Synergistic effect of metformin and medroxyprogesterone 17-acetate on the development of endometrial cancer

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Abstract. Accumulating data indicate that insulin resistance and unopposed estrogen are important risk factors of endometrial cancer (EC). Medroxyprogesterone 17-acetate (MPA) has been used in the treatment of EC for many years. However, the therapeutic effect of this agent on EC has not been satisfactory. 36 arMetformin was recently reported to be a promising agent for the treatment of malignant diseases including EC. However, information on the synergistic effect of the two agents in EC is limited. With the aim to evaluate the synergistic effect of metformin and MPA, we conducted the present study *in vitro* and *in vivo*. We found that the combined application of metformin and MPA significantly inhibited the proliferation of the Ishikawa cells and arrested the cells in the G0/G1 phase. Furthermore, the apoptosis rate of the Ishikawa cells was significantly increased. In the animal study, the development of the xenograft tumors was significantly suppressed by the combined application of the two agents. Further investigation revealed that the synergistic inhibitory effect of the two agents on EC can be at least partly, explained by the decreased expression of cyclin D1 and cyclin E. The results of the current study provide novel insights into the treatment of EC.

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Introduction

Endometrial cancer (EC) is a common gynecological malignancy in developed countries. Unfortunately, the etiology of EC remains unclear. Traditionally, 'unopposed estrogen' is considered responsible for the carcinogenesis of EC (1,2). According to this hypothesis, without enough progestin, estrogen urges the malignant transformation of the endometrium by stimulating the proliferation and inhibiting the apoptosis of endometrial cells. Research supporting this hypothesis has revealed that postmenopausal women receiving hormone replacement therapy (HRT) containing estrogen alone, face increased risk of developing EC, but, this risk decreased when progestin was added (3). However, 'unopposed estrogen' alone, cannot totally explain the pathogenesis of EC. Recently, accumulating evidence has revealed that insulin resistance is probably an important risk factor of EC (4,5). In the state of insulin resistance, elevated levels of insulin stimulate the development of EC by activating phosphoinositide 3-kinase/protein kinase B (PI3K/PKB) and mitogen-activated protein kinase/extracellular regulated kinase (MAPK/ERK) signaling pathways (6,7). Furthermore, insulin was found to be an independent risk factor of EC for both pre- and postmenopausal women (8,9). Since estrogen and insulin are established risk factors for the development of EC, they appear to be targets for the treatment of EC.

To date, medical treatment for EC consists primarily of surgery, including total abdominal hysterectomy, bilateral salpingo-oophorectomy and additional lymph node dissection (10). However, the surgical operations add a potential threat to approximately 20% of patients with EC who are premenopausal early-phase patients wishing to maintain their fertility (11). In addition, for some patients with EC, surgical treatment appears to be unsuitable because of morbid obesity and other serious complications. Recently, several studies on the non-surgical treatment of EC have revealed new insights into this malignancy (12,13). Progestin and metformin are promising candidates as therapeutic agents for EC treatment (14). Progestin has been employed in the treatment of EC for many years. However, the effect of progestin on the treatment of EC is poor (15). Metformin is a popular insulin-sensitizing agent used in the treatment of type II diabetes mellitus. Furthermore,

the antitumor effects of metformin have attracted scientific attention (16). In addition, the results provided by some studies reported that metformin plays an important role in the treatment of several malignant diseases including EC (17).

However, there is limited knowledge about the synergistic effect of progestin and metformin on EC. In the present study, we examined the combined effects of progestin-MPA, a synthetic progestin- and metformin on EC *in vitro* and *in vivo*. Furthermore, the mechanism of the effect was also explored.

