Synergistic therapeutic effects of metformin at a clinically relevant dosage in combination with chemotherapy via the AKT/mTOR pathway on ovarian cancer

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Abstract

Background Ovarian cancer is the leading cause of cancer-related death among women. Metformin has antitumor effects in many cancer models. However, many studies have shown that metformin has cytotoxic activity at higher concentrations than those used for diabetic patients; this effect may be derived from high concentrations directly inducing the death of tumor cells. The synergism of metformin at clinically relevant dosages and chemotherapy in ovarian cancer remains unclear.

Methods We applied two clinical databases to survey metformin use and ovarian cancer survival rate. The Cancer Genome Atlas dataset, an L1000 microarray with Gene Set Enrichment Analysis (GSEA) analysis, western blot analysis and an animal model were used to study the activity of the AKT/mTOR pathway in response to the synergistic effects of metformin and chemotherapy.

Results We found that ovarian cancer patients treated with metformin had significantly longer overall survival than did patients treated without metformin. The gene profile induced by metformin in ovarian cancer predominantly involved the AKT/mTOR pathway. Micromolar concentrations of metformin alone and in combination with chemotherapy also reduced cell viability via the AKT/mTOR pathway in vitro and in vivo . Both neoadjuvant and concurrent therapy protocols showed good synergistic effects.

Conclusions This study shows that metformin at clinically relevant dosages is efficacious in treating ovarian cancer, and the results can be used to guide clinical trials.

Background

Ovarian cancer is the fifth leading cause of mortality in developed countries [1]. In the United States, an estimated 22,240 women were diagnosed with ovarian cancer in 2018, and 14,070 deaths due to ovarian cancer occurred [2]. Complete cytoreductive surgery followed by standard first-line platinum-taxane chemotherapy has been shown to improve the survival rate. However, the majority of patients experience relapse, and the 5-year survival rate is approximately 45% [3]. Chemoresistance to platinum-based treatment remains a major challenge in the successful treatment of ovarian cancer [4], and the mechanisms underlying platinum resistance are multifactorial. Various cellular processes are observed in resistant cells, and activation of the PI3K/AKT pathway is believed to be a determinant of resistance in ovarian cancer [5, 6]. Thus, the development of an improved treatment to overcome acquired resistance in cancer cells or decrease the side effects of platinum-based treatment is needed to fight ovarian cancer.

Metformin (N0,N0-dimethylbiguanide), a biguanide, is an oral hypoglycemia agent that is widely used as an antidiabetic drug to treat type 2 diabetes mellitus (DM); it is also widely used to treat polycystic ovarian syndrome [7]. Metformin has been shown to reduce cancer development in type 2 DM patients and inhibit growth in several cancer models [8, 9] either alone or in combination with cytotoxic agents [10, 11]. The major target of metformin in cancer cells is the tumor suppressor LKB1/AMP-activated protein kinase (AMPK) pathway, which serves as a metabolic checkpoint to arrest cell growth when intracellular ATP levels are low, such as in nutrient-poor conditions [12]. After activating AMPK, metformin

phosphorylates tuberous sclerosis complex 2 (TSC2) and then binds with its obligate partner TSC1. TSC2 leads to the accumulation of Rheb–GDP and the inhibition of mTORC1, which influence eukaryotic translation initiation factor 4e-binding protein 1 (4eBP1) and ribosomal S6 kinase (S6K1), respectively. Shank et al. [13] showed that metformin can restrict the growth and proliferation of ovarian cancer stem cells. Yasmeen et al. [14] revealed that metformin induces apoptosis in ovarian cancer cell lines in an AMPK-independent manner by activating caspases 3/7, downregulating Bcl–2 and Bcl-xL expression, and upregulating Bax and Bad expression, which resulted in cell cycle arrest in the S and G2/M phases. Rattan et al. [15] identified metformin as an antiproliferative therapeutic that can act through both AMPK-dependent and AMPK-independent pathways; via these pathways, metformin inhibited cell proliferation in both wild-type and AMPK null mouse embryo fibroblasts as well as in AMPK-silenced ovarian cancer cells. In addition, metformin has been shown to inhibit PI3K/AKT/mTOR signaling in lung cancer [16, 17], breast cancer [18], pancreatic cancer [19], and hepatic cancer [20].

