ORIGINAL RESEARCH

MET Inhibits the Proliferation of EC Cells by Increasing MPA Sensitivity

Fang Sun, MM; Jiayun Zhou, BM; Yongxin Hao, BM; Lei Kuang, MM; Bei Zhang, PhD

ABSTRACT

Context • The high resistance rate and high recurrence rate of progesterone only as a treatment for endometrial cancer (EC) limit its clinical application. Metformin (MET) may have antitumor ability. Combining MET and medroxyprogesterone acetate (MPA) may strengthen their inhibitory effects on proliferation of EC cells, but MET's mechanisms remain unclear.

Objective • The study intended to identify the specific molecular mechanism that MET combined with MPA uses against EC progression.

Design • The research team performed a controlled animal study.

Setting • The study took place at Xuzhou Medical University in Xuzhou, China.

Animals • The animals were16 female non-obese diabetic-severe combined immunodeficient (NOD-SCID) nude mice, about 12 to 16 g in weight.

Interventions • The research team divided randomly, the mice into four groups and induced EC in all groups, four in each group: (1) The control group which received received normal saline, (2) the MPA group, which received 100 mg/kg of MPA; (3) the MET group, which received metformin at the rate of 200 mg/kg, each gavage volume was 0.1ml; (4) the MET+MPA group, which received 100 mg/kg of MPA and 200 mg/kg of MET.

Outcome measures • The research team: (1) used a CCK-8 kit, an EdU assay, and a flow-cytometry assay to measure cancer-cell proliferation, count, and viability; determine the cell cycle; and measure apoptosis; (2) performed a Western blot analysis to determine the expression of the PR, CD133, pAkt, totalAkt, p-mTOR, and totalTOR antibodies; and (3) determined the size and volume of tumors in vivo and used immunohistochemical staining to determine expression of the Ki67 protein.

Results • The MET+MPA group had a significantly lower number of cancer cells than the MET or MDA groups (both $P < .001$). That group also had significantly more stagnated cancer cells in the G0/G1 phase and significantly fewer cancer cells in the S phase or G2/M phase control, MET, or MPA groups (all $P < .01$). The MET+MPA group's PCNA and Ki-67 protein expression was significantly lower than that of the MET and MPA group. The EDU assay yielded similar results. Additionally, the MET+MPA group had significantly higher PR expression than that of to MET or MPA group (both $P < .001$). The MET and MPA groups' expression of CD133, p-Akt, and p-mTOR were significantly lower than those of the control group, while the MET+MPA group's levels were significantly lower than those of the MET and MPA groups. In-vivo experiments revealed that the MET and MPA groups did show decreased tumor size and volume. The MET+MPA group had tumor weights that were significantly lower and tumor volumes were significantly smaller than those of the MET and MPA groups (all $P < .001$). Immunohistochemical analysis revealed that the MET+MPA group's levels of the Ki-67 antigen were significantly lower than those of the MET and MPA groups.

Conclusions • MET inhibited the proliferation of EC cells by increasing MPA-sensitivity, which was dependent on the inhibition of the CD133 expression and the Akt/mTOR pathway. In addition, if MET acts as an effective progestin sensitizer, it certainly offers promising therapeutic prospects for patients with early-stage EC or overgrown endometrium who have fertility requirements. (*Altern Ther Health Med.* 2023;29(5):334-341).

Fang Sun, MM, Associate chief physician; **Lei Kuang, MM**, Resident Doctor; and **Bei Zhang, PhD**, Chief Physician; Department of Obstetrics and Gynecology, Xuzhou Central Hospital, Xuzhou, China. **Jiayun Zhou, BM; Yongxin Hao, BM;** Affiliated Xuzhou Clinical College of Xuzhou Medical University, Xuzhou, Xuzhou, China.

Corresponding author: Bei Zhang, PhD
E-mail: bettyzhang10@163.com

Endometrial cancer (EC) is a common gynecologic tumor.EC was diagnosed in 417 367 women in 2020, worldwide. The incidence of EC is rapidly increasing.[1] Classified as either a type I estrogen-dependent or a type II non-estrogen-dependent cancer, 70% to 80% of ECs are type I.[2] In recent years, the proportion of young EC patients who haven't given birth has increased, and the types of EC in these young patients are mostly early estrogen-dependent type I.[3]

A hysterectomy is the first-line treatment for EC. Women with low-grade endometrioid EC and no evidence of MMI on imaging who wish to preserve their fertility can be considered for a non-surgical approach. High-dose oral progesterone and/or levonorgestrel-release intrauterine devices (LNG- IUDs) can be used.[4]

Progesterone

In one meta-analysis of oral medroxyprogesterone acetate, a response rate of 75.2% and a live birth rate of 28% was reported.[5] One study demonstrated a response rate of 75% in patients with complex atypical hyperplasia and early-grade endometrioid EC. However, although response rates are high with these treatments, relapse rates are also high.[6] Using oral MPA, Garzon et al found its recurrence rate of 30.7%.[7]

As an effective drug for patients with early-stage EC who receive conservative treatment of progesterone only, the high resistance rate and high recurrence rate of progesterone limit its clinical application.[8] Progesterone's specific mechanism in the treatment of EC and drug resistance is still unclear. Traditionally, progesterone binds to the progesterone receptor (PR) in cytoplasm and nuclei to regulate transcription-factor synthesis of specific proteins and to inhibit tumor-cell proliferation.[9]

Kim et al's recent study found that long-term treatment with large amounts of progesterone for EC or excessive endometrial hyperplasia can result in downregulation of the PR, accompanied by progesterone-promoting proliferation.[10]

Reis et al and Zeberg et al found that the PR mediates the physiological effects of progesterone, and the ultimate effects of progesterone in vivo depend not only on the secretion and metabolism of progesterone itself but also on the expression and function of the PR gene.[11,12]

