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Original paper

Mechanism of mTOR inhibitors mediated DDP resistance in pancreatic cancer through the mTOR pathway

YULAN PAN[1#], FEI HE[2#], YIYANG ZHANG[3], CHUNMING FEI[4*]

[1]Liuhe Branch of Nanjing Gulou Hospital, Nanjing 211500, PR China
[2]Department of Gastroenterology, Liuhe Branch of Nanjing Gulou Hospital, Nanjing 211500, PR China
[3]Department of Gastroenterology, Nanjing Gulou Hospital, School of Medicine, Nanjing University, Nanjing 210008, PR China
[4]Department of Gastroenterology, Liuhe Branch of Nanjing Gulou Hospital, Nanjing 211500, PR China
[#]These authors contributed equally to this work as co-first author

Abstract

Mechanism of mTOR inhibitor rapamycin mediated cis-Dichlorodiamine Platinum (DDP) resistance in pancreatic cancer was explored. PANC-1, BxPC-3 and SW1990 cells cultured were used for wound healing assay and MTT assay. Western blot was used to see expression levels of mTOR signal pathway associated protein PI3K, p-PI3K, AKT, p-AKT, mTOR, and p-mTOR inhibited by rapamycin. The pancreatic cancer cell lines of PANC-1, BxPC-3 and SW1990 were all resistant to DDP. After the application of DDP combined with rapamycin, the migration and invasive ability of the three cell lines decreased significantly (P<0.01). Moreover, after different concentrations of rapamycin were added, the survival rate of the three cell lines decreased significantly, so did the IC_{50} (P<0.01). Western blot assay showed that rapamycin significantly down-regulated the expression of PI3K, AKT and mTOR proteins in PANC-1 and BxPC-3 cells, inhibited the phosphorylation of PI3K, AKT and mTOR, decreased p-mTOR protein expression, but its effect on the expression of mTOR protein in SW1990 cells was weaker. Rapamycin can reduce the migration and invasion of pancreatic cancer cells, increase the sensitivity of pancreatic cancer to DDP, and reverse the resistance of DDP. This process is achieved by inhibiting expression and phosphorylation of mTOR signaling pathway related proteins.

Keywords mTOR inhibitors, pancreatic cancer, mTOR signaling pathway, drug resistance.

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*Corresponding author: CHUNMING FEI, 28 Yan'an Road, Liuhe District, Nanjing City, Jiangsu Province, PR China
E-mail: ribin212@yandex.com ujb53685@126.com

Introduction

Pancreatic cancer (PC) is a common digestive tract tumor with a high degree of deterioration, most of which originate from ductal adenocarcinoma (1). The disease is characterized by short course of disease, rapid deterioration, difficult early stage diagnose, low cure rate and less than 1% 5-year survival rate. It is one of the malignant tumors with the worst prognosis (2). At present, the chemotherapy regimen of pancreatic cancer is mainly based on platinum drugs and cis-Dichlorodiamine Platinum (DDP) is one of the representative drugs (3). DDP is a nonspecific anti-tumor drug in cell cycle. After DDP enters human body, it is hydrated and binds to DNA of PC tumor cells and inhibits their replication and transcription, promotes apoptosis of tumor cells, and thereby kills tumor cells (4). However, a large number of studies have found that PC cells have developed drug resistance to DDP, leading to the failure of PC chemotherapy. The mTOR signaling pathway is a nutrient sensing mechanism coupled to mTOR, the mammalian or mechanistic target of rapamycin. mTOR is a component of two complexes, TORC1 and TORC2, Only TORC1 is acutely sensitive to rapamycin which inhibits some, but not all, of TORC1 functions. the TORC 1 pathway appears to play a role in activated pancreatic stellate cells where it mediates effects of insulin to enhance collagen synthesis and fibrosis. TORC1 activity is increased in many pancreatic ductal adenocarcinomas (PDAC) in part due to mutations in upstream regulatory molecules including PTEN, AKT and TSC1/2. The change of mTOR signaling pathway in tumor cells is one of the causes of drug resistance (5, 6). Rapamycin is a novel immunosuppressant of macrolides with high efficiency and low toxicity. It was first used to alleviate rejection reaction of organ transplantation and to treat autoimmune diseases. The mechanism of impaired DDP accumulation is unknown, and the mechanism by which DDP enters or exits from cells remains poorly defined. DDP efflux is characterized by an initial very rapid phase followed by long terminal half-life. An increased rate of efflux has been reported in some DDP-resistant cell lines and in cells that overexpress glutathione GS-X pump. In recent years, accumulating evidences suggest that rapamycin is also a mTOR signaling pathway inhibitor, which can reduce the resistance of tumor cells to DDP (7, 8). In this study, three kinds of pancreatic cancer cell lines were selected to further study the mechanism of rapamycin inhibiting mTOR signaling pathway in PC tumor cells.

