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Metformin reduces pancreatic cancer cell proliferation and increases apoptosis through MTOR signaling pathway and its dose-effect relationship

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Abstract. – OBJECTIVE: To study the influences of metformin on the proliferation and apoptosis of pancreatic cancer cells and its dose-effect relationship and crucial molecular mechanism.

MATERIALS AND METHODS: With human pancreatic cancer cell line PANC-1 as the study object, different concentrations of metformin were added for intervention. Then, the proliferation of PANC-1 cells was detected *via* methyl thiazolyl tetrazolium (MTT) assay to determine the dose-effect relationship of metformin in PANC-1 cell proliferation. PANC-1 cells were treated with metformin at three appropriate concentrations as Metformin treatment groups, and an equal amount of dimethyl sulfoxide (DMSO) was added in Control group. Flow cytometry was performed to detect PANC-1 cell cycle and apoptosis, and the apoptosis of PANC-1 cells was also evaluated *via* terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Caspase-3 protein localization and expression in PANC-1 cells were detected using immunofluorescence assay. Besides, the expressions of the apoptosis-associated proteins Caspase-3, B-cell lymphoma 2 (Bcl-2), and Bcl-2 associated X protein (Bax) and phosphatidylinositol 3-hydroxy kinase (PI3K), phosphorylated protein kinase B (p-Akt), and p-mammalian target of rapamycin (mTOR) proteins related to the mTOR pathway were detected using Western blotting.

RESULTS: Metformin repressed the proliferation of human pancreatic cancer PANC-1 cells in a concentration-dependent and time-dependent manner. Compared with Control group, Metformin treatment groups (0, 20 and 40 mM) exhibited a higher proportion of PANC-1 cells in G0/G1 phases, and a lower proportion of PANC-1 cells in S phase ($p<0.05$), and the change in the proportion of cells in G2/M phase was not statistically significant ($p>0.05$). Moreover, Metformin treatment groups (0, 20, and 40 mM) had more apoptotic PANC-1 cells, higher expression levels of pro-apoptosis proteins Caspase-3 and Bax and lower expression levels of anti-apoptosis protein Bcl-2 and the mTOR pathway-related proteins PI3K, p-Akt, and p-mTOR in cells than Control group ($p<0.05$).

CONCLUSIONS: Metformin modulates the mTOR signaling pathway to reduce the proliferation of pancreatic cancer cell, but increase their apoptosis.

Key Words:
Metformin, Pancreatic cancer, PANC-1, Apoptosis, mTOR.

Introduction

Pancreatic cancer is a highly malignant digestive system tumor with a poor prognosis, and its 5-year survival rate is about 5%[1]. Radical surgery and postoperative chemotherapy and radiotherapy are the conventional treatments for pancreatic cancer, but clinical evidence has revealed that pancreatic cells, especially pancreatic cancer stem cell subsets, are insensitive to conventional chemotherapy and radiotherapy agents, and their invasiveness is even enhanced, which is one important cause of the high recurrence and metastasis rates of pancreatic cancer after treatment[2]. Therefore, the current hotspot of research is effective inhibition of proliferation and increase in apoptosis in pancreatic cancer cells.

Metformin, as a first-line drug for clinically treating type 2 diabetes, reduces gluconeogenesis and promotes glucose utilization[3]. Some studies[4,5] have demonstrated that metformin has a certain inhibitory effect on the proliferation and differentiation of lung cancer, breast cancer, gastric cancer, intestinal cancer, ovarian cancer,

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and prostate cancer cells. It has been clinically evidenced that diabetic patients taking metformin have a lower risk of pancreatic cancer than those not taking metformin and that the incidence rate of pancreatic cancer declines in the induced mice administered with metformin[6,7]. The mammalian target of rapamycin (mTOR) pathway modulates cell proliferation and apoptosis, cell cycle, protein synthesis, and cell migration and has been confirmed to be aberrantly activated in liver cancer, lung cancer, breast cancer, and cervical cancer, thereby participating in the development and progression of cancers[8]. Moreover, metformin is able to promote cell apoptosis by regulating the mTOR pathway[9]. This study, therefore, preliminarily explored the correlations of metformin with the proliferation and apoptosis of pancreatic cancer cells and its dose-effect relationship and mechanism, so as to provide theoretical and practical bases for the treatment of pancreatic cancer.

