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Metformin inhibits proliferation and migration of endometrial cancer cells through regulating PI3K/AKT/MDM2 pathway

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Abstract. – OBJECTIVE: To investigate the influences of metformin on the proliferation and migration of endometrial cancer (EC) Ishikawa cells and its mechanism.

MATERIALS AND METHODS: After the EC Ishikawa cells were treated with metformin at a concentration of 10 mM for 24 h, the proliferation of cancer cells was detected via XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-car-boxanilide] assay and colony formation assay, and the migration and invasion of cancer cells were detected via wound healing assay and transwell assay. In addition, the expressions of epithelial-mesenchymal transition (EMT)-related proteins, E-cadherin and Vimentin, were detected via Western blotting, and immunofluorescence staining was performed for E-cadherin in cancer cells. Finally, the protein expression level of phosphatidylinositol 3-hydroxy kinase/protein kinase B/murine double minute 2 (PI3K/AKT/MDM2) signaling pathway in cancer cells was detected via Western blotting.

RESULTS: Metformin inhibited the proliferation of Ishikawa cells in a concentration-dependent manner (0-10 mM) ($p<0.05$). Moreover, metformin (10 mM) also inhibited the proliferation of Ishikawa cells in a time-dependent manner (0-72 h) ($p<0.05$). The results of colony formation assay revealed that metformin (10 mM) could significantly inhibit the colony formation of Ishikawa cells ($p<0.05$). The results of wound healing assay and transwell assay showed that metformin (10 mM) significantly inhibited the migration and invasion of Ishikawa cells ($p<0.05$). According to further studies, metformin (10 mM) inhibited the EMT process in Ishikawa cells. Western blotting results manifested that the activation of PI3K/AKT/MDM2 signaling pathway was inhibited by metformin ($p<0.05$).

CONCLUSIONS: Metformin can inhibit the proliferation and migration of EC cells by inhibiting the activation of PI3K/AKT/MDM2 signaling pathway. Therefore, metformin is expected to be a new drug for the clinical treatment of EC.

Key Words:
Metformin, Endometrial cancer cells, Proliferation, Migration

Introduction

Endometrial cancer (EC) is a kind of common tumor in the female reproductive system, as well as the third major cause of gynecological cancer death[1]. In Europe and America EC has the highest morbidity rate in female[2]. The level of leptin or adiponectin in many women is increased due to modern lifestyles, thus raising the incidence rate of EC[3]. In 1983 EC was divided into two types by Bokhman according to its microstructure, clinical features and epidemiological features, namely type 1 and type 2. Most EC patients are diagnosed after menopause, but there are still about 14% EC patients suffering from it at the age of 404. At present, the standard therapeutic methods for EC are chemotherapy using cytotoxic drugs and staging operations, including hysterectomy, bilateral salpingo-ovariotomy and lymph node dissection. However, whether EC patients treated with operation or chemotherapeutic drugs are complicated with severe complications remains unknown[5]. Therefore, searching the cheap and efficient anti-EC drugs is of great significance in the clinical prevention and treatment of EC. Metformin is widely applied in the treatment of type 2 diabetes mellitus, which can significantly inhibit the insulin resistance and reduce the diabetes-related morbidity and mortality rates[5,6]. According to the

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population-based studies, metformin can reduce the risk of cancer in a dose-dependent manner[7]. Moreover, metformin treatment also increases the drug sensitivity after neoadjuvant chemotherapy for breast cancer, indicating that it may function as an anti-cancer drug[8]. Type 2 diabetes mellitus is correlated with the increased insulin resistance and insulin level, and increased cancer-related mortality rate, which may be related to the activation of insulin and insulin-like growth factor signaling pathway and estrogen receptor by metformin[9, 10]. Therefore, reversing these processes via oral administration of metformin to reduce insulin resistance may be a new therapeutic strategy for cancer. In this study, EC Ishikawa cells were treated with metformin at different concentrations, after which the proliferation and migration of EC cells were observed and detected, and the possible molecular mechanism of metformin in affecting the biological behaviors of EC cells was explored, so as to provide some references for the prevention and treatment of EC in the future.

