

Original Research Article

Role of Antidiabetic Drug Metformin in Ovarian Cancer Cells in Vitro

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Abstract: Ovarian cancer is one of the most common lethal gynecological malignancies. Despite an initial 70-80% response rate, most patients relapse within 1-2 years and develop chemo resistance. Tumor cells have the capacity to respond to chemotherapy through multiple cell death pathways such as apoptosis and autophagy. In this study, we have attempted to evaluate the effect of metformin (an antidiabetic drug) in ovarian cancer because of its promising effect in other solid tumors. In this study, the human ovarian cancer cell line SKOV3 cell line was used to assess the effect of metformin on apoptosis and autophagy. The cancer cells were treated with metformin. MTT, Flowcytometry and Western blotting were used to characterize the effects of treatments. Metformin inhibited proliferation of SKOV3 ovarian cancer cells, caused cell cycle arrest in G0/G1 and S phase, induced apoptosis by modulating apoptotic proteins Bcl-2 and Bax but was not able to induce autophagy as seen by decreased Beclin expression. These data illustrated that induction of apoptosis and inhibition of autophagy by metformin may enhance the cell-killing effect of metformin in multidrug-resistant human ovarian cancer cells like SKOV3. Hence, it may represent a novel approach to deal with chemo-resistance by using metformin as an anticancer modality.

Keywords: Metformin, ovarian cancer, SKOV3, apoptosis, autophagy.

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) (<http://globocan.iarc.fr/>), Platinum/paclitaxel-based chemotherapy after surgical staging and resection is the current standard of treatment. Despite the advances in chemotherapy, the prognosis still remains poor due to abdominal or pelvic recurrence that is resistant to further chemotherapy. Tumor cells have the ability to respond to chemotherapy through several growth arrest and cell death pathways [1]. Different modes of cell death have been elucidated, such as necrosis, apoptosis and autophagic cell death. Modulating apoptosis, the type I programmed cell death, has long been one of the most important strategies for cancer treatment.

However, apoptosis accounts for only 20% of cases [2]. While, autophagy, the type II programmed cell death, has been reported to play vital role in the development of cancer. Autophagy is a conserved catabolic process for the degradation and recycling of misfolded or aggregated proteins and excess or defective organelles, primarily a response to the stress of chemotherapeutic agents [3], starvation [4], hypoxia

or viral infection, withdrawal of growth factors [5]. Autophagy is considered to be a double-edged sword either promoting cell survival or leading to cell death. Hence, elucidating role of autophagy at different stages of cancer progression is a challenging task and better understanding of its regulation and impact on treatment outcomes would provide novel anticancer therapeutic.

Metformin, the most common antidiabetic drug, has been found to possess anticancer properties. Numerous reports have demonstrated metformin's growth inhibitory potential in various gynecological cancer cells such as ovary [6,7]. Metformin activates AMPK, a critical cellular energy sensor. Activation of AMPK suppresses the mTOR and leads to reduced protein synthesis and cell proliferation and induces apoptosis [8]. Autophagy is also promoted by AMP activated protein kinase (AMPK) [9]. Whether metformin induces other forms of cell death is still under research. Metformin has been seen to induce apoptosis in some cancers and autophagy in other, including ovary, melanoma, lymphoma, and colon cancer, prostate, esophageal cancer cells, microvascular endothelial cells [10-15]. Apoptosis and autophagy have multiple functional relationships in cancer cells. Thus, a better understanding of the crosstalk between

apoptosis and autophagy may play a crucial role in cancer therapeutics.

