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RESEARCH ARTICLE

Assessing the antitumor effects of metformin on ovarian clear cell carcinoma

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Abstract

Developing novel therapies that outperform the existing chemotherapeutic treatments is required for treatment-resistant ovarian clear cell carcinoma. We investigated the antitumor effect of metformin on ovarian clear cell carcinoma, enhancement of the antitumor effect by its combination with chemotherapy, and its molecular regulatory mechanism. First, we evaluated the viability of ovarian clear cell carcinoma lines using the water-soluble tetrazolium-1 assay and found that metformin suppressed cell viability. Cell viability was significantly suppressed by co-treatment with cisplatin and metformin. In contrast, co-treatment with paclitaxel and metformin showed no significant difference in viability compared with the group without metformin. Western blot analysis showed increased phosphorylation of AMP-activated protein kinase in some cell lines and suppressed phosphorylation of the mammalian target of rapamycin in a particular cell line. Flow cytometry analysis revealed a significant increase in the rate of apoptosis in the metformin-treated group and rate of cell cycle arrest at the G2/M phase in a particular cell line. These results indicated that metformin may be effective against cultured ovarian clear cell carcinoma cells, particularly in combination with cisplatin.

Keywords AMP-activated protein kinases · Apoptosis · Cell cycle checkpoints · Carcinoma · Metformin

Introduction

Ovarian cancer (OC) is the leading cause of death among gynecological cancers and the fourth leading cause of cancer-related deaths among women in developed countries [1]. Owing to the lack of symptoms in the early stages of OC and the difficulty in early detection, approximately 40% of patients are diagnosed only at advanced stages (stage III and IV) [2]. Ovarian clear cell carcinoma (OCCC) is a more common histological type in Asians, accounting for less than 10% of OCs in the West [3]. In particular, OCCC accounts for approximately 25% of OCs in Japan [4]. Patients with advanced OC are initially treated with tumor-reducing surgery, followed by a combination of standard chemotherapy with platinum agents and paclitaxel. However, because OCCC is chemotherapy-resistant, its prognosis is worse than that of other histologic types. Data from the US showed that the median survival of patients with other histologic types of advanced OC was 45 or 56 months, whereas that of patients with OCCC was 24 months [5]. Therefore, developing novel therapies for OCCC that exceed the efficacy of the existing therapies and overcome chemotherapy resistance is required.

Metformin (1,1-dimethylbiguanide) is an oral biguanide that has been used as a first-line treatment of type II diabetes for decades. Beginning with a report by Evans et al. [6] in 2005, it became clear that patients with diabetes mellitus taking metformin are less likely to have cancer. Several meta-analyses have reported that metformin may reduce the overall cancer incidence by 10–40% and cancer-specific mortality [6–10]. This inhibitory effect on carcinogenesis has also been observed in patients without diabetes mellitus [11], with some reports showing a reduced risk of developing various carcinomas [12–15]. Metformin has also been reported to enhance sensitivity to chemotherapy in basic and animal research [16–21]. The mechanism of inhibition of tumors by metformin is currently under investigation. This mechanism is thought to include cell cycle arrest, induction of apoptosis, inhibition of cell proliferation, and inhibition of

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angiogenesis via phosphorylation of AMP-activated protein kinase (AMPK; or downstream antiphosphorylation of mammalian target of rapamycin [mTOR]) in cancer cells [22, 23].

The antitumor and synergistic effects of metformin with chemotherapy have also been reported for OC [24–30]. However, in vitro studies have mainly focused on ovarian serous carcinoma and little is known about its effect on OCCC. If metformin can be shown to have a growth-inhibitory effect on OCCC, or if it can be shown to enhance the antitumor effect in combination with antitumor drugs in OCCC, which is resistant to drug therapy, it could be used for future therapeutic strategies. Therefore, we investigated the antitumor effects of metformin on OCCC cell lines, its enhancement in combination with chemotherapy, and its molecular regulatory mechanisms.

Materials and methods

Reagents and antibodies

Fetal bovine serum (FBS) was purchased from Corning Inc. (Corning, NY, USA). Ham's F12 culture medium, RPMI 1640 culture medium, penicillin–streptomycin, 0.25% trypsin–EDTA solution, and WST-1 reagent were purchased from Sigma–Aldrich (St. Louis, MO, USA). Metformin was purchased from Cayman Chemical (Ann Arbor, MI, USA). Cisplatin was purchased from Tokyo Kasei Kogyo (Chuo-ku, Tokyo, Japan). Paclitaxel was purchased from FUJIFILM Wako Pure Chemicals Co (Osaka-city, Osaka, Japan). APC-Annexin V Apoptosis Detection Kit with 7-AAD (Cat. No. 640930), a reagent used for apoptosis evaluation by flow cytometry (FCM), was purchased from BioLegend (San Diego, CA, USA). The Cell Cycle Assay Solution Deep Red (C548), a reagent used for cell cycle evaluation in FCM, was purchased from Dojin Chemical Co. (Mashiki, Kumamoto, Japan). The antibodies used for western blotting: a mouse monoclonal IgG Actin antibody (sc-47778), a mouse monoclonal IgG mTOR antibody (sc-517464), a mouse monoclonal IgG p-mTOR antibody (Ser-2448:sc-293133), a mouse monoclonal IgG AMPK alpha 1/2 antibody (sc-74461), a goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (sc-2004), and a goat anti-mouse IgG conjugated with HRP (sc-2005) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). A rabbit monoclonal IgG p-APMK antibody (#2535) was purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell line

Human OCCC-derived cell lines, RMG-I and OVISE, were purchased from the JCRB Cell Bank (Ibaraki, Osaka, Japan). RMG-I cells (JCRB0172, rot: 07052011) were cultured in Ham's F12 medium supplemented with 10% FBS and antibiotics (penicillin, 50 U/ml and streptomycin, 50 μg/ml) at 37 °C and 5% CO_2. OVISE cells (JCRB1043, rot; 03022020) were cultured in RPMI 1640 medium supplemented with the same composition of FBS and streptomycin. Cell cultures were passaged every 3–5 days.

WST-1 assay

For the WST-1 assay, which reflects cell viability, was performed by seeding 100 μl of RMG-I and OVISE at a concentration of 2.0×10^4 cells/well in 96-well plates. After culture for 1 day, the medium was changed and metformin (1–100 mM) was added for 48 h. Then 10 μl water-soluble tetrazolium-1 (WST-1) reagent was added to each well and absorbance was measured after 1 h using an i Mark microplate reader (BIO-RAD, Hercules, CA, USA).

For co-treatment with antitumor drugs (cisplatin and paclitaxel), RMG-I and OVISE were similarly spread in 96-well plates at a concentration of 2.0×10^4 cells/well in 100 μl. After 1 day of culture, metformin (1 mM) and antitumor drugs (Cisplatin: 5 or 10 μg, Paclitaxel: 10^{-7} or 10^{-6} M) were added after changing the medium. After 4 h, the cells were cultured in medium without antitumor drugs for 44 h (metformin group continued to be incubated with metformin). Thereafter, absorbance was measured in the same manner as described above. Eight samples were used in all experiments. Additionally, the absorbance was evaluated as a relative value divided by the mean absorbance of the control.