Materials and Methods

Materials and Cells

EC Ishikawa cell lines were purchased from Shanghai Kanglang Biological Technology Co., Ltd. (Shanghai, China), metformin from Sigma-Aldrich, (St. Louis, MO, USA), fetal bovine serum (FBS) and Dulbecco's modified Eagle' medium (DMEM)/F12 medium from Gibco (Rockville, MD, USA).

Cell Treatment

Ishikawa cells were inoculated into a 96-well plate (5×104/mL) and treated with metformin at different concentrations (0.1, 1, 4, 8 and 10 mM) after 48 h. After 72 h, 50 mL XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-car-boxanilide] were added for incubation at 37°C for 2 h, and then the absorbance was read at 450 nm.

Western Blotting

(1) The culture solution in the medium was discarded first, and cells were washed with phosphate-buffered saline (PBS) for 3 times. (2) 1000 μL lysis buffer were added into every dish and fully vibrated for 20 min. (3) The cells at the bottom of dish were scraped off using a brush and placed into the Eppendorf (EP) tube. (4) The cells collected were lysed using an ultrasonic pyrolyser for about 15 s. (5) After standing for 15 min, the cells were centrifuged at 12,000 rpm for 0.5 h. (6) The supernatant was taken and placed into the EP tubes, the protein concentration was detected via ultraviolet spectrometry, and all the protein samples were adjusted to be the same concentration. (7) The protein was sub-packaged and placed in the refrigerator at -80°C. After the total protein was extracted from EC cells, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Next, the protein in the gel was transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and incubated with the primary antibody at 4°C overnight, and then incubated again with the goat anti-rabbit secondary antibody in a dark place for 1 h. The protein band was scanned and quantified using the Odyssey scanner, and the level of protein to be detected was corrected using glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Colony Formation Assay

The cells in each group were cultured till logarithmic growth phase, and digested with 0.25% trypsin into single cell suspension (the proportion of single cells was more than 95%). After that, the suspension was inoculated into a 6-well plate (about 500 cells/well) and added with 2 mL 1640 medium per well, and the medium was replaced once every 48 h. After 10 d, the cells were fixed with formaldehyde and stained with crystal violet, and the number of colonies in each well was counted.

Wound Healing Assay

The cells in logarithmic growth phase were inoculated into the 96-well plate (about 5×104 cells/well). After 24 h, the plate was scratched in the center using the pipette tip, the shedding cells were washed away with PBS, and the medium was replaced with serum-free medium. After 24 h, the cell migration status was photographed and recorded under a high-power microscope.

Invasion Assay

The Matrigel (diluted at 1:8) was used to coat the transwell chamber (8 μm) and incubated in the incubator at 37°C for 2 h to gelatinize. After that, the cell lines were diluted into single cell suspension using the serum-free medium and inoculated into the upper transwell chamber (5×104/100 μL), and the medium containing 10% fetal bovine serum (FBS) was added into the lower chamber,

followed by culture for 24 h. The cells passing through the member were fixed with 5% glutaraldehyde, stained with 0.1% crystal violet and photographed.

Immunofluorescence Staining

First, Ishikawa cells were inoculated into a 24-well plate containing the slide, treated with metformin (10 mM) and after 24 h, collected for immunofluorescence staining. The cells were fixed with formaldehyde, the cell membrane was disrupted using 0.2% Triton, and the cells were sealed with goat serum (diluted at 1:200 with PBS), followed by incubation with anti-E-cadherin antibody (diluted at 1:200 with PBS) at 4°C overnight. The cells were washed with PBS on a shaking table for 4 times, and incubated with FITC-secondary antibody at 37°C for 1 h, followed by nuclear staining with 4',6-diamidino-2-phenylindole (DAPI). After color development, 6 samples were randomly selected in each group and 5 visual fields were randomly selected in each sample, followed by photography under a fluorescence microscope (×200).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for the analysis of all data. Measurement data were expressed as mean ± standard deviation, and t-test was used for the comparison of data between two groups. $p<0.05$ suggested that the difference was statistically significant.