However, most studies showing that metformin alleviates cancer have used higher doses *in vitro* than those used in human body for diabetic patients [8]. These high concentrations may directly cause the death of tumor cells. In the present study, we tested a micromolar concentration as the effective dose, which was a clinically relevant dose. The effects of micromolar metformin on AKT/mTOR signaling in ovarian cancer remain unclear.

The aim of the present study was to examine the effects of a combination of metformin at clinically relevant dosages and chemotherapy on ovarian cancer via the AKT/mTOR pathway. We found that metformin reduced ovarian cancer death in two clinical datasets and predicted that the effect of metformin in ovarian cancer was mediated by the AKT/mTOR pathway using a bioinformatics model. Then, we demonstrated that the micromolar concentration of metformin inhibited the growth of a mouse ovarian surface epithelial cell line (MOSEC), a mouse ovarian cancer cell line and a human ovarian cancer cell line and that it had a synergistic effect in combination with chemotherapy via the AKT/mTOR pathway both *in vitro* and *in vivo*. Both neoadjuvant and concurrent application of metformin with carboplatin in chemotherapy protocols yielded beneficial synergistic effects in ovarian cancer. The results provide insight into the potential of metformin to augment the efficacy of existing cancer therapeutics.

Methods

Patient Samples from Taipei Veteran General Hospital Medical Center

A total of 797 patients were diagnosed with primary ovarian cancer in the Department of Gynecology and Obstetrics, Taipei Veteran General Hospital, from 1995 to 2012. After a review of the patients' clinical and drug histories, 737 patients who underwent complete surgery and were treated with platinum-based therapy plus paclitaxel were included for analysis. Of these patients, 32 were identified as having taken metformin, either during admission or in the outpatient clinic. The overall survival (OS) was measured from the date of diagnosis to death or was censored at the date of the last follow-up. All documents were collected under protocols approved by the institutional review board of the hospital.

Patient Samples from the National Health Insurance Taiwanese Dataset

The reimbursement data of Taiwanese female patients with a new diagnosis of type 2 DM between 2000 and 2010 (n = 38,886) were retrieved from the National Health Insurance database. Among these patients, none used only insulin or only metformin. Therefore, we compared two groups: (1) those who received metformin and insulin (n = 24,033) and (2) those who received neither metformin nor insulin (n = 14,853). Then, we followed the two groups for newly diagnosed ovarian cancer from 2000 to 2011. Thirty-seven patients across the two groups were diagnosed with ovarian cancer.

Microarray Analysis

The microarray experiments were conducted following the L1000 Operating Procedure (L1000 SOP) [21]. Briefly, the human ovarian cancer cell line ES-2 was left untreated (control) or treated with micromolar concentrations of metformin (0.5 mM), 50 μ M carboplatin, or a combination in a microplate. After 6 hours of drug treatment, the medium was removed, and lysis buffer was added (included in the L1000 kit) to the wells for 30 minutes. After cell lysis, the lysate was stored at 80°C for at least one night before being transferred to a 384-well plate, which was performed using the protocol available at http://s3.amazonaws.com/support.lincsccloud.org/protocols/data_generation/L1000_SOP.pdf. Gene expression profiles were detected by L1000 array technology. Up and down probesets were selected by performing two sample t-tests; genes with expression differences significant at a p value < 0.01 and with fold changes > 1.5-fold were included. The *up and down* probesets were input into GSEA software for analysis and to interpret the transcriptional profile data of the four groups by GSEA methods [22, 23].

Analysis of Ovarian Cancer in the Cancer Genome Atlas (TCGA) Genomics Data

Clinical data and protein expression data of ovarian cancer from TCGA were downloaded from the cBioPortal website (<http://www.cbioportal.org/>) [24, 25]. Patients in the ovarian cancer (cBioPortal TCGA, provisional, ovarian cancer genomics, n = 606) dataset were categorized into low and high protein expression groups by a half-division approach. These two groups of patients were input as “User-defined Case List” to assess the total and phospho-protein levels, as evaluated by RPPA z-score, of ± 0 , including key proteins involved in the AKT/mTOR pathway and AMPK. Kaplan-Meier analyses were performed to assess the correlations among the indicated proteins (AKT [total and pSer473], mTOR [total and pSer2448], AMPK [total and pThr172]).