In addition, Yu et al found that progesterone can activate the phosphoinositide 3-kinase (P13K)/ protein kinase B (AKT) pathway,[13] which Zhang et al found to be closely related to hormone resistance.[14]

Metformin (MET)

MET is the most frequently used drug for the treatment of type 2 diabetes.[15] MET can reduce insulin resistance by controlling body weight, enhancing insulin action, and inhibiting gluconeogenesis.[16]

Gynecological clinics widely use MET combined with oral contraceptives, mainly for the treatment of insulin resistance, ovulation promotion, and improvement in assisted-reproduction rates in patients with polycystic ovary syndrome (PCOS).[17] Del et al found that MET has antitumor ability.[18]

Podhorecka et al and Sun et al found that MET can prohibit the growth of breast, prostate, pancreatic, and ovarian, and endometrial cancers.[19,20]

Pabona et al found that MET could block G1 cell-cycle arrest in EC cells.[21] The regulation of the cyclin-dependent kinase cycle may cause inhibition of cell proliferation; it's involved in the anticancer effects of progesterone.[22] Nevertheless, whether MET induces apoptosis in tumor cells is controversial.

PI3K/Akt/mTOR Pathway

Pedroza et al reported that some growth factor signaling pathways—epidermal growth factor (EGF), heregulin (HRG), and insulin—can directly downregulate the expression levels of PR through the AKT/ mammalian target of the rapamycin (mTOR) pathway, which is independent of estrogen.[23] An important target downstream of Akt, mTOR regulates the basic transduction pathway, is involved in coupling cells, and stimulates cell-cycle progression.[24]

Three studies found that MET can suppress tumor proliferation, induce apoptosis, and arrest the cell cycle through regulation and inhibition of the PI3K/Akt/mTOR pathway.[20,25,26] Kalender et al found that MET can directly inhibit the mTOR pathway, independently of the 5' adenosine monophosphate-activated protein kinase (AMPK).[27]

Bitelman et al found that MET can suppress the growth of breast, prostate, and pancreatic cancers and endometrial tumors through inhibiting glucose metabolism and the PI3K/Akt/mTOR pathway.[28] Patel et al found that progesterone resistance was related to the Akt/mTOR signaling pathway.[29]

CD133

CD133 is the first surface marker for labeling and isolating EC stem cells.[30] Recent studies have shown that CD133+ EC cells have biological characteristics of cancer stem cells with high tumor initiation, proliferative potential, and tolerance to conventional chemotherapeutic agents.[31]

Current Study

Based on previous studies, the current research speculated that MET may enhance the progesterone sensitivity of EC cells through the PI3K/Akt/mTOR pathway. The study intended to identify the specific molecular mechanism that MET combined with MPA uses against EC progression.

METHODS

Animals

The research team performed an animal study, which took place at Xuzhou Medical University in Xuzhou, China. The animals were 16 female non-obese diabetic-severe combined immunodeficient (NOD-SCID) nude mice, about 12 to 16 g in weight. The research team purchased them from Shanghai Bangyao Biotech (Shanghai, China). The team kept the mice in a pathogen-free environment, with a room

temperature of 20 ± 2°C and a relative humidity of 45 to 65%. The mice received food and water ad libitum.The Animal Care and Use Committee of the Xuzhou Medical University Ethics Committee approved the study's protocols.

Procedures

Groups. The research team divided Randomly, the mice into four groups and induced EC in all groups: (1) the control group, which received no treatments; (2) the MPA group, which received 100 mg/kg of MPA; (3) the MET group, which received metformin at the rate of 200 mg/kg, each gavage volume was 0.1 ml; (4) the MET+MPA group, which received 100 mg/kg of MPA and 200 mg/kg of MET.

EC induction. The research team injected Ishikawa cells (ATCC, Shanghai, China), an endometrial adenocarcinoma cell line, subcutaneously, 5 × 106, into the forelimbs of the mice; (2) assessed the tumor sizes every five days; and (3) after 30 days, euthanized the mice and gathered and stored the tumor tissues at -80°C.

Cell culture and treatment. The research team measured the Ishikawa cell were cultured in RPMI 1640 medium with 10% FBS and 1% penicillin and streptomycin mixture. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C. (1) Ishikawa cells were seeded in 6-well plates at a density of 5-10 × 103 cell per well and treated with different concentrations(0–40 mmol/L) of MET for 24–72 h 25 mmol/L MET for 48 h was used because this dose showed the maximal inhibition effect on Ishikawa cell growth in vitro. Ishikawa cells were treated with MPA (0, 0.1, 1, 5, 10, 20, 30 μM) of different dose gradients alone for 48h, and shikawa cells were treated with metformin (2.5 mM) combined with MPA above concentration for 48h. The cell proliferation of the two groups was stained with CCK8 following the manufacturer's instruction, (2) performed a 5-ethynyl-2'-deoxyuridine (EDU) assay using an EdU kit (Guangzhou RiboBio, Guangzhou, China), following the manufacturer's instructions; and (3) took pictures, using a fluorescence microscope (1X53, Japan Olympus Corporation). According to the doses of MET that prostate and colon cancer studies have used (0.01-5 mM),[32,33] untreated, non-diabetic patients with EC received metformin 500 mg tid from diagnostic biopsy to surgery. Fasting plasma insulin, insulin-like growth factor 1 (IGF-1) and based on our proliferation experiment screening, the treatment concentration of MET is 2.5 mM, and the MPA concentration is 10 um.

