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Mechanism of metformin enhancing the sensitivity of human pancreatic cancer cells to gem-citabine by regulating the PI3K/Akt/mTOR signaling pathway

H.-Y. ZHOU, X.-M. YAO, X.-D. CHEN, J.-M. TANG, Z.-G. QIAO, X.-Y. WU

Department of Gastroenterology, Affiliated Wujiang Hospital of Nantong University, Suzhou, China

Abstract. – OBJECTIVE: To investigate the effect of metformin (MET) on enhancing the sensitivity of human pancreatic cancer cells to gemcitabine (GEM) by regulating the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway.

MATERIALS AND METHODS: The GEM-resistant human pancreatic cancer PANC-1/GEM cell line was established, and the proliferation ability of PANC-1 and PANC-1/GEM cell lines was detected using the Cell Counting Kit-8 (CCK-8), which was then detected by flow cytometry after they were labeled by Ki67. Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) and Western blotting were adopted to detect the difference in the mTOR expression between PANC-1 and PANC-1/GEM cell lines. The proliferation ability of PANC-1/GEM/MET and PANC-1/GEM cell lines was determined using CCK-8 after drug-resistant cell lines were treated with 20 mmol/L MET combined with 0.4 μmol/L GEM or 0.4 μmol/L GEM alone for 48 h. Colony formation assay was applied to detect the proliferation ability of cells. The difference in the expression of mTOR/PI3K/Akt between PANC-1/GEM/MET and PANC-1/GEM cell lines was tested via qRT-PCR and Western blotting, respectively.

RESULTS: Compared with PANC-1 cells, PANC-1/GEM cells had significantly enhanced proliferation ability ($p<0.01$). Flow cytometry results showed that the proliferation ability of PANC-1/GEM cells was notably enhanced ($p<0.01$). The expression level and phosphorylation level of mTOR in drug-resistant cell lines were increased ($p<0.01$). After the drug-resistant cell lines were treated with 20 mmol/L MET for 48 h, the proliferation ability of PANC-1/GEM/MET cells was evidently decreased compared with that of PANC-1/GEM cells ($p<0.01$). The messenger ribonucleic acid (mRNA) and protein expression levels of mTOR/PI3K/Akt were markedly down-regulated ($p<0.01$).

CONCLUSIONS: MET can regulate the PI3K/Akt/mTOR signaling pathway to enhance the sensitivity of human pancreatic cancer cells to GEM.

Key Words:
Metformin, PI3K/Akt/mTOR signaling pathway, Human pancreatic cancer cell, Gemcitabine.

Introduction

Pancreatic cancer is a tumor with an extremely high degree of malignancy and poor therapeutic effect and prognosis, and its incidence rate is increasing year by year[1]. In Western countries, it has become the fourth leading cause of death from malignant tumors[2]. In addition to operation, chemotherapy is an important adjuvant treatment, especially for unresectable tumors. Gemcitabine (GEM) is a nucleoside analogue which is widely used in the clinic and is the best drug to treat pancreatic cancer so far. GEM plays an important role in alleviating symptoms, prolonging survival time and improving the quality of life, especially in the treatment of advanced pancreatic cancer[3]. However, in the course of tumor chemotherapy, the existence of drug resistance, especially acquired drug resistance, seriously hinders the use of this chemotherapeutic drug[4,5]. Some studies have shown that the mechanism of drug resistance of GEM is related to abnormal regulation of the phosphatidylinositol-3-kinase (PI3K) signaling pathway. The expression of the PI3K/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway is out of balance in various human tumors. Abnormal activation of the PI3K/Akt/mTOR signaling pathway can lead to abnormal proliferation and differentiation of cells and promote the occurrence and development of tumors[6,7].