Materials and Methods

Materials

Human pancreatic cancer cell line PANC-1 was purchased from Shanghai Institutes for Biological Sciences (Shanghai, China), Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) from Gibco (Rockville, MD, USA), metformin, methyl thiazolyl tetrazolium (MTT) and dimethyl sulfoxide (DMSO) from Sigma-Aldrich (St. Louis, MO, USA), cell cycle assay kit and Annexin V-fluorescein isothiocyanate (FITC) apoptosis assay kit from Becton Dickinson (Franklin Lakes, NJ, USA), terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) apoptosis assay kit from Wuhan Beyotime Biotechnology Co., Ltd. (Shanghai, China), Caspase-3, B-cell lymphoma 2 (Bcl-2), Bcl-2 associated X protein (Bax), phosphatidylinositol 3-hydroxy kinase (PI3K), phosphorylated protein kinase B (p-Akt), Akt, p-mTOR, mTOR, and β-actin antibodies from Abcam (Cambridge, MA, USA), and horseradish peroxidase-labeled goat anti-rabbit/rat secondary antibodies from Beijing Applygen Technologies Inc. (Beijing, China).

Cell Culture and Drug Intervention

Human pancreatic cancer PANC-1 cells were cultured using DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL of streptomycin in an incubator with 5% CO_2 and saturated humidity at 37°C. When covering 95% of the flask bottom, the cells were digested by 0.25% trypsin. With the trypsin removed, the resulting cells were added with fresh medium, pipetted into single suspended cells, and sub-cultured in a new culture flask. The cells in the logarithmic growth phase were harvested for experiments. After being stimulated by metformin at different concentrations for 24, 48, and 72 h, the PANC-1 cells were collected to measure the dose-effect relationship of metformin. Subsequently, three different concentrations of metformin were selected from the above results for stimulation, and 48 h later, the proliferation and apoptosis, as well as the mTOR pathway, were detected in PANC-1 cells.

Detection of Cell Proliferation Via MTT Assay

Human pancreatic cancer cells in the logarithmic growth phase were seeded into a 96-well plate at 5×10^3 cells/well, and at 24 h after adherence, different concentrations of metformin (1, 2.5, 5, 10, 20, 40 and 60 mM) were added to stimulate the cells for 24, 48, and 72 h. Then, the supernatant was aspirated, and the cells were added with 90 μL of fresh medium and 10 μL of MTT solution and incubated in the incubator with 5% CO_2 and saturated humidity at 37°C for 4 h. Subsequently, the supernatant was discarded, and each well was added with 150 μL of DMSO and shaken at a low speed to fully dissolve the crystals. Afterwards, the absorbance (A) value of cells in each well was measured using an enzyme-linked immunosorbent assay reader. Cell growth inhibition rate = $(1-A_{\text{Experiment well}}/A_{\text{Control well}})\times100\%$. Finally, three drug concentrations were selected around the half maximal inhibitory concentration (IC_{50}) for subsequent experiments.

Detection of Cell Cycle Using Flow Cytometry

Human pancreatic cancer cells were inoculated into a 6-well plate at 3×10^5 cells/well, and when the adherent cells covered 70% of the flask bottom, they were stimulated using 10, 20, or 40 mM metformin for 48 h, harvested, and fixed with pre-cooled 70% alcohol overnight. After being washed using pre-cooled phosphate-buffered saline (PBS), the cells were stained with 300 μL of staining solution containing 100 μg/mL RNase A, 50 μg/mL propidium iodide (PI), and 0.2% Triton X-100 at room temperature in the

dark for 30 min. Finally, cell cycle was detected using Flow cytometer (FACSCalibur; BD Biosciences; San Jose, CA, USA).

Detection of Apoptosis Using Flow Cytometry

Human pancreatic cancer cells were first inoculated into a 6-well plate at 3×10^5 cells/well. Then, the cells attaching to the wall and covering 70% of the flask bottom were stimulated by 10, 20, or 40 mM metformin for 48 h, collected, washed using pre-cooled PBS, and suspended in 300 μL of binding buffer. Subsequently, the resulting cells were added with 5 μL of Annexin V-FITC and 5 μL of PI, mixed evenly and reacted in the dark for 15 min. Finally, cell apoptosis was detected using the flow cytometer.

TUNEL Apoptosis Assay

Human pancreatic cancer cells were first seeded into a 6-well plate 3×10^5 cells/well. When attaching to 70% of the flask bottom, the cells were stimulated by 0, 20, or 40 mM metformin, and harvested 48 h later. Then, 50 μL of TUNEL staining solution was prepared according to the instructions of the TUNEL apoptosis assay kit, added into cells and mixed evenly. Following reaction in the dark for 30 min, the resulting cells were observed under a fluorescence microscope, and the percentage of apoptotic cells in 200 cells was calculated.