In this study, the human ovarian cancer cell line SKOV3 was used to assess the effect of metformin, the crosstalk of autophagy and apoptosis were used to explore the effect and the potential mechanism. Deletion of a single nucleotide at position 267 (codon 90) blocks p53 protein expression makes SKOV3 is p53(-/-) ovarian cancer cell line [16] Aberrations in the repair mechanism in which the wild type TP53 as “the guardian of genome” [17] contributes directly and indirectly, might lead to unrestrained proliferation and neoplasia and chemo resistance making SKOV3 multidrug resistant. Hence, the present study would contribute some insight to improve the treatment efficacy for chemoresistant ovarian cancer cell and bring new insights for cancer development

MATERIALS AND METHODS

Cells lines and treatment

The ovarian cancer cell line SKOV-3 was obtained from NCCS, Pune, grown in 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.

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 phosphatase-conjugated anti-mouse Ig G, reagents were obtained from Santa Cruz, USA.

MTT assay for cell viability/proliferation

The dose of Metformin was standardized using MTT assay. Approximately, 5x10⁴ cells/well were plated in 96-well culture plates. After overnight incubation, the cells were treated with varying concentrations of metformin for 24, 48, 72 hours. The cells were incubated with 100 µl of 5 mg/ml MTT for 4hrs at 37°C. The formazan crystals thus formed were dissolved in DMSO and the absorbance was measured at 570nm using an ELISA reader and 620nm as the reference wavelength. The doses of 15mM metformin were used for treatment of SKOV-3 cells after standardization.

Flow cytometry

SKOV-3 cells were treated with 15mM Metformin for duration of 48 hrs and then harvested.

After treatment with metformin, adherent cells were collected using trypsin Ethylene diamine tetra acetic acid (EDTA) while floating cells by centrifugation. The collected cells were washed twice with ice-cold phosphate-buffered saline (PBS). To determine the percentage of apoptotic cells and cell cycle analysis, after collection and washing, the cells were fixed in 70% ethanol. The cells were then again 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). After 30 minutes at room temperature, the cell cycle distribution was determined by flow cytometry (BD Faces, USA) using Win Mdi 2.9 software [18].

Protein extraction and Western blot analysis

Cells were lysed in Radioimmunoprecipitation assay buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail tablets (G biosciences, USA). The total protein content was determined according to the Bradford method. The protein lysates (60-100µg) were resolved electrophoretically on 10%-15% denaturing SDS-polyacrylamide gels followed by transfer to nitrocellulose membranes. Next, after blocking in 5% nonfat milk, membranes were probed with the primary antibodies specific to Bcl-2, Bax and β-actin. Immuno blotted proteins were visualized using Alkaline Phosphatase-conjugated secondary antibodies. Final detection was performed with 5-bromo-4-chloro-3'-indolylphosphate/nitro-blue tetra zolium chloride (BCIP/NBT) substrate (Promega, USA). Appropriate positive and negative controls were run simultaneously. The bands were analyzed and quantitated using Alpha imager scanning densitometer. The protein expression is expressed 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 [18].

STATISTICAL ANALYSIS

Data are presented as mean ±SEM of at least 3 independent experiments. All groups were studied in parallel and differences between groups were analyzed by ANOVA, as appropriate, and Bonferroni post hoc tests for multiple unpair wise comparisons of means. The level of significance adopted was P<0.05.

RESULTS

Effect of metformin on viability

To examine the effects of metformin on cancer cell growth we treated SKOV-3 cell line with metformin. Cell viability was determined with increasing doses of metformin for 24, 48 and 72 hours and measured by MTT assay. As shown in Figure 1

metformin inhibited cell viability as related to control.

The dose was standardized at 15mM for 48hrs.

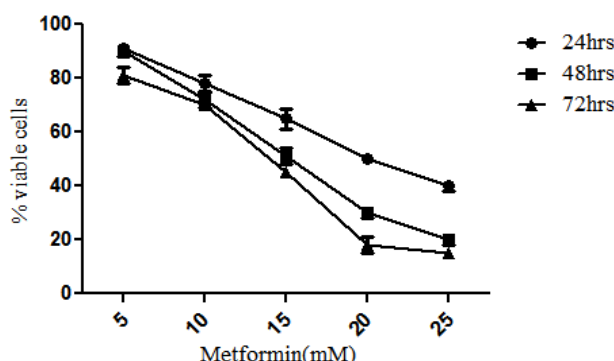


Fig 1: Cell viability of SKOV-3 cells treated with increasing doses of metformin for 24,48 and 72 hours measured by MTT assay.