Materials and Methods

Cell lines and main reagents

PANC-1 cells, BxPC-3 cells, SW1990 cells (all purchased from Chinese cell line resource bank); rapamycin (purchased from Beyotime Biotechnology Institute); cis-Dichlorodiamine Platinum (DDP), MTT, dimethyl sulfoxide (DMSO) (purchased from Sigma company); Transwell chamber, anti-PI3K, anti-p-PI3K, anti-AKT, anti-p-AKT, anti-mTOR, anti-p-mTOR, anti-β-actin and Goat anti Rabbit IgG antibodies (all purchased from Thermo Fisher Scientific company); RPMI-1640, fetal bovine serum (FBS) (Gibco company, USA); trypsase, penicillin streptomycin double antibody (purchased from Thermo Fisher Scientific company).

Experimental method

PANC-1, BxPC-3 and SW1990 were cultured in 5% CO_2 incubator at 37°C with 10% fetal bovine serum and 1×10^5 U/L RPMI-1640. Then the cells in logarithmic growth phase were received for the experiment.

(1) Wound healing assay: Three kinds of cells were inoculated on 6-well plate, scratched and took photos when the cell fusion degree was 80%. The medium was removed, 2.5% serum medium (serum medium containing 20 mg/L DDP or 20 mg/L DDP + 10 μmol/L rapamycin) was added, then cultured for 24 hours and took photos. Scratch repair rate = (0 h scratch width – 24 h scratch width) / 0 h scratch width × 100%.

(2) Transwell assay: The cells were digested with trypsin, the culture medium was removed, the cells were washed twice by phosphate buffer solution. The cell density was adjusted to 15×10^4 by adding serum-free medium, the upper chamber was inoculated with 200 μL cell fluid, and the lower chamber was supplemented with 600 μL 10% fetal bovine serum. The cells were treated with 20 mg/L DDP or 20 mg/L DDP + 10 μmol/L rapamycin for 12 h, fixed with methanol, stained with crystal violet, then observed under microscope and counted the number of perforating cells.

(3) MTT assay: The cells were digested with trypsin, then inoculated in 96-well plate with 5×10^3 cells per well for 12 h. After the cells were adhered to the cell wall, the culture medium was removed, DDP and 200 μL of rapamycin serum culture medium with the concentration of 0, 5, 10, 20 μmol/L was added, and the culture medium was changed every 24 hours. After 48 hours, 20 μL of MTT was added to culture medium and cultured for 4 h, then the culture medium was removed, 150 μL of DMSO was added and shaked for 10 min without light. 100 μL of the cells were extracted, the absorbance was measured at 490 nm and the semi-inhibitory concentration (IC_{50}) of DDP on cell lines was calculated. Repeat 3 times for each group of 6 wells and take the average value.

(4) Western blot: The total protein was extracted, 10 μg of the protein was sampled and isolated by SDS-PAGE. Then transferred to the polyvinylidenfluorid (PVDF) membrane and blocked by bovine serum albumin (BSA) for 1 h. The primary antibody was added, then incubated overnight at 4°C. The membrane was washed 3 times by TBST, then incubated with the second antibody for 2 h. ECL luminescence solution was added, photo was taken, and band gray scale was analyzed.

Statistical analysis

All the data collected in this study were statistically analyzed by SPSS 19.0 software. The measured data were expressed as mean ± standard deviation ($\overline{x} \pm s$). T test was used for comparison between the two groups and one-way ANOVA was used for multi-group contrast. $P < 0.05$

indicated the difference was statistically significant, and $P < 0.01$ represented highly significant difference.

