours. The diagram represents mean of three independent experiments ±SD. *P < 0.05 metformin treated versus control in SKOV-3 cancer cells, # P < 0.05 metformin treated versus control OAW-42 cancer cells. (b) Representative experiment showing flow cytometric analysis for percentage apoptosis in (i) control SKOV3 (ii) treated SKOV3 and (iii) control OAW42 (iv) treated OAW42 ovarian cancer cells. In The horizontal margin drawn from Y axis to peak represents % of apoptotic cells. (c) Mean percentage apoptosis induced in control and treated SKOV3 and OAW42 ovarian cancer cells as measured by flowcytometry. The diagram represents mean of three independent experiments±SEM. **p < 0.01metformin versus control in SKOV3 cancer cells, ## p < 0.01 metformin versus control in OAW-42 ovarian cancer cells.

3.3 Effect of metformin on pro-survival and anti-survival proteins of the Bcl-2 family in ovarian cancer cells.

The p53 status of the ovarian cancer cells were seen by western blotting in which SKOV3 showed no band whereas a distinct band was seen in OAW42 cancer cells blots (Figure 3a). Similarly, we measured the levels of proapoptotic and antiapoptotic proteins in the presence of metformin. (Figure 3b). We found that the protein expression of Bcl-2 decreased by 1.5 fold and1.9 fold with metformin in SKOV3 and OAW42 cells respectively. Similarly, protein expression of Bax was increased by 2.5 and 2.2 fold in SKOV3 and OAW42 cells respectively (Figure 3c). These pro and anti-apoptotic proteins regulate the permeability of outer mitochondrial membrane and hence apoptosis. The protein expression of Bax increases and Bcl-2 decreases in both SKOV3 and OAW42 irrespective of p53 status.

4. Discussion

Ovarian cancer is a heterogeneous disease with inter and intra-tumor heterogeneity and has a high mortality rate. Despite advances in surgical and radiation treatments, chemotherapy continues to be an important therapeutic option for different malignancies. Increasing chemo resistance and lack of successful new treatments evoke the need of comprehensive genomic analysis to identify genetic abnormalities in ovarian tumors that could influence the pathophysiology of the disease and chemotherapeutic response.[28,29,30] TP53 is the most commonly mutated gene in ovarian tumors. The guardian of genome p53 normally protects against cancer through protein-protein interactions, cell cycle arrest, apoptosis, autophagy and DNA damage repair.

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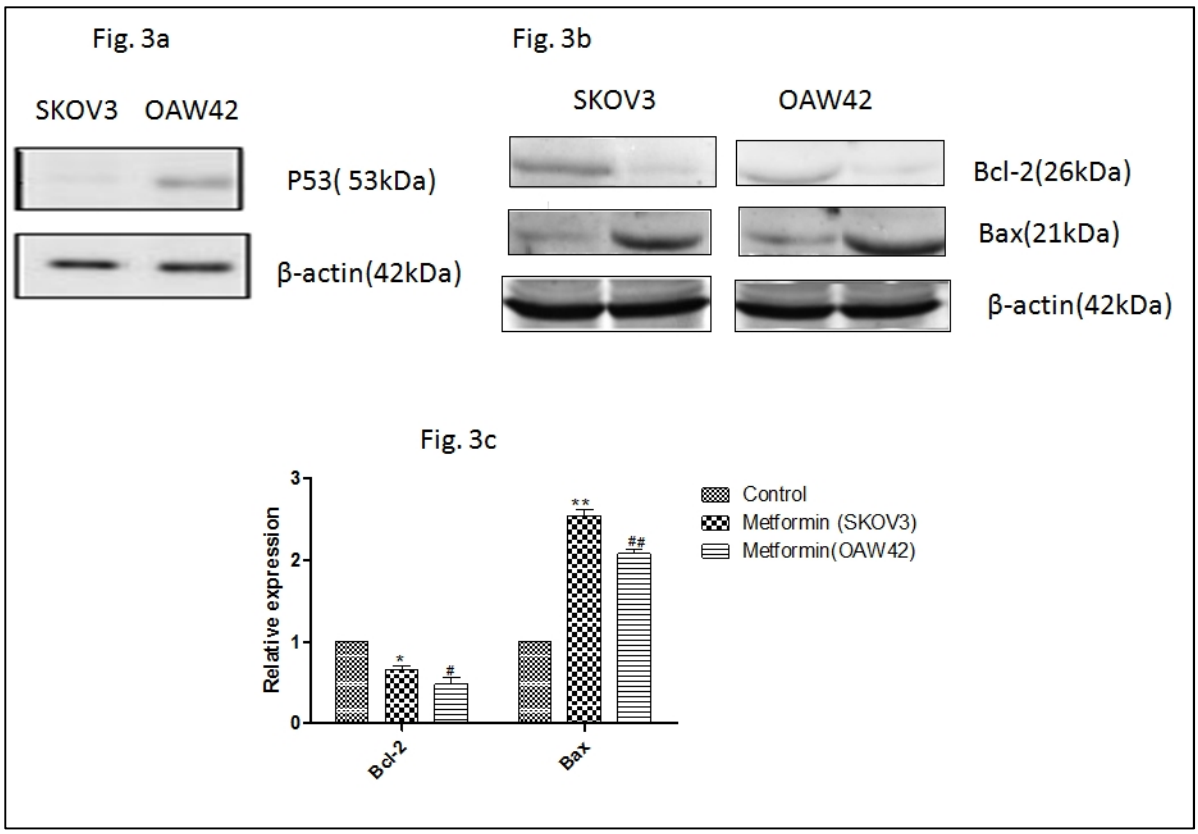

Figure 3: (a and b) Effect of metformin on the expression of anti-apoptotic and pro-apoptotic proteins (p53, Bcl-2, Bax and β Actin) in SKOV3 and OAW42 ovarian cancer cells treated with 15 mM and 10 mM metformin for 48hrs respectively. Cell lysates were subjected to Western blot, one representative blot out of three is shown. (c) Densitometric analysis of protein expression of Bcl-2 and Bax ovarian cancer cells in control and treated SKOV3 and OAW42 cells as measured by western blot analysis. The diagram represents the mean of three independent experiments ± SEM. *P < 0.05, **P < 0.01 metformin versus control in SKOV3 cancer cells, # P < 0.05, ## P < 0.01 metformin versus control in OAW42 cancer cells.