Results

Metformin Inhibited the Proliferation of Ishikawa Cells in a Concentration-Dependent Manner

First, Ishikawa cells were treated with metformin at different concentrations (0.1, 1, 4, 8 and 10 mM) for 24 h, and then the cell proliferation in each group was detected via XTT assay. The results revealed that metformin at a concentration of 1, 4, 8 and 10 mM could significantly inhibit the proliferation of Ishikawa cells ($p<0.05$), while metformin at a concentration of 0.1 mM had no influence on the proliferation of Ishikawa cells ($p>0.05$) (Figure 1). Therefore, metformin at the concentration of 10 mM was selected for verification in the subsequent experiments.

Metformin Inhibited the Proliferation of Ishikawa Cells in a Time-Dependent Manner

After treatment with metformin (10 mM) for 24, 48 and 72 h, the proliferation of Ishikawa cells significantly declined compared with that in control group ($p<0.05$) (Figure 2). Therefore, metformin at the concentration of 10 mM was selected in the subsequent experiments, and Ishikawa cells were stimulated for 24 h.

Metformin Inhibited the Colony Formation of Ishikawa Cells

Furthermore, the proliferation of Ishikawa cells was detected via colony formation assay. The results manifested that the number of colonies in metformin group (99.56±12.31) was significantly smaller than that in control group (243.22±14.98) ($p<0.05$) (Figure 3), indicating that metformin can reduce the colony formation ability of EC cells.

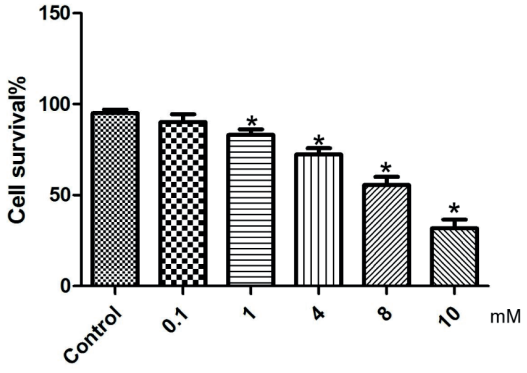

Figure 1. Metformin inhibits the proliferation of Ishikawa cells in a concentration-dependent manner. Control: Control group. *$p<0.05$: there is a statistically significant difference compared with Control group.

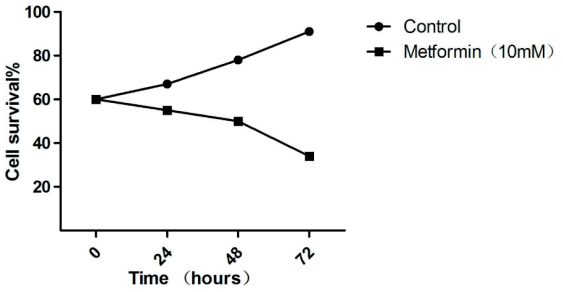

Figure 2. Metformin inhibits the proliferation of Ishikawa cells in a time-dependent manner. Control: Control group, Metformin: metformin group. *$p<0.05$: there is a statistically significant difference at 24, 48 and 72 h.

