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Original Article

Metformin Affects Endometrial Carcinoma Cell Proliferation and Apoptosis by Regulating the Prognostic Marker S100a2 Expression

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ABSTRACT

Background: To investigate the effects of metformin on the proliferation and apoptosis of human endometrial carcinoma cells, along with elucidating the potential underlying mechanisms, we conducted a study wherein the candidate prognostic gene associated with endometrial carcinoma progression was identified through bioinformatics analysis. **Materials and Methods:** Endometrial carcinoma cells Ishikawa were treated with metformin at concentrations of 5, 10 and 20 mmol/L. Cell proliferation and apoptosis were assessed using Cell Counting Kit-8 (CCK-8) assays, clone formation tests and flow cytometry analysis. Additionally, immunofluorescence staining was performed to detect the expression of the apoptotic marker Caspase-1. The mRNA levels of S100 calcium binding protein A2 (S100A2), Phosphatidylinositol-4,5-bisphosphate 3-Kinase (PI3K), serine/Threonine Kinase 1 (AKT), BCL2 associated x (Bax) and B-cell lymphoma 2 (Bcl-2) were quantified by Real-Time quantitative Reverse Transition PCR (qRT-PCR). **Results:** The results indicated that S100A2 is a potential prognostic gene influencing the pathogenesis of endometrial carcinoma. Metformin inhibited the proliferation and clone formation of Ishikawa cells in a dose-dependent manner, while also significantly increasing the apoptotic rate of these cells ($p<0.05$). Furthermore, metformin dose-dependently upregulated Caspase-1 protein expression and downregulated the mRNA levels of S100A2, PI3K, AKT and Bcl-2, as well as increasing the expression of Bax mRNA ($p<0.05$). **Conclusion:** We concluded that metformin inhibits cell proliferation and promotes apoptosis in endometrial carcinoma, potentially through the regulation of S100A2 expression. These findings may offer new insights into the development of therapeutic targets for endometrial carcinoma in the future.

Keywords: Endometriosis (EMS), Human endometrial stromal cells (HESCs), Proliferation, Invasion, Wnt/β-catenin.

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INTRODUCTION

Endometrial carcinoma is a prevalent gynecological malignancy, yet its pathogenesis remains elusive.[1] Obesity and diabetes are notable risk factors for this disease, sharing a common pathophysiological foundation in hyperinsulinemia and insulin resistance.[2] Metformin, a biguanide compound, is widely employed in clinical practice to treat type 2 diabetes by effectively enhancing insulin sensitivity and improving blood glucose levels.[3] Recent studies have demonstrated that metformin can rectify insulin resistance in patients, subsequently influencing the onset and progression of endometrial carcinoma.[4,5] Furthermore, numerous reports have indicated that the anticancer effects of metformin are mediated through the regulation of diverse molecular signaling pathways, encompassing direct targets or other downstream effectors, thereby inhibiting tumor cell proliferation.[6,7] However, the precise mechanism of metformin's action on endometrial carcinoma remains to be fully elucidated.

Through the intersection of GSE17025 and GSE63678 datasets, coupled with subsequent analysis using Gene Expression Profiling Interactive Analysis (GEPIA) and Kaplan-Meier plotter, S100A2 was validated as a prognostic gene implicated in the progression and outcome of endometrial carcinoma. Given its crucial role in the disease's advancement, S100A2 has emerged as a diagnostic and prognostic biomarker for endometrial carcinoma.[8] In this study, the Ishikawa endometrial carcinoma cell line served as the model to explore whether metformin modulates the malignant behaviors of these cells by regulating S100A2. Our findings may

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unveil a novel regulatory mechanism underlying the influence of S100A2 in endometrial carcinoma under metformin therapy.

MATERIALS AND METHODS

Gene screening

We obtained two gene datasets, GSE17025 and GSE63678, from the NCBI website. The criteria for screening and identifying up-regulated mRNAs were set at a log2 Fold Change (FC) greater than 2 and a *p*-value less than 0.05.

Cell culture and drug treatment

Ishikawa cells, an endometrial carcinoma cell line, were cultivated in RPMI-1640 medium (Gibco, USA) at 37°C with 5% CO_2, supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin. Metformin (PHR1084, Sigma, USA) was dissolved in RPMI-1640 medium and diluted to concentrations of 5, 10 and 20 mmol/L. Subsequently, Ishikawa cells were treated with these metformin concentrations for 24 hr. Control cells, in contrast, were treated with PBS buffer.