Metformin (MET) was initially widely applied in clinic as a safe and effective drug for diabetes mellitus type 2[8]. With the deepening of research, more and more research reports have revealed that MET

Corresponding Author: Xiaoying Wu, MM; e-mail: xiangyun19881015@126.com

can significantly inhibit the progression of tumor cells such as breast cancer and lung cancer cells, and exert a significant anti-cancer effect[9]. However, there is no report on whether MET can regulate the PI3K/Akt/mTOR signaling pathway and enhance the sensitivity of human pancreatic cancer cells to GEM.

In this study, therefore, whether MET can regulate the PI3K/Akt/mTOR signaling pathway and enhance the sensitivity of human pancreatic cancer cells to GEM was investigated through the establishment of the GEM-resistant human pancreatic cancer PANC-1/GEM cell line and treatment of drug-resistant cell lines with 20 mmol/L MET + 0.4 μmol/L GEM or 0.4 μmol/ L GEM alone for 48 h, to explore a more effective treatment regimen for pancreatic cancer.

Materials and Methods

Reagents and Instruments

Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan), Ki67, AV/PI, the bicinchoninic acid (BCA) protein quantification kit, cell lysate (Hanbio Co., Ltd., Shanghai, China), the ribonucleic acid (RNA) extraction kit (Invitrogen, Carlsbad, CA, USA), the quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), the primer synthesis kit (Hanbio Co., Ltd., Shanghai, China) and PI3K, Akt, mTOR and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primary and secondary antibodies (Cell Signaling Technology, Danvers, MA, USA).

Cell Culture and Treatment

The GEM-resistant human pancreatic cancer PANC-1/GEM cell line was established and cultured in an incubator with 5% CO_2 at 37°C. When the cell fusion reached about 80%, the single cell suspension was digested with trypsin and inoculated and cultured in the corresponding culture plate for subsequent experiments. When the cell fusion reached 60-70%, drug-resistant cell lines were treated with 20 mmol/L MET combined with 0.4 μmol/L GEM or 0.4 μmol/L GEM alone for 48 h, and subsequent experiments were carried out.

Detection of Cell Proliferation Activity via CCK-8

Cell proliferation activity was detected by CCK-8 after 48 h of cell transfection: 100 μL cells at a concentration of 1×10^5/mL were inoculated in each well of a 96-well plate. Three repeated wells were set in each group, and the medium was replaced after incubation with 5% CO_2 at 37°C for 48 h. 10 μL of CCK-8 detection solution was added to each well, and after 30 min, the optical density (OD) value at the wavelength of 562 nm in each well was measured with a microplate reader.

Detection of Cell Proliferation Activity via Flow Cytometry

When the fusion of PANC-1 cells reached 60-70% in a 6-well plate, the cells were treated with trypsin and collected after 48 h of administration. Then 300 μL of binding buffer was added, gently shaken and fully mixed, and Ki67 was added for labeling after punching with 1% Triton. Finally, the proliferation of cells was detected by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA).

Colony Formation Assay

In a 6-well plate, 1,000 PANC-1/GEM cells were inoculated into each well, and after they adhered to the wall, the drug-resistant cell lines were treated with 20 mmol/L MET combined with 0.4 μmol/L GEM or 0.4 μmol/L GEM alone for 7 d. Subsequently, the cells were stained with crystal violet and photographed, and the colonies formed by the cells were observed.

Detection of the Expression Level of Related Messenger RNAs (mRNAs) via qRT-PCR

The treated cells in a 6-well plate were added with 500 μL of TRIzol (Invitrogen, Carlsbad, CA, USA) in each well and let stand for 5 min. Then, they were added with chloroform, shaken violently for 15 s and let stand for 15 min. After that, the water phase layer was taken to another batch of new Eppendorf (EP; Eppendorf, Hamburg, Germany) tubes, added with isopropanol and turned upside down several times, followed by centrifugation. The white precipitate at the bottom of the tubes was RNAs, whose concentration and purity were detected after they were washed and dissolved in water. The results were considered qualified when the ratio of absorbance $(A)_{260}/A_{280}$ was 1.8-2.0. After complementary DNAs (cDNAs) were obtained through room temperature (RT), fluorescence Real Time-quantitative analysis was conducted to detect the mRNA expression level, and primer sequences are shown in Table I. Reaction conditions: at 94°C for 5 min, amplification at 94°C for 30 s, 57°C for 30 s and 72°C for 30 s

Table I. RT-PCR primer sequences.