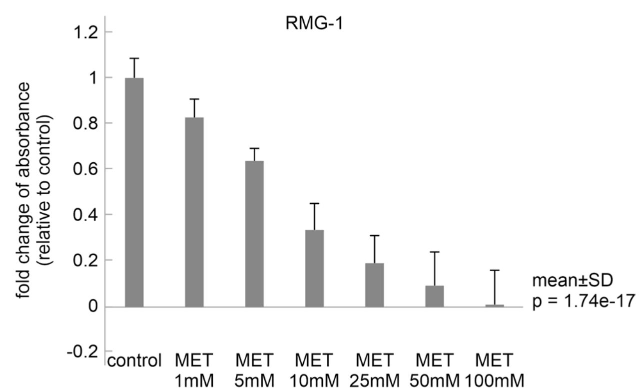


Fig. 1 Effect of metformin on survival in RMG-I. RMG-I cells are cultured with metformin at concentrations ranging between 1 and 100 mM and a non-added group (control) for 48 h. WST-1 reagent is added, and absorbance is measured after 1 h. The mean absorbance of the control group is set to 1. The average absorbance of the non-supplemented group is set to one and plotted. Metformin decreases RMG-I cell viability in a concentration-dependent manner ($*p = 1.74e-17$). Each value is shown as the mean ± SD of eight samples. Abbreviations: *MET* metformin, *WST-1* water-soluble tetrazolium-1

Western blot (AMPK, p-AMPK and mTOR, p-mTOR)

RMG-I and OVISE were spread in 6-well plates at a concentration of 2.0×10^5 cells/well. After 1 day of culture, the medium was changed; metformin (1 mM) was added to the metformin-incubated group. Thereafter, the cells were cultured for 48 h. The cultured cells were washed twice with ice-cold PBS and lysed in lysis buffer. Insoluble substances were removed by centrifugation at 15,000×*g* for 2 min at 4 °C. To 30 µL of the supernatant, 4 × Laemmli buffer containing 200 mM dithiothreitol was added and heated at 95 °C for 5 min. The extracted proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes, which were then incubated with 0.1% Tween-20 and 3% bovine serum albumin for 1 h at 25 °C. After blocking with Tris-buffered saline (10 mM Tris and 140 mM NaCl, pH 7.4), the membranes were incubated with primary antibodies—AMPK and p-AMPK antibodies (1:1000), mTOR and p-mTOR(Ser-2448) antibodies (1:1000)—at 4 ℃ overnight. The target proteins were then reacted with secondary antibodies and visualized using enhanced chemiluminescence (Immobilon Western, Millipore). Images were captured using the LAS 4000 system (GE Healthcare, UK). Images were analyzed using ImageJ software (version 1.41, NIH, MD, USA).

Flow cytometry (cell cycle evaluation)

RMG-I and OVISE were spread in 10-ml plates at a concentration of 1.0×10^5 cells/well. After 1 day of culture, the medium was changed and metformin (1 mM) was added to the metformin-incubated group for 24 h. Cultured cells were washed twice with ice-cold PBS; thereafter, 2 ml of 0.25% trypsin solution was added and incubated at 37 ℃ for 3 min. After adding 8 ml of culture medium, the cells were transferred to 15 ml centrifuge tubes and centrifuged at 1000 rpm for 5 min. To the aspirated supernatant pellet, 0.5 ml of ice-cold PBS was added, Cell Cycle Assay Solution Deep Red was added and mixed, and the pellet was incubated in the dark at 37 ℃ for 15 min. The samples were then filtered through a 40 µm cell strainer (pluriStrainer mini 43-10040: pluriSelect, Deutscher, Leipzig, Germany). Measurements were performed using the BD FACSLyric system (BD Biosciences, Becton Drive, NJ, USA). Data were analyzed using BD FlowJo (version 10) software (BD Biosciences).

Flow cytometry (evaluation of apoptosis)

RMG-I and OVISE were spread in 10-ml plates at a concentration of 1.0×10^5 cells/well. After 1 day of culture, the medium was changed and metformin (1 mM) was added to the metformin-fed group for 4 h. Pellets were obtained as described for the cell cycle evaluation. To the aspirated supernatant pellet, 100 µl of Annexin-V buffer in the APC-Annexin V Apoptosis Detection Kit with 7-AAD, mix was added; then 5 µl of each of APC-Annexin V and 7-AAD viability staining solution was added. The mixture was left in the dark at 25 ℃ for 15 min, 400 µl of Annexin-V buffer was added, mixed, and filtered through a 40 µm cell strainer (pluriStrainer mini). The measurements were performed using a BD FACSLyric. BD FlowJo was used for data analysis.

Statistical analysis

All values are expressed as the mean ± standard deviation (SD). The Jonckheere–Terpstra test was used to test the concentration-dependent trends among multiple groups. The Wilcoxon signed-rank test was used to compare the cell cycle assessment between the control and control groups. The Mann–Whitney *U* test was used to compare the control and subject groups for the WST-1 assay, apoptosis assessment, and western blotting. All statistical analyses were performed using IBM SPSS Statistics software (version 21, IBM, Tokyo, Japan). A p-value < 0.05 was considered significant difference.

Results

Effect of metformin on survival in RMG-I and OVISE

When RMG-I cells were treated with metformin for 48 h, the absorbance values on the WST-1 assay decreased in a