Materials and methods

Cell line and reagents. The well-differentiated human EC cell line Ishikawa (a kindly gift from Professor Fengxia Xue), expressing estrogen and progestin receptors was used in the present study. The cells were maintained in phenol red-free DMEM/F12 with 10% fetal bovine serum (FBS) at 37˚C in 5% CO_2. We passaged the Ishikawa cells every 3 to 5 days. Metformin, medroxyprogesterone 17-acetate (MPA) and MTT dye were purchased from Sigma-Aldrich (St. Louis, MO, USA). Caspase-3 ELISA kit was purchased from Cell Signaling Technology (Beverly, MA, USA). All primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA): β-actin monoclonal antibody (sc-47778), cyclin D1 monoclonal antibody (sc-450), cyclin E monoclonal antibody (sc-247), phosphor-Akt monoclonal antibody (sc-514032), phosphor-ERK monoclonal antibody (sc-7383) and secondary antibody (mouse IgGκ light chain binding protein) (sc-516102).

Proliferation assay. The effects of metformin and (or) MPA treatment on Ishikawa cell proliferation were determined by an MTT assay. EC cells were plated and grown in 96-well plates at a concentration of about 8,000 cells/μl for 24 h. The EC cells were then treated with metformin and (or) MPA for 72 h at a concentration of 5 and 1 μM, respectively. The cells treated with phosphate-buffered saline (PBS) were considered as the control group. Cell densities at different time-points (1, 12, 24, 48 and 72 h) were determined by metabolic conversion of the MTT dye. We added MTT (5 mg/ml) to the 96-well plates at 10 μl in every well, and then incubated those plates for an additional hour. Finally, we ended the MTT reaction through the addition of 100 μl of dimethyl sulfoxide (DMSO). Subsequently, the MTT assay results were examined by determining absorption at 595 nm. The effect of metformin and (or) MPA on the proliferation of Ishikawa cells was assessed as a percentage of the control cell-growth obtained from the PBS-treated cells grown in the same 96-well plates. All experiments were performed in triplicate and repeated three times.

Apoptosis assay. Cells were cultured in 6-well plates at a concentration of ~$2x10^5$ cells/well for 24 h and then treated with metformin (5 μM) and (or) MPA (1 μM) in 0.5% stripped serum for an additional 72 h. Cells treated with PBS were considered as the control group. Caspase-3 kit was used to determine the apoptosis rate of the Ishikawa cells at different time-points (1, 12, 24, 48 and 72 h). Reagents were added according to the manufacturer's instructions and the ELISA plate was examined by assessing absorption at 450 nm.

All experiments were performed in triplicate and repeated three times.

Flow cytometry. In order to explore the mechanism of the effects of metformin and (or) MPA on Ishikawa cells, we examined the cell cycle profile. The cells were plated at a concentration of $2x10^5$ cells/well in 6-well plates for 24 h. Subsequently, the cells were starved overnight and then, treated with 15% serum for 72 h with metformin (5 μM) and (or) MPA (1 μM). The cells were then collected and washed with PBS, fixed in a 90% methanol solution and stored at -20˚C until the flow cytometric analysis was performed. In the analysis, the Ishikawa cells were firstly washed and centrifuged with cold PBS, suspended in 100 μl PBS and 10 μl of RNase. Then the solution (250 μg/ml) was incubated at 37˚C for 30 min. After incubation, 110 μl of propidium iodide (PI) stain (100 μg/ml) were added to each tube and incubated at 4˚C for at least 30 min before the examination. Flow cytometric analysis was performed on a CyAn machine (Beckman Coulter, Inc., Miami, FL, USA). ModFit (Verity Software House, Topsham, ME, USA) was used for the analysis for dead cells and cell debris.

Animal study. Four-week old female Balb/C nude mice with a mean body mass of 15-18 g were purchased from Charles River Laboratories (Beijing, China). After one week to adapt to the new environment, the mice were injected subcutaneously into the right flank with ~$5x10^6$ Ishikawa cells. One week after cell implantation, all the tumors became palpable. The mice were randomly divided into four groups (*n*=8 for each group). One group received no treatment and served as the control group. The other three groups were treated with metformin (250 mg/kg, daily, *per os*) and (or) MPA (1 mg in 0.1 ml volume, weekly, intramuscular). Tumor dimensions were measured twice a week. Tumor volume was calculated using the following formula: $V=a$ x b x $b/2$ (*a* is the longest axis and *b* the shortest axis of the tumor). On day 60, the animals were sacrificed. The tumors were excised and frozen for further analysis. All procedures involving animals in the present study were approved by the Animal Care and Use Committee of Yantai Yuhuangding Hospital Affiliated to Qingdao University. The welfare of the animals was well-ensured in the present study (18).