Cell-cycle detection. The research team: (1) placed Ishikawa cells in six-well plates and treated them with MET(2.5 mM)and MPA(10 uM), alone or in combination for 48 h; (2) collected the cells, using 0.1% dimethyl sulfoxide (DMSO) medium as a negative control; (3) fixed them overnight at 4°C with pre-cooled 70% ethanol; (4) added 100 μL of RNase A to each well; (5) incubated them with water at 37°C for 30 min; (6) incubated the cells with 400 μL of propidium iodide (PI) dye for 30 min at 4°C, away from light; (7) detected the OD values using flow cytometry; and (8) analyzed the results using ModfiT software (LSRFortessa 164TM X-20; BD Biosciences, San Jose, NJ, USA) to analyze the cell cycle.

Western blot assay. The research team measured extracted protein concentrations using the Bradford assay (Bio-Rad, Hercules, CA, USA): (1) separated 30 mg of proteinsfrom Ishikawa cell line reated with different drugs in the six-well plate t, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); (2) used a polyvinylidene difluoride (PVDF) membrane to transfer the proteins; and (3) blocked them with 5% bovine albumin (BSA) from Sigma-Aldrich (St. Louis, MO, USA) for 15 min.

The team then incubated the membranes with antibodies in each group separately, antibodies from Abcam (Cambridge, MA, USA): (1) primary anti-β-actin, ab8227 at 1:2000; (2) anti-[5] (PCNA), ab18197 at 1:2000); (3) anti-Ki67 antigen, ab15580 at 1:2000; (4) anti-cell-cycle-related protein (p21), ab109520 at 1:2000; (5) anti-B-cell leukemia/lymphoma 2 (Bcl-2) protein, ab32124 at 1:2000; (6) anti-caspase-3, ab32351 at 1:2000; (7) anti-CD133 antigen, ab32351 at 1:2000; (8) anti-progesterone receptor (PR), ab32085 at 1:2000; and (9) The membranes were incubated with horseradish peroxidase-233conjugated anti-rabbit or anti-mouse IgG for 2 h at room temperature and 234immunoreactive bands were detected by ECL horseradish peroxidase substrateusing 235X-ray film. Image J computer software (Software Inquiry; Quebec, Canada) was used 236 to analyzed the quantification of the bands)

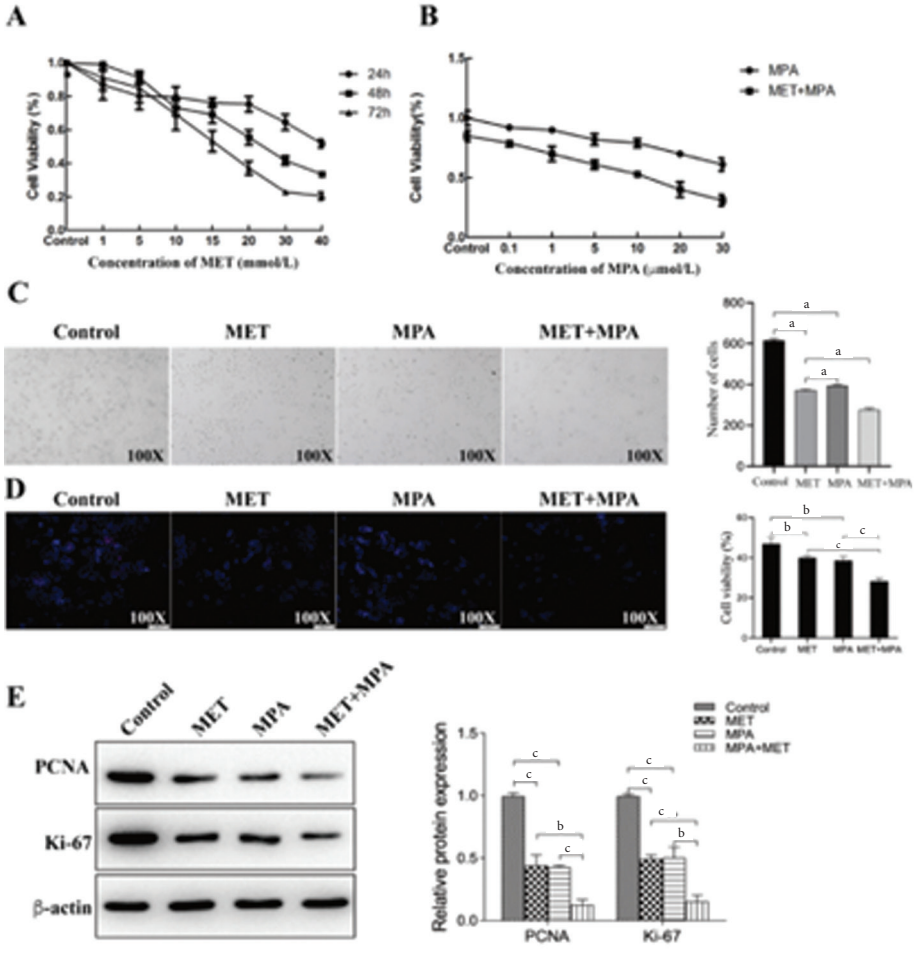
Flow cytometry analysis. The research team: (1) harvested treated cells and suspended them; (2) treated them with fluorescein isothiocyanate (FITC)- annexin (A) V and PI for 15 min; (3) added binding buffer to the cell suspension in each tube; and (4) detected apoptosis using flow cytometry.(LSRFortessa 164TM X-20; BD Biosciences, San Jose, NJ, USA).

Immunohistochemical staining. The research team: (1) treated the tissue sections with primary antibody of Ki-67 and a secondary antibody; (2) counterstained the nuclei with hematoxylin; and (3) observed the sections under a microscope (OlympusRX51; 176 Tokyo, Japan).

Sphere-formation assay. The research team: (1) harvested the treated Ishikawa cells; (2) cultured them in six-well plates with stem-cell condition medium (Bioworld Inc., USA); and (3) counted the sphere numbers under a light microscope (OlympusRX51; 176 Tokyo, Japan) after 7 days of incubation.