Effect of metformin on cell cycle distribution

To further evaluate the mechanism of growth inhibition by metformin the cell cycle profile was analyzed by flowcytometry after treatment with metformin. Majority of cells presented in the G1-phase of the cell cycle, and small part in the G2/M-phase and the rest of the cells were found to be in the S-phase in control. Thus, it was seen that at the cellular level,

metformin interferes with cell cycle leading to G0/G1 or S phase arrest. Treatment of metformin for 48h showed a dose dependent increase in G0/G1 and S phase cells. The percentage of apoptotic cells was determined by cell flow cytometric analysis following PI staining. Compared with untreated cells, cells treated with metformin for 48h resulted increased apoptosis in a time dependent manner. (Figure 2, 3).

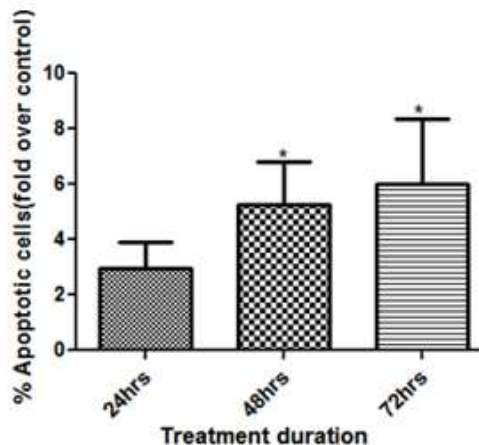
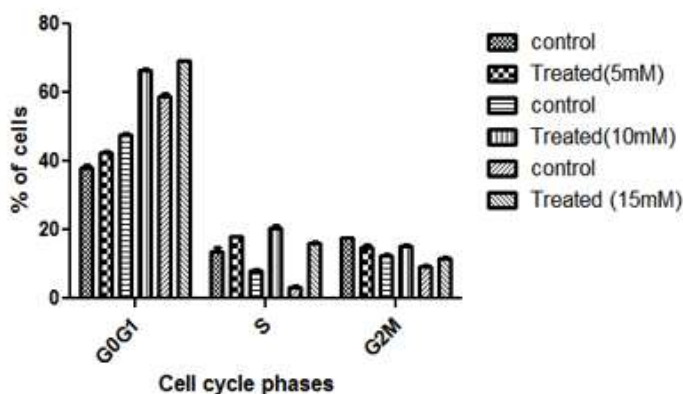


Fig 2: Cell cycle distribution in control and metformin (15mM) treated SKOV-3 cells. Figure 3: Mean apoptosis (fold over control) in treated SKOV-3 ovarian cancer cells as measured by flowcytometry. The diagram represents mean of three independent experiments ±SEM.* p<0.05 versus control

Effect of metformin on pro-survival and anti-survival proteins of the Bcl-2 family and Beclin in SKOV-3 cell line

Level of Bcl-2 was downregulated and Bax upregulated in SKOV-3 cells treated with 15mM

metformin compared to control. However, there was a decrease in expression of Beclin. The protein expression of Bcl-2 decrease by 1.6-fold, Bax increased by 1.5 compared to control, Beclin decreased by 1.06 compared to control (Figure 4a, 4b).