Western blot analysis. In the present study, the expression of cyclin D1, cyclin E and phosphorylated Akt and ERK was examined by western blot analysis. Frozen tumor tissues were thawed and lysed in RIPA buffer (1% NP 40, 0.5 sodium deoxycholate and 0.1% SDS). Lysates (10 μg of protein) were separated by gel electrophoresis and transferred onto the nitrocellulose membranes. Subsequently, the membranes were blocked by 5% non-fat dry milk and 0.1% Tween-20 to saturate non-specific sites. The primary antibodies were diluted (1:1,000) and incubated overnight at 4˚C. The secondary antibody was diluted (1:4,000) and incubated at room temperature for 60 min. The signals were detected using the ECL reagent (GE Healthcare, Chicago, IL, USA) and the ImageQuant LAS 4000 system (GE Healthcare). The gray value of each band in the imaging data was analyzed using Quantity One software (Bio-Rad Laboratories, Inc., Hercules,

Table I. Gray values in the western blot analysis.

Proteins	Gray value			
	Control	Metformin	MPA	Metformin+MPA
Cyclin D1	1.21±0.11	0.88±0.08	0.95±0.08	0.55±0.10
Cyclin E	0.98±0.12	0.76±0.06	0.80±0.07	0.48±0.09
p-Akt	0.90±0.08	0.89±0.09	0.88±0.08	0.84±0.08
p-ERK	0.81±0.07	0.77±0.10	0.80±0.09	0.76±0.09

CA, USA). The ratios of the gray value of the target band and β-actin used in the analysis are listed in Table I.

Statistical analysis. The data were analyzed using SAS software package (version 9; SAS Institute, Cary, NC, USA). Significance of difference between the variables, except for flow-cytometry data, was examined by the Student's t test. Mann-Whitney U test was used to analyze the data of flow cytometry. All P-values were two-sided among which a value <0.05 was considered to indicate a statistically significant difference.

Results

Effect of metformin and (or) MPA on cell proliferation. The effect of metformin and (or) MPA on the proliferation of cancer cells was determined at different time-points in the present study. As displayed in Fig. 1, one hour after the treatment, there was no significant difference among the proliferation rate of the cells treated with PBS, metformin, MPA or metformin + MPA. At the time-points of 12, 24, 48 and 72 h after treatment, the proliferation rate of the cells treated with metformin (P-value=0.047, 0.041, 0.026 and 0.020, respectively) MPA (P-value=0.048, 0.043, 0.030 and 0.022, respectively) and metformin + MPA (P-value=0.044, 0.038, 0.019 and 0.016, respectively) was significantly lower than that of the cells treated with PBS. Furthermore, the proliferation inhibitory effect of metformin + MPA was found significantly stronger than that of metformin (P-value=0.045, 0.042 and 0.039, respectively) or MPA (P-value=0.040, 0.037 and 0.026, respectively) used alone at 24, 48 and 72 h after treatment.