Results

The experimental results of wound healing assay

The results of wound healing assay showed that the three cell lines of PANC-1, BxPC-3 and SW1990 with DDP resistance had stronger migration ability when using DDP alone, in which PANC-1 had the strongest migration ability, followed by SW1990 and BxPC-3. In combination with rapamycin, the migration ability of the three drug resistant strains was significantly lower than that without rapamycin ($P < 0.01$), as shown in Table 1 and Figure 1.

Table 1. The results of wound healing assay ($\overline{x} \pm s$, $n = 6$, %)

Groups	PANC-1	BxPC-3	SW1990
DDP (20 mg/L)	92.15±5.42	80.75±4.39	86.41±5.15
DDP(20mg/L)+rapamycin(10 μmol/L)	40.24±2.21	48.35±4.34	54.15±3.68
t	21.724	12.856	12.484
P	<0.01	<0.01	<0.01

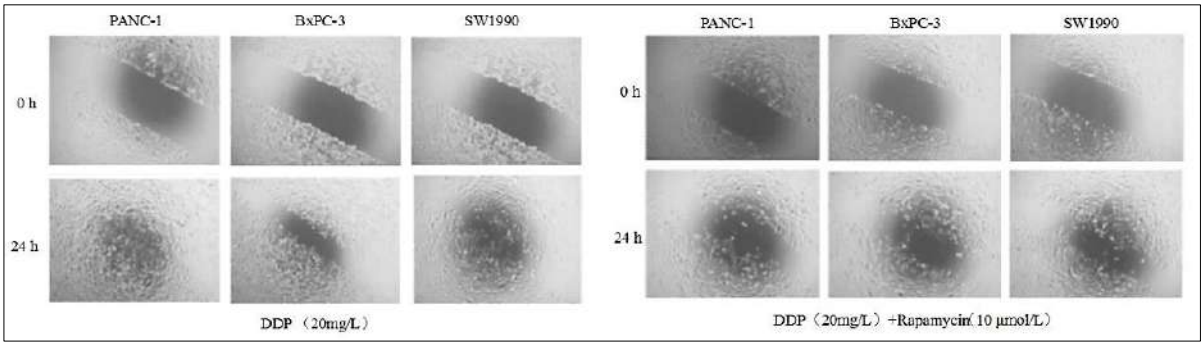

Figure 1. The results of wound healing assay

The experimental results of Transwell

Transwell assay indicated that all three DDP resistant cell lines of PANC-1, BxPC-3 and SW1990 had strong invasion ability when using DDP alone. In combination with rapamycin, the invasive ability of three drug-resistant strains was significantly decreased ($P < 0.01$), as shown in Table 2 and Figure 2.

Table 2. The results of the Transwell ($\overline{x} \pm s$, $n = 6$)

Groups	PANC-1	BxPC-3	SW1990
DDP (20 mg/L)	226.56±45.37	190.78±36.74	248.28±40.25
DDP(20 mg/L)+rapamycin(10 μmol/L)	140.45±15.68	100.38±12.75	120.16±13.98
t	4.394	5.694	7.365
P	0.001	<0.01	<0.01