Cell Lines, Cell Culture, Chemicals, and Antibodies

The MOSEC line was a kind gift from Dr. Honami Naora (The University of Texas MD Anderson Cancer Center). Stable MOSEC lines were generated as previously described [26]. The human ovarian cancer SKOV3 and clear-cell ES-2 cell lines were provided by Dr. Gordon Mills (The University of Texas MD Anderson Cancer Center) and Dr. Patrice Morin (National Institute on Aging, Baltimore, Maryland, USA), respectively. The MOSEC lines were cultured in DMEM medium, and the SKOV3 and ES-2 cell lines were cultured in McCoy's 5A medium [27]. All cell culture reagents used were obtained from Invitrogen (Thermo

Fisher Scientific Inc., Waltham, MA, USA). Metformin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMEM containing 10% fetal bovine serum (FBS) at the indicated concentration. Carboplatin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in water and diluted in DMEM to various concentrations. Primary antibodies against AMPK α , phospho-AMPK α (Thr172), AKT, phospho-AKT (Ser473), mTOR, phospho-mTOR (Ser2448) S6, phospho-S6 (Ser235–236), 4EBP1, phospho-4EBP1 (Thr37/46) and β -actin were obtained from Cell Signaling Technology (Danvers, MA, USA). All other chemicals were purchased from Sigma-Aldrich (MO, USA).

In Vitro Cell Viability Assays and Cell Proliferation Assay

To assay cell viability following 0.5 mM metformin treatment, we seeded MOSECs in 12-well plates (2×10^4 per well), cultured the cells for 1 to 5 days in medium containing 0.2% FBS, and collected and stained the cells with trypan blue for quantification at different time points. Day 0 represents the day of treatment. Cell proliferation was measured with the MTT assay [28] or the sulforhodamine B (SRB) assay [29]. Briefly, MOSECs and human ovarian cancer cells (ES-2 and SKOV3) were seeded in 96-well plates and treated with different concentrations of metformin, carboplatin or both for the indicated times. The results were analyzed as described by Chou [30] using the CompuSyn program downloaded from <http://www.combosyn.com/>. The IC₅₀ values for each drug were determined by interpolation from the dose–response curves. The resulting combination index (CI) is a quantitative measure of the degree of interaction between different drugs. CI = 1 denotes additivity; if CI > 1, it denotes antagonism; and if CI < 1, it denotes synergism. For interpretation, the combination was plotted as the log₁₀(CI) versus the fraction affected (Fa; defined as 1–survival fraction). On these plots, additivity was defined as log(CI) = 0, synergy was defined as log₁₀(CI) < 0; and antagonism was defined as log₁₀(CI) > 0. All of the results were experimentally reproducible.

Western Blotting Analysis

Cancer cells were treated with control vehicle, metformin, carboplatin, or a combination for 48 hours, and the cells were pelleted by centrifugation and rinsed with PBS. The cell pellets were then lysed in RIPA buffer followed by sonication. Lowry assays (Bio-Rad) were performed to determine the protein concentration. Equal amounts of protein were loaded in each lane and resolved by 10% to 12% gradient Bis-Tris gels. All western blot analyses were performed using whole-cell lysates prepared as described above. SDS-PAGE and western blotting were performed using standard methods.

Tumor Xenografts in a Mouse Model

C57BL/6 (B6) mice (4 weeks of age) were purchased from Taiwan National Laboratory Animal Center and LASC0 laboratory. The research protocol was approved, and the mice were maintained in accordance with the Institutional Guidelines of Taipei Medical Center and Taipei Veteran General Hospital. MOSECs (1×10^6 cells) were subcutaneously injected into the right flank of B6 mice (6 weeks of age). One week post-injection, mice were randomly divided into 4 groups: a control group, an oral metformin (150 mg/kg once per day) group, an IP carboplatin (30 mg/kg twice a week) group, and a combined-treatment

(metformin+carboplatin) group. There were 5 mice per group (20 total). Drugs were applied one week after tumor injection, which was designated week 0 in all groups. Tumor length and width were measured using a caliper, and tumor volume was calculated using the following formula: volume = $[\text{length} \times \text{width}^2]/2$. The change in tumor size is expressed as the fold change in tumor volume. The fold change in tumor size each week was calculated as follows: fold change in tumor size = (week)n/tumor size initial (week 1). At the end of the experiment, the mice were sacrificed, and tumor samples from each group were collected for western blotting analysis.