Effect of metformin and (or) MPA on cell apoptosis. The apoptosis assay was performed by examining the activity of caspase-3 which is a biomarker for cell apoptosis. As displayed in Fig. 2, twelve hours after treatment, the apoptosis rate of cells treated with metformin + MPA was significantly higher than that of the other three groups (P=0.013 for the metformin group, 0.011 for the MPA group and 0.006 for the control group). At the time-points of 24, 48 and 72 h after treatment, the apoptosis rate of the cells treated with metformin (P=0.041, 0.037 and 0.033, respectively), MPA (P=0.048, 0.042 and 0.038, respectively) and metformin + MPA (P=0.002, <0.001 and <0.001, respectively) were all significantly higher than that of the cells treated with PBS. Furthermore, at 24, 48 and 72 h after treatment, the proliferation inhibitory effect of metformin + MPA was found significantly stronger than that
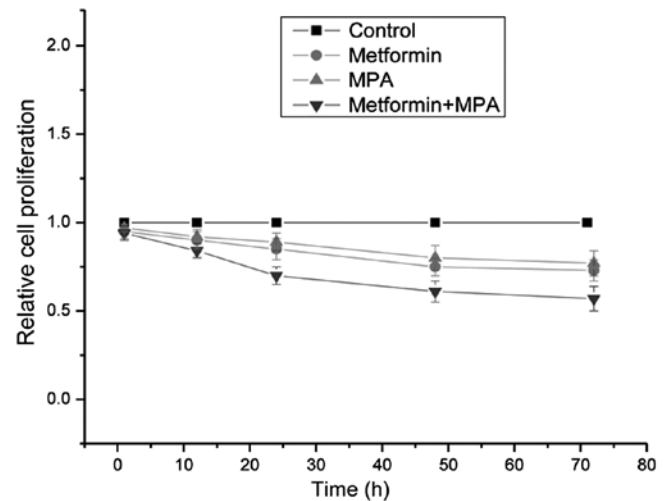

Figure 1. Effect of metformin and (or) MPA on the proliferation of Ishikawa cells. Metformin, MPA and metformin + MPA all inhibited the proliferation of Ishikawa cells in a time-dependent manner. The combined use of metformin and MPA exhibited significantly stronger inhibitory effect than either of the other agents used alone. The results are presented as the mean ± SE.
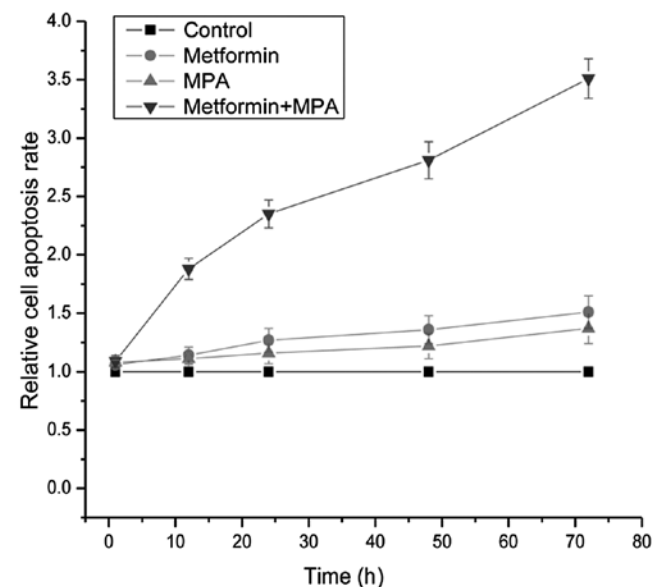