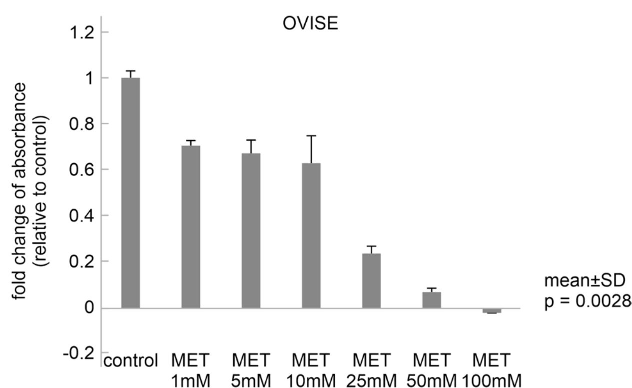
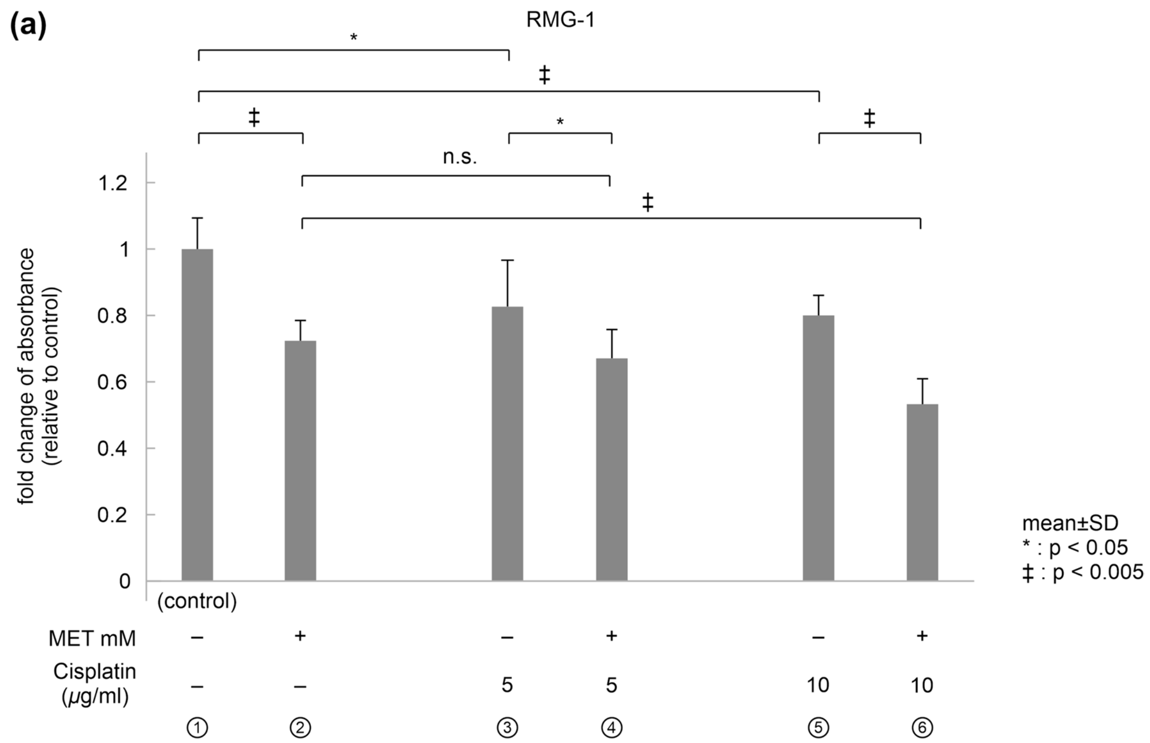
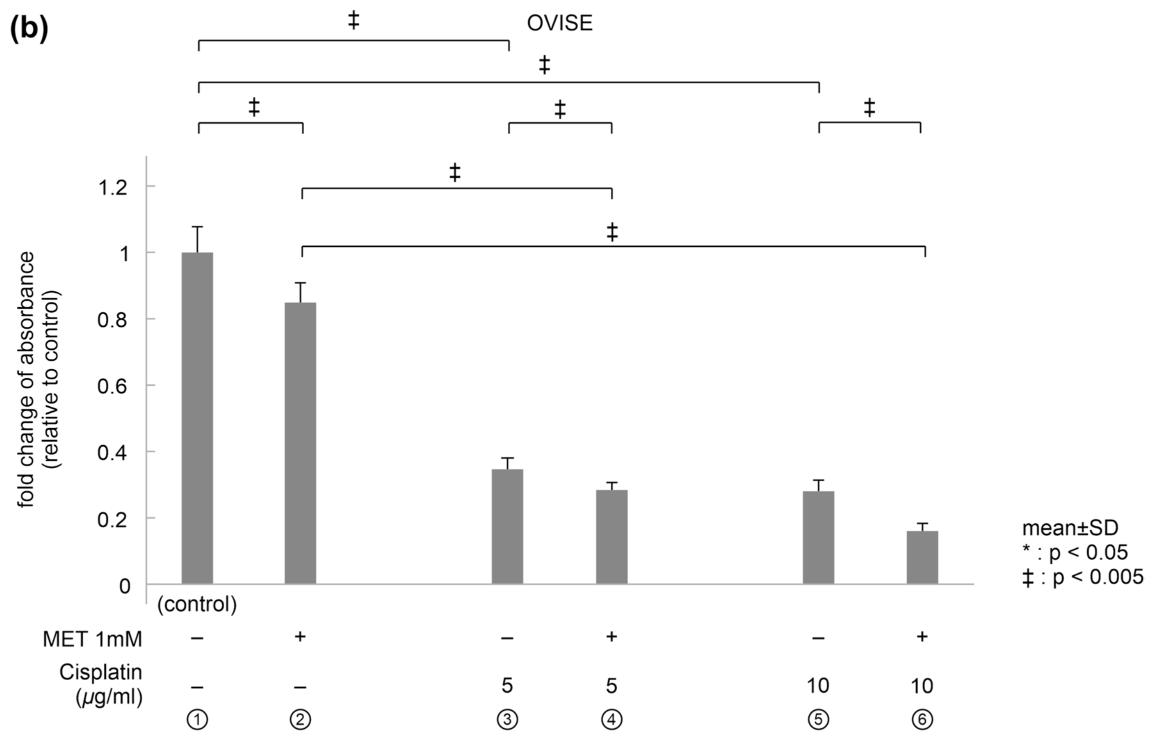


Fig. 2 Effect of metformin on survival in OVISE. OVISE is cultured with metformin at concentrations ranging between 1 and 100 mM and a non-added group (control) for 48 h. WST-1 reagent is added, and absorbance is measured 1 h later. The average absorbance of the non-supplemented group is set to one and plotted. Metformin decreases the survival of OVISE cells in a concentration-dependent manner ($*p = 1.19e-14$). Each value is shown as the mean ± SD of eight samples. Abbreviations: *MET* metformin, *WST-1* water-soluble tetrazolium-1





concentration-dependent manner; cell viability was suppressed ($p = 1.74e - 17$) (Fig. 1). Similar results were obtained with OVISE ($p = 0.0028$) (Fig. 2).

Effect of metformin on survival under cisplatin in RMG-I and OVISE

The addition of cisplatin to RMG-I inhibited cell viability in a concentration-dependent manner, measured using the WST-1 assay. When cisplatin was added at each

◂**Fig. 3** Effect of metformin on survival in RMG-I and OVISE under cisplatin. RMG-I and OVISE are prepared in groups with 5 µg of cisplatin, 10 µg of cisplatin, and no cisplatin, and also in groups with 1 mM of metformin and no metformin. Four hours after the addition of metformin, the cells are incubated for 44 h in medium without cisplatin (the metformin group continues to be incubated with metformin). The WST-1 reagent is added, and the absorbance is measured after 1 h. The mean absorbance of the non-treated group (control) for both drugs is taken as 1, and the relative values are plotted. **a** In RMG-I, the addition of cisplatin inhibited viability in a concentration-dependent manner (*$p < 0.05$, ‡$p < 0.005$). At each cisplatin concentration, survival is significantly suppressed in the metformin and non-metformin groups (*$p < 0.05$, ‡$p < 0.005$). **b** In OVISE, the addition of cisplatin suppresses cell viability in a concentration-dependent manner ($p < 0.005$). At each cisplatin concentration, survival is significantly suppressed in the metformin and non-metformin groups (‡$p < 0.005$). Each value is presented as the mean ± SD of eight samples. Abbreviations: *MET* metformin, *WST-1* water-soluble tetrazolium-1

concentration, cell survival was significantly suppressed in the metformin- and non-metformin-treated groups. The viability was not significantly different between the cisplatin-free and cisplatin (5 µg/ml) groups under metformin treatment. However, the viability was significantly different between the cisplatin-free and cisplatin (10 µg/ml) groups under metformin treatment (Fig. 3a).

Similar experiments were performed in OVISE (Fig. 3b), where the addition of cisplatin suppressed cell viability in a concentration-dependent manner. The viabilities of the metformin-treated and metformin-free groups were significantly different at each cisplatin concentration. The viability was significantly suppressed in the cisplatin-free group compared with the cisplatin (5 µg/ml) and cisplatin (10 µg/ml) groups under metformin treatment.

Effect of metformin on survival under paclitaxel in RMG-I and OVISE

The addition of paclitaxel to RMG-I resulted in concentration-dependent suppression of cell viability. No significant difference in absorbance was observed between the metformin- and non-metformin-treated groups at any paclitaxel concentration. Comparing the paclitaxel-free group with the paclitaxel (10^{-7} M and 10^{-6} M) groups under metformin treatment, survival was significantly suppressed (Fig. 4a). The same experiment was performed using OVISE (Fig. 4b); the addition of paclitaxel suppressed survival in a concentration-dependent manner. No significant difference in absorbance was observed between the metformin- and non-metformin-treated groups at the respective paclitaxel concentrations. Comparing the paclitaxel-free and paclitaxel (10^{-7} M and 10^{-6} M) groups under metformin administration showed that survival was significantly suppressed.