Western Blotting

Human pancreatic cancer cells were collected from each treatment group, and lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) to extract total proteins therein. Then, the concentration of the total proteins was measured by bicinchoninic acid (BCA) colorimetry (Pierce, Rockford, IL, USA). Subsequently, the prepared proteins were mixed with sodium dodecyl sulphate (SDS)-loading buffer, and boiled at 95°C for 3 min, and the same volume of total proteins was separated using 8-10% polyacrylamide gel electrophoresis (Beijing Applygen Technologies, Inc., Beijing, China) and transferred onto a nitrocellulose membrane. After being blocked using 10% skim milk, the proteins were incubated with primary antibodies on a shaking table at 4°C overnight, washed using Tris-buffered saline with Tween-20 (TBST) for 3 times, incubated with the corresponding secondary antibodies on the shaking table at room temperature for 1 h, rinsed with TBST for 3 times and added with enhanced chemiluminescence (ECL) solution for exposure and image development. Finally, the relative expression level of the target protein was analyzed using ImageJ software.

Immunofluorescence Staining

Likewise, human pancreatic cancer cells were first inoculated into a 6-well plate at 3×10^5 cells/well. When the adherent cells covered 70% of the flask bottom, they were stimulated using 10, 20, or 40 mM metformin for 48 h, and collected. Then, the cells were washed using PBS, fixed in 4% paraformaldehyde for 1 h, rinsed using PBS twice, penetrated with 0.2% Triton X-100 for 20 min, washed again with PBS twice, and blocked by goat serum for 1 h. After the blocking solution was removed, the resulting cells were incubated with Caspase-3 primary antibody at 4°C overnight. On the next day, the cells were washed using PBS twice, incubated with fluorescence-labeled secondary antibody at room temperature for 30 min, rinsed by PBS twice, added with DAPI solution to stain the cell nuclei for 5 min, and washed with PBS once. Finally, 20 mL of cell mixture was added dropwise to a glass slide, and covered by the coverslip, and the expression of Caspase-3 in cells was observed under the fluorescence microscope.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA) was used for analysis, and measurement data were presented as mean ± standard deviation. Intergroup comparisons were made using One-way analysis of variance, and *t*-test was performed for pairwise comparisons. $p<0.05$ denoted that the differences were statistically significant.

Results

Influence of Metformin on PANC-1 Cell Proliferation and its Dose-Effect Relationship

First, human pancreatic cancer PACN-1 cells were treated with metformin at different concentrations (1, 2.5, 5, 10, 20, 40, and 60 mM), and then, the cell proliferation was detected *via* MTT assay. According to the results (Figure 1), metformin inhibited the proliferation of the PACN-1 cells in a concentration-dependent and time-dependent manner. In other words, with the extending of time and increase in concentration,

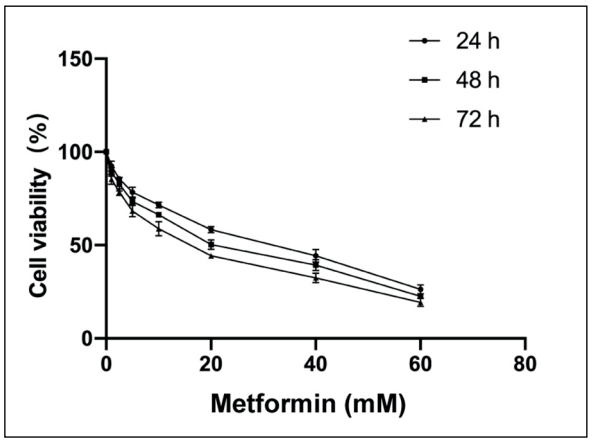

Figure 1. Proliferation of PANC-1 cells treated with different concentrations of metformin.

metformin had a more evident inhibition effect on the proliferation of PANC-1 cells. When PANC-1 cell inhibition rate was 50%, the concentration of metformin was 20 mM.

Effect of Metformin on PANC-1 Cell Cycle

After PANC-1 cells were treated with different concentrations of metformin, flow cytometry was performed to detect cell cycle, and the results showed that Metformin treatment groups (10, 20, and 40 mM) had a higher proportion of G_0/G_1 phase cells, and a lower proportion of S phase cells than the Control group ($p<0.05$), and the change in the proportion of G_2/M phase cells was not statistically significant ($p>0.05$) (Figure 2, Table I).