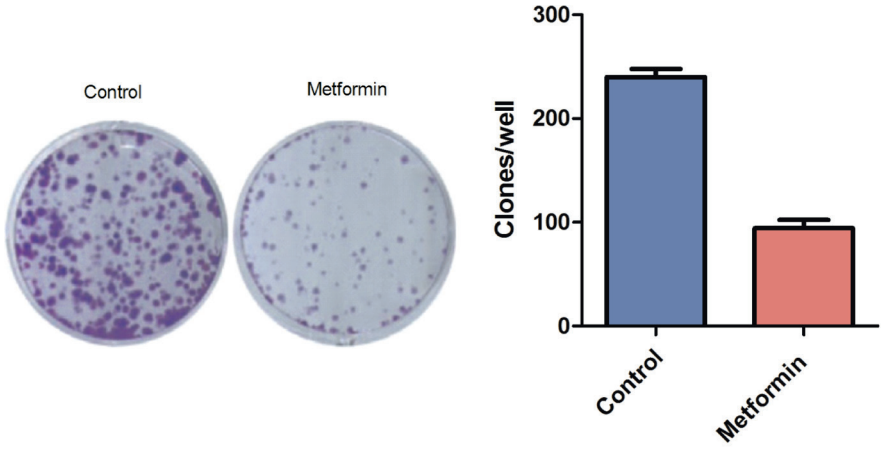

Figure 3. Metformin inhibits the colony formation of Ishikawa cells. Control: Control group, Metformin: metformin group. *p<0.05: there is a statistically significant difference compared with Control group.

Metformin Inhibited the Migration of Ishikawa Cells

The results of wound healing assay showed that metformin significantly inhibited the wound healing ability of Ishikawa cells. The wound-healing rate was (63.12±1.34)% and (14.78±2.11)%, respectively, in control group and metformin group (p<0.05) (Figure 4), suggesting that metformin exerts a certain inhibitory effect on the migration of cancer cells.

Metformin Inhibited the Invasion of Ishikawa Cells

At the same time, the effect of metformin on the invasion of EC cells was also detected. It was found that the number of invasive Ishikawa cells in control group was about 4.76 times that in metformin group (p<0.05) (Figure 5).

Metformin Inhibited Epithelial-Mesenchymal Transition (EMT) in Ishikawa Cells

Considering that EMT is one of the major mechanisms of cancer cell proliferation and migration, the EMT-related protein markers were further detected via Western blotting and immunofluorescence staining. It was found that metformin treatment could significantly inhibit the expression of Vimentin and increase the expression of E-cadherin (p<0.05) (Fi-

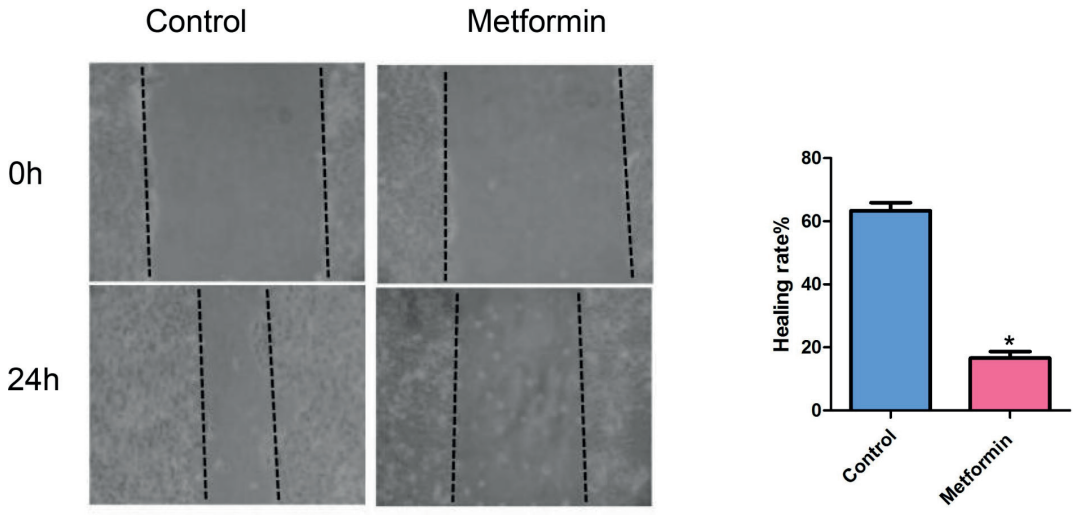

Figure 4. Metformin inhibits the migration of Ishikawa cells. Control: Control group, Metformin: metformin group. *p<0.05: there is a statistically significant difference compared with Control group.