CCK-8 assay

Ishikawa cells were treated with the specified concentrations of metformin. Following incubation for 24, 48 and 72 hr, the cells were exposed to CCK-8 solution for 4 hr at 37°C. Afterwards, the Optical Density (OD) at 490 nm was measured using an Enzyme-Linked Immunosorbent Assay (ELISA) reader (Thermo Fisher, USA).

Clonal formation experiment

Ishikawa cells were cultured for 15 days, subsequently fixed with 4% paraformaldehyde and stained with 1% crystal violet. Then, colonies consisting of more than 50 cells (considered as one clone) were counted in five randomly chosen fields using a microscope.

Immunofluorescence staining

Cells underwent standard fixation and permeabilization procedures. After a blocking step for 1 hr, they were incubated with the primary antibody against Caspase-1 (1:200 dilution) overnight at 4°C. Subsequently, the cells were treated with the secondary antibody for 2 hr and then counterstained with 4',6-Diamidino-2-Phenylindole (DAPI). The resulting images were captured using an Olympus FSX100 microscope (Japan).

Apoptosis measurement

Following the specified treatment, the cells were harvested and resuspended in binding buffer. Subsequently, 5 µL of Annexin V-FITC and Propidium Iodide (PI) solutions were added to the cells and the mixture was incubated in the dark. After the incubation period, the apoptotic rate was precisely analyzed by flow cytometry, in strict accordance with the manufacturer's instructions.

qRT-PCR

After extracting total RNA from Ishikawa cells, cDNA was synthesized using a reverse transcription kit. Further analysis was conducted on an ABI 7500 PCR system. β-actin served as the internal control and the relative gene expressions were accurately quantified using the $2^{\Delta\Delta Ct}$ method.

Statistical analysis

GraphPad Prism software was employed for statistical analysis. The experimental data were presented as Mean±SD and comparisons were made using one-way ANOVA, with a significance threshold set at $p<0.05$.

RESULTS

Abnormal expression of S100A2 in endometrial carcinoma

The up-regulated genes linked to endometrial carcinoma were identified using the gene expression profiles GSE17025 and GSE63678 and visually represented in a volcano plot (Figure 1A). Notably, S100A2 exhibited high expression levels in both profiles. Further analysis using GEPIA and Kaplan-Meier plotter revealed that S100A2 is a potential gene involved in the progression and prognosis of endometrial carcinoma (Figure 1B-C). These findings indicate that S100A2 is aberrantly up-regulated in endometrial carcinoma.

Effect of metformin on the proliferation of endometrial carcinoma cells

Ishikawa cells were treated with 5, 10 and 20 mmol/L of metformin. The CCK-8 assay revealed that an increasing concentration of metformin significantly decreased cell proliferation in a dose-dependent manner (Figure 2A). Additionally, the colony-forming ability of the cells was notably reduced in the metformin-treated group, exhibiting a gradual decline (Figure 2B-C). These findings suggest that metformin exerts an inhibitory effect on the proliferation of endometrial carcinoma cells.

Effect of metformin on the apoptosis of endometrial carcinoma cells

As shown in Figure 3A, the immunofluorescent staining data indicated that metformin increased the Caspase-1 protein level in Ishikawa cells in a dose-dependent manner. Furthermore, flow cytometry results demonstrated that the apoptotic rate of Ishikawa cells was elevated after 24 hr of co-culture with metformin, also in a dose-dependent manner (Figure 3B-C). These findings suggest that metformin promotes the apoptosis of endometrial carcinoma cells.

Effect of metformin on PI3K/AKT signaling in endometrial carcinoma cells

As shown in Figure 4A, the mRNA expressions of S100A2, PI3K and AKT in Ishikawa cells were reduced in a dose-dependent manner following metformin treatment. Furthermore, metformin significantly decreased Bcl-2 mRNA levels and increased Bax mRNA levels, also in a dose-dependent fashion (Figure 4B). These results suggest that metformin inhibits the S100A2/PI3K/AKT pathway and promotes cell apoptosis.