Outcome measures. The research team: (1) used a CCK-8 kit, an EdU assay, and a flow-cytometry assay to measure cancer-cell proliferation, count, and viability; determine the cell cycle; and measure apoptosis; (2) performed a Western blot analysis to determine the cell-cycle-related protein (p21) and apoptosis-related protein(Bcl-2, cleaved-caspase-3), and PR and C133 proteins and of the p-Akt/AKT and p-mTOR /mTOP antibodies; (3) Measure and record the size and volume of tumors in vivo, used immunohistochemical staining to determine expression of the Ki67 protein.

Figure 1. Inhibition Through Co-treatment With Metformin (MET) and Medroxyprogesterone Acetate (MPA) of Cancer Cell Growth in Endometrial Carcinoma for the Control, MPA, MET, and MET+MPA Groups Figure 1A shows the effects of MET only on the viability of Ishikawa cells, as detected using CCK-8; Figure 1B shows the effects of MET combined with MPA on the viability of Ishikawa cells, as detected using CCK-8; Figure 1C shows the cell count for the four groups, as observed under a microscope; Figure 1D shows the cell proliferation, as detected using an EDU assay; and Figure 1E shows the expression of the proliferation-related indicators, PCNA and Ki67 antigens, as detected using Western blot.



[a]$P < .001$, indicating that the MET and MPA groups' cell numbers were significantly lower than those of the control group and that the MET+MPA group's cell numbers were significantly lower than those of the MET and MPA groups

[b]$P < .05$, indicating that the MET and MPA groups' Ishikawa cells viability were significantly lower than that of the control group, that the MET+MPA group's PCNA protein expression was significantly lower than that of the MET group, and that the MET+MPA group's Ki-67 protein expression was significantly lower than that of the MPA group

[c]$P < .01$, indicating that the MET+MPA group's Ishikawa cells viability was significantly lower than those of the MET and MPA groups, that the MET and MPA groups PCNA and Ki-67 protein expression was significantly lower than that of the control group, that the MET+MPA group's PCNA protein expression was significantly lower than that of the MPA group, and that the MET+MPA group's Ki-67 protein expression was significantly lower than that of the MET group

Abbreviations: CCK-8, cell counting kit-8 (CCK-8); EDU, 5-ethynyl-2'-deoxyuridine; MPA, medroxyprogesterone acetate; PCNA, proliferating cell nuclear antigen.

Statistical Analysis

The research team used GraphPad Prism 8.0, (La Jolla, CA, USA) and Statistical Product and Service Solutions (SPSS) 13.0 (IBM, Chicago, IL, USA) to analyze the data. The team: (1) expressed all data as means ± standard deviations (SDs), (2) used Student's *t* test to compare means between the groups, and (3) used an analysis of variance (ANOVA) to compare means between three or more groups. $P < .05$ was considered to be significant.

RESULTS

Cancer-cell Growth

The cancer-cell viability didn't change significantly with time when the MET concentration was 10 mmol/L or lower (Figure 1A). As the MET concentration increased, the cell viability gradually decreased, and the cell viability at 72 h was significantly lower than that at 48 h and 24 h. In addition, MPA had a similar concentration-dependent effect on the Ishikawa cells.

As Figure 1B shows that a co-treatment with 2.5 mmol/L of MET and (0, 0.1, 1, 5, 10, 20, 30 μM) of MPA further inhibited cancer-cell viability, especially at a concentration of 10 mmol/L of MPA.

Furthermore, Figure 1C shows that the MET and MPA groups' cancer-cell numbers were significantly lower after incubation than those of the control group (both $P < .001$), and the MET+MPA group's cancer-cell numbers were significantly lower than those of the MET and MPA groups (both $P < .001$).

Moreover, the EDU assay yielded similar results related to cell-cancer viability (Figure 1D). The MET and MPA groups' cancer-cell viability was significantly lower than that of the control group (both $P < .05$) and that the MET+MPA group's cancer-cell viability was significantly lower than those of the MET and MPA groups (both $P < .01$).

Figure 1E shows that the MET, MPA, and MET+MPA groups showed significantly restricted

expression of PCNA and Ki-67, the proliferation-related genes. The MET+MPA group's PCNA protein expression was significantly lower than that of the MET group ($P < .05$) and the MDA group ($P < .01$), and that the MET+MPA group's Ki-67 protein expression was significantly lower than that of the MET group ($P < .01$) and MPA group ($P < .05$).