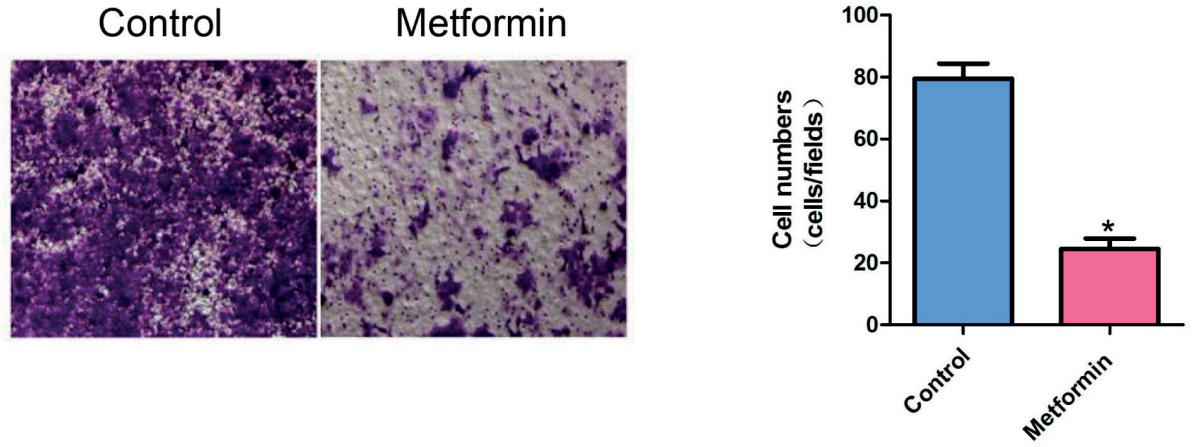

Figure 5. Metformin inhibits the invasion of Ishikawa cells. Control: Control group, Metformin: metformin group. *p<0.05: there is a statistically significant difference compared with Control group.

gure 6), proving that the inhibitory effects of metformin on cancer cell migration and invasion are related to its regulation on Vimentin and E-cadherin.

Metformin Inhibited the Activation of Phosphatidylinositol 3-Hydroxy Kinase/Protein Kinase B/Murine Double Minute 2 (PI3K/AKT/MDM2) Signaling Pathway in Ishikawa Cells

The activation of the PI3K/AKT/MDM2 signaling pathway is one of the main mechanisms of occurrence and development of tumor, so it was explored whether the inhibitory effects of metformin on the biological behaviors of Ishikawa cells are related to PI3K/AKT/MDM2. The main proteins of this pathway were detected via Western blotting, and it was found that the phosphorylation of AKT and MDM2 in metformin group was remarkably inhibited compared with that in control group (p<0.05), indicating that the inhibitory effect of metformin on EC may be related to its inhibition on PI3K/AKT/MDM2 signaling pathway (Figure 7).

Discussion

EC is one of the most common malignant tumors in the female reproductive system in developed countries, which has a high mortality rate[11,12]. The treatment of EC includes surgical resection supplemented by radiotherapy, chemotherapy and hormone therapy or targeted therapy (rapamycin)[13]. However, the patients with advanced metastatic or recurrent EC miss the best operation opportunity, so the treatment failure rate is still high[14]. With the molecular mechanism of EC gradually clarified in recent years, increasingly more molecular targeted drugs have been applied in clinic, obtaining satisfactory efficacy[15]. Therefore, designing drugs based on key target genes or proteins in the occurrence and development of EC is of great significance in the accurate diagnosis and individualized treatment of EC in the future. EMT can promote the motor activity and invasion ability of tumor cells[16]. The down-regulation of cell surface marker proteins, such as E-cadherin, is one of the earliest pathological changes in metastasis of tumor cells[17]. E-cadherin can exert an anti-cancer effect through inhibiting invasion and metastasis of tumor cells[18]. In the occurrence and development of EMT, a variety of signaling pathways are abnormally activated or inhibited, thus regulating the E-cadherin expression. For example, the activation of the PI3K/AKT/mTOR and Ras/ERK signaling pathways can inhibit the E-cadherin expression[19]. The PI3K signals are generally activated in various tumors, and the phosphorylated AKT can further phosphorylate multiple downstream effectors, such as mTOR and MDM2, once PI3K is activated. The activation of these signaling pathways can affect the expression of EMT-related marker proteins (E-cadherin and Vimentin), ultimately altering the migration and proliferation ability of tumor cells[20]. Metformin was initially applied in the treatment of type 2 diabetes mellitus, whose effect on the tumors, cardiovascular diseases, cerebrovascular diseases, nervous system diseases and en-