Influence of Metformin on PANC-1 Cell Apoptosis

PANC-1 cells were first treated with different concentrations of metformin, and the cell apoptosis was detected *via* flow cytometry. It was found that the apoptosis rates in Metformin treatment groups (0, 20, and 40 mM) were (14.27±0.26)%, (16.36±0.19)% and (31.02±0.41)%, respectively, higher than that in Control group [(2.25±0.13)%] ($p<0.05$) (Figure 3). Besides, based on the TUNEL apoptosis assay results (Figure 4), compared with that in Control group, the proportion of TUNEL-positive cells was increased in Metformin treatment groups (0, 20, and 40 mM) ($p<0.05$).

Impacts of Metformin on the Expression Levels of Apoptosis-Associated Proteins in PANC-1 Cells

PANC-1 cells were treated with metformin at different concentrations, and the expression of Caspase-3 was detected using immunofluorescence assay. The results revealed that the expression intensity of fluorescent Caspase-3 in Metformin treatment groups (0, 20, and 40 mM) was higher than that in Control group (Figure 5). Moreover, the expression levels of the apoptosis-associated proteins were determined *via* Western blotting, and according to the results (Figure 6) and compared with Control group, Metformin treatment groups (0, 20, and 40 mM) exhibited increased expressions of Caspase-3 and Bax, but decreased Bcl-2 expression ($p<0.05$).

Influence of Metformin on the mTOR Pathway in PANC-1 Cells

PANC-1 cells were first treated with different concentrations of metformin, and then, the expression levels of the proteins related to the mTOR pathway were measured *via* Western blotting. It was discovered that the expression levels

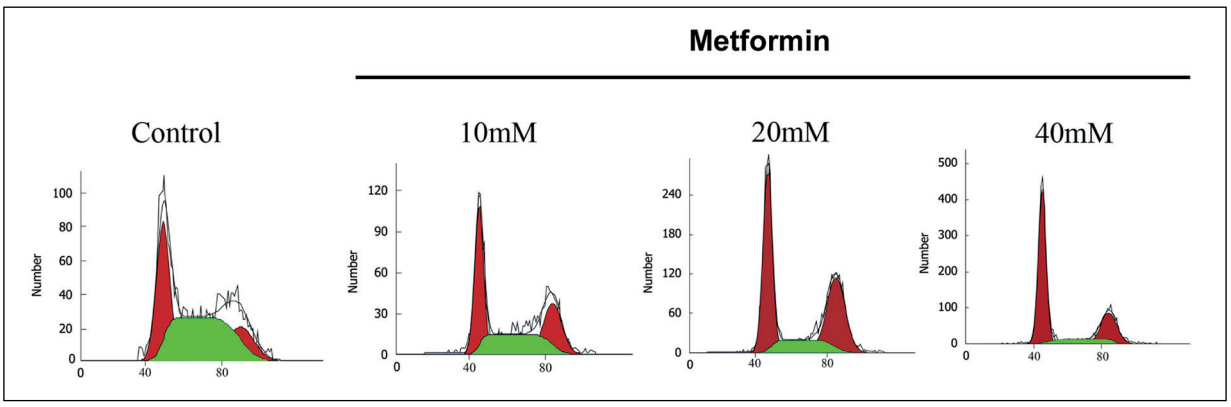

Figure 2. Cell cycles in Metformin treatment groups (10, 20, and 40 mM) and in Control group detected by a flow cytometer.

Table I. Changes in cell cycle under different treatment conditions [%, n=5, ($\bar{x} \pm s$)].

Group	G_0/G_1	G_2/M	S
Control group	41.32 ± 2.02	25.81 ± 2.87	32.85 ± 2.77
Metformin 10 mM	50.05 ± 1.74[a]	25.36 ± 2.16	24.63± 2.54[a]
Metformin 20 mM	53.15 ± 1.56[a]	28.18 ± 3.63	18.73 ± 3.94[a]
Metformin 40 mM	67.33 ± 2.51[a]	18.36 ± 2.56	14.37 ± 2.64[a]

Note: [a]$p<0.05$ *vs.* Control group.

of PI3K, p-Akt, and p-mTOR in Metformin treatment groups (0, 20, and 40 mM) were lower than those in Control group ($p<0.05$) (Figure 7).