Statistical Analysis

Statistical analysis was carried out using the PASW package (PASW Statistics V18, Chicago, IL, USA). Survival analysis was based on the Kaplan–Meier method. Comparisons of clinical characteristics between two groups were performed by Student's t-test, Chi-square test or Fisher's exact test. Comparisons between survival curves were performed using the log-rank or Breslow test. Comparisons of relative fold-changes in tumor cell survival among different treatment groups were performed by 2-way ANOVA with Bonferroni post-tests. A value of $p < 0.05$ was considered statistically significant.

Results

The Effect of Metformin on Survival in Ovarian Cancer Patients

We investigated the impact of metformin on human ovarian cancer by analyzing a clinical dataset. In total, 797 patients were diagnosed with primary ovarian cancer in the Department of Gynecology and Obstetrics, Taipei Veteran General Hospital, from 1995 to 2012. After their clinical and drug histories were reviewed, 737 patients who underwent complete surgery and were treated with carboplatin were included for analysis. Thirty-two of these patients took metformin either during admission or in the outpatient clinic. The clinical characteristics of the 737 patients are listed in Table 1. OS was measured from the date of diagnosis to death or was censored at the date of the last follow-up. The OS of patients with metformin treatment ($n = 32$) was significantly higher than that of patients without metformin ($n = 705$) ($p = 0.03$) (Fig. 1a). Figure 1b shows the ovarian cancer-free incidence of female DM patients ($n = 24,033 + 14,853$) from the National Health Insurance Taiwanese Dataset. Ovarian cancer was less frequent among metformin(+)/insulin(+) users ($n = 24,033$) than among metformin(-)/insulin(-) users ($n = 14,853$) ($p = 0.034$). The use of metformin or insulin may help prevent ovarian cancer in female DM patients. We next investigated the cellular effects of metformin on ovarian cancer. The expression profiles of differentially expressed genes in response to treatment with metformin, chemotherapy or both were retrieved from the L1000 study. GSEA was performed using the up and down gene expression datasets from L1000. GSEA revealed that the gene expression induced by control and metformin treatment was similar to that of the KEGG pancreatic cancer pathway (http://www.genome.jp/dbget-bin/www_bget?pathway+hsa05212) (Additional File 1: Figure S1), which predominantly involves the AKT/mTOR pathway. By proteomic investigation, metformin may involve the alternation of phosphorylation in the AKT/mTOR pathway in ovarian cancer cells, without affecting the total amount of protein (Fig. 1c). We

further investigated the clinical role of the AKT/mTOR pathway using TCGA ovarian cancer dataset. The patients with upregulated protein expression of AKT_pSer473 or mTOR_pSer2448 had significantly poor OS; the expression of total protein was not associated with clinical importance in ovarian cancer (Fig. 1d, Additional File 2: Figure S2). These findings highlight the value of metformin in inhibiting ovarian tumor cells via the phosphorylation of the AKT/mTOR pathway, which indeed plays an essential role in the prognosis of ovarian cancer.

Table 1. The clinical characteristics of the study population

Characteristic	No metformin use n=705 (96%)	Metformin use n=32 (4%)	p value
Diabetes rate [%]	19 (2.7%)	32 (100%)	<0.001 ***
Stage ^{a%}			
Early (I~II)	339 (51.1%)	16 (59.3%)	0.3168
Late (III~IV)	324 (48.9%)	11 (40.7)	
Histology ^{b%}			
Epithelial	542 (81.1)	24 (77.4)	0.5958
Other types ^c	126 (18.9)	7 (22.6)	
CA-125 ^{d#}	1250±273	767±565	0.4109
Death rate [%]	239 (33.9%)	5 (15.6%)	0.0045 **
Overall survival ^{eS}	57.3±4.2	69.7±22.5	0.0338 *

^aFIGO stage: *International Federation of Gynecology and Obstetrics*, surgical staging of ovarian cancer; missing values not included in the statistical test

^bMissing value not included in the statistical test

^cincluding *primary peritoneal serous carcinoma* (PPSC)

^dbefore surgery, mean±s.d. (U/ml)

^eoverall survival months, mean±s.d.