Figure 2. Effect of metformin and (or) MPA on the apoptosis of Ishikawa cells. Metformin, MPA and metformin + MPA all promoted the apoptosis of Ishikawa cells in a time-dependent manner. The combined use of metformin and MPA demonstrated significant stronger apoptosis-promoting effect than either of the agents used alone. The results are presented as mean ± SE.
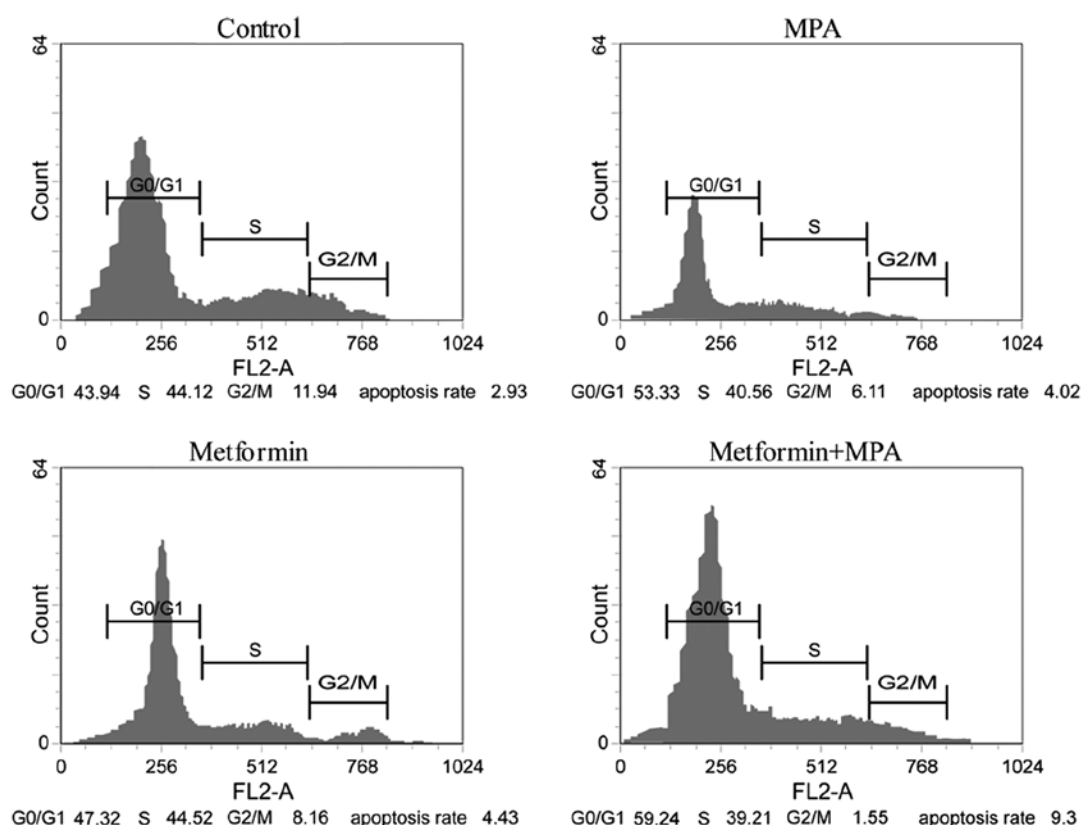
Figure 3. Metformin and (or) MPA induce cell cycle arrest in Ishikawa cells. Ishikawa cells were treated with PBS, metformin, MPA and metformin + MPA for 72 h. Cells treated with PBS were considered as the control group. Metformin, MPA and metformin + MPA treatments all showed significantly stronger G0/G1 phase arresting effects compared with the control group. Furthermore, the G0/G1 phase arresting effect of metformin + MPA was significantly stronger than either of them used alone.
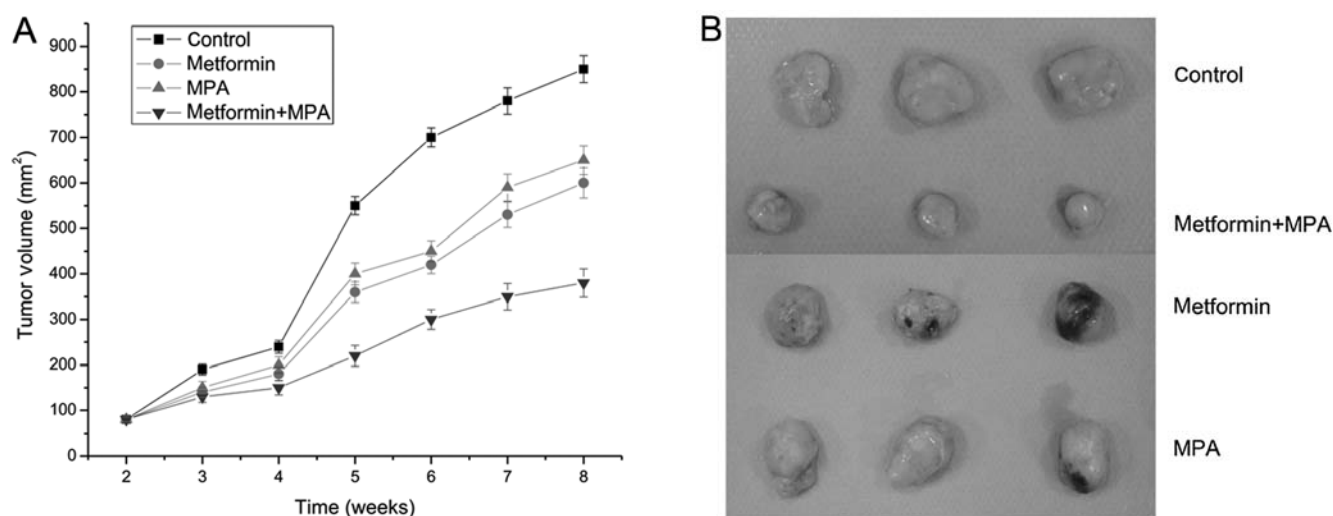