Discussion

Pancreatic cancer is a ductal adenocarcinoma arising from the epithelium of pancreatic ducts with the features of high malignancy, recurrence rate and metastasis rate, difficulty in treatment and poor prognosis[10]. With the elevation of the morbidity and mortality rates in recent years, pancreatic cancer is expected to rank 2nd among malignancies in the mortality rate by 2030[11]. Radical surgery is the primary treatment for pancreatic cancer. Although a great stride has been made in surgical techniques, the patients still have a poor prognosis and a 5-year survival rate below 10%[12]. As the disease is delved well into, the treatment of pancreatic cancer gradually transforms from surgical treatment alone to combined treatments, of which the postoperative combinations of chemotherapy, radiotherapy, and immunotherapy can prolong the survival time of pancreatic cancer patients and decrease the mortality rate in them[13]. However, according to clinical evidence, pancreatic cancer cells are prone to developing resistance to chemotherapeutic agents, thereby reducing the efficiency of chemotherapy

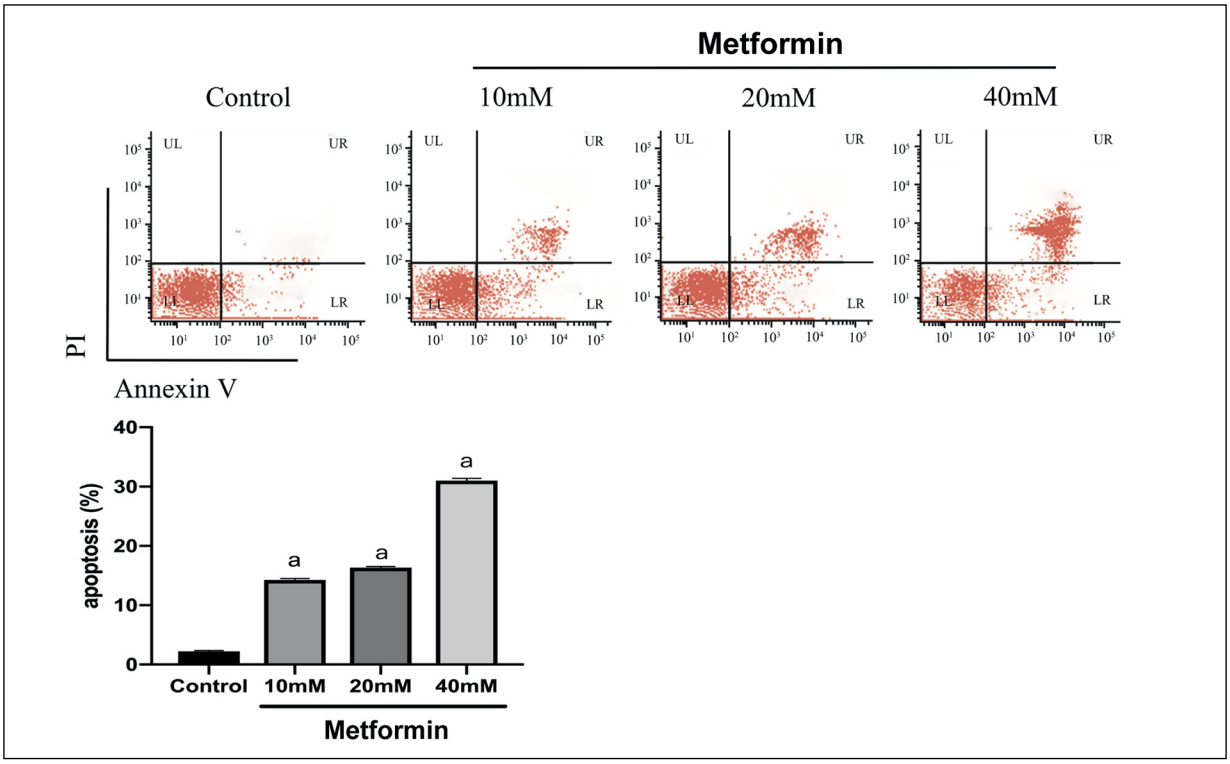

Figure 3. Cell apoptosis in Metformin treatment groups (10, 20 and 40 mM) and Control group detected by a flow cytometer. Note: [a]$p<0.05$ *vs.* Control group.