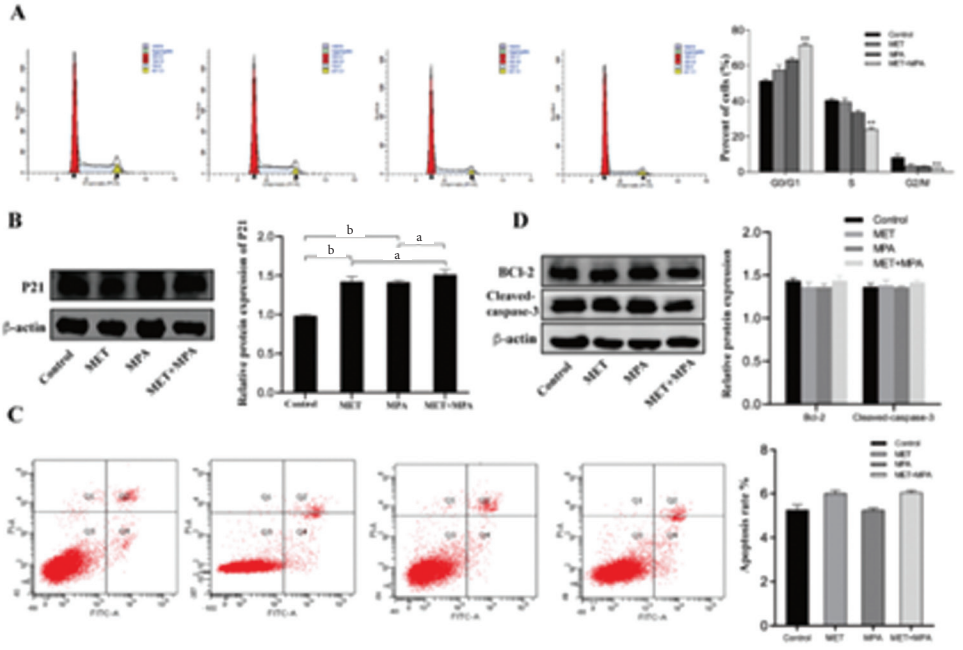
Cell Cycle and Apoptosis

Figure 2A shows that MET+MPA group, compared with control, MET, and MPA groups, had significantly more stagnated cancer cells in the G0/G1 phase and significantly fewer cancer cells in the S phase or G2/M phase (all $P < .01$).

Figure 2B shows that the MET and MPA groups had significantly greater inhibition of the expression of P21 related to the cell cycle than the control group did (both $P < .01$). The MET+MPA group had significantly greater inhibition of the expression of P21 than the MET and MPA groups did ($P < .05$)

No significant differences existed in apoptosis (Figure 2C) or in the expression of Bcl-2 and cleaved-caspase-3, the apoptosis-related proteins (Figure 2D), among the three groups.

Figure 2. Inhibition Using Metformin (MET) Combined With Medroxyprogesterone Acetate (MPA) of the Cell Cycle in Ishikawa cells for the Control, MPA, MET, and MET+MPA Groups Figure 2A shows metformin combined with MPA could increase the G1 phase blocking effect of MPA on Ishikawa cells, Compared with MPA alone, as detected using flow cytometry; Figure 2B shows MET's inhibition of the cell-cycle-related protein (p21), as detected using the Western blot; Figure 2C The results showed that MET + MPA had no significant effect on the apoptosis of Ishikawa cells compared with the control group. Figure 2D shows that the effects of MET and MPA on apoptosis-related proteins Bcl-2 and aved-caspase-3 were not significant, as detected using the Western blot.



[a]$P < .05$, indicating that the MET+MPA had significantly greater inhibition of the expression of P21 related to the cell cycle than the MET and MPA groups did

[b]$P < .01$, indicating that the MET+MPA group had significantly more stagnated Ishikawa cells in the G0/G1 phase and significantly fewer cancer cells in the S phase or G2/M phase than the control, MET, and MPA groups did and that the MET and MPA groups had significantly greater inhibition of the expression of P21 related to the cell cycle than the control group did

Abbreviations: BCl-2, B-cell leukemia/lymphoma 2 protein; FITC-A, fluorescein isothiocyanate-annexin; G2, gap stage; M, mitosis stage.

PR Expression

Figure 3 shows that the MET and MPA groups had PR protein expression that was significantly higher than that of the control group (both $P < .001$). The MET+MPA group's PR protein expression was significantly higher than that of the MPA or MET group (both $P < .001$). This phenomenon was dose-dependent, and when the research team halved the doses of MET and MPA, the expression of PR protein also significantly decreased compared to that of the MET+MPA group ($P < .001$).

CD133 Expression

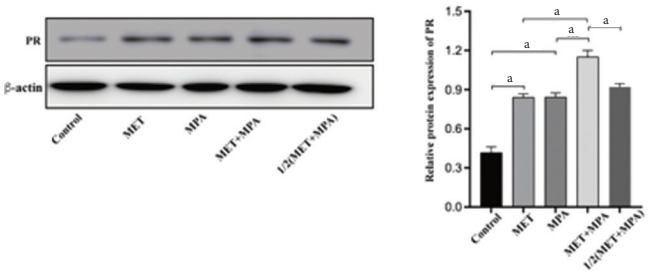
Figure 4A shows that the MET and MPA groups' CD133 protein levels were significantly lower than those of the control group, with $P < .001$ and $P < .05$, respectively, and that the MET+MPA group's CD133 protein expression was significantly lower than those of the MET and MPA groups (both $P < .001$). In contrast, the 1/2(MET+MPA) group had a significantly higher CD133 expression than that of the MET+MPA group ($P < .01$).

Figure 4B shows that the MET and MPA groups' sphere numbers were significantly lower than those of the control group, with $P < .001$ and $P < .05$, respectively, and the MET+MPA group's sphere numbers were significantly lower than those of the MET or MPA groups, with $P < .05$ and $P < .001$, respectively.

Akt/mTOR Pathway

No significant difference existed in the expression of total Akt or mTOR in cells for the MET, MPA or MET+MPA groups (data not shown). Figure 5 shows that both the MET and MPA groups had significantly lower expression of p-Akt,

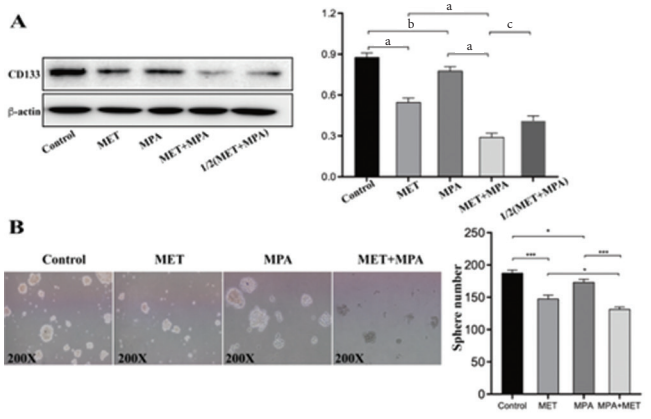
Figure 3. Promotion of Progesterone Sensitivity in Ishikawa cells Using Metformin (MET) and Medroxyprogesterone Acetate (MPA) Through an Increase in PR Expression for the Control, MPA, MET, and MET+MPS Groups