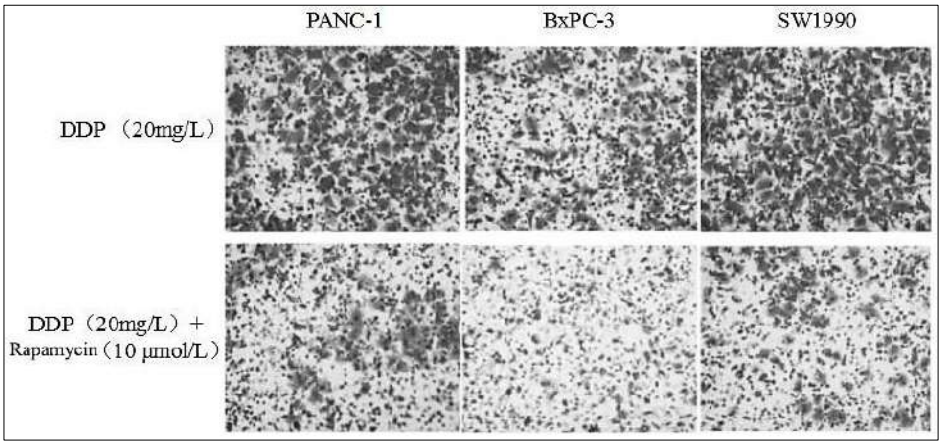

Figure 2. The results of the Transwell

The experimental results of inhibition rate

The wound healing assay and Transwell test showed that 10 μmol/L rapamycin could inhibit the migration and invasion abilities of PANC-1, BxPC-3 and SW1990 resistant strains. Therefore, DDP was further designed in combination with rapamycin at different concentrations to investigate the changes of IC_{50} in three cell lines treated with DDP. The results showed that IC_{50} of the three cell lines was higher when rapamycin was not added, and IC_{50} of the three cell lines decreased significantly with the addition of 5, 10 and 20 μmol/L rapamycin ($P < 0.01$), as shown in Table 3.

Table 3. Effect of rapamycin on IC_{50} of three cell lines ($\overline{x} \pm s$, $n = 6$)

Groups	PANC-1	BxPC-3	SW1990
DDP	42.31±4.68	37.58±4.24	33.69±3.57
DDP+rapamycin (5 μmol/L)	34.59±4.22[a]	30.24±3.52[a]	27.21±3.28[a]
DDP+rapamycin (10 μmol/L)	23.38±4.24[ab]	18.34±4.01[ab]	18.34±3.21[ab]
DDP+rapamycin (20 μmol/L)	13.78±3.11[abc]	10.36±3.24[abc]	12.24±3.14[abc]
F	55.90	62.01	49.36
P	<0.01	<0.01	<0.01

Note: Compared with the control group, [a]$P < 0.01$; compared with the low dose group, [b]$P < 0.01$; compared with the medium dose group, [c]$P < 0.01$.

Rapamycin inhibits the protein expression of PI3K/AKT/mTOR signaling pathway in PANC-1 cell line

Western blot assay showed that the expression of PI3K, p-PI3K, AKT, p-AKT, mTOR and p-mTOR protein in PANC-1 resistant strain was relatively high. Moreover, the expression of PI3K, p-PI3K, AKT, p-AKT, mTOR and p-mTOR protein in PANC-1 resistant strain decreased significantly after the addition of 5, 10 and 20 μmol/L rapamycin ($P < 0.01$), as shown in Table 4 and Figure 3.

Table 4. Inhibition effect of rapamycin on expression of PI3K/AKT/mTOR signaling pathway related proteins in PANC-1 cell line ($\overline{x} \pm s$, $n = 6$)

Groups	PI3K	p-PI3K	AKT	p-AKT	mTOR	p-mTOR
DDP	0.951±0.012	0.634±0.011	0.588±0.010	0.617±0.011	0.487±0.009	0.429±0.009
DDP+rapamycin (5 μmol/L)	0.842±0.010[a]	0.513±0.010[a]	0.553±0.011[a]	0.515±0.010[a]	0.366±0.008[a]	0.362±0.009[a]
DDP+rapamycin (10 μmol/L)	0.503±0.011[ab]	0.457±0.008[ab]	0.525±0.009[ab]	0.453±0.008[ab]	0.342±0.009[ab]	0.342±0.011[ab]
DDP+rapamycin (20 μmol/L)	0.350±0.008[abc]	0.403±0.009[abc]	0.439±0.008[abc]	0.384±0.010[abc]	0.291±0.011[abc]	0.183±0.010[abc]
F	4439.11	641.98	265.42	609.64	477.72	680.40
P	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Note: Compared with the control group, [a]$P < 0.01$; compared with the low dose group, [b]$P < 0.01$; compared with the medium dose group, [c]$P < 0.01$.