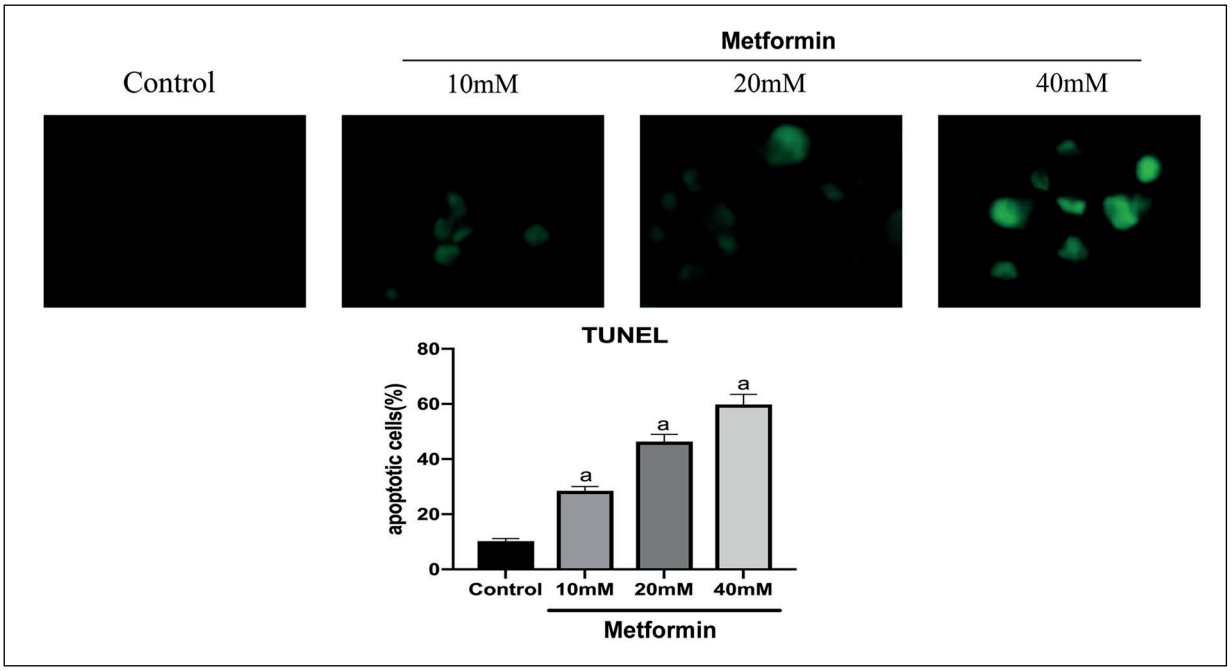

Figure 4. Cell apoptosis in Metformin treatment groups (10, 20, and 40 mM) and Control group observed *via* TUNEL assay (magnification: 40×). Note: [a]$p<0.05$ *vs.* Control group.

and raising the recurrence and metastasis rates of pancreatic cancer[14]. Therefore, the treatment of pancreatic cancer remains greatly difficult, and further research is warranted to search for more efficacious treatment means.

Metformin, a common drug for the treatment of type 2 diabetes, has its safety, efficaciousness, and economic efficiency clinically confirmed. Moreover, it can not only enhance the function of insulin to increase the ability of insulin to reduce blood glucose, but also promote the glucose utilization of the muscle and liver, thereby effectively lowering blood glucose[15]. According to the surprising findings in a retrospective study of diabetic patients, the risk of cancer is significantly decreased in the diabetic patients receiving metformin treatment, and myriads of subsequent studies[16,17] have uncovered that metformin has an inhibitory effect on the morbidity rates of such cancers as lung cancer, breast cancer, gastric cancer, intestinal cancer, and ovarian cancer. It has been found in the research into pancreatic cancer that the diabetic patients taking metformin has a lower risk of pancreatic cancer than those not taking metformin, and that the incidence rate of pancreatic cancer declines in the mice induced and administered with metformin[6,7]. However, there has been a lack of basic research. Since the influences of metformin on pancreatic cancer cell proliferation and apoptosis and the critical pathway therein have not yet been elucidated, the present study preliminarily explored the direct correlations of metformin with pancreatic cancer cell proliferation and apoptosis and the pivotal pathway. First, the pancreatic cancer PANC-1 cells, as the study object, were treated with different doses of metformin, and the changes in the proliferation of the PANC-1 cells were detected. The results showed that after metformin treatment, the proliferation of PANC-1 cells evidently declined in a time and concentration manner. Three concentrations were selected around IC_{50} for subsequent investigation, and it was found that the majority of PANC-1 cells were arrested in G_0/G_1 phase, while few cells in G_2/S and M phases, with increases in cell apoptosis and expressions of apoptosis-associated proteins in Metformin treatment groups. The above results suggest that metformin can reduce the proliferation of human pancreatic cancer PANC-1 cells and increase their apoptosis. MTOR is a threonine/serine protein kinase that can be activated by the upstream PI3K/Akt pathway to regulate cell proliferation, apoptosis, cell cycle, protein synthesis, and cell migration[18]. The studies[10,19] on lung cancer, liver cancer, intestinal cancer, and gastric cancer have found that the aberrantly activated mTOR pathway is involved in the development and progres-