[%]calculated by *Chi-square test*

[#]calculated by Student's t-test

- calculated by Kaplan-Meier log-rank test

Metformin at a Clinically Relevant Dosage Inhibits Ovarian Cancer Growth through the AKT/mTOR Pathway

The AKT/mTOR pathway is typically involved in cellular proliferation. To mimic the clinically relevant dosages in the human body, we used micromolar concentrations of metformin for the experiment. We calculated the cell growth of metformin in 0.5 mM-treated mouse and human ovarian cancer cell lines to evaluate the growth-inhibitory effect of metformin at clinically relevant doses. As shown in Fig. 2a, 0.5 mM metformin reduced cell growth in both ovarian cancer cell lines after 3~4 days of treatment. We further investigated the targeted pathway after micromolar metformin treatment. Metformin inhibited the AKT/mTOR pathway in a dose-dependent manner (Fig. 2b), as shown by western blot analysis, although our doses were lower than those in other studies. We found that micromolar doses, e.g., 0.25 mM and 0.5 mM, both could influence the AKT/mTOR pathway in ovarian cancer; the effect of 0.5 mM metformin was stronger than that of 0.25 mM. Furthermore, 0.5 mM metformin treatment beginning from day 2 to day 6 reduced cell viability, and cell viability recovered from day 6 to day 8 after discontinuing treatment (Fig. 2c). The inhibitory effect of micromolar metformin on the AKT/mTOR pathway could be shown during metformin treatment (day 2 to day 6). The phospho-protein expression was inhibited during metformin treatment; these expression levels could be restored when suspending metformin (Fig. 2d). The aforementioned data suggested that metformin at micromolar concentrations could inhibit cell growth in ovarian cancer cell lines through inhibition of the AKT/mTOR pathway, as supported by the GSEA.

The Synergic Effects of Metformin and Chemotherapy on Ovarian Cancer In Vitro and In Vivo

Although standard first-line platinum-based protocols improve survival in ovarian cancer, strengthening these chemotherapy regimens is warranted. We evaluated the antiproliferative effects of different doses of metformin alone or in combination with carboplatin. We assessed the growth-inhibitory effects of 0.5 mM metformin alone or in combination with 50 μ M carboplatin (Fig. 3a). As shown in Figure 3a, both metformin (0.5 mM) and carboplatin (50 μ M) inhibited cell viability, and their combination yielded the strongest inhibition. Forty-eight-hour exposure to both metformin and carboplatin (ranging from 5–50 μ M) resulted in a clear synergistic effect, with negative log (CI) values in MOSECs (Fig. 3b) and the human ovarian cancer cell line ES–2 (Additional File 3: Figure S3a). However, carboplatin at higher concentrations (100–1000 μ M) did not show synergism with metformin. We further investigated the *in vivo* antitumor activity of metformin in B6 mice bearing MOSECs that were grown subcutaneously as tumor xenografts. Treatment with metformin and carboplatin as single agents caused a decrease in tumor size relative to that of control untreated mice. Treatment with the combination of metformin and carboplatin significantly reduced tumor growth (Fig. 3c).

The activated forms of AKT are key intracellular mediators of growth, cell survival and platinum response. Determining the activation state of the AKT/mTOR pathway is important for understanding the synergistic mechanism of action of micromolar metformin and carboplatin in ovarian cancer. Western

blot analysis demonstrated that both carboplatin alone and metformin alone reduced the levels of phosphorylated AKT without affecting the total amount of AKT protein (Fig. 3d). The combination of carboplatin and metformin produced a stronger inhibition of pAKT and the AKT downstream effectors pmTOR (Ser2448), pS6 kinase (Ser235/236) and p4E-BP1 (Thr37/46) than did metformin or carboplatin alone. In contrast, the total amounts of mTOR, S6 kinase and 4E-BP1 were unaffected by treatment, and AMPK phosphorylation was not reduced by treatment with metformin or carboplatin, either alone or in combination.

Some DM patients treated with metformin cannot achieve good blood sugar control and require other medications. We investigated the cellular effects of metformin treatment on tumors in patients with poor blood sugar control. As shown in Figure 3e (left panel), MTT assays indicated that cells cultured in high-glucose medium (4500 mg/L) showed accelerated cell proliferation relative to that of cells in control medium (1000 mg/L); metformin treatment could diminish this effect induced by high glucose. The combined effect of carboplatin and metformin in high-glucose medium was antagonistic, with a positive log(CI) (Fig. 3e, right panel). To determine whether high glucose induces the AKT/mTOR pathway in MOSECs, we treated the cells with metformin alone, carboplatin alone or a combination of carboplatin and metformin in high-glucose medium and control medium for 48 hours (Additional File 3: Figure S3b). High-glucose medium resulted in marked increases in pAKT, pmTOR, pS6 kinase and p4E-BP1 levels compared to control medium. Treatment with metformin and carboplatin in high-glucose medium reduced the protein level of pAKT but not the protein levels of pMTOR, pS6 kinase and p4E-BP1 relative to the levels in control medium. These results revealed that high glucose or poor blood sugar control may diminish the antitumor effects of metformin and carboplatin, although they still have effects on pAKT.