Figure 4. (A) Xenograft tumor growth curve. (B) Effect of metformin and (or) MPA on xenograft tumors. Metformin, MPA and metformin + MPA all inhibited the growth of the xenograft tumors in a time-dependent manner. The combined use of metformin and MPA exhibited significant stronger inhibitory effect than either of the agents used alone. The results are presented as mean ± SE.

of metformin ($P < 0.001$, < 0.001 and < 0.001, respectively) or MPA ($P < 0.001$, < 0.001 and < 0.001 respectively) used alone.

Effect of metformin and (or) MPA on the cell cycle. As displayed in Fig. 3, metformin, MPA and combined application of them all significantly inhibited the proliferation of the Ishikawa cells. Compared with the control group, more cells were arrested in the G0/G1 phase, whereas, less cells were found in the G2/M phase when stimulated with metformin and (or) MPA. This synergistic arresting effect of the two agents was significantly stronger than that of metformin or MPA applied alone.
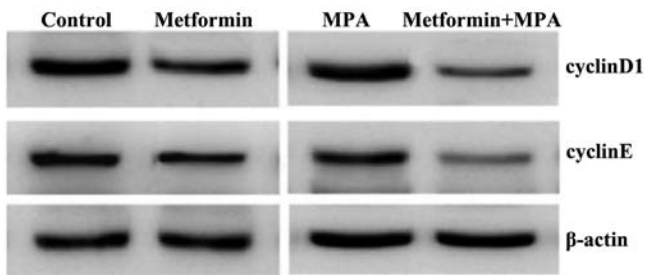
Figure 5. Effect of metformin and (or) MPA on the expression of cyclin D1 and cyclin E. Metformin and MPA treatment both significantly inhibited the expression of cyclin D1 and cyclin E. The inhibitory effect of metformin + MPA treatment was significantly stronger than that of metformin or MPA treatment alone.
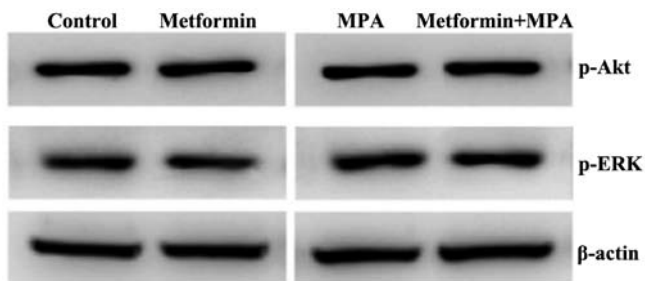

Figure 6. Effect of metformin and (or) MPA on PI3K/PKB and MAPK/ERK signaling pathways. There was no significant difference of the expression of p-Akt and p-ERK among the groups with different treatment.