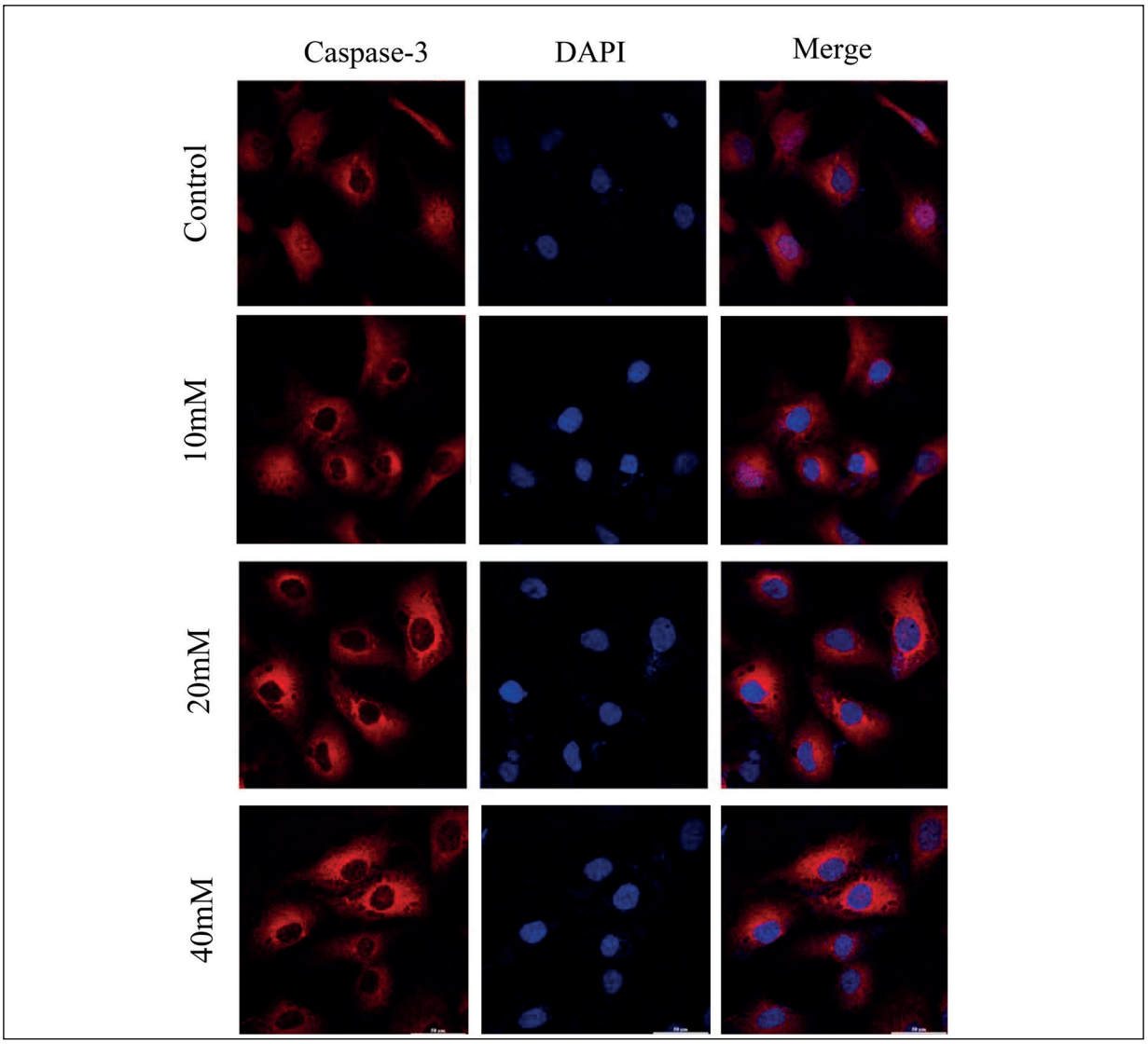

Figure 5. Caspase-3 expression and localization in Metformin treatment groups (10, 20, and 40 mM) and Control group observed *via* immunofluorescence (magnification: 400×).