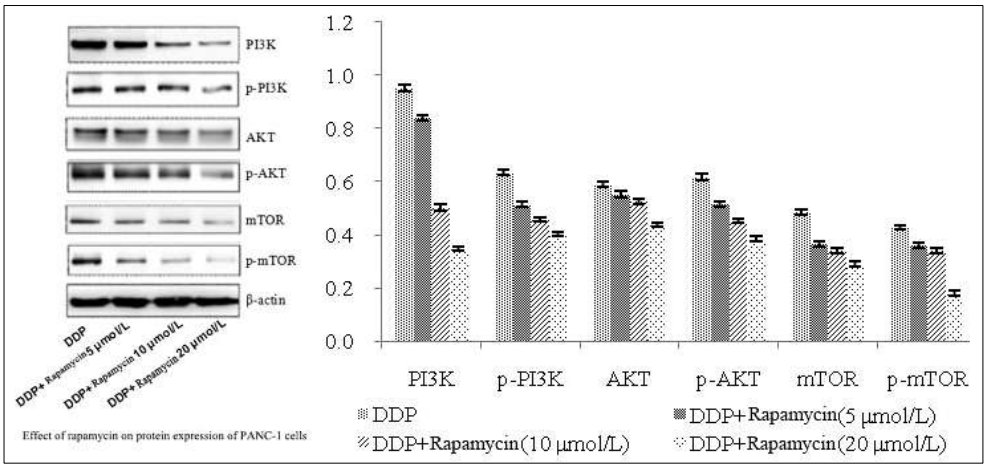

Figure 3. Effect of rapamycin on protein expression of PANC-1 cell

Rapamycin inhibits the protein expression of PI3K/AKT/mTOR signaling pathway in BxPC-3 cell line

Western blot assay showed that the expression of PI3K, p-PI3K, AKT, p-AKT, mTOR and p-mTOR protein in BxPC-3 resistant strain was relatively high when 20 mg/L DDP was added only. Furthermore, the expression of PI3K, p-PI3K, AKT, p-AKT, mTOR and p-mTOR protein in BxPC-3 resistant strain decreased significantly after the addition of 5, 10 and 20 μmol/L rapamycin ($P < 0.01$), as shown in Table 5 and Figure 4.

Table 5. Inhibition effect of rapamycin on expression of PI3K/AKT/mTOR signaling pathway related proteins in BxPC-3 cell line ($\overline{x} \pm s$, $n = 6$)

Groups	PI3K	p-PI3K	AKT	p-AKT	mTOR	p-mTOR
DDP	0.926±0.012	0.717±0.013	0.858±0.012	0.458±0.013	0.668±0.012	0.458±0.013
DDP+rapamycin (5 μmol/L)	0.883±0.011[a]	0.655±0.011[a]	0.755±0.010[a]	0.405±0.011[a]	0.604±0.008[a]	0.393±0.009[a]
DDP+rapamycin (10 μmol/L)	0.854±0.009[ab]	0.482±0.009[ab]	0.386±0.011[ab]	0.222±0.012[ab]	0.326±0.010[ab]	0.374±0.010[ab]
DDP+rapamycin (20 μmol/L)	0.455±0.008[abc]	0.356±0.010[abc]	0.314±0.013[abc]	0.169±0.008[abc]	0.223±0.011[abc]	0.192±0.008[abc]
F	2790.73	1378.33	3240.28	939.84	2574.07	753.25
P	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Note: Compared with the control group, [a]$P < 0.01$; compared with the low dose group, [b]$P < 0.01$; compared with the medium dose group, [c]$P < 0.01$.

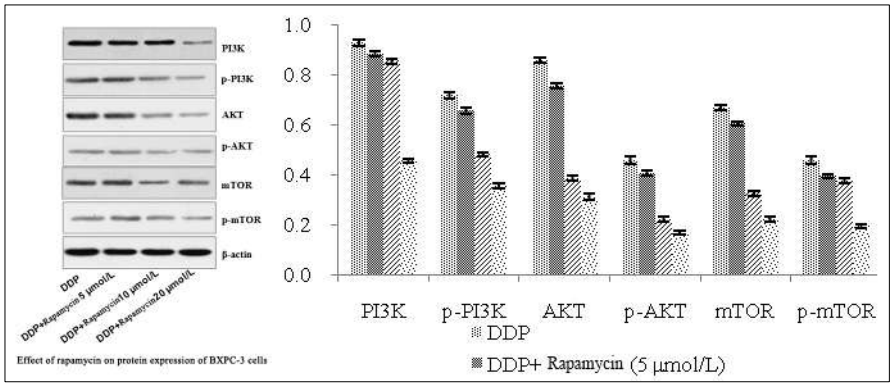

Figure 4. Effect of rapamycin on protein expression of BxPC-3 cell

Rapamycin inhibits the protein expression of PI3K/AKT/mTOR signaling pathway in SW1990 cell line