The Synergistic Effects of Combination Treatment Under Different Protocols

We further investigated the synergistic effects of low concentrations of metformin and carboplatin under different treatment regimens commonly used in clinical settings, as shown in Figure 4a. Synergistic effects were observed in both protocol 1 (neoadjuvant therapy) and protocol 2 (concurrent therapy) but not in protocol 3 (adjuvant therapy). Western blot analysis indicated that protocols 1–3 reduced the activities of pAKT, pmTOR, pS6 kinase and p4E-BP1, with protocols 1 and 2 having superior effects compared to protocol 3 (Fig. 4b). These results indicate that metformin should be used before or concurrent with chemotherapy to enhance the antitumor effect.

Schematics of the intracellular effects of treatment with metformin, carboplatin, or their combination on ovarian cancer are shown in Figure 4c. Metformin and carboplatin produced synergistic effects, inhibiting AKT and its downstream pathway. A high-glucose environment, such as that in poorly controlled type 2 DM patients, may increase activated AKT levels. Thus, for patients with poor glucose control, the combination of metformin and carboplatin may be slightly superior to carboplatin alone and not as efficacious as that in patients with good glucose control.

Discussion

Diabetes is strongly associated with an increased incidence of cancer [31]. Many studies have shown that metformin can reduce the risk of cancer, including breast, colon, liver, and pancreatic cancers, and improve outcomes over those obtained with other antidiabetic treatments (sulfonylurea, insulin) in diabetic patients [9]. Whether metformin can reduce the risk of ovarian cancer has been examined [32–34], but few studies have focused on the effects of metformin combined with commonly used first-line chemotherapeutic drugs, such as carboplatin, and the underlying mechanisms [35]. In this study, we evaluated the synergistic effects of carboplatin and micromolar metformin in ovarian cancer via the AKT/mTOR pathway both *in vitro* and *in vivo* and found that poor glucose control diminished the synergistic, antitumor effects of combination treatment.

A previous case-control study that used the UK-based General Practice Research Database [33] revealed that the adjusted odds ratio of metformin use vs. non-use for ovarian cancer incidence was not significant in non-diabetic patients but was significant in diabetic patients. In another recent meta-analysis, which included one observational study and two clinical trials, the pooled odds ratio (95% CI) of metformin use for ovarian cancer incidence was 0.67 (0.44–1.04) [36]. Recently, a study was performed using reimbursement databases of the National Health Insurance (NHI) to evaluate metformin use in Taiwanese women with type 2 DM. Metformin decreased the incidence of ovarian cancer, and the overall fully adjusted hazard ratio (95% CI) for ever-users versus never-users was 0.658 (0.593–0.730). In the present study, the results were similar to those of a previous report [34], in which ovarian cancer was less likely to occur in metformin(+)/insulin(+) users (n = 24,033) than in metformin(-)/insulin(-) users (n = 14,853). This previous study similarly used reimbursement databases of the NHI, although the data were from different years. The hospital cohort data showed that ovarian cancer patients treated with metformin had a significantly longer OS than did patients not treated with metformin. This finding is similar to that of another case-control study, in which an association between metformin use and improved survival of ovarian cancer patients was identified [37]. These results indicated that metformin may reduce ovarian cancer incidence in type 2 DM patients and improve survival after a diagnosis of ovarian cancer.

Most studies examining the effects of metformin on cancer have used doses (1–10 mM) higher than those used clinically for diabetic patients [8, 38], yielding metformin plasma concentrations between 6 and 30 $\mu\text{mol/L}$. Few studies have focused on the impact of metformin at clinically relevant dosages [35]. In the present study, we tested a low concentration of 0.5 mM (500 $\mu\text{mol/L}$) as the effective dose. The cellular events observed *in vitro* suggest that this dose is safe and can be translated to *in vivo* conditions. The dosage of metformin given to mice was 150 mg/kg/day, which is equivalent to 720 mg/day for a 60 kg person according to a formula suggested by the National Institute of Health (U.S. A.) [39]. This equivalent dosage is 3 times lower than the maximum safe dosage of 2550 mg/day recommended in the Physician's Desk Reference.