Gene name		Primer sequences
PI3K	Forward	GCTCTCTCACTGCATACATTGT
	Reverse	AGTCACAGCTGTATTGGTCG
mTOR	Forward	ACGCACGACGTCTTCCAGTA
	Reverse	CCACCTGGTTCAACTCACTCC
GAPDH	Forward	GGATATTGTTGCCATCAATGACC
	Reverse	AGCCTTCTCCATGGTGGTGAAGA

for a total of 40 cycles and 72°C for 5 min. Microsoft Excel software was used to process the data, and with GAPDH as a control gene, the relative level was calculated using $2^{-\Delta\Delta Ct}$ according to the following formula: ΔCt (target gene) = Ct (target gene) - Ct (control gene) and ΔΔCt = ΔCt (target gene) - ΔCt (standard value). The relative expression level of the target gene was $2^{-\Delta\Delta Ct}$.

Western Blotting Detection

100 μL of radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) lysate was added to the treated cells in each well of a 6-well plate, lysed on ice for 20 min, scraped off and centrifuged at 12000 rpm and 4°C for 10 min. Then, the supernatant was collected into another batch of new EP tubes. The concentration of the extracted proteins was measured and the proteins were quantified using the BCA kit. 20 μg of proteins were taken and detected by Western blotting. After electrophoretic separation, the separated proteins were electrified in an electrophoretic buffer and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Then proteins were sealed in 5% skim milk at room temperature for 2 h, washed on a shaker and incubated in an incubation box containing the primary antibody (1:1000) at 4°C overnight. After the membrane was fully washed with Tris-Buffered Saline and Tween (TBST; Sigma-Aldrich, St. Louis, MO, USA), the second antibody (1: 5000) was added for incubation at room temperature for 1 h, the color was developed using electrochemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) in a dark room, and the images were scanned and recorded using a gel imager (Bio-Rad Laboratories, Hercules, CA, USA). Ultimately, grayscale analysis and comparison were carried out with GAPDH as an internal reference.

Statistical Analysis

All the data were expressed as mean ± standard deviation, processed using Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA) and statistically analyzed by one-way analysis of variance. $p<0.05$ suggested that the difference was statistically significant.

Results

Proliferation Activity of the GEM-Resistant Human Pancreatic Cancer PANC-1/GEM Cell Line

CCK-8 detection was performed after 5,000 cells were inoculated into the 96-well plate. As shown in Figure 1, the GEM-resistant human pancreatic cancer PANC-1/GEM cell line had significantly increased proliferation activity compared with the human pancreatic cancer PANC-1 cell line ($p<0.01$).

Proliferation of Cell Lines Labeled by Ki67 Detected via Flow Cytometry

It was found in Figure 2 that, compared with that of the human pancreatic cancer PANC-1 cell line, the proliferation activity of the GEM-resistant human pancreatic cancer PANC-1/GEM cell line was notably enhanced ($p<0.01$), indicating that the proliferation activity of drug-resistant cell lines is higher than that of ordinary cell lines.