DISCUSSION

Metformin is commonly prescribed in gynecology to address insulin resistance and hyperinsulinemia in patients.[9,10] Endometrial carcinoma ranks as the sixth most prevalent tumor globally and is the most common malignancy affecting women.[11] This type of cancer is linked to hyperinsulinemia in the body and metformin has been shown to alleviate this condition and inhibit the growth of endometrial carcinoma cells in laboratory settings.[7,12,13] However, its anti-tumor mechanism remains to be further studied. Our findings indicate that metformin can suppress cell proliferation and promote apoptosis in endometrial carcinoma cells, potentially through the regulation of S100A2 expression. These insights may offer a fresh perspective for the development of therapeutic targets in endometrial carcinoma treatment.

Uncontrolled proliferation, blocked apoptosis and distant metastasis are hallmark biological characteristics of malignant tumors, including endometrial carcinoma.[14,15] Numerous anti-tumor drugs exert their therapeutic effects by influencing one or more of these biological processes. In this study, we observed that metformin, at concentrations of 5, 10 and 20 mmol/L, significantly inhibited the proliferation of Ishikawa cells and with the proliferative rate decreasing as the metformin dose

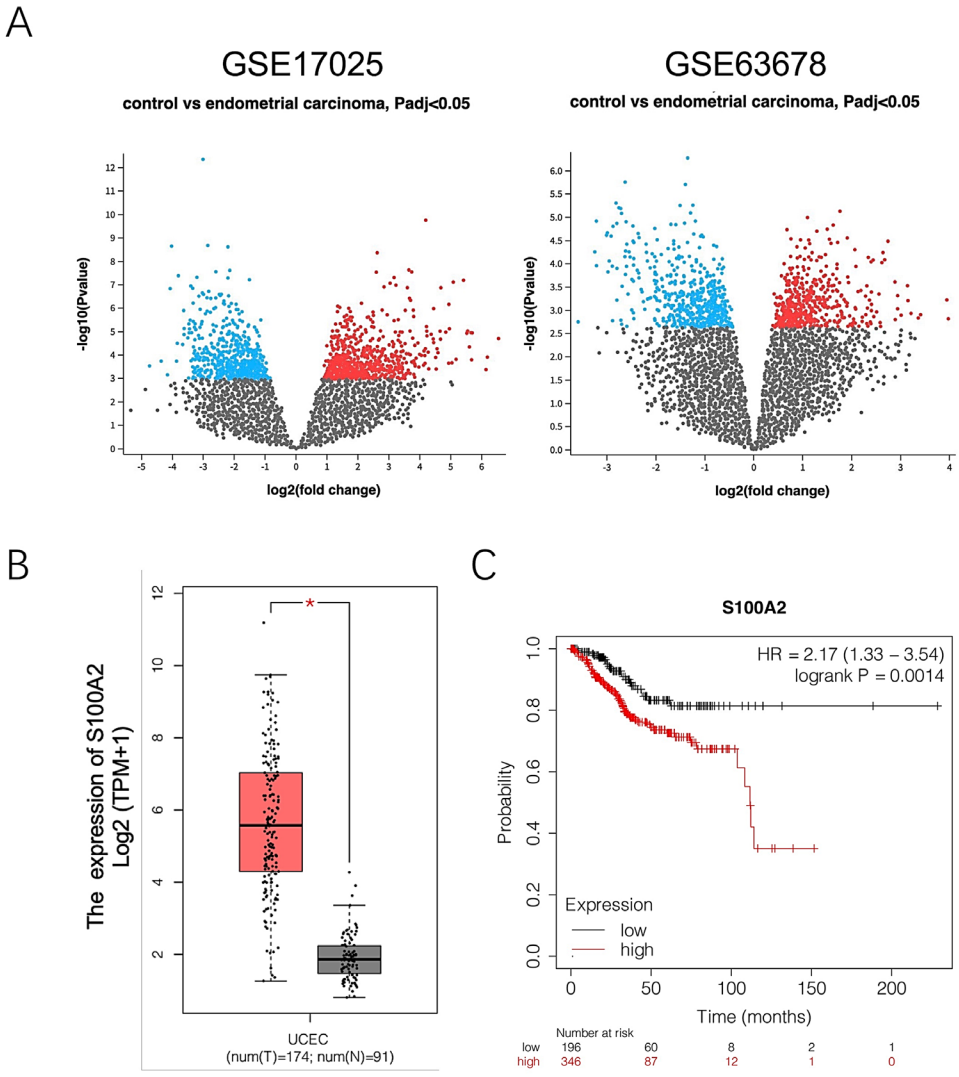

Figure 1: Abnormal expression of S100A2 in endometrial carcinoma. Note: (A) volcano plot. (B) GEPIA analysis. (D) Kaplan-Meier plotter analysis.