Metformin activates AMPK via LKB1, which leads to the inhibition of mTOR signaling and its major downstream effectors, the 4E-BPs and p70S6Ks, and the inhibition of global protein synthesis and proliferation in various different cancer cell lines [6, 40]. In previous studies, metformin tested on ovarian

cancer was found to induce cell cycle arrest, apoptosis [41], angiogenesis and decreased pmTOR expression [41], p38 MAPK pathway activity [42] and cancer stem cell activity [43]. In this study, we showed for the first time the effects of metformin on gene expression patterns using GESA and the L1000 system and found that the effects of metformin on gene expression in ovarian cancer are similar to those following activation of the KEGG pancreatic cancer pathway (Additional File 1: Figure S1). In previous studies, following metformin treatment, phospho-AKT levels decreased in two pancreatic cancer cell lines, A549 and PANC1. We analyzed TCGA data and found that the phospho-AKT/mTOR pathway is a determinant of clinical survival in ovarian cancer [5, 6]. We also demonstrated, for the first time, the effects of metformin in combination with carboplatin, a first-line chemotherapy drug, and showed that the synergism of these drugs is due to the inhibition of the AKT/mTOR pathway, which is independent of AMPK at micromolar concentrations of metformin (0.5 mM). These results are consistent with those of Rattan et al. [15], who showed that metformin as an antiproliferative therapeutic can act through both AMPK-dependent and AMPK-independent pathways. Metformin can be a “very-very-very” cheap drug compared with AKT/mTOR inhibitors, and it may also have antitumor effects. Knowledge of this mechanism may be useful in clinical trials to adjust the dosage of platinum-based therapy or further overcome the chemoresistance to platinum in the future.

Metformin can be added at different times (before, during and after adjuvant chemotherapy), and the effects of these addition time points need to be investigated. In the present study, a synergistic effect ($CI < 1$) was observed in the neoadjuvant (protocol 1) and concurrent (protocol 2) protocols but not in protocol 3 (adjuvant). These results are similar to those of Erices et al. [35], although they did not test the adjuvant use of metformin. These results provide cellular evidence that can guide the design of clinical trials. The differences among protocols observed in this study may be related to the cytotoxic effects of metformin on cancer stem cells [43], which can enhance the efficacy of neoadjuvant and concurrent chemotherapy by preventing the establishment of chemoresistant clones.

Some patients treated with metformin continue to show poor blood sugar control, and high blood glucose may diminish the antitumor effect of metformin. Karnevi et al. [19] reported that metformin can significantly reduce the proliferation of several pancreatic cancer cell lines under normal glucose conditions; however, they found that hyperglycemia reduced metformin-induced growth inhibition by enhancing the IGF-I response and activating AKT, which stimulated AMPK-Ser485 phosphorylation and impaired AMPK-Thr172. Zhuang et al. [44] obtained similar results in breast cancer and ovarian cancer cell lines, reporting that cancer cells became less responsive to metformin when glucose was increased to 10 mM. In a breast cancer cell line, under low-glucose conditions, metformin significantly decreased the phosphorylation of AKT and various targets of mTOR, whereas phospho-AMPK was not significantly altered. In the present study, we used an ovarian cancer cell line and demonstrated that high-glucose medium decreased the response to metformin. The synergistic effects of carboplatin and metformin were abolished. The phosphorylation of AKT in low-glucose conditions (1000 mg/L) was substantially reduced by metformin, and phosphorylation levels of targets of mTOR (S6K and 4EBP1) were decreased relative to those in high-glucose conditions (25 mM). In ovarian cancer, phospho-AMPK was not significantly altered. The response to metformin was substantially altered in low-glucose conditions. Based on

previous studies and our observations, we hypothesize that high glucose fuels glycolytic metabolism, which maintains cellular ATP levels when metformin blocks mitochondrial function. When glucose is limiting, cancer cells lack sufficient fuel to maintain glycolytic metabolism. Additionally, mTOR signaling is blocked in an AMPK-independent manner, enhancing metabolic deficiency. Cellular ATP is depleted, leading to energy collapse and cell death [44].

