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ORIGINAL ARTICLE

A study on the mechanism of rapamycin mediating the sensitivity of pancreatic cancer cells to cisplatin through PI3K/AKT/mTOR signaling pathway

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Summary

Purpose: *To study the mechanism of rapamycin mediating the sensitivity of pancreatic cancer cells to cisplatin through phosphatidylinositol 3-kinase (PI3K)/serine-threonine kinase (AKT)/mammalian target of rapamycin (mTOR) signaling pathway in vitro.*

Methods: *SW1990 cells were cultured in vitro and treated with rapamycin, cisplatin, and rapamycin combined with cisplatin, respectively, with dimethyl sulphoxide (DMSO) as the control. Cell Counting Kit-8 (CCK-8) and flow cytometry were adopted for determination of cell proliferation and apoptosis levels, respectively. The changes in PI3K/AKT/mTOR signal transmission were detected via Western blotting and reverse transcription polymerase chain reaction (RT-PCR), respectively.*

Results: *1: Compared with those in DMSO blank control group, the proliferation level of pancreatic cancer cells was markedly decreased and the cell apoptosis rate was remarkably increased in simple drug group ($p<0.05$). 2: The combined administration group had markedly decreased proliferation level and remarkably increased cell apoptosis rate of human pancreatic cancer cells, compared with those in the rapamycin alone group or cisplatin alone group ($p<0.05$). 3: Rapamycin combined with cisplatin could inhibit the expressions of PI3K, AKT and phosphorylated mTOR (p-mTOR) in pancreatic cancer cells ($p<0.05$).*

Conclusions: *Rapamycin combined with cisplatin can alter the PI3K/AKT/mTOR signal transduction pathway which leads to markedly increased cell apoptosis rate, indicating that rapamycin can mediate the sensitivity of pancreatic cancer cells to cisplatin.*

Key words: *pancreatic cancer, rapamycin, cisplatin, PI3K/AKT/mTOR*

Introduction

Pancreatic cancer is a highly lethal disease, ranking fourth in mortality among solid tumors [1,2]. Among patients with locally advanced or metastatic pancreatic cancer, 80-85% are unable to undergo radical resection and have poor prognosis. Although great progress has been made in the detection and treatment techniques, the 5-year overall survival rate is still as low as 4% [3]. In addition, pancreatic cancer responds poorly to most chemotherapeutic drugs. As targeted therapies produce mild adverse reactions due to their specificity and targeted inhibition, they have become the preferred treatments for cancers in recent years.

Rapamycin, as a lipophilic macrolide antibiotic, was originally developed as a fungicide and an immunosuppressant [4]. It has been reported in previ-

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ous studies that rapamycin has an antiproliferative effect on certain tumors [5-11]. mTOR is a downstream molecule of phosphatidyl-inositol 3-kinase (PI3K)/serine-threonine kinase (AKT) signal transduction that can be recognized and bound by rapamycin [12]. mTOR plays a key role in the regulation of cell growth by integrating signals from stress events, nutrients and growth factors [13], therefore is considered as a major effector of cell growth and proliferation controlling protein synthesis through a large number of downstream targets [14,15].

Malignant tumors, due to their very complicated pathogenesis and being affected by many factors *in vivo* and *in vitro*, are often the result of multiple signals and multiple target interactions which lead to the disorder and failure of regulatory mechanisms. Therefore, the mechanisms of tumorigenesis should be deeply understood, and comprehensive intervention is necessary for multiple targets in the process of tumorigenesis, in order to obtain better therapeutic efficacy. On the basis of this purpose, this study aimed to evaluate the antitumor effect of mTOR in human pancreatic cancer SW1990 cells and to elucidate the possible molecular mechanisms of this molecule in inducing cell apoptosis and autophagy.

Methods

Cell culture and treatment

Human pancreatic cancer SW1990 cells in the logarithmic growth phase were digested by 0.25% trypsin and inoculated into 6-well well plates . Cells in the experimental groups (mTOR group) were treated with mTOR at a working concentration of 30 μg/L, 3 mg/L cisplatin , or the combination of both. Cells in the control group (DMSO group) received 0.5% dimethyl sulphoxide (DMSO group) and were cultured for 24, 48 and 72 h for the subsequent experimental study.