Difference in the Expression of PI3K/Akt/mTOR Between PANC-1 and PANC-1/GEM Cell Lines Detected via qPCR

Real Time-quantitative Polymerase Chain Reaction (qPCR) detection results manifested that compared with that in the PANC-1 cell line,

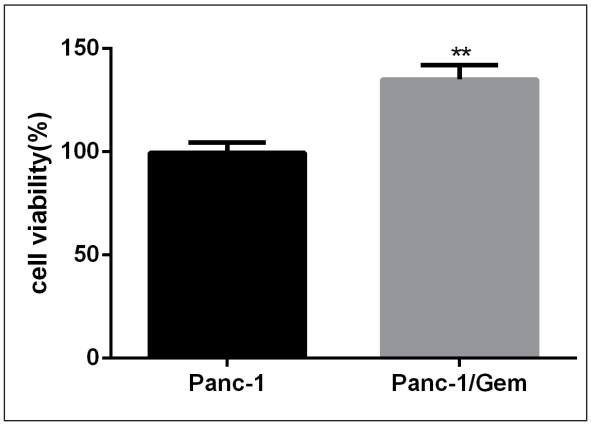

Figure 1. Proliferation activity of the human pancreatic cancer PANC-1/GEM cell line detected via CCK-8. *$p<0.01$ *vs.* PANC-1 group.

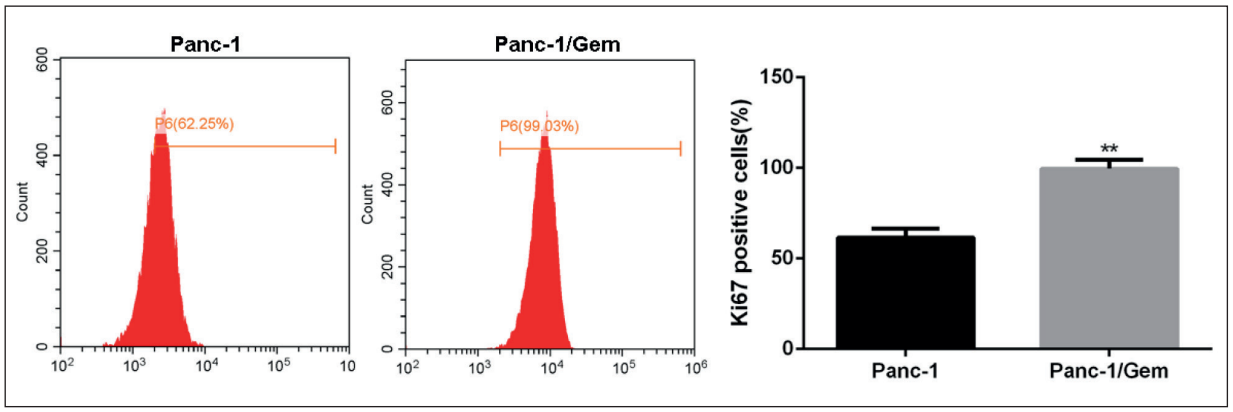

Figure 2. Cell line proliferation detected via flow cytometry. **p<0.01 *vs.* PANC-1 group.

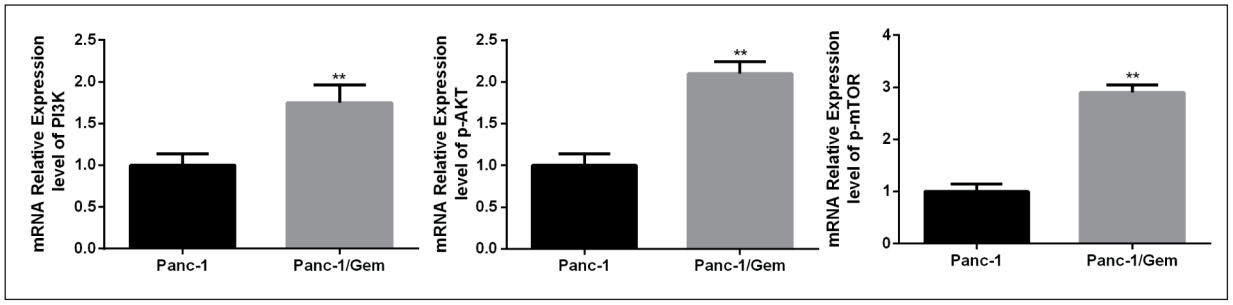

Figure 3. Difference in the expression of PI3K/Akt/mTOR between PANC-1 and PANC-1/GEM cell lines detected via qPCR. **p<0.01 *vs.* PANC-1 group.

the expression level of PI3K/Akt/mTOR in the PANC-1/GEM cell line was markedly increased (p<0.01), suggesting that the drug resistance of the PANC-1/GEM cell line may be related to the abnormal expression of the PI3K/Akt/mTOR signaling pathway.