[a]$P < .001$, indicating that the MET and MPA groups' protein expression of PR were significantly higher than that of the control group, that the MET+MPA group's protein expression of PR was significantly higher than those of the MET and MPA groups, and that the 1/2(MET+MPA) group's protein expression of PR was significantly lower than that of the MET+MPA group

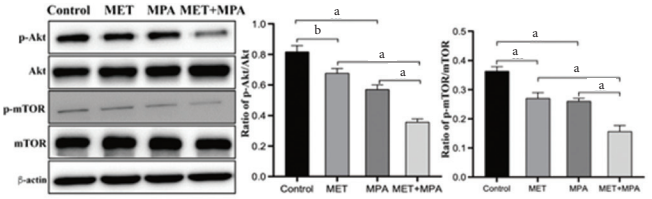
Abbreviations: PR, progesterone nuclear receptor.

Figure 4. Promotion of Progesterone Sensitivity in Ishikawa cells Using Metformin (MET) to Inhibit Expression of the CD133 Antigen for the Control, Medroxyprogesterone Acetate (MPA), MET, and MET+MPS Groups Figure 4A shows the expression of CD133 proteins after treatment with MET and MPA, and Figure 4 B shows the effects on the spherification process of MET and MPA.



[a]$P < .001$, indicating that the MET group's expression of PR proteins and sphere numbers were significantly lower than those of the control group, that the MET+MPA group's expression of PR proteins was significantly lower than that of the MET and MPA groups, and that the MET+MPA group's expression of PR proteins and sphere numbers were significantly lower than those of the MPA group
[b]$P < .05$, indicating that the MPA group's expression of PR proteins and sphere numbers were significantly lower than those of the control group and that the MET+MPA group's sphere numbers were significantly lower than those of the MET group
[c]$P < .01$, indicating that the 1/2(MET+MPA) group's expression of PR proteins was significantly higher than that of the MET+MPA group

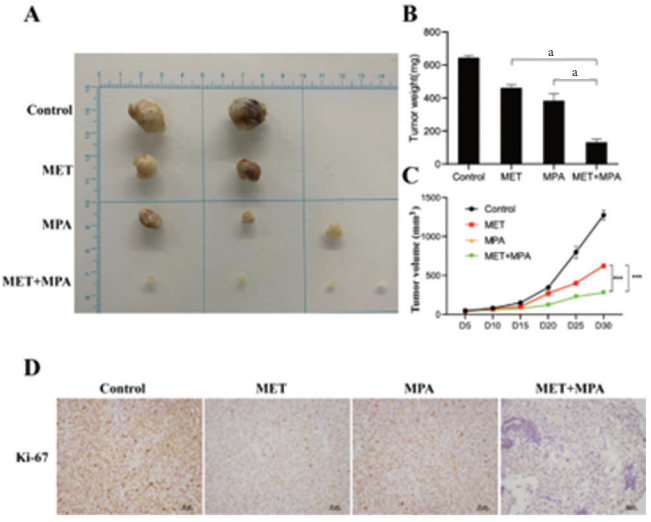
Figure 5. Enhancement of Progesterone Sensitivity in Ishikawa cells With Metformin (MET) Through the Akt/mTOR Pathway for the Control, Medroxyprogesterone Acetate (MPA), MET, and MET+MPA Groups The figure shows the Akt/mTOR-pathway-related protein expression as affected by MET and MPA, detected using the Western blot.



[a]$P < .001$, indicating that the MET group had significantly lower expression of p-mTOR than the control group did, that the MPA group had significantly lower expression of p-Akt and p-mTOR than the control group did, and that the MET+MPA group had significantly lower expression of p-Akt and p-mTOR than the MET and MPA groups did
[b]$P < .01$, indicating that the MET group had significantly lower expression of p-Akt than the control group did

Abbreviations: Akt, protein kinase B; mTOR, mammalian target of rapamycin.

Figure 6. Use of Metformin (MET) to Boost to the Sensitivity of Progesterone In Vivo in Ishikawa cells for the Control, Medroxyprogesterone Acetate (MPA), MET, and MET+MPS Groups ,The figures show in-vivo results. Figure 6A shows the tumor growth after Ishikawa cells induction in the mice; Figures 6B and 6C) shows the tumor volumes and weights of the mice at the end of 30 days; and Figure 6D shows the representative Ki67 antigen in the subcutaneous tumors after Ishikawa cells induction in the mice.



[a]$P < .001$, indicating that the MET+MPA group's tumor weights were significantly lower and tumor volumes were significantly smaller than those of the MET and MPA groups

with $P < .05$ and $P < .001$, respectively, and significantly lower expression of p-mTOR (both $P < .001$) than the control group did. The MET+MPA group had significantly lower expression of p-Akt and p-mTOR (both $P < .001$).

Sensitivity of MPA to EC

Figures 6A6B, and 6C show that the MET and MPA alone did decrease the tumor size and volume, The MET+MPA group's tumor weights were significantly lower and tumor volumes were significantly smaller than those of the MET and MPA groups (all $P < .001$).

The immunohistochemistry showed that the MET+MPA group's levels of the Ki-67 antigen were significantly lower than those of the MET and MPA groups (Figure 6D).

DISCUSSION

The current research team conducted a study to determine whether MET increases the sensitivity of Ishikawa cells to progesterone and initially investigated its mechanisms of action.