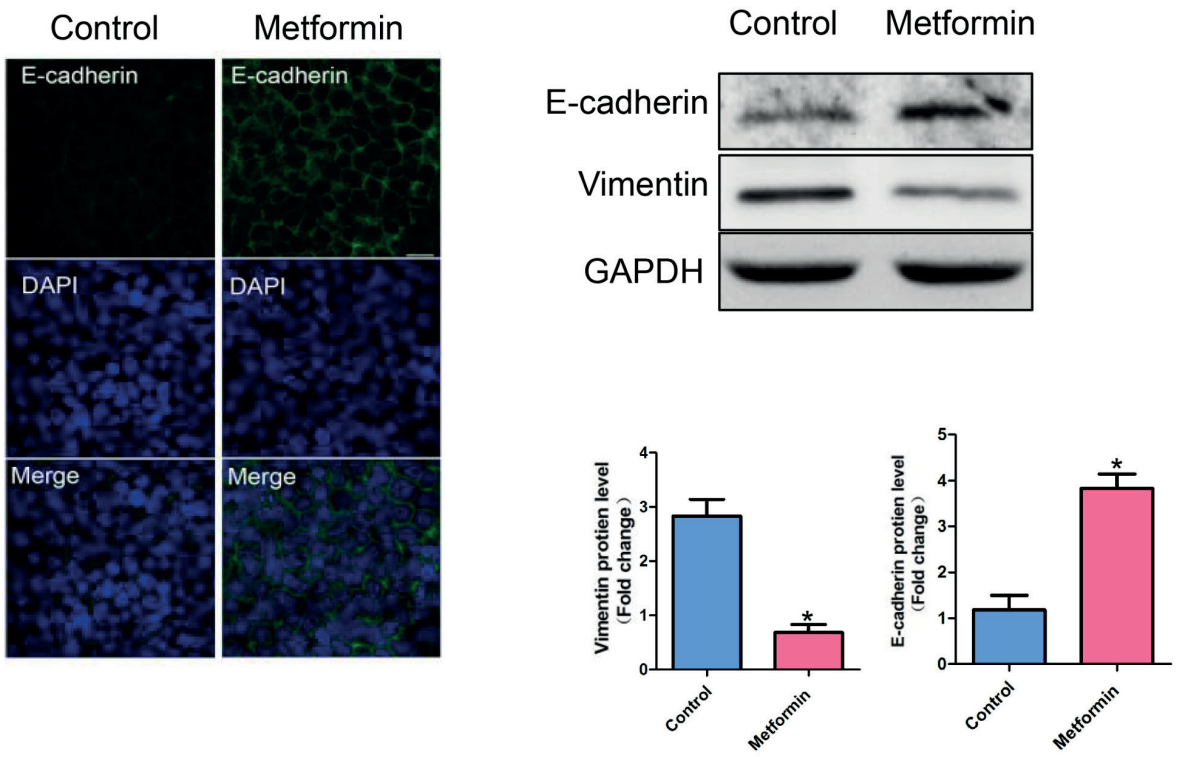

Figure 6. Metformin inhibits EMT in Ishikawa cells. Control: Control group, Metformin: metformin group. *p<0.05: there is a statistically significant difference compared with Control group.