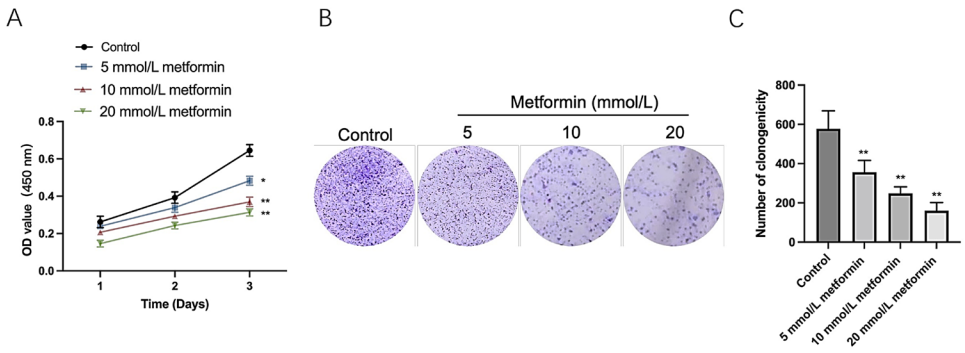

Figure 2: Effect of metformin on the proliferation of endometrial carcinoma cells. Note: (A) Cell proliferation measured by CCK-8 assay. (B) Results of clone formation. (C) Quantitative data of clones. ***p*<0.01, suggested a remarkable difference with the control group.

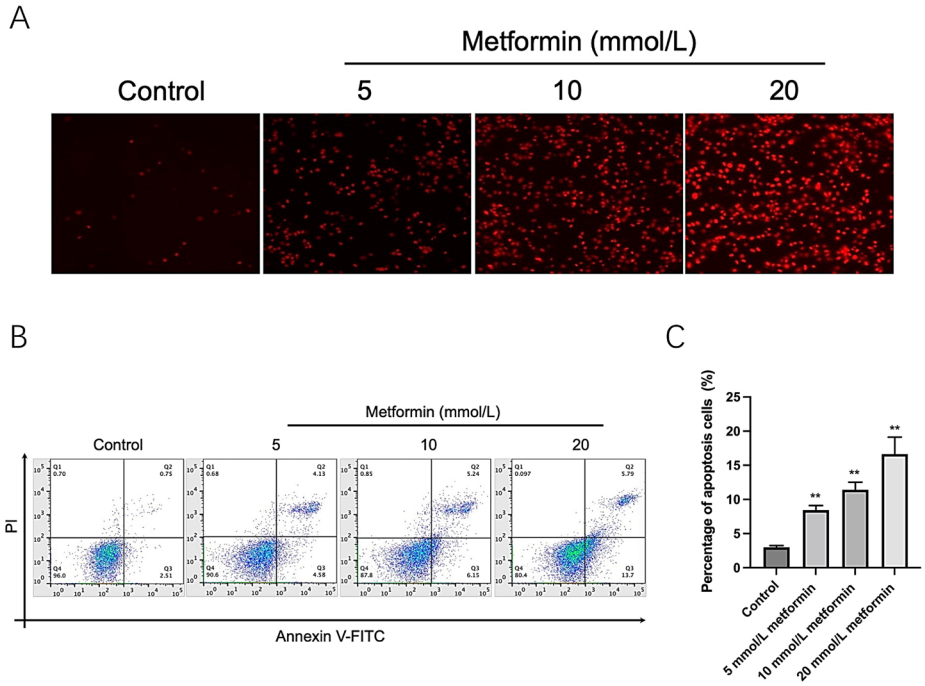

Figure 3: Effect of metformin on the apoptosis of endometrial carcinoma cells. Note: (A) Immunofluorescent staining of Caspase-1 protein. (B) Flow cytometry detection of cell apoptosis. (C) Quantitation of apoptotic rate. ***p*<0.01, suggested a remarkable difference with the control group.