Effect of metformin on AMPK phosphorylation in RMG-I and OVISE

The effect of metformin on AMPK phosphorylation in RMG-I cells was confirmed by western blotting. The density of p-AMPK/AMPK in the metformin-treated group was significantly higher than that in the control group (Fig. 5a, b). Similar experiments were performed using OVISE, and the density of p-AMPK/AMPK in the metformin-treated group was significantly higher than that in the control group (Fig. 5c, d).

Effect of metformin on mTOR phosphorylation (Ser2448) in RMG-I and OVISE

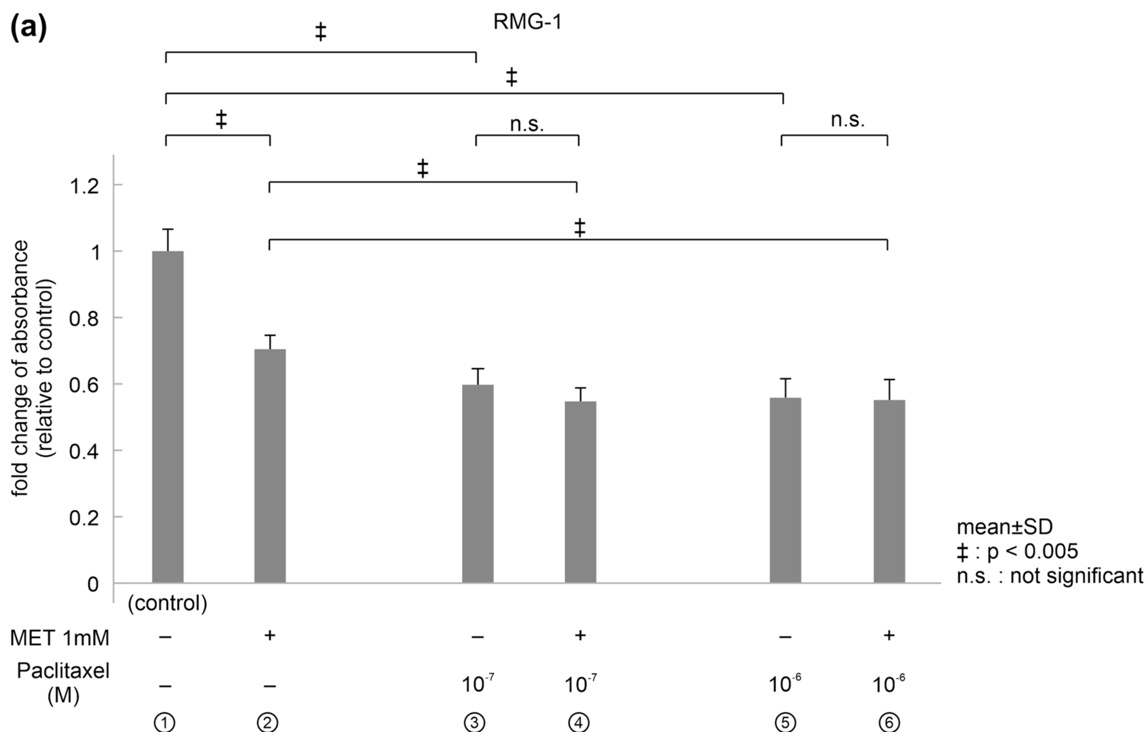
The effect of metformin on mTOR phosphorylation in RMG-I cells was confirmed by western blotting. The density of p-mTOR/mTOR in the metformin-treated group was significantly lower than that in the control group (Fig. 6a, b). However, in OVISE, the density of p-mTOR/mTOR in the metformin-supplemented group were not significantly different from those in the control group (Fig. 6c, d).

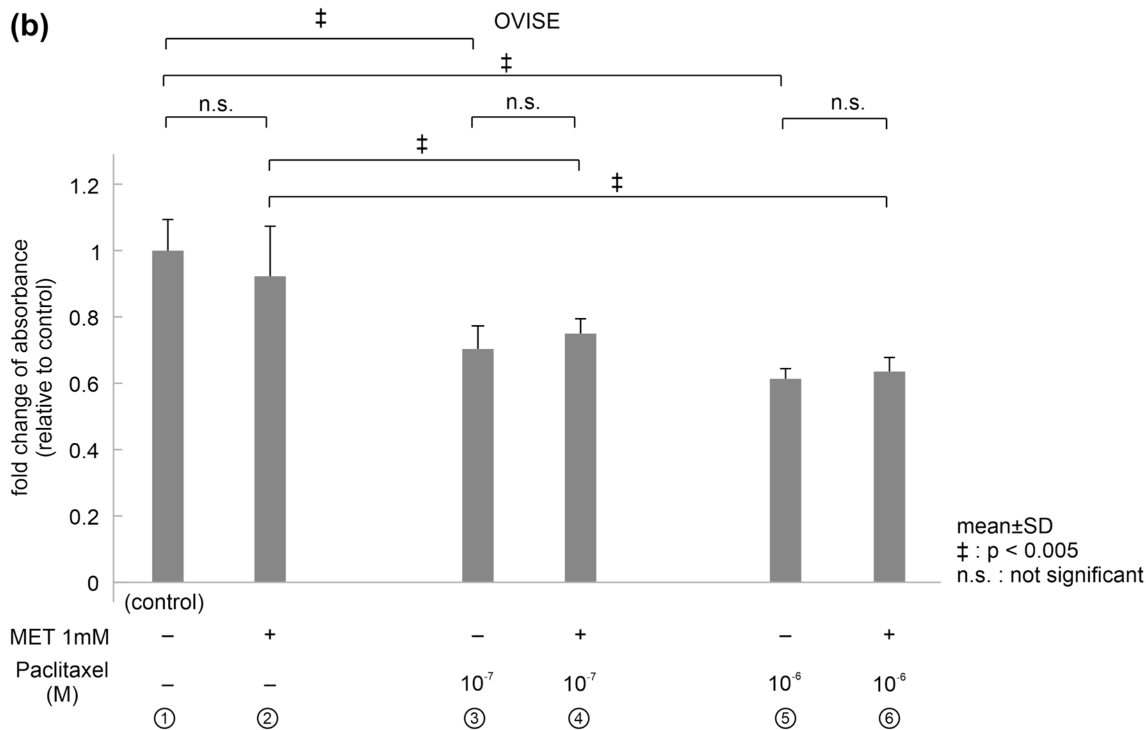
Effect of metformin on cell cycle arrest in RMG-I and OVISE

The effects of metformin on the cell cycle of RMG-I cells were evaluated by flow cytometry using APC-Cy7 staining. The percentage of cells at the G2/M phase was significantly higher in the metformin-supplemented group than that in the control group (Fig. 7). However, in OVISE, the metformin-supplemented group showed no significant difference at the G2/M phase compared with the control group (Fig. 8).

Effect of metformin on apoptosis in RMG-I and OVISE

The growth-inhibitory effect of metformin on RMG-I cells was evaluated by flow cytometry with APC-Annexin V and 7AAD staining. The sum of the ratio of Q3 (corresponding to early apoptosis) and Q2 (corresponding to late apoptosis) was defined as “Apoptosis rate” [31]. The percentage of Q3 and the apoptosis rate were significantly higher in the metformin group compared with the control group ($n = 4$, $p < 0.05$), despite showing no significant difference in Q2 (Fig. 9). In OVISE, the metformin group showed no significant difference in Q2 and Q3 percentage or apoptosis rate compared with the control group (Fig. 10).