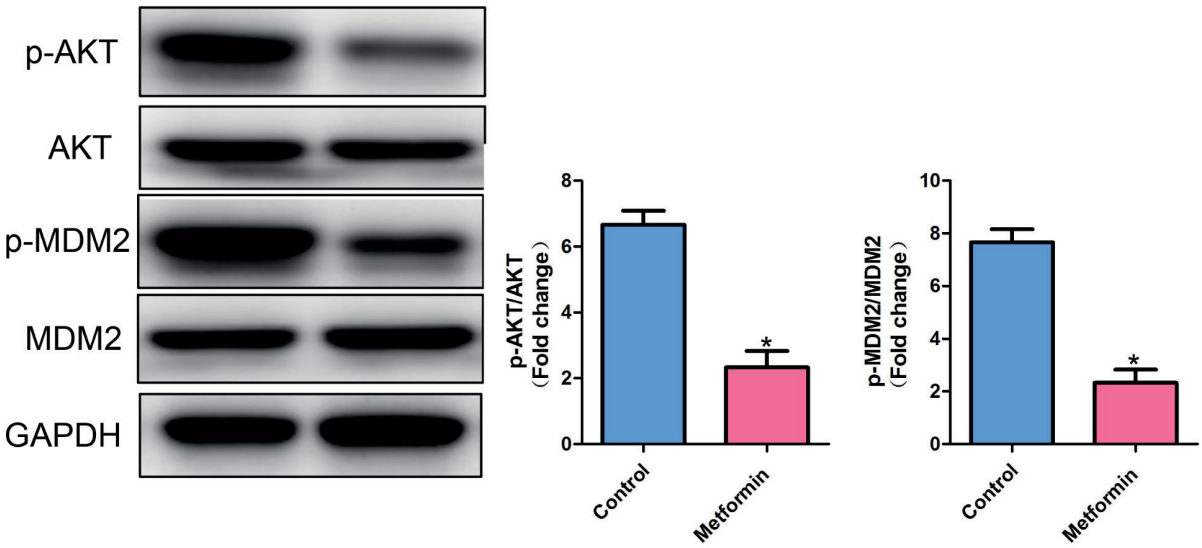

Figure 7. Metformin inhibits the activation of PI3K/AKT/MDM2 signaling pathway in Ishikawa cells. Control: Control group, Metformin: metformin group. *p<0.05: there is a statistically significant difference compared with Control group.

docrine diseases has been gradually revealed in recent years[21]. For example, metformin can inhibit the proliferation and promote the apoptosis of tumor cells, inhibit the angiogenesis and increase the sensitivity of tumor cells to chemotherapeutic drugs, ultimately inhibiting the occurrence and development of a variety of tumors[9]. For example, metformin can inhibit

triple-negative breast cancer stem cells through its targeted inhibition on the activation of PKA-GSK3β-KLF5 signaling pathway[22]. Moreover, metformin can inhibit the proliferation of breast cancer cells and tumorigenesis through up-regulating miR-200 and down-regulating AKT2[23]. In addition, metformin can also inhibit the progression of refractory breast cancer and increase its sensitivity to chemotherapeutic drugs[24]. In this study, it was proved for the first time that metformin could inhibit the proliferation and colony formation ability of EC cells in a time- and dose-dependent manner. Besides, metformin could inhibit the migration and invasion of EC cells, up-regulate the E-cadherin expression and down-regulate the Vimentin expression, thereby inhibiting EMT of EC cells. Finally, it was found that the inhibitory effects of metformin on migration, invasion and proliferation of EC cells were mediated by the PI3K/AKT/MDM2 signaling pathway. However, there were still some deficiencies in this experiment: (1) the inhibition assay was not performed to verify that the anti-cancer effect of metformin depends on the PI3K/AKT/MDM2 signaling pathway, (2) the conclusion was not proved via animal experiments, and (3) only one kind of EC cell line was used.

Conclusions

We showed that metformin can suppress the proliferation and migration of EC cells through inhibiting EMT of EC cells, ultimately exerting an anti-cancer effect. The anti-cancer effect of metformin may be related to its inhibition on PI3K/AKT/MDM2 signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Funding Acknowledgements

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