Tumor growth assay in vivo. In this section, we evaluated the effect of metformin and (or) MPA on the growth of the xenograft tumor model. As displayed in Fig. 4, between the fifth to the eighth week, metformin (P=0.034, 0.032, 0.039 and 0.030, respectively), MPA (P=0.039, 0.035, 0.036 and 0.033 respectively) and metformin + MPA (P=0.014, 0.011, 0.019 and 0.018, respectively) all significantly inhibited the growth of the xenograft tumors. Furthermore, between the fifth to the eighth week, the synergistic suppressive effect of metformin combined with MPA on tumor growth was significantly stronger than metformin (P=0.020, 0.028, 0.025 and 0.024, respectively) or MPA (P=0.017, 0.021, 0.019 and 0.020, respectively), alone. Although tumor growth appeared more severely suppressed by metformin than MPA (Fig. 4B), the difference was not statistically significant.

Western blot analysis of the xenograft tumors. Cyclin D1 and cyclin E play important roles in cell-cycle progression. The expression of these two proteins is reported as being positively associated with cell proliferation. The PI3K/Akt and MAPK/Ras signaling pathways are also significant factors in regulating cell proliferation and apoptosis. Therefore, the expression of Cyclin D1, cyclin E and phosphorylated Akt and ERK in xenograft tumors was evaluated. As displayed in Fig. 5, the expression of cyclin D1 and cyclin E was significantly decreased by metformin and (or) MPA compared with the control group. Although the inhibitory effect of metformin appeared stronger than that of MPA, the difference was not statistically significant. Furthermore, the combined effect of metformin and MPA was found significantly stronger than

either of them used alone. Subsequently, we examined the phosphorylation of Akt and ERK in the xenograft tumors. Notably, as dipslayed in Fig. 6, we observed no difference in the key-protein phosphorylation among the groups with different treatments.

Discussion

The results provided by the present study demonstrated that both metformin and MPA have potent inhibitory effects on the development of EC cells. Furthermore, the synergistic effect of these two agents was significantly stronger than either of them used alone. Through arresting cancer cells in the G0/G1 phase, the agents promoted the apoptosis and inhibited the proliferation of the Ishikawa cells. Furthermore, these two agents potently inhibited the development of the xenograft tumors *in vivo* and the combined effect of them displayed greater inhibitory effect. The inhibition of the expression of cyclin D1 and cyclin E is probably one of the mechanisms of the synergistic effect of these two agents. Our findings indicated that the combined use of metformin and MPA may be a more effective strategy for the treatment of EC.

The anti-estrogen strategy had been applied in the treatment of EC for many years. MPA is one of the most popular agents clinically used in the treatment of EC. By binding to its receptor, especially the B subtype, progestin regulates multiple signaling pathways related to cell proliferation, apoptosis and differentiation (19). This inhibitory effect of MPA on cancer cells was revealed in a cell cycle phase-specific mode (20). The decreased expression and (or) inactivation of c-Myc, cyclin and the associated cyclin-dependent kinases (CDKs) appeared to play significant roles in the cell cycle-phase arresting effect (21-24). Cyclin is a family of proteins playing an important role in cell cycle progression. CDK family is also a key factor in cell cycle progression the activation of which is positively associated with the expression of cyclins. Enough cyclin binding to CDK is pivotal for cells to pass through the G1-phase. As indicated in our results, MPA effectively inhibited the proliferation of the Ishikawa cells *in vitro* and the G0/G1-phase arrest was probably one of the mechanisms. Subsequently, the animal study revealed that the MPA management significantly inhibited the growth of the xenograft tumors. The expression of cyclin D1 and cyclin E of the xenograft tumors treated with MPA was significantly lower than that of the controls. The results of the current study were similar to previous studies (25-27). Accordingly, it was indicated that MPA inhibits the development of EC through the cell cycle arrest.