Discussion

The results of our study revealed that metformin had an inhibitory effect on survival in several OCCC cell lines. In the same cell lines, combining metformin with cisplatin enhanced the inhibitory effect on survival. However, the combination of metformin and paclitaxel did not enhance the inhibitory effect on survival. Further, AMPK phosphorylation was enhanced by metformin in RMG-I and OVISE, whereas mTOR phosphorylation (Ser2448) was suppressed in RMG-I. Metformin treatment induced cell cycle arrest at

◂**Fig. 4** Effect of metformin on survival in RMG-I and OVISE under paclitaxel. RMG-I and OVISE are prepared in groups with 10^{-7} M of paclitaxel, 10^{-6} M of paclitaxel, and no paclitaxel, as well as in groups treated with 1 mM of metformin and no metformin. After 4 h, the cells are incubated in medium without paclitaxel (metformin group is maintained with metformin) for 44 h. The WST-1 reagent is added, and the absorbance is measured after 1 h. The mean absorbance of the untreated group (control) for both drugs is set to one, and the relative values are plotted. **a** In RMG-I, the addition of paclitaxel inhibited viability in a concentration-dependent manner ($^{‡}p<0.005$). Comparison of the metformin- and non-paclitaxel-treated groups at each paclitaxel concentration reveals that survival is significantly suppressed in the non-paclitaxel-treated group ($p<0.005$). However, in the paclitaxel (10^{-7} M and 10^{-6} M) groups, the metformin-supplemented group shows no significant difference in survival values compared with the non-supplemented group. **b** In OVISE, the addition of paclitaxel suppresses cell viability in a concentration-dependent manner ($p<0.005$). Comparing between the metformin- and non-paclitaxel-treated groups at each concentration of paclitaxel, the viability is significantly suppressed in the non-paclitaxel-treated group ($^{‡}p<0.005$). However, in the paclitaxel (10^{-7} M and 10^{-6} M) groups, the metformin-supplemented group shows no significant difference in survival values compared with the non-supplemented group. Each value is presented as the mean ± SD of eight samples. Abbreviations: *MET* metformin, *WST-1* water-soluble tetrazolium-1

the G2/M phase in RMG-1. Finally, apoptosis was induced by adding metformin.

We demonstrated the inhibitory effect of metformin on the survival of RMG-I and OVISE. The effects of metformin on OCCC cell lines have been reported using the ES-2 cell line [32]. However, the effect of metformin on RMG1 and OVISE has not been previously reported. Additionally, the survival-suppressive effect of metformin was observed in the three OCCC cell lines, which was consistent with the study by Tang et al. [32], suggesting that the effect is not specific to a cell line but common to OCCC.

Subsequently, the inhibitory effect of metformin on cell viability in RMG-I and OVISE was similar to that of cisplatin addition but not to that of paclitaxel addition. In RMG-I, a significant difference in viability was observed between the control and cisplatin (10 μg/ml) groups; in OVISE, a significant difference in viability was observed between the control and cisplatin (5 μg/ml) and (10 μg/ml) groups. These results indicated that combining metformin with cisplatin enhanced the inhibitory effect of cisplatin on the viability of RMG-I cells. OVISE also showed enhanced survival inhibition with the combination of metformin and cisplatin.

Previous studies on co-treatment of metformin with platinum preparations, including cisplatin, have reported varying effects and mechanisms of action in various cell lines, depending on the synergistic effects of inhibition of viability, growth inhibition, cell cycle arrest, and induction of apoptosis reported[18–21]. In addition to in vitro experiments[28, 29], animal experiments were conducted [33].

In contrast to platinum-based drugs, there are few reports on co-treatment with paclitaxel and metformin [34]. In an in vitro study, Dos Santos Guimarães et al. reported an increased sensitivity to paclitaxel in an ovarian endometrial carcinoma cell line cultured with metformin [28]. However, at drug concentrations similar to our study (metformin, 1 mM and paclitaxel, 10 nM), they found no significant difference. There are several possible explanations for why metformin did not enhance the inhibitory effect of paclitaxel on survival in our study, although combination with metformin enhanced the inhibitory effect of cisplatin. The possible explanations are that metformin and paclitaxel were added at low concentrations, and the cell cycle is possibly involved, as discussed later.

We then examined the molecular mechanisms underlying the inhibitory effects of metformin on cell survival. Using western blotting, we showed that metformin affected AMPK phosphorylation in RMG-I and OVISE and inhibited Ser-2448 phosphorylation, which is involved in mTOR complex I, among TOR phosphorylation in RMG-1. The main mechanism of the original efficacy of metformin as a therapeutic agent for diabetes mellitus is through Thr-172 phosphorylation in AMPK [35]. Similarly, the antitumor effect of metformin is known to start mainly from AMPK phosphorylation; in the case of the insulin-independent pathway, it leads to various antitumor effects, in part, through inhibiting downstream mTOR phosphorylation (Ser-2448) [22, 26].

Metformin activates AMPK phosphorylation and inhibits mTOR phosphorylation in several cancer cell lines, including a previous study of OCCC [32]. Second, the FCM results indicated that metformin arrested the cell cycle at the G2/M phase in RMG-I cells. In basic experiments with OC and other carcinomas, metformin arrested the cell cycle at the G0/G1 [17, 34] and G2/M phases [29]. Cell cycle arrest induced by metformin involves a cyclin-dependent kinase inhibitor, which is absent or downregulated in many cancers [23], leading to differences in findings among reports. Our results are consistent with those of the latter reports on cell cycle arrest at the G2/M phase. Cisplatin is a cell cycle-independent drug, whereas paclitaxel arrests cell division at the G2/M phase and exhibits antitumor activity. In addition to the theory described above, the lack of growth inhibition by metformin in the presence of paclitaxel, unlike cisplatin, suggests that metformin and paclitaxel may be acting to arrest the cell cycle at the same phase.

FCM results also suggested that metformin-induced apoptosis in RMG-I cells. In vitro experiments showed that metformin-induced apoptosis in various cancer cells. Many reports have suggested that this mechanism involves apoptosis induction dependent on the regulation of the Bcl-2 protein family, starting with AMPK activation and proceeding through P53 activation [17, 29, 33]. However, the apoptosis-inducing effect of metformin has also been observed in p-53-deficient colon cancer cell lines[36]. As mentioned above, AMPK-independent induction of