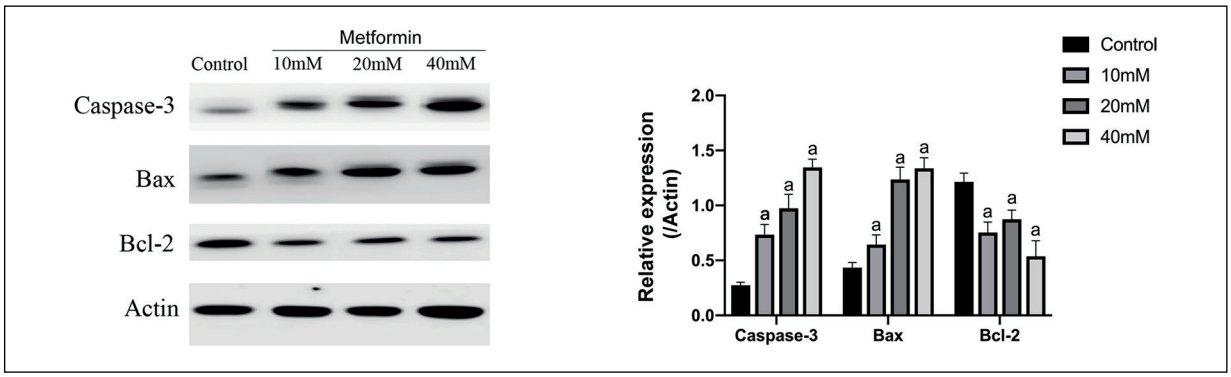

Figure 6. Expressions of apoptosis-associated proteins in Metformin treatment groups (10, 20, and 40 mM) and Control group detected *via* Western blotting. Note: [a]$p<0.05$ *vs.* Control group.

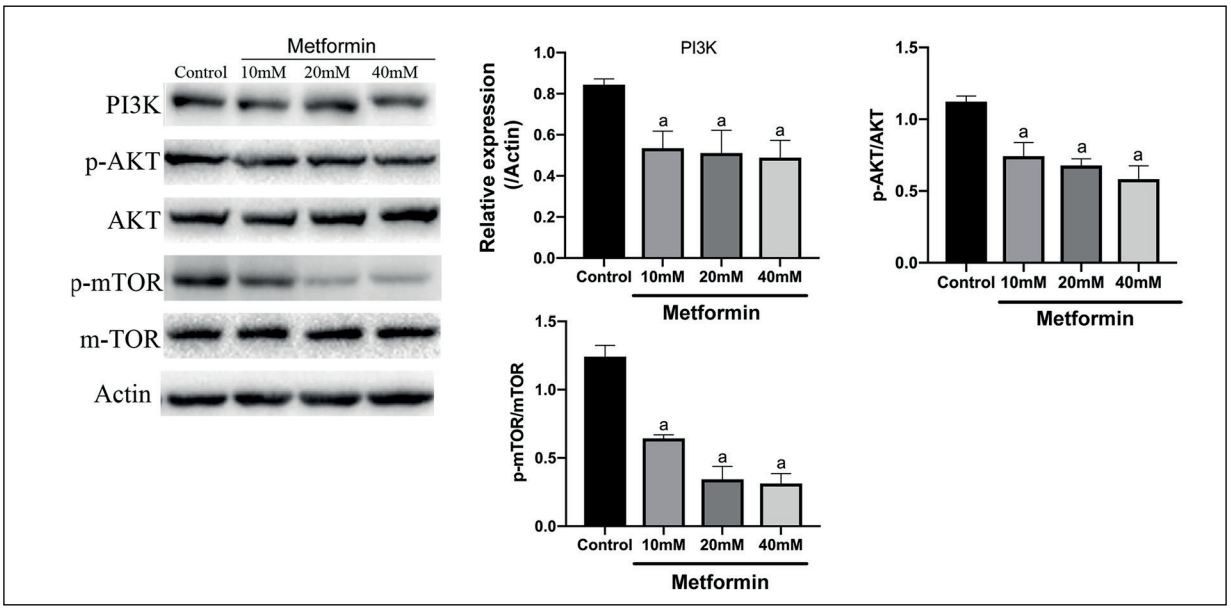

Figure 7. Expressions of the mTOR pathway-related proteins in Metformin treatment groups (10, 20, and 40 mM) and Control group detected *via* Western blotting. Note: [a]$p<0.05$ *vs.* Control group.

sion of cancers. The present study, therefore, explored whether the PI3K/Akt/mTOR pathway is pivotal for metformin in increasing the apoptosis of PANC-1 cells, and the results revealed that metformin decreased the protein expressions of PI3K, p-Akt, and p-mTOR, and repressed the abnormal activation of the mTOR pathway, thereby reducing PANC-1 cell proliferation.

Conclusions

In summary, metformin reduces the activation of the mTOR pathway to increase the apoptosis of PANC-1 cells and decrease their proliferation, which provides theoretical and practical bases for pancreatic cancer treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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