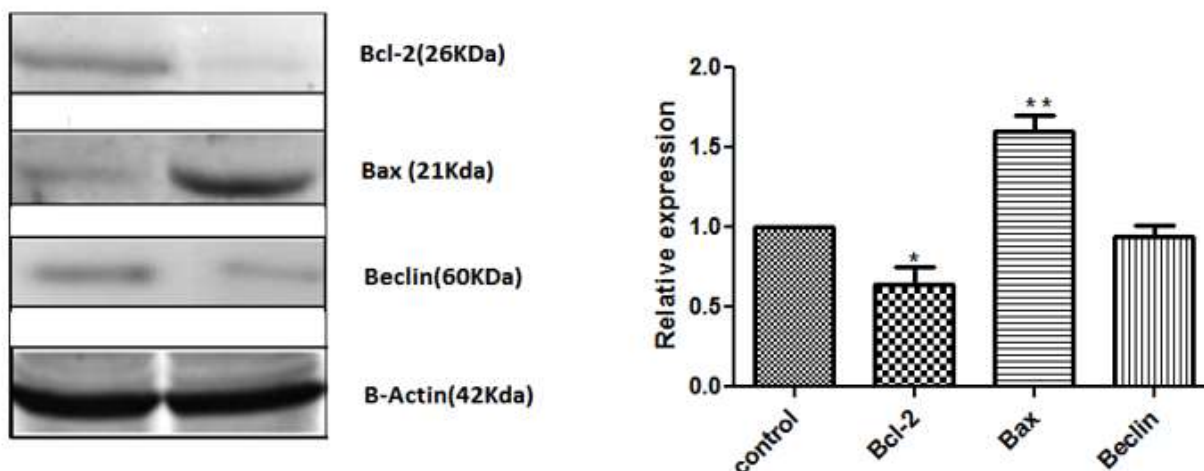


Fig 4a: Effect of metformin on the expression of proteins (Bcl-2, Bax, Beclin and β Actin) in SKOV-3 ovarian cancer cells treated with 15 mM metformin for 48hrs. Cell lysates were subjected to Western blot, one representative blot out of three is shown. **4b:** Densitometric analysis of protein expression of Bcl-2, Bax, Beclin in control and treated SKOV-3 cells as measured by western blot analysis. The bar diagram represents the mean of three independent experiments \pm SEM. *P<0.05 versus control

DISCUSSION

Despite the significant advances in our understanding of various aspects of ovarian cancer initiation, progression and metastasis we have achieved limited clinical success. It still remains the most lethal gynecological malignancy, with an alarmingly poor prognosis attributed to late detection and chemo resistance. Initially, most tumors respond to chemotherapy but eventually relapse unraveling the need of development of new drugs or targeting new pathways by old drugs.

Tumor cells respond in different ways through different pathways. Hence, we ought to study role of apoptosis and autophagy in ovarian cancer cells. Apoptosis has long been known to play an important role in the response to several chemotherapeutic agents; however, the importance of treatment-induced autophagic cell death in tumor regression has only recently been recognized [19]. Furthermore, numerous studies have demonstrated anti-cancer effects of metformin [8], leading to an explosion of interest in evaluating this agent in human cancer.

To determine whether the processes of autophagy and apoptosis are linked, we performed several experiments following the treatment with metformin. These results are based on in vitro studies only, and further in vivo studies are necessary. We have evaluated the effects of metformin on cell cycle distribution and progression. Concurrently, ovarian cancer cells were blocked in G0/G1 and S phases on exposure to metformin. However different studies reveal difference in cell cycle distribution probably due to existing polymorphisms of the metformin transporter, OCT1 (organic cation transporter) [20]. The role of

OCT1 in metformin uptake by ovarian cancer cells is unknown at the moment and is under investigation.

Further the percentage apoptotic cells were measured by Flowcytometry. We found dose and time dependent increase in apoptosis. These findings were further confirmed by evaluating effect of metformin in pro and antiapoptotic proteins. Our result shows significant decrease in antiapoptotic protein Bcl-2 and increase in proapoptotic protein Bax.

Next, we tried to find the underline mechanism which would contribute to metformin's anticancer role in SKOV-3 cells besides apoptosis, which would contribute to find more target for treatment. Hence, to determine type of cell death, autophagy or apoptosis, would play predominant roles in killing effect we studied Beclin expression besides pro and antiapoptotic proteins.