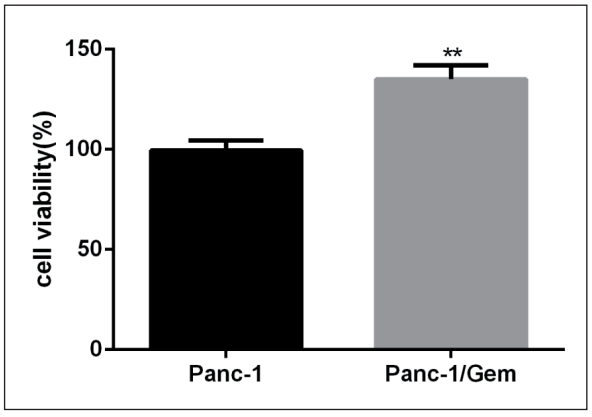

Figure 4. Proliferation activity of the human pancreatic cancer PANC-1/GEM cell line detected via CCK-8. **p<0.01 *vs.* PANC-1 group.

Effect of MET Combined with GEM in the Treatment of the Drug-Resistant PANC-1/GEM Cell Line on the Proliferation Activity

CCK-8 detection was performed after 5,000 cells were inoculated into the 96-well plate, According to the results (Figure 4), after drug-resistant cells lines were treated with 20 mmol/L MET combined with 0.4 μmol/L GEM or 0.4 μmol/L GEM alone for 48 h, the cell proliferation activity in the MET + GEM group was evidently reduced compared with that in the MET group (p<0.01), indicating that MET can enhance the sensitivity of human pancreatic cancer cell lines to GEM.

Effect of MET Combined with GEM in the Treatment of the Drug-Resistant PANC-1/GEM Cell Line on Clone Formation

As shown in Figure 5, after drug-resistant cells lines were treated with 20 mmol/L MET combined with 0.4 μmol/L GEM or 0.4 μmol/L GEM alone for 7 d, the colony formation ability in the MET + GEM group remarkably declined compared with that in the MET group (p<0.01),

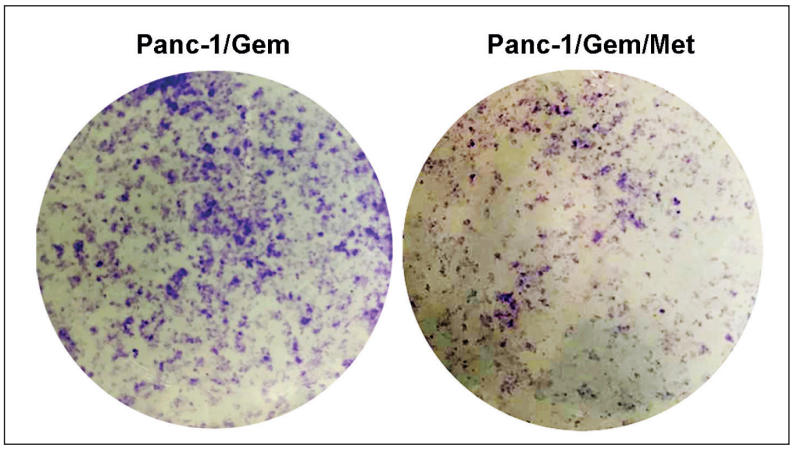

Figure 5. Cell proliferation ability detected via colony formation assay (magnification: 40×).

indicating that MET can improve the sensitivity of human pancreatic cancer cell lines to GEM.