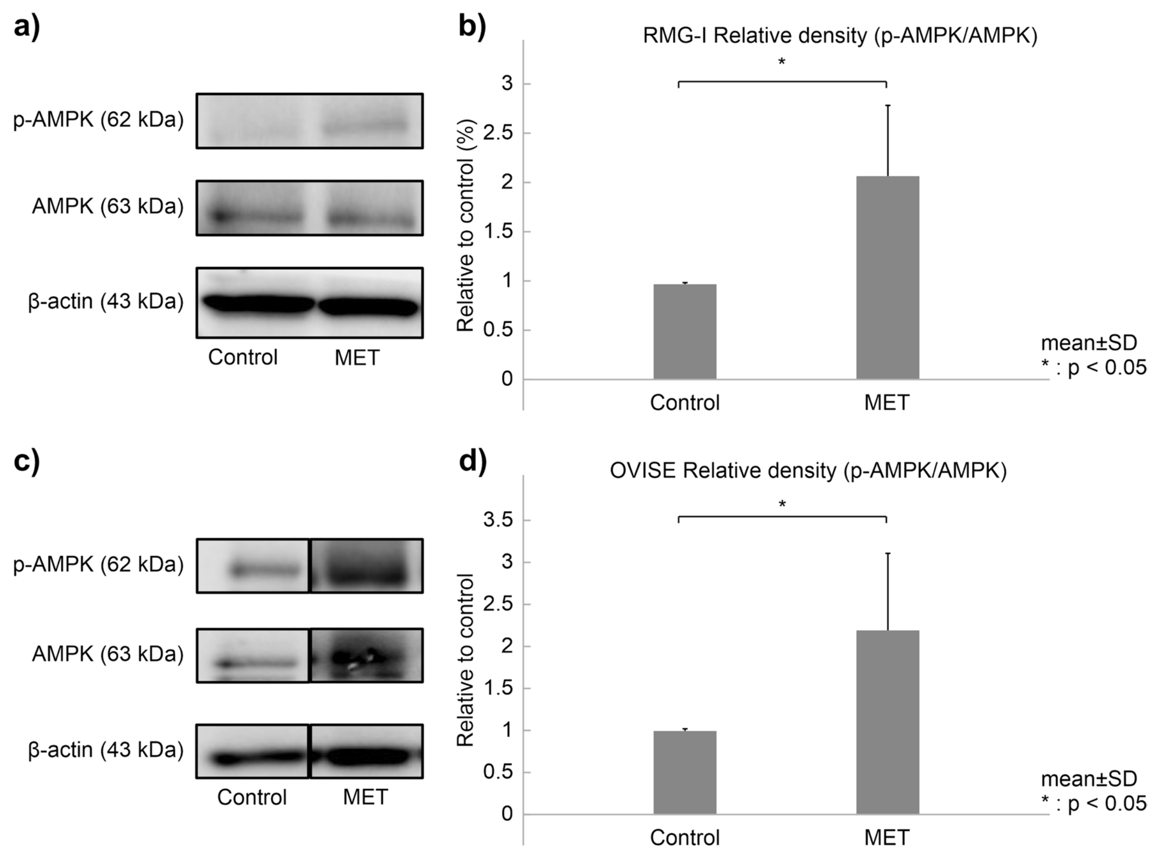


Fig. 5 Effect of metformin on AMPK phosphorylation in RMG-I and OVISE. **a** RMG-I is prepared with 1 mM metformin (MET) or without metformin (Control) and cultured for 48 h. Cells are lysed and extracted proteins were analyzed by SDS-PAGE followed by western blotting using AMPK and p-AMPK antibodies. AMPK and p-AMPK are shown as bands of approximately 63 kDa and 62 kDa, respectively. β-actin antibody is used as a control. **b** The densitometric value of the p-AMPK band in (**a**) is divided by the densitometric value of the AMPK band, and the relative values are shown in the graph with the value of the control group as 1. The MET-added group has significantly higher p-AMPK/AMPK values than does the non-added group ($*p<0.05$). Each value is shown as the mean ± SD of three independent experiments. **c** The same process as in **a** is performed in OVISE, and **d** is shown graphically as in (**b**). p-AMPK/AMPK values were significantly higher ($*p<0.05$) in the MET-added group than in the non-added group. Each value is shown as the mean ± SD of three independent experiments. Abbreviations: *MET* Metformin, *(p-)AMPK* (phospho-)AMP-activated protein kinase, *SDS-PAGE* sodium dodecyl sulfate polyacrylamide gel electrophoresis

apoptosis was reported [29]. There are multiple possible pathways through which metformin induces apoptosis; further studies are expected to reveal these. It has also been hypothesized that cell cycle arrest during the G2/M phase induces apoptosis owing to the inability of chromosome replication to induce mitotic cell division [29]. Therefore, it is possible that the two results, cell cycle arrest and induction of apoptosis, are not independent events, but rather a series of cellular changes.

The strength of this study lies in its demonstration of the antitumor effects of metformin in several OCCC cell lines with different genetic mutation patterns. The OCCC cell lines used in this study were tested for genetic mutations as described by Kashiyama et al. and Kolendowski et al. [37, 38]. To summarize the two reports, RMG-I showed mutations only in *NOTCH3*, OVISE had mutations in *ARIDA* and *PIK3CA*. ES-2 used in a previous report [32] showed mutations only in *NOTCH3*, similar to RMG-I. In OCCC, a combination of mutations in *ARID1A* and *PIK3CA* is more frequent [38] because metformin was found to be effective in both OVISE, which has these mutations, and RMG-I, which does not. This highlights the possibility of using metformin as an antitumor drug targeting OCCC.

The novelty of this study lies in the relatively low concentration of metformin used. In a previous study investigating the same effect [32], the concentration of metformin used was 10 mM, which is ten times the concentration used in this study. The fact that an antitumor effect was observed at relatively low concentrations in our study supports its clinical application. Observational studies of ovarian cancer and metformin in clinical practice [25, 27] have shown divergent findings in terms of efficacy and inefficacy according to the respective reports; future clinical trials are warranted. Over the years, phase I–III