First, we conducted a proliferation experiment of CCK8 cells in vitro , and the results showed that metformin could inhibit the proliferation of Ishikawa cells. metformin at 25mM showed obvious inhibitory effect After 48 h of treatment, when the cells were in good condition However, the effective dose of MET as an antitumor medication isn't clear. Metformin (2.5 mM) combined with different concentration (0, 0.1, 1, 5, 10, 20, 30 μM) MPA can increase the inhibitory effect of progesterone on the proliferation of Ishikawa cells. Metformin (2.5 mM) combined with MPA (10 μM) treated Ishikawa cells for 48h, the inhibitory effect was obvious and the cells were in good condition . We use this concentration for our experiment by my study and the current study's results.In my study,.Ishikawa cells treated with MET (2.5 mM), MPA (10 μM) or the combination for 48h caused blocked G1 cell-cycle arrest, which was consistent with previous reports about EC cells[22]; however, none of drug-treated groups exhibited significant apoptosis.

In the current study, the MET significantly upregulated PR expression, and even more so when combined with MPA, and this combined synergistic effect was dose dependent. This upregulation effect was reduced when met combined with mpa dose was halved.

Down-regulation of PR expression is an important reason for insensitivity to progesterone therapy. The current study found that both MET and MPA could inhibit AKT/ mTOR phosphorylation, and the inhibition was more pronounced after using the combination of drugs. Therefore, the current research team needs to further study the relationship between the upregulation of PR expression and the decrease in AKT/mTOR phosphorylation level.

The current study found that both MET and MPA downregulated CD133 protein expression in the Ishikawa cells, and the inhibitory effect was more pronounced in the MET+MPA group. In addition, the expression of CD133 protein was upregulated in the MET+MPA group after the dose was halved. These results indicate that MET may increase the sensitivity of Ishikawa cells to progesterone through downregulating CD133.

MET inhibited proliferation by regulating the expression of PR and CD133 via the AKT/ mTOR pathway and increased the sensitivity of Ishikawa cells to progesterone.

The current study found that MET repressed the Akt/ mTOR pathway and had a synergistic inhibitory effect with MPA. The study also found a synergistic blocking effect of MET and MPA on the Ishikawa cell cycle G1, which may be related to the synergistic inhibition of mTOR phosphorylation, and consequently, cell proliferation.

MET may be a targeted drug for Akt/mTOR signaling pathway and CDl33+ cells to increase the sensitivity of EC to progesterone and is expected to be an effective progesterone sensitizer.

The current study had two major limitations that the research team could be address in future research. First, study performed only in-vitro studies on proliferation and apoptosis of one cell line, so more cell-line studies and in-vivo studies must confirm the results. Second, the study lacked an examination of the interactions between Akt/ mTOR pathway, CD133, and the research team needs to perform more experiments to find their possible interactions.

CONCLUSIONS

MET inhibited the proliferation of EC cells by increasing MPA-sensitivity, which was dependent on the inhibition of CD133 expression and the Akt/mTOR pathway. In addition, if MET acts as an effective progestin sensitizer, it certainly offers promising therapeutic prospects for patients with early-stage EC or overgrown endometrium who have fertility requirements.

DATA AVAILABILITY

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

AUTHORS' DISCLOSURE STATEMENT

The authors declare that they have no conflicts of interest related to the study.

REFERENCES

1. Kim S, Kim J, Lu KH, Broaddus RR. Endometrial Cancer.
2. Braun MM. Diagnosis and Management of Endometrial Cancer. *Endometrial Cancer*. 2016;93(6).
3. Rees M, Angioli R, Coleman RL, et al. European Menopause and Andropause Society (EMAS) and International Gynecologic Cancer Society (IGCS) position statement on managing the menopause after gynecological cancer: focus on menopausal symptoms and osteoporosis. *Maturitas*. 2020;134:56-61. doi:10.1016/j.maturitas.2020.01.005
4. Erickson BK, Martin JY, Shah MM, Straughn JM Jr, Leath CA III. Reasons for failure to deliver National Comprehensive Cancer Network (NCCN)-adherent care in the treatment of epithelial ovarian cancer at an NCCN cancer center. *Gynecol Oncol*. 2014;133(2):142-146. doi:10.1016/j.ygyno.2014.02.006
5. Gallos ID, Yap J, Rajkhowa M, Luesley DM, Coomarasamy A, Gupta JK. Regression, relapse, and live birth rates with fertility-sparing therapy for endometrial cancer and atypical complex endometrial hyperplasia: a systematic review and metaanalysis. *Am J Obstet Gynecol*. 2012;207(4):266.e1-266.e12. doi:10.1016/j.ajog.2012.08.011
6. Pal N, Broaddus RR, Urbauer DL, et al. Treatment of Low-Risk Endometrial Cancer and Complex Atypical Hyperplasia With the Levonorgestrel-Releasing Intrauterine Device. *Obstet Gynecol*. 2018;131(1):109-116. doi:10.1097/AOG.0000000000002390
7. Garzon S, Uccella S, Zorzato PC, et al. Fertility-sparing management for endometrial cancer: review of the literature. *Minerva Med*. 2021;112(1):55-69. doi:10.23736/S0026-4806.20.07072-X
8. Li W, Wang S, Qiu C, et al. Comprehensive bioinformatics analysis of acquired progesterone resistance in endometrial cancer cell line. *J Transl Med*. 2019;17(1):58. doi:10.1186/s12967-019-1814-6
9. You Y, Tan W, Guo Y, et al. Progesterone promotes endothelial nitric oxide synthase expression through enhancing nuclear progesterone receptor-SP1 formation.
10. Kim JJ, Kurita T, Bulun SE. Progesterone action in endometrial cancer, endometriosis, uterine fibroids, and breast cancer. *Endocr Rev*. 2013;34(1):130-162. doi:10.1210/er.2012-1043