Effect of MET Combined with GEM in the Treatment of the Drug-Resistant PANC-1/GEM Cell Line on the mRNA Expression Level of PI3K/Akt/mTOR

After drug-resistant cells lines were treated with 20 mmol/L MET combined with 0.4 μmol/L GEM or 0.4 μmol/L GEM alone for 48 h, the mRNA expression level of PI3K/Akt/mTOR in the MET + GEM group was remarkably down-regulated compared with that in the MET group (p<0.01; Figure 6), suggesting that MET can enhance the sensitivity of human pancreatic cancer cell lines to GEM by inhibiting the PI3K/Akt/mTOR signaling pathway.

Effect of MET Combined with GEM in the Treatment of the Drug-Resistant PANC-1/GEM Cell Line on the Protein Expression Level of PI3K/Akt/mTOR

After drug-resistant cells lines were treated with 20 mmol/L MET combined with 0.4 μmol/L GEM or 0.4 μmol/L GEM alone for 48 h, the protein expression level of PI3K/Akt/mTOR in the MET + GEM group was notably decreased compared with that in the MET group (p<0.01; Figure 7), suggesting that MET can enhance the sensitivity of human pancreatic cancer cell lines to GEM by inhibiting the PI3K/Akt/mTOR signaling pathway.

Discussion

Pancreatic cancer ranks second in the digestive tract tumors, with a high degree of malignancy, thus endangering human health, and its morbidity and mortality rates are comparable to those of colorectal cancer[10]. The hidden incidence of pancreatic cancer makes patients easily miss a better operation opportunity, and it has a high recurrence rate, so the treatment of pancreatic cancer is still faced with difficulties. As a first-line chemotherapy drug for pancreatic cancer, GEM has a certain curative effect on the clinical treatment of advanced pancreatic cancer, but there is no significant curative effect and improvement in survival rate and overall survival[11]. Therefore, improving the sensitivity of

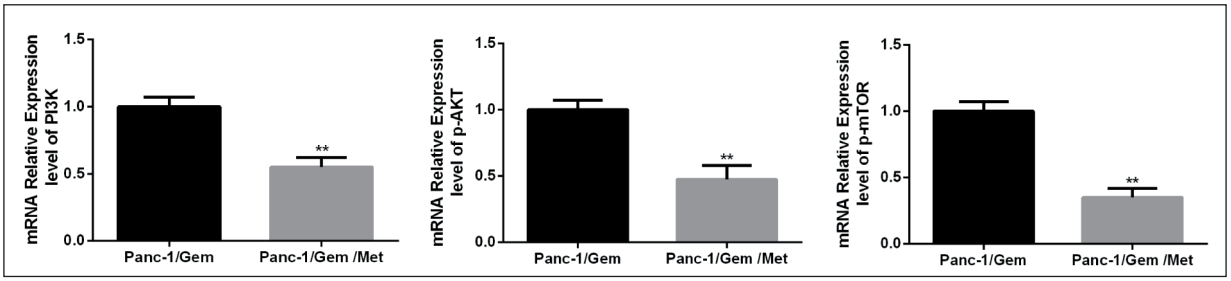

Figure 6. MRNA expression level of PI3K/Akt/mTOR detected via qRT-PCR. **p<0.01 *vs.* PANC-1/GEM group.