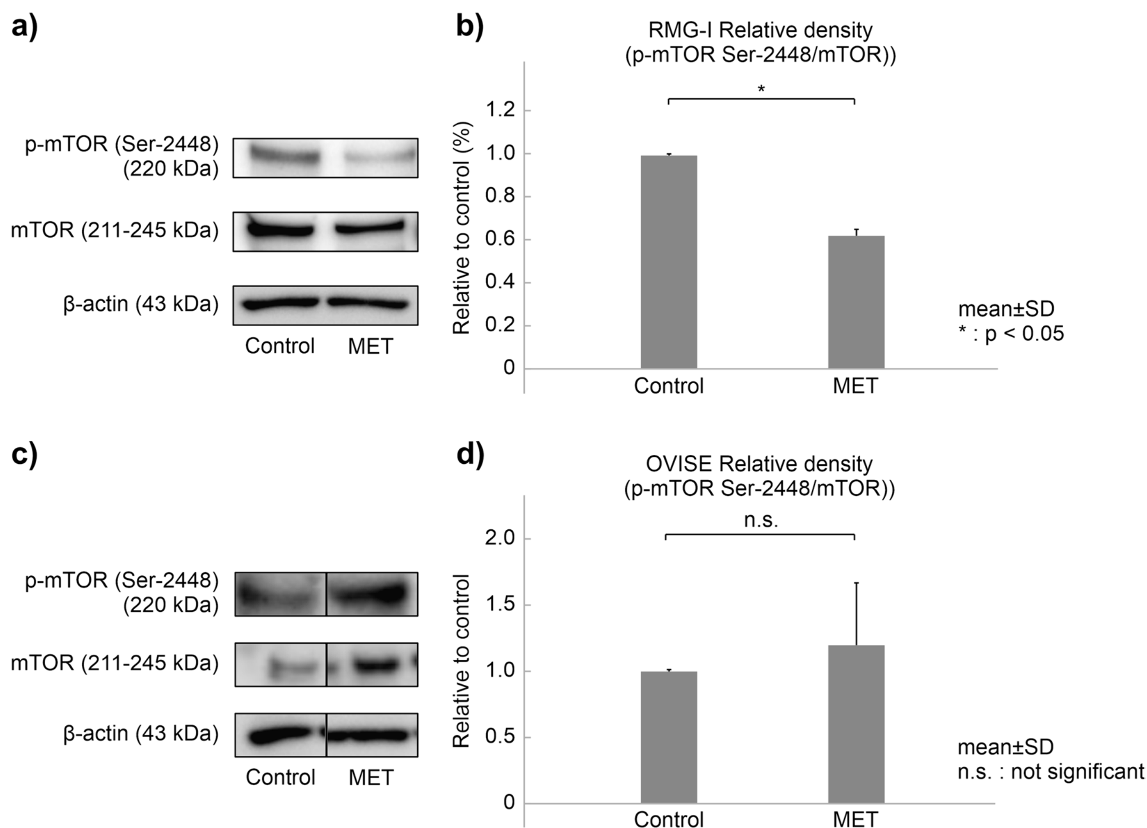


Fig. 6 Effect of metformin on mTOR phosphorylation (Ser2448) in RMG-I and OVISE. **a** RMG-I is cultured with 1 mM metformin (MET) or without metformin (Control) for 48 h. The cells are then lysed and the extracted proteins are analyzed by SDS-PAGE followed by western blotting using mTOR and p-mTOR antibodies. mTOR and p-mTOR are shown as bands of approximately 221–245 kDa and 220 kDa, respectively. β-actin antibody is used as a control. **b** The densitometric value of the p-mTOR band in **a** is divided by the densitometric value of the mTOR band, and the relative values are shown in the graph with the value of the control group as one. The MET-added group has significantly lower values of p-mTOR/mTOR compared with the non-added group ($*p < 0.05$). Each value is shown as the mean ± SD of three independent experiments. **c** The same process as in **a** is performed in OVISE, and **d** is shown on the graph in (**b**). There is no significant difference in p-mTOR/mTOR values in the MET-added group versus the non-added group ($*p \geq 0.05$). Each value is shown as the mean ± SD of three independent experiments. Abbreviations: *MET* Metformin, *(p-)mTOR(C)* (phospho-)mammalian target of rapamycin(complex), *SDS-PAGE* sodium dodecyl sulfate polyacrylamide gel electrophoresis

clinical trials of OC and metformin have been conducted. However, these trials are being conducted in Europe, the United States, and Australia, regions where the percentage of OCCC is low, and the target patients are “advanced stage OC,” “serous carcinoma” and “endometrial carcinoma”; there are no clinical trials specifically on OCCC [39]. Although our study used lower concentrations of metformin than previously reported, this is a limitation. The concentration of 1 mM used in this study was relatively high compared with the blood concentration in clinical settings. The blood concentration of metformin in patients with type 2 diabetes is approximately 50 µM, which is one-twentieth of the concentration used for our experiment [40]. However, metformin has been reported to accumulate in tissues, with up to 8 mM metformin found in the livers of mice administered therapeutic doses of metformin [41]. Based on the accumulated concentrations in the livers of mice, we suggest that the concentrations used in our experiments could be applied clinically.

As a further prospect, we would like to investigate the compatibility of MET with drugs frequently used in the treatment of ovarian cancer, such as bevacizumab and PARP inhibitors.

There have been case reports of significant efficacy in endometrial carcinoma when used in combination with bevacizumab [42], and there have been reports of its use in combination with PARP inhibitors in ovarian cancer [43], but there is not enough information on both of these combinations in OCCC, so this is an issue for the future.

We also hope that mTOR inhibitors other than metformin (such as everolimus and rapamycin) will also show antitumor effects on OCCC. Clinical trials of these drugs have already been reported in other gynecological oncology fields

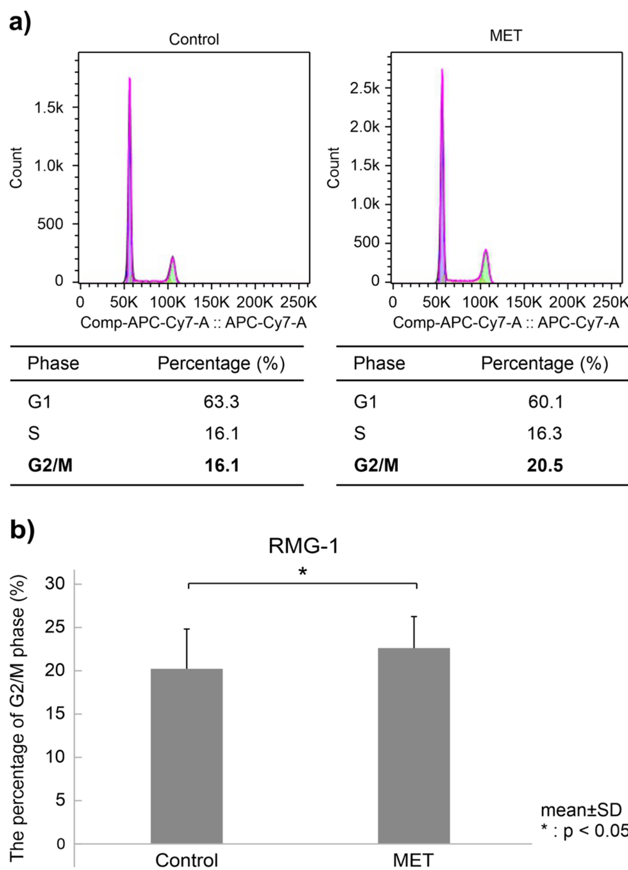


Fig. 7 Effect of metformin on cell cycle arrest in RMG-I. **a** RMG-I treated with 1 mM metformin for 24 h (MET) and those without (Control) are measured by flow cytometry with APC-Cy7 staining. The horizontal axis represents the APC-cy7 value and the vertical axis represents the number of cells. The estimated cell cycle is analyzed and assigned by analysis software. **b** The percentage of cells in the G2/M phase as a percentage of the total cell count is compared between the CONT and MET groups, and the addition of MET significantly increases the percentage of cells (*$p < 0.05$). Each value is shown as the mean ± SD of five independent experiments. Abbreviations: *MET* Metformin, *APC* allophycocyanin, *Cy7* cyanin7

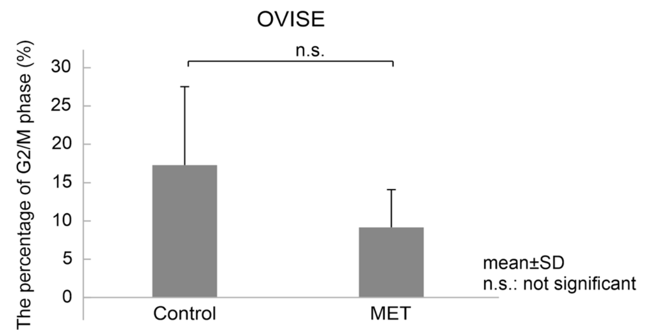


Fig. 8 Effect of metformin on cell cycle arrest in OVISE. RMG-I cells treated with 1 mM metformin for 24 h (MET), and those without metformin (control) are analyzed by flow cytometry with APC-cy7 staining. The cell cycle estimated by the analysis software is analyzed and assigned: the proportion of cells in the G2/M phase to the total number of cells is compared between the CONT and MET groups, and no significant change in cell number is observed with the addition of MET ($p \geq 0.05$). Each value is shown as the mean ± SD of three independent experiments. Abbreviations: *MET* Metformin, *APC* allophycocyanin, *Cy7* cyanin7

[44], and we would like to investigate which drugs, including metformin, are effective for OCCC.