Western blot assay showed that the expression of PI3K, p-PI3K, AKT, p-AKT, mTOR and p-mTOR protein in SW1990 resistant strain was relatively high when 20 mg/L DDP was added only. The expression of mTOR protein in SW1990 resistant strain decreased after the addition of 5, 10 and 20 μmol/L rapamycin, but there was no significant difference among the three groups ($P > 0.05$). Moreover, the expression of PI3K, p-PI3K, AKT, p-AKT and p-mTOR protein in SW1990 resistant strain was significantly decreased ($P < 0.01$), as shown in Table 6 and Figure 5.

Note: Compared with the control group, [a]$P < 0.01$; compared with the low dose group, [b]$P < 0.01$; compared with the medium dose group, [c]$P < 0.01$.

Table 6. Inhibition effect of rapamycin on expression of PI3K/AKT/mTOR signaling pathway related proteins in SW1990 cell line ($\overline{x} \pm s$, $n = 6$)

Groups	PI3K	p-PI3K	AKT	p-AKT	mTOR	p-mTOR
DDP	0.915±0.012	0.886±0.009	0.957±0.013	0.566±0.012	0.518±0.013	0.517±0.012
DDP + rapamycin (5 μmol/L)	0.724±0.009[a]	0.712±0.010[a]	0.852±0.011[a]	0.452±0.011[a]	0.454±0.011[a]	0.424±0.008[a]
DDP + rapamycin (10 μmol/L)	0.452±0.008[ab]	0.420±0.010[ab]	0.552±0.012[ab]	0.143±0.010[ab]	0.446±0.010[a]	0.342±0.011[ab]
DDP + rapamycin (20 μmol/L)	0.366±0.011[abc]	0.333±0.011[abc]	0.463±0.010[abc]	0.114±0.008[abc]	0.452±0.011[a]	0.253±0.010[abc]
F	3716.07	3928.93	2503.10	2828.88	53.78	712.62
P	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Note: Compared with the control group, [a]$P < 0.01$; compared with the low dose group, [b]$P < 0.01$; compared with the medium dose group, [c]$P < 0.01$.