Beclin 1 is a tumor suppressor gene product that allosterically activates the class III phosphatidylinositol 3-kinase (PI3KC3), which is essential for the recruitment of other autophagy-related gene (Atg) proteins to the phagophore assembly site (PAS) to initiate autophagosome formation [21,22]. We found a decrease in protein expression of Beclin suggesting metformin might not be able to induce autophagy in SKOV3 cancer cells. Similarly, results were seen in prostate cancer cells. However, it is not conclusive since some studies suggest that autophagy can also occur in a Beclin1-independent manner and in this case PI3K inhibitors fails to suppress it [23-24]. Hence, in the present scenario we cannot clearly delineate autophagy from apoptosis which needs further study.

Thus, it may be hypothesized that metformin could induce apoptosis in SKOV3 cell through modulation of apoptotic protein but no significant change in expression of Beclin suggesting apoptosis might be the predominant mechanism after metformin treatment rather than autophagy.

Metformin is an AMPK activator hence may induce autophagy. Nevertheless, metformin has also been reported to inhibit autophagy by decreasing Beclin 1 expression and inducing AMPK-dependent apoptosis [25] as seen in prostate cancer cells. Similarly, metformin promotes apoptosis but suppresses autophagy in hepatocellular carcinoma cells [26]. In the present study metformin, could not induce autophagy and concomitantly induces apoptosis in cell line. Moreover, SKOV3 is p53 (-/-) ovarian cancer cell line (deletion of a single nucleotide at position 267 (codon 90) blocks p53 protein expression; Milner et al 1993). Buzzai et al got the same results and found p53-/- cells were unable to undergo autophagy which p53+/+ cells can normally do hence metformin was selectively toxic to p53-deficient cells by forcing a metabolic conversion autophagy that p53 -/- cells are unable to execute [27]. Thus, a potential link between metformin and p53 induced autophagy becomes important both mechanistically and at the clinical level.

In SKOV-3 cells lack of autophagy induction, triggered the up-regulation of apoptosis, suggesting there might be a molecular switch between autophagy and apoptosis in SKOV-3 cells. In recent years, more and more researches suggested that the apoptosis and autophagy could be mutually antagonistic or promoted in some scenario. The same induction factors can play a positive or negative role for the two kinds of programmed cell death in different cells. For example, metformin could inhibit autophagy at the same time promote or inhibit apoptosis depending on p53 status of cell and localization. p53 may play a dual role, nuclear p53 induces autophagy through transcriptional effects and cytoplasmic p53 represses autophagy [28].

Expanding our knowledge of the molecular pathways that regulate tumor cell death is crucial in guiding the successful design of future anticancer therapeutics. Moreover, the complex interrelationship between apoptosis and autophagy has been reported to be affected by various biochemical processes via different pathways [29,30] and growing number of studies suggested the presence of crosstalk between autophagy and apoptosis; Autophagy may be indispensable for apoptosis, may antagonize, occur independently, or molecular switch between them determining the fate of cell. However, this therapy needs to be further explored for clinical application with large scale multicentric collaboration. As autophagy is a

complex, dynamic process it warrants the need of further studies to measure, quantify and develop new techniques for assessing autophagy to accentuate progress in this field of investigation. The multifaceted nature of autophagy and its diverse crosstalk with other biological processes must be carefully considered when the autophagic system is targeted for anticancer benefit. Taken together, the role of metformin, p53, autophagy and apoptosis in cancer has developed into a tightly knit, exciting and rapidly changing field.

CONCLUSIONS

Thus, metformin may not only inhibit autophagy, but also cause cell cycle arrest and induce apoptosis in SKOV-3 cells, ultimately leading to the increase of cell death. These data illustrated that inhibition of autophagy may enhance the cell-killing effect of metformin in multidrug-resistant human ovarian cancer cells, which may represent a novel approach to increase the efficacy of chemotherapeutics by using metformin as a chemo adjuvants as an anticancer modality. However, our increased understanding of the role of metformin and p53 in autophagy and tumor metabolism will hopefully provide a prospective strategy for cancer if only we can unravel some of the complexities.