Conclusion

These results suggested that metformin may be useful as an adjuvant therapy for OCCC in combination with chemotherapy, particularly cisplatin. Further studies on the mechanism of action and development of animal models and clinical studies for clinical applications are desirable.

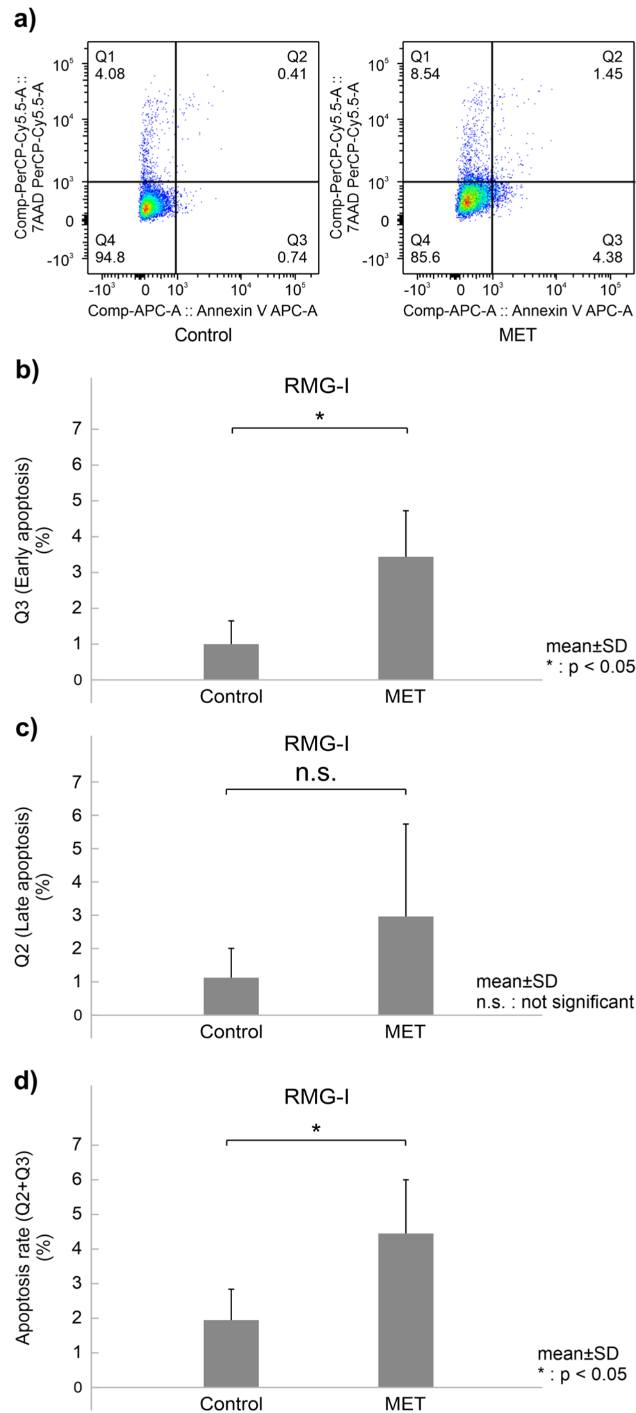


Fig. 9 Effect of metformin on apoptosis in RMG-I. **a** RMG-I with 1 mM metformin for 4 h (MET) and without (CONT) are measured by flow cytometry with APC-Annexin V and 7-AAD staining. The horizontal axis represents the value of APC-Annexin V and the vertical axis represents the value of 7-AAD. The cells are divided into 4 parts by an arbitrary value, and the sum of the percentages of Q3 (corresponding to early apoptosis) and Q2 (corresponding to late apoptosis) is defined as the "Apoptosis rate". **b–d** The percentage of cells in each of Q3, Q2, and Q3 + Q2 (Apoptosis rate) is compared between the CONT and MET groups. There is no significant difference between the two groups in Q2; in Q3 and apoptosis rate, the addition of MET significantly increases the percentage of cells (*$p<0.05$). Each value is presented as the mean ± SD of four independent experiments. Abbreviations: *MET* Metformin, *CONT* Control, *APC* Allophycocyanin, *7-AAD* 7-Aminoactinomycin D

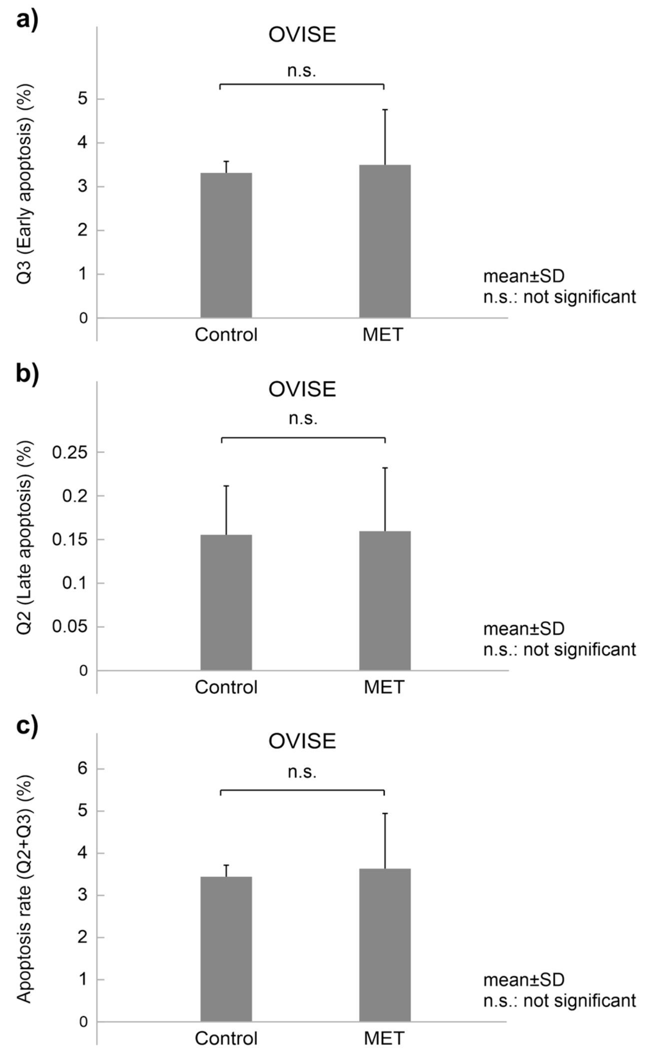


Fig. 10 Effect of metformin on apoptosis in OVISE. The same experiment shown in Fig. 9 is performed using OVISE. **a–c** Percentages of Q3, Q2, and Q3 + Q2 (apoptosis rate) cells in the CONT and MET groups are compared. There is no significant difference between the two groups in both Q2, Q3 and Apoptosis rate ($p \geq 0.05$). Each value is presented as the mean ± SD of three independent experiments. Abbreviations: *MET* Metformin, *CONT* Control, *APC* Allophycocyanin, *7-AAD* 7-Aminoactinomycin D

Author contributions ST and YK conceived the idea of the study. TM developed the statistical analysis plan and conducted statistical analyses. All authors contributed to the interpretation of the results. ST drafted the original manuscript. YK supervised the conduct of this study. All authors reviewed the manuscript draft and revised it critically on intellectual content. All authors approved the final version of the manuscript to be published.

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Data availability The data that support the findings of this study are available from the corresponding author, Tohru M, upon reasonable request.

Declarations

Conflict of interest The authors declare no conflicts of interest.

Ethical approval Not applicable.

Informed consent Not applicable.

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