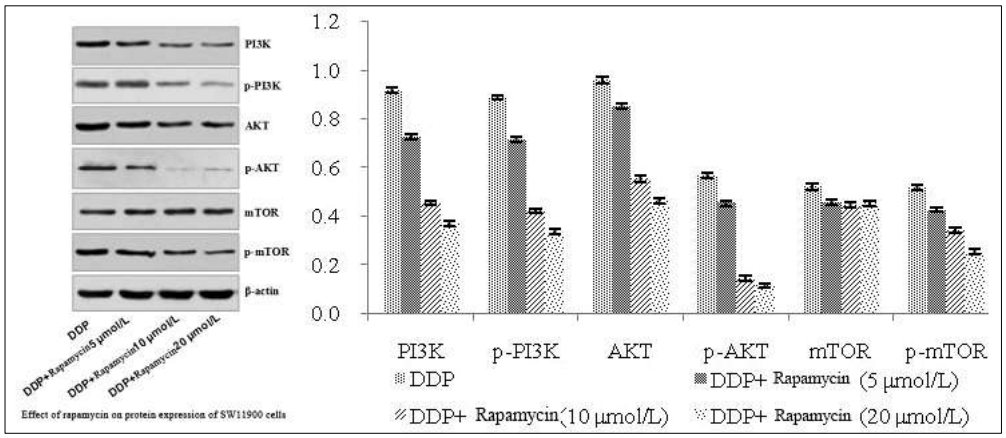

Figure 5. Effect of rapamycin on protein expression of SW1990 cell

Discussion

It has been demonstrated that the multidrug resistance of tumor cells is an important reason for the failure of chemotherapy. According to the mechanism of drug resistance, it can be divided into primary drug resistance (PDR) and multidrug resistance (MDR). PDR is only resistant to induction drugs and MDR is not only resistant to induction drugs, but also resistant to drugs with different structures and different mechanisms, which is the primary reason for the failure of tumor chemotherapy (8, 9). The most important mechanisms by which cells develop resistance are as follows: ① Intracellular drug concentration decreased: changes of tumor cell membrane make it difficult for drugs to enter cells, or tumor cells increase drug efflux, and ultimately decrease the concentration of drugs in cells and fail to reach the minimum effective concentration (10, 11). ② Changes of apoptosis process in tumor cells: tumor cell injury activates the PI3K/AKT/mTOR signaling pathway, enhances the repair mechanism of DNA damage, and changes the gene activity associated with the signaling pathway (12, 13).

mTOR signaling pathway is mainly composed of PI3K, AKT and mTOR, which affects cell proliferation, apoptosis, metabolism and other physiological activities (14). Insulin-like growth factor (IGF) activates PI3K, which lead to phosphorylation of PIP2 to form PIP3. PIP3 stimulates AKT to be phosphorylated by PDK on the surface of cell membrane to form p-AKT, which activates mTOR in the inhibitory state. Phosphorylated p-mTOR activates downstream related molecules, promotes cell translation and synthesis, regulates cell metabolism and other physiologic function (15, 16). In recent years, more and more studies have confirmed that mTOR signaling pathway can affect the growth and metabolism of tumor cells, promote cell proliferation, inhibit cell apoptosis and enhance cell invasion ability. High expression of AKT1 was found in lung cancer cell line A549/CDDP with DDP resistant and inhibition of AKT1 expression could reverse DDP resistance of lung cancer cell line (17, 18). Wang Yuliang (19, 20) has found that the protein encoded by CA916798 gene exists in three phosphorylation sites of mTOR signaling pathway, and mTOR pathway can regulate gene expression at transcriptional level and induce DDP resistance.

In this study, it was found that the three pancreatic cancer cell lines of PANC-1, BxPC-3 and SW1990 were resistant to DDP. When DDP was used alone, the three cell lines had strong ability of cell migration and invasion, and IC_{50} was relatively high. In combination with rapamycin, the migration and invasion ability of the three cell lines decreased significantly ($P < 0.01$), which was consistent with the results of Zhu Fang (21). When different concentrations of rapamycin were added, the survival rate and IC_{50} of the three cell lines decreased significantly ($P < 0.01$), which suggested that rapamycin could enhance the sensitivity of resistant strains to DDP. Further western blot assay showed that rapamycin could down-regulate the expression of PI3K, AKT and mTOR protein and inhibit the phosphorylation of PI3K, AKT and mTOR in PANC-1 and BxPC-3 cells. Although the effect of rapamycin on the expression of mTOR in SW1990 cell was weak, the phosphorylation of related proteins was significantly inhibited, suggesting that rapamycin might reverse the mechanism of DDP resistance in pancreatic cancer cell lines by inhibiting the mTOR pathway. mTOR has two kinds of complex, mTORC1 and mTORC2, which have different effects on proliferation, differentiation, growth and metastasis of tumor cells. Fei Shijiang (22) and others found that the expression of mTORC2 in drug-resistant cells was significantly higher than that in sensitive cells, and the activity of mTORC1 was higher in sensitive cells. However, in both kinds of cells, the mTOR signaling pathway was highly activated, suggesting that there may be a dynamic balance between mTORC1 and mTORC2. When the upstream signal changes, it causes mTORC1 to transform into mTORC2, resulting in drug resistance. This may be the reason why rapamycin has no obvious inhibition on mTOR protein in SW1990 resistant strain, suggesting that there may be other pathways affecting the expression of mTOR protein, and the specific mechanism of its resistance to DDP should be further studied.

In conclusion, rapamycin as a mTOR inhibitor can significantly reduce the migration, invasion and survival rate of pancreatic cancer cells, increase the sensitivity of pancreatic cancer to DDP, and reverse DDP resistance. The possible mechanism is to inhibit the expression of mTOR signaling pathway related protein, providing new ideas for clinical treatment of pancreatic cancer and overcoming DDP resistance.

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Conflict of Interest

The author(s) declare that they have no conflict of interest.

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