11. Pedroza DA, Rajamanickam V, Subramani R, Bencomo A, Galvez A, Lakshmanaswamy R. Progesterone receptor membrane component 1 promotes the growth of breast cancers by altering the phosphoproteome and augmenting EGFR/PI3K/AKT signalling. *Br J Cancer*. 2020;123(8):1326-1335. doi:10.1038/s41416-020-0992-6
12. Zeberg H, Kelso J, Pääbo S. The Neandertal Progesterone Receptor. Falush D, ed. *Molecular Biology and Evolution*. 2020;37(9):2655-2660. doi:10.1093/molbev/msaa119
13. Yu P, Zhang Z, Li S, et al. Progesterone modulates endothelial progenitor cell (EPC) viability through the CXCL12/CXCR4/PI3K/Akt signalling pathway. *Cell Prolif*. 2016;49(1):48-57. doi:10.1111/cpr.12231
14. Zhang Z, Kang X, Guo Y, et al. Association of circulating galectin-3 with gestational diabetes mellitus, progesterone, and insulin resistance. *J Diabetes*. 2021;13(1):54-62. doi:10.1111/1753-0407.13088
15. Flory J, Lipska K. Metformin in 2019. *JAMA*. 2019;321(19):1926-1927. doi:10.1001/jama.2019.3805
16. Metformin HL, Metabolism S. *Trends Pharmacol Sci*. 2020;41(11):868-881. doi:10.1016/j.tips.2020.09.001
17. Al Khalifah RA, Florez ID, Dennis B, Thabane L, Bassilious E. Metformin or Oral Contraceptives for Adolescents With Polycystic Ovarian Syndrome: A Meta-analysis. *Pediatrics*. 2016;137(5):e20154089. doi:10.1542/peds.2015-4089
18. Del Barco S, Vazquez-Martin A, Cufí S, et al. Metformin: multi-faceted protection against cancer. *Oncotarget*. 2011;2(12):896-917. doi:10.18632/oncotarget.387
19. Podhorecka M, Ibanez B, Dmoszyńska A. Metformin – its potential anti-cancer and anti-aging effects. *Postepy Hig Med Dosw*. 2017;71(1):0-0. doi:10.5604/01.3001.0010.3801
20. Sun R, Zhai R, Ma C, Miao W. Combination of aloin and metformin enhances the antitumor effect by inhibiting the growth and invasion and inducing apoptosis and autophagy in hepatocellular carcinoma through PI3K/AKT/mTOR pathway. *Cancer Med*. 2020;9(3):1141-1151. doi:10.1002/cam4.2723
21. Pabona JMP, Burnett AF, Brown DM, et al. Metformin Promotes Anti-tumor Biomarkers in Human Endometrial Cancer Cells. *Reprod Sci*. 2020;27(1):267-277. doi:10.1007/s43032-019-00019-2
22. Huang X, Zhong R, He X, et al. Investigations on the mechanism of progesterone in inhibiting endometrial cancer cell cycle and viability via regulation of long noncoding RNA NEAT1/microRNA-146b-5p mediated Wnt/β-catenin signaling. *IUBMB Life*. 2019;71(2):223-234. doi:10.1002/iub.1959
23. Reis FM, Coutinho LM, Vannuccini S, Batteux F, Chapron C, Petraglia F. Progesterone receptor ligands for the treatment of endometriosis: the mechanisms behind therapeutic success and failure. *Hum Reprod Update*. 2020;26(4):565-585. doi:10.1093/humupd/dmaa009
24. Llauradó M, Ruiz A, Majem B, et al. Molecular bases of endometrial cancer: new roles for new actors in the diagnosis and the therapy of the disease. *Mol Cell Endocrinol*. 2012;358(2):244-255. doi:10.1016/j.mce.2011.10.003
25. Guo J, Wang Z, Xue M, et al. Metformin protects against abdominal aortic aneurysm by Atg7-induced autophagy. *Adv Clin Exp Med*. 2022;31(1):59-69. doi:10.17219/acem/142026
26. Kim TS, Lee M, Park M, et al. Metformin and Dichloroacetate Suppress Proliferation of Liver Cancer Cells by Inhibiting mTOR Complex 1. *Int J Mol Sci*. 2021;22(18):10027. doi:10.3390/ijms221810027
27. Kalender A, Selvaraj A, Kim SY, et al. Metformin, independent of AMPK, inhibits mTORC1 in a rag GTPase-dependent manner. *Cell Metab*. 2010;11(5):390-401. doi:10.1016/j.cmet.2010.03.014
28. Bitelman C, Sarfstein R, Sarig M, et al. IGF1R-directed targeted therapy enhances the cytotoxic effect of chemotherapy in endometrial cancer. *Cancer Lett*. 2013;335(1):153-159. doi:10.1016/j.canlet.2013.02.009
29. Patel BG, Rudnicki M, Yu J, Shu Y, Taylor RN. Progesterone resistance in endometriosis: origins, consequences and interventions. *Acta Obstet Gynecol Scand*. 2017;96(6):623-632. doi:10.1111/aogs.13156
30. Hubbard SA, Friel AM, Kumar B, Zhang L, Rueda BR, Gargett CE. Evidence for cancer stem cells in human endometrial carcinoma. *Cancer Res*. 2009;69(21):8241-8248. doi:10.1158/0008-5472.CAN-08-4808
31. Nakamura M, Zhang X, Mizumoto Y, et al. Molecular characterization of CD133+ cancer stem-like cells in endometrial cancer. *Int J Oncol*. 2014;44(3):669-677. doi:10.3892/ijo.2013.2230
32. Schuler KM, Rambally BS, DiFurio MJ, et al. Antiproliferative and metabolic effects of metformin in a preoperative window clinical trial for endometrial cancer. *Cancer Med*. 2015;4(2):161-173. doi:10.1002/cam4.353
33. Laskov I, Drudi L, Beauchamp MC, et al. Anti-diabetic doses of metformin decrease proliferation markers in tumors of patients with endometrial cancer. *Gynecol Oncol*. 2014;134(3):607-614. doi:10.1016/j.ygyno.2014.06.014