Conflict of interest

The authors declare that they have no conflicts of interest.

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REFERENCES

1. Jin Z, El-Deiry WS. Overview of cell death signaling pathways. *Cancer biology & therapy*. 2005 Feb 2; 4(2):147-71.
2. Verheij M, Bartelink H. Radiation-induced apoptosis. *Cell and tissue research*. 2000 Jun 20; 301(1):133-42.
3. Kondo Y, Kanzawa T, Sawaya R, Kondo S. The role of autophagy in cancer development and response to therapy. *Nature Reviews Cancer*. 2005 Sep 1; 5(9):726-34.
4. Tallóczy Z, Jiang W, Virgin HW, Leib DA, Scheuner D, Kaufman RJ, Eskelinen EL, Levine B. Regulation of starvation- and virus-induced autophagy by the eIF2 α kinase signaling pathway. *Proceedings of the National Academy of Sciences*. 2002 Jan 8; 99(1):190-5.
5. Martinet W, De Meyer GR, Andries L, Herman AG, Kockx MM. In situ detection of starvation-induced autophagy. *Journal of Histochemistry & Cytochemistry*. 2006 Jan; 54(1):85-96.

6. Irie H, Banno K, Yanokura M, Iida M, Adachi M, Nakamura K, et al. Metformin: A candidate for the treatment of gynecological tumors based on drug repositioning (Review). *Oncology Letters*. 2016; 11:1287–93.
7. Li L, Qi X, Xu M, Ding X, Zhou X, Zhang C, Fan Z, Zhao Y, Sheng H, Zhang Y. The effects of metformin on ovarian cancer: an updated systematic review and meta-analysis. *INTERNATIONAL JOURNAL OF CLINICAL AND EXPERIMENTAL MEDICINE*. 2016 Jan 1; 9(9):17559-68.
8. Dowling RJ, Goodwin PJ, Stambolic V. Understanding the benefit of metformin use in cancer treatment. *BMC medicine*. 2011 Apr 6;9(1):33.
9. Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nature cell biology*. 2011 Feb 1; 13(2):132-41.
10. Yasmeen A, Beauchamp MC, Piura E, Segal E, Pollak M, Gotlieb WH. Induction of apoptosis by metformin in epithelial ovarian cancer: involvement of the Bcl-2 family proteins. *Gynecologic oncology*. 2011 Jun 1; 121(3):492-8.
11. Tomic T, Botton T, Cerezo M, Robert G, Luciano F, Puissant A, Gounon P, Allegra M, Bertolotto C, Bereder JM, Tartare-Deckert S. Metformin inhibits melanoma development through autophagy and apoptosis mechanisms. *Cell death & disease*. 2011 Sep 1; 2(9):e199.
12. Shi WY, Xiao D, Wang L, Dong LH, Yan ZX, Shen ZX, Chen SJ, Chen Y, Zhao WL. Therapeutic metformin/AMPK activation blocked lymphoma cell growth via inhibition of mTOR pathway and induction of autophagy. *Cell death & disease*. 2012 Mar 1; 3(3):e275.
13. Sahra IB, Laurent K, Giuliano S, Larbret F, Ponzio G, Gounon P, Le Marchand-Brustel Y, Giorgetti-Peraldi S, Cormont M, Bertolotto C, Deckert M. Targeting cancer cell metabolism: the combination of metformin and 2-deoxyglucose induces p53-dependent apoptosis in prostate cancer cells. *Cancer research*. 2010 Mar 15; 70(6):2465-75.
14. Feng Y, Ke C, Tang Q, Dong H, Zheng X, Lin W, Ke J, Huang J, Yeung SC, Zhang H. Metformin promotes autophagy and apoptosis in esophageal squamous cell carcinoma by downregulating Stat3 signaling. *Cell death & disease*. 2014 Feb 1; 5(2):e1088.