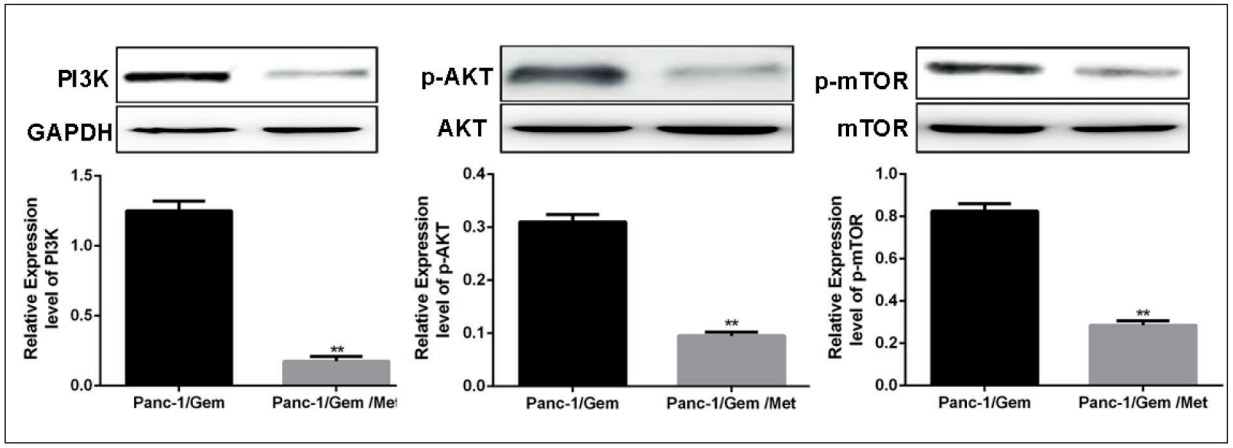

Figure 7. Protein expression level of PI3K/Akt/mTOR detected via Western blotting. $^{**}p<0.01$ *vs.* PANC-1/GEM group.

pancreatic cancer cells to chemotherapeutic drugs will become an important measure for the treatment of pancreatic cancer.

PI3K, as a heterodimer, consists of P85 regulatory subunit and P110 catalytic subunit, and is an important member of the growth factor receptor tyrosine kinase signaling pathway[12,13]. After PI3K is activated, downstream proteins can be phosphorylated, and serine protein kinase Akt can be recruited and phosphorylated. Phosphorylated Akt can further phosphorylate the target protein mTOR of rapamycin, an important target downstream molecule, and then make this signaling pathway continue to be transduced[14]. S6 ribosomal protein kinase and eukaryotic translation initiation factor are target downstream proteins of mTOR. When S6K and 4E binding protein 1 are phosphorylated by mTOR[15], the transcription and translation mediated by various downstream regulatory factors are activated, resulting in abnormal proliferation and deterioration of tumor cells. The PI3K/Akt/mTOR signaling pathway plays an important role in tumor development and has become an important research object in tumor treatment[16,17]. MET, as a safe hypoglycemic agent, has been shown by more and more studies[18] to have an anti-tumor effect with mild adverse reactions. Ricchi et al[19] have confirmed that MET, in combination with chemotherapeutic drugs, produces enhanced chemotherapeutic efficacy with reduced side effects.

In this work, the GEM-resistant human pancreatic cancer PANC-1/GEM cell line was established, and it was found that the proliferation ability of PANC-1/GEM cells was significantly enhanced compared with that of PANC-1 cells. Flow cytometry results revealed that the proliferation ability of PANC-1/GEM cells was remarkably enhanced, and the expression level and phosphorylation level of mTOR in drug-resistant cell lines were increased. In addition, after drug-resistant cell lines were treated with 20 mmol/L MET for 48 h, the proliferation ability of PANC-1/GEM/MET cells were notably decreased compared with that of PANC-1/GEM cells, and mRNA and protein expression levels of mTOR/PI3K/Akt were significantly down-regulated, but the apoptosis rate was significantly increased. Similarly, previous studies have shown that paclitaxel combined with MET can significantly inhibit the proliferation and progression of lung cancer and human breast cancer cells. It has also been found that in the paclitaxel + MET group, the mTOR of cells is inhibited, resulting in stronger cell cycle arrest and apoptosis, and the inhibition rate on transplanted tumors was markedly increased compared with those in the paclitaxel group[20].

Conclusions

MET can regulate the PI3K/Akt/mTOR signaling pathway to enhance the sensitivity of human pancreatic cancer cells to GEM, which is expected to become a new approach in the treatment of pancreatic cancer.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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