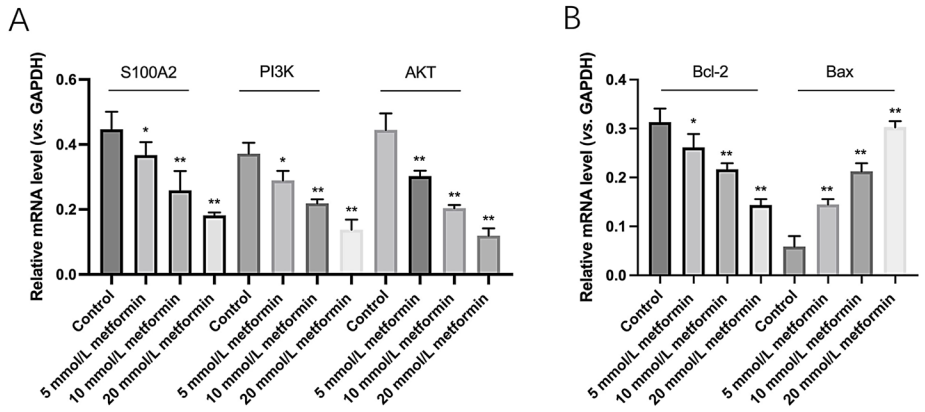

Figure 4: Effect of metformin on S100A2/PI3K/AKT pathway in endometrial carcinoma cells. Note: (A) qRT-PCR detection of S100A2, PI3K and AKT mRNA. (B) qRT-PCR detection of Bcl-2 and Bax mRNA. ***p*<0.01, suggested a remarkable difference with the control group.

increased. The clonal formation rate reflects the survival rate of inoculated cells, indicating the number of adherent cells that survive and form clones after inoculation.[16] Our data showed that metformin reduced the colony-forming ability of Ishikawa cells. Furthermore, we found that metformin dose-dependently induced Caspase-1 protein and Bax mRNA expression, decreased Bcl-2 mRNA levels and increased the apoptotic rate of Ishikawa cells, suggesting that metformin promotes apoptosis in endometrial carcinoma cells. Collectively, these results indicate that metformin inhibits cell proliferation and induces cell apoptosis in endometrial carcinoma.

To investigate the potential regulatory mechanisms, we analyzed the intersection of the GSE17025 and GSE63678 datasets, combined with GEPIA and Kaplan-Meier plotter analyses. These analyses confirmed that S100A2 is a prognostic marker and plays a role in the progression of endometrial cancer. The PI3K/AKT pathway is a crucial cellular signaling pathway that regulates various physiological functions, including cellular metabolism and proliferation.[17,18] Studies have highlighted the essential role of the PI3K/AKT signaling pathway in the development of endometrial carcinoma.[19-21] Our results demonstrated that the mRNA levels of PI3K and AKT in Ishikawa cells were reduced in a dose-dependent manner following metformin treatment. These findings suggest that metformin may affect the migration activities of Ishikawa cells by modulating the PI3K/AKT signaling pathway. Overall, our results reveal a significant inhibitory effect of metformin on the transcription of PI3K and AKT in endometrial carcinoma cells.

CONCLUSION

To summarize, the influence of metformin on the proliferation and apoptosis of endometrial cancer cells may partially be attributed to the inhibition of the PI3K/AKT pathway. Nonetheless, given that metformin is a multi-target drug, further research is necessary to elucidate its specific actions and mechanisms in combating endometrial cancer.

ABBREVIATIONS

EMS: Endometriosis; **HESCs:** Human endometrial stromal cells.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Yang Liu were responsible for conception and design. Boer Deng was responsible for manuscript writing. Dan Luo and Weimin Kong were responsible for collection and assembly of data. Jianjun Zhang and Weimin Kong were responsible for data analysis and interpretation. All authors were responsible for manuscript writing. All authors were responsible for the final approval of the manuscript.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

The ethics are approved and agreed by Beijing Obstetrics and Gynecology Hospital.

SUMMARY

In summary, while the suppression of the PI3K/AKT pathway may partly account for metformin's impact on the proliferation and apoptosis of endometrial cancer cells, this multi-target drug's precise actions and mechanisms in combating endometrial cancer require further investigation.

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