There is also a need to develop new drugs or reposition drugs for treating ovarian cancer. Originating from the French Lilac plant (Galega officinalis), metformin has been able to reduce cancer risk[31] and mortality[32] besides inhibiting cancer cells in vitro and vivo. Hence, in this study we sought to find whether metformin could induce apoptosis in ovarian cancer cells in p53 deficient and mutant cells. Metformin causes molecular activation of AMPK and inactivation of mTOR signaling in cancer cells thereby exhibiting antiproliferative effect whereas chemotherapeutic drugs through activation of p53 yields similar effects to metformin.[33] In this study, we have seen that metformin has antiproliferative effect and induces cell cycle arrest in vitro. The effect of metformin on different stages of cell cycle was first studied to understand its anticancer potential.

There were more cells in G0/G1 and S phase and G2M phase cells in metformin treated SKOV3 and OAW42 cultures respectively compared with those in control cultures after 48hrs of treatment (Figure 2a) suggesting arrest of the cell cycle by metformin at two points. It may be due to p21 expression which can be induced by both p53 dependent and independent mechanism. p21 is a inhibitory regulator of the G1/S transition as well as inhibitor of the cyclin dependent kinase (CDK1)/cyclin B complex that causes G2/M arrest.[34,35,36] p53 mutations are one of the most common mutations and play an integral role in pathogenesis and complexity of cancer implicating the necessity of studying its role. SKOV3 cell line has a p53 null mutation and OAW42 silent mutation. In this study, we found that metformin induces cell cycle arrest in ovarian cancer cells both at G0/G1, S (in SKOV3) and G2/M (in OAW42) irrespective of p53 status. Similarly, metformin induced cell cycle arrest in endometrial cancer cells in G1 and G2/M via a p53-independent pathway as reported by Takahashi *et al.*[37] Quieroz *et al.*[38] have reported cell cycle arrest in G0/G1 phase in breast cancer cells. This difference in cell cycle arrest may also be attributed to difference in cell cycle specific conditions, incubation time and dosages of metformin or existing polymorphisms of the metformin transporter, OCT1 (organic cation transporter).[39] The role of OCT1 in metformin uptake by ovarian cancer cells is under investigation.

Further to study apoptotic potential of metformin, cells were incubated with or without metformin (15 or 10 mM) for 48 h, the proportion of apoptotic cells was measured by flow cytometry. Metformin treatment led

to increase in proportion of apoptotic cells in SKOV3 and OAW42 cultures compared with that in controls (Figure 2b, 2c).

We next sought to evaluate the effect of metformin on various pro and anti apoptotic proteins of the Bcl-2 family. The exact molecular mechanism underlying the apoptotic response to metformin remains unclear. Metformin may induce Bcl-2 phosphorylation, which may be less capable of forming heterodimers with (the proapoptotic) Bax protein. This may lead to increased formation of Bax:Bax homodimers, driving the cell towards apoptosis. Our results showed downregulation of antiapoptotic and upregulation of apoptotic proteins with metformin treatment in both SKOV3 and OAW42 cancer cells (Figure 3c). Metformin was able to induce apoptosis of SKOV3 and OAW42 ovarian cancer cells. These findings were in congruence with other studies that have reported apoptotic potential of metformin in prostate ovary, breast, colon, endometrial cancer cells and esophageal squamous cell carcinomas[15,18,40,41,42,43] using different mechanisms..

In our experiments we observed metformin irrespective of p53 status (OAW 42 cells and SKOV3 cell line) was able to inhibit cell viability and modulate apoptotic proteins. However, additional studies are required for suggesting that p53 independent apoptosis is likely the predominant mechanism of cytotoxic action of metformin in SKOV3 cells. Besides, more studies are required to understand the molecular basis for this differential response to enhance the effectiveness of metformin in the treatment of patients with malignant disease.

Moreover, it has been seen that metformin is selectively toxic to p53 deficient cells. In the presence of metformin, p53+/+ cells, but not p53-/-cells activated autophagy. Autophagy is a cellular survival pathway necessary to maintain homeostasis in normal cells as well as in times of metabolic stress with nutrient recycling.[41] Metformin was able to induce apoptosis in p53 -/- cancer cell line SKOV3 comparable to OAW42. It may do so either by preventing autophagy or inducing apoptosis in a p53 independent pathway in SKOV3 cell line and p53 dependent pathway in OAW42 cell line. Hence, further autophagic potential of metformin needs to be studied.

TP53 plays an important role in complexity and heterogeneity in ovarian cancer pathogenesis, chemotherapeutics and prognosis. In the case of metformin, the crosstalk with autophagy, apoptosis, chemotherapeutic drugs induced signaling pathways may result in direct interactions between these drug induced signaling systems at the level of AMPK.[23] Hence it is essential to study the effect of current and novel treatment strategies like metformin in the light of important and frequent mutations like p53.

5. Conclusion

Hence, in this study we have tried to demonstrate that metformin promotes the elimination of ovarian cancer cells through regulation of apoptosis independent of p53 status suggesting its future role as chemo adjuvant. These results are based on in vitro studies only and further in vivo studies are necessary.

Conflict of interest

The authors declare that they have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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