15. Samuel SM, Ghosh S, Triggle CR. Metformin Sensitizes Glucose Starved Microvascular Endothelial Cells to Death by Inhibition of the mTOR Pathway and Autophagy. *FASEB J*. 2016 Apr 1; 30(1 Supplement):948.2-948.2.
16. Hamroun D, Kato S, Ishioka C, Claustres M, Bérout C, Soussi T. The UMD TP53 database and website: update and revisions. *Human mutation*. 2006 Jan 1; 27(1):14-20.
17. Lane DP. Cancer. p53, guardian of the genome. *Nature*. 1992; 358:15-6.
18. Patel S, Kumar L, Singh N. Metformin and epithelial ovarian cancer therapeutics. *Cellular Oncology*. 2015 Oct 1; 38(5):365-75.
19. Jing K, Song KS, Shin S, Kim N, Jeong S, Oh HR, Park JH, Seo KS, Heo JY, Han J, Park JI. Docosahexaenoic acid induces autophagy through p53/AMPK/mTOR signaling and promotes apoptosis in human cancer cells harboring wild-type p53. *Autophagy*. 2011 Nov 1; 7(11):1348-58.
20. Takane H, Shikata E, Otsubo K, Higuchi S, Ieiri I. Polymorphism in human organic cation transporters and metformin action.
21. Kihara A, Kabeya Y, Ohsumi Y, Yoshimori T. Beclin–phosphatidylinositol 3-kinase complex functions at the trans-Golgi network. *EMBO reports*. 2001 Apr 1; 2(4):330-5.
22. Scarlatti F, Maffei R, Beau I, Codogno P, Ghidoni R. Role of non-canonical Beclin 1-independent autophagy in cell death induced by resveratrol in human breast cancer cells. *Cell Death & Differentiation*. 2008 Aug 1; 15(8):1318-29.
23. Tian S, Lin J, Zhou J, Wang X, Li Y, Ren X, Yu W, Zhong W, Xiao J, Sheng F, Chen Y. Beclin 1-independent autophagy induced by a Bcl-XL/Bcl-2 targeting compound, Z18. *Autophagy*. 2010 Nov 16; 6(8):1032-41.
24. Chu CT, Zhu J, Dagda RK. Beclin 1-independent pathway of damage-induced mitophagy and autophagic stress: implications for neurodegeneration and cell death. *Autophagy*. 2007 Nov 26; 3(6):663-6.
25. Sahra IB, Laurent K, Giuliano S, Larbret F, Ponzio G, Gounon P, Le Marchand-Brustel Y, Giorgetti-Peraldi S, Cormont M, Bertolotto C, Deckert M. Targeting cancer cell metabolism: the combination of metformin and 2-deoxyglucose induces p53-dependent apoptosis in prostate cancer cells. *Cancer research*. 2010 Mar 15; 70(6):2465-75.
26. Park DB. Metformin Promotes Apoptosis but Suppresses Autophagy in Glucose-Deprived H4IIE Hepatocellular Carcinoma Cells. *Diabetes Metab J*. 2015 Dec; 39:518–27.
27. Buzzai M, Jones RG, Amaravadi RK, Lum JJ, DeBerardinis RJ, Zhao F, Viollet B, Thompson CB. Systemic treatment with the antidiabetic drug metformin selectively impairs p53-deficient tumor cell growth. *Cancer research*. 2007 Jul 15; 67(14):6745-52.

28. Green DR, Kroemer G. Cytoplasmic functions of the tumour suppressor p53. *Nature*. 2009 Apr 30; 458(7242):1127-30.
29. Moretti L, Cha YI, Niermann KJ, Lu B. Switch between apoptosis and autophagy: radiation-induced endoplasmic reticulum stress? *Cell cycle*. 2007 Apr 1; 6(7):793-8.
30. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nature reviews Molecular cell biology*. 2007 Sep 1; 8(9):741-52.