Conclusions

In conclusion, metformin treatment of patients with ovarian cancer may have antitumor effects and synergistic effects when used in combination with carboplatin through the AKT/mTOR pathway. Future prospective clinical trials in patients with ovarian cancer are required to investigate the beneficial effects of metformin in augmenting the efficacy of existing cancer therapeutics.

Abbreviations

DM: Diabetes Mellitus; AMPK: AMP-activated Protein Kinase; TCGA: The Cancer Genome Atlas; OS: Overall Survival; GSEA: Gene Set Enrichment Analysis; MOSEC: Mouse Ovarian Surface Epithelial Cell Line; TSC2: Tuberous Sclerosis Complex 2; 4eBP1: 4e-Binding Protein 1; S6K1: Ribosomal S6 Kinase; L1000: L1000 Operating Procedure.

Declarations

Ethics Approval and Consent to Participate

To collect patient data and clinical characteristics, we sent the IRB application form for the protocol approved by the Ethics and Research Committee of the Taipei Veterans General Hospital (VGH 9032IC).

Consent for Publication

Not applicable.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no competing interests.

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Authors' Contributions

KCW, PLS and MHL contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript. AW contributed to the animal study. PCC, JHL, PHW and CYH supervised the findings of this work. MHL and SCY contributed to the research, analysis and writing of the manuscript.

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Figures

Figure 1

The effect of metformin on survival in clinical ovarian cancer patients. a Kaplan–Meier OS of ovarian cancer patients with (n=32) or without (n=705) metformin use. b The ovarian cancer-free incidence in female DM patients: metformin(+) (ever used)/insulin(+) (ever used) users (n=24,033) and metformin(-) (never used)/insulin(-) (never used) users (n=14,853) from the National Health Insurance Taiwanese Dataset. c Western blot analyses of the indicated proteins in the AKT/mTOR pathway of mouse ovarian cancer cells treated with different concentrations of metformin. d Comparison of the death rates between groups with low and high protein expression by the half-division approach. Kaplan-Meier analysis assessed the correlations of the indicated proteins (AKT [total and pSer473], mTOR [total and pSer2448], AMPK [total and pThr172]) with the overall survival of patients; data from the cBioPortal TCGA database

(TCGA Provisional, ovarian cancer genomics, n=606). A log-rank p-value less than 0.05 indicated a significant difference in overall prognosis (*p<0.05, **p<0.01, ***p<0.001).

way ANOVA. b Western blot analyses of the indicated proteins in the AKT/mTOR pathway of cells treated with different micromolar metformin. c and d Cell viability and protein analyses of the AKT/mTOR pathway under treatment with micromolar metformin from day 2 to day 6 and when treatment was suspended from day 6 to day 8.

Figure 3

The Synergic Effects of Metformin and Chemotherapy on Ovarian Cancer In Vitro and In Vivo. a The inhibitory effects of micromolar metformin combined with carboplatin on cell viability. *: $p < .05$, by two-way ANOVA. b Synergic effects of metformin combined with carboplatin at different concentrations. c MOSECs were injected into B6 mice ($n=20$), which were divided into 4 groups, and subcutaneous tumor size was measured after different treatments (control, metformin or carboplatin alone, combination). Subcutaneous tumors at the end of the experiment. d The effects of carboplatin alone, metformin alone, or combined treatment assessed by the indicated antibodies in western blot analysis. e MTT assays showed that cells cultured in high-glucose medium (4500 mg/L) exhibited greater growth than did those in control medium (1000 mg/L). Blue squares represent cells cultured in high-glucose medium, and red solid circles represent those cultured in control medium.

Figure 4

The synergistic effects of combination treatment under different protocols. a and b Protocol 1: a neoadjuvant protocol, MOSECs treated with metformin alone for 1 day and then with a combination of metformin and carboplatin for 2 days. Protocol 2: a concurrent protocol, MOSECs treated with both metformin and carboplatin from day 2 for 2 days. Protocol 3: an adjuvant protocol, MOSECs treated with carboplatin alone from day 1 for 1 day and then with a combination of metformin and carboplatin for 1

day. Black unfilled circles represent $\log(\text{CI})$ values under protocol 1, blue squares represent those under protocol 2, and red circles represent those under protocol 3. c The model of synergistic inhibitory effects by micromolar concentration of metformin combined with carboplatin in the AKT pathway.

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