Metformin is a common insulin-sensitizing agent used to treat type II diabetes. However, accumulating evidence indicated that metformin could be applied in the treatment of some malignant diseases including EC (28-30). Firstly, as an insulin-sensitizing agent, metformin decreased the circulating insulin levels by inhibiting the hepatic glucose and lipid synthesis, as well as by increasing muscle glucose uptake. As a result, the insulin induced proliferation-promoting and apoptosis-inhibiting effects were weakened by metformin. Sex hormone binding globulin (SHBG) is a significant serum sex hormone-concentration regulator which tightly binds to sex hormones. It was reported that insulin inhibited the

production of SHBG (31) leading elevated free-estrogen levels to promote the development of EC. Since metformin downregulates serum insulin levels, circulating SHBG levels will be increased, resulting in less free-estrogen to stimulate the pathogenesis of EC. Secondly, insulin acts as an anti-tumor agent directly suppressing the development of EC. It is well-known that metformin phosphralytes LKB-1 and then AMP-activated protein kinase (AMPK) is activated which leads to the inactivation of mammalian target of rapamycin (mTOR)-signaling pathway (32). Furthermore, metformin was found to exert cell cycle-inhibitory effect on cancer cells by downregulating the expression of the related key proteins such as cyclin D1 and cyclin E (33-36). This cell cycle-arresting effect greatly impaired cell proliferation. These results were in line with that of the present study. In our study, metformin significantly inhibited the proliferation of the Ishikawa cells and arrested cancer cells in the G0/G1-phase. Furthermore, the growth of the xenograft tumors was significantly inhibited by metformin management. The expression of cyclin D1 and cyclin E were significantly lower. The data provided by the present study indicated that cell cycle phase-arrest can be used to explain the inhibitory effects of metformin on the development of EC.

To date, only a small number of studies have reported the combined application of metformin and progestin on the treatment of EC. It was revealed that metformin inhibited the expression of glyoxalase which is a key regulator of progestin-resistance to strengthen the therapeutic effect of progestin on EC cells (37). Furthermore, the inhibitory effect of metformin on the mTOR signaling pathway was associated with the upregulation of the expression of the progestin receptor in EC cells (38). These studies indicated that the combined application of metformin and progestin had stronger inhibitory effect on the EC cells than using either of the agents alone. However, the role played by metformin appeared adjuvant, but not synergistic. Furthermore, the common targets of the two agents were not provided. In the present study, the combined use of metformin and progestin exhibited stronger inhibitory effect on the development of EC than either of the two agents used alone. Furthermore, we revealed that cyclin D1 and cyclin E were the common targets of these two agents. The synergistic inhibitory effect of metformin and progestin on the expression of cyclin D1 and cyclin E caused cancer cell-arrest in the G0/G1 phase. As a result, the development of EC was severely delayed. In the present study, the expression of phosphorylated Akt and ERK was also evaluated. However, the differences were not significant among different groups. The data presented in the study indicated that G0/G1 phase-arrest induced by the down-regulation of the expression of cyclin D1 and cyclin E is at least partly responsible for the synergistic effect of metformin and progestin on EC.

Although the present study provided interesting results, there are still some weak points. Firstly, only one dose of each agent was used in the present study, therefore it remains unknown whether the synergistic inhibitory effect is dose-dependent. Furthermore, the most optimal dose combination of the two agents was not provided. Secondly, since the observation time for the animal study was short, it is unknown whether there are any adverse impacts of the combination use of these two agents. Thirdly, only cyclin D1 and cyclin E were identified as the common targets of metformin and MPA, however the whole signaling network was not explored.

In conclusion, the present study provided novel insights into the treatment of EC. The combined application of metformin and MPA inhibited the development of EC in a synergistic manner. The downregulation of cyclin D1 and cyclin E was identified as one of the mechanisms of the synergistic inhibitory effect. Future studies are warranted to further evaluate the combined application of metformin and MPA and the mechanisms underlying this synergistic inhibitory effect.

Acknowledgements

Competing interests

The authors declare that they have no competing interests.

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