Western blotting detection

Cells cultured for different times were lysed to extract proteins, the concentration of which was determined by bicinchoninic acid (BCA) method. The extracted proteins were separated by electrophoresis, transferred onto a membrane and blocked. Antibodies such as phosphatase and tensin homolog deleted on chromosome 10 (PTEN), p-mTOR, phosphorylated AKT (p-AKT), PI3K, and β-actin were added for incubation overnight. After the membrane was washed WITH phosphate buffered saline (PBS), secondary antibodies were added for color development.

Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells cultured for different times, followed by reverse transcription and amplification detection All expressions were calculated using the $2^{-\Delta\Delta Ct}$ method (Table 1).

Table 1. Primers used in fluorescent quantitative PCR

Name	*Primer pair*
PTEN	F:5'CAGAGCGAGGGCATCAC 3'
	R:5'GCAGGAAATCCCATAGCAATAA 3'
AKT	F:5'GTGCTGGAGGACAATGACTA 3'
	R:5'AGCAGCCCTGAAAGCAAGGA 3'
PI3K	F:5'ATGGGGATGATTTACGGC 3'
	R: 5'TCTCCTTTGTTCTTGTCTTTGA5'
mTOR	F: 5'TGGCTTCTAAGTCTACCACGACAG 5'
	R:5'GAGGTCCTTGACATTCCCTGATT 3'
β-actin	F: 5'CTTCCTTCCTGGGCATG 3'
	R: 5'GTCTTTGCGGATGTCCAC 3'

Detection of cell proliferation inhibition by Cell Counting Kit-8 (CCK-8)

Cells from 6-well plates were evaluated with CCK-8 assay, and the absorbance value was measured at a wavelength of 450nm. The proliferation inhibition rate (%) = (1-optical density $(OD)_{experimental\ group}$ / $OD_{DMSO\ group}$) × 100%.

Flow cytometry

The apoptotic cells and living or necrotic cells were detected by using Annexin V-FITC combined with propidium iodide (PI) [16]. The sample was washed WITH PBS twice. Ten μL Annexin V-FITC and 10 μL PI (20 μg/mL) were added respectively for incubation in the dark at room temperature for at least 20 min for further flow cytometry.

Statistics

All data were statistically analyzed by Student's *t*-test using SPSS 20.0 software (IBM, Armonk, NY, USA). $P<0.05$ suggested that the difference was statistically significant.

Results

Effects of mTOR alone, cisplatin alone or their combined administration on the apoptosis rate of pancreatic cancer SW1990 cells

The results of apoptosis of human pancreatic cancer SW1990 cells by flow cytometry are shown in Figure 1 and Table 2. Compared with the DMSO control group, the treatment of mTOR alone significantly increased cell apoptosis, and the apoptosis rate was clearly dependent on the duration of drug exposure, among which the early and late apoptosis rates of cells at 72 h were 8.16±0.67% and 3.79±0.45%, respectively ($p<0.05$). Compared with the control group, the cell apoptosis rate in the cisplatin alone group was also remarkably increased ($p<0.05$), with early and late apoptosis rates of cells at 72 h 10.25±1.23% and 5.36 ± 0.62%, respectively ($p<0.05$). Compared with mTOR alone

group, the apoptosis rate was increased in the cisplatin alone group, but without significant difference. The apoptosis rate of SW1990 cells was remarkably increased in the combined administration group, and the early and late apoptosis rates of cells at 72 h were 21.18±1.35% and 10.24±0.9% respectively, compared to single use of mTOR or cisplatin ($p<0.05$). Compared with other groups, the combined administration group had significantly increased number of apoptotic cells at different time points ($p<0.05$).

Effects of single or combined administration of drugs on the growth inhibition of SW1990 cells

The results of SW1990 cell proliferation assay are shown in Table 3. The effect of single use of mTOR on inhibiting cell growth and proliferation was lowest at 24 h (6.39±0.58%) and the inhibition rate reached 12.37±0.86% at 72 h . The effect of single treatment of cisplatin was lowest at 24 h (7.09±0.89%) and the inhibition rate reached 2.74±0.18% at 72 h . The inhibition rate of cell proliferation in the combined administration group was 19.36±2.17% at 24 h and 62.26±6.24% at 72h, indicating the single or combined use of mTOR could significantly reduce cell growth and proliferation, compared with that in the control group ($p<0.05$). The above results indicated that mTOR combined with cisplatin has a very notable synergistic effect in human pancreatic cancer SW1990 cells, which can increase the chemosensitivity of SW1990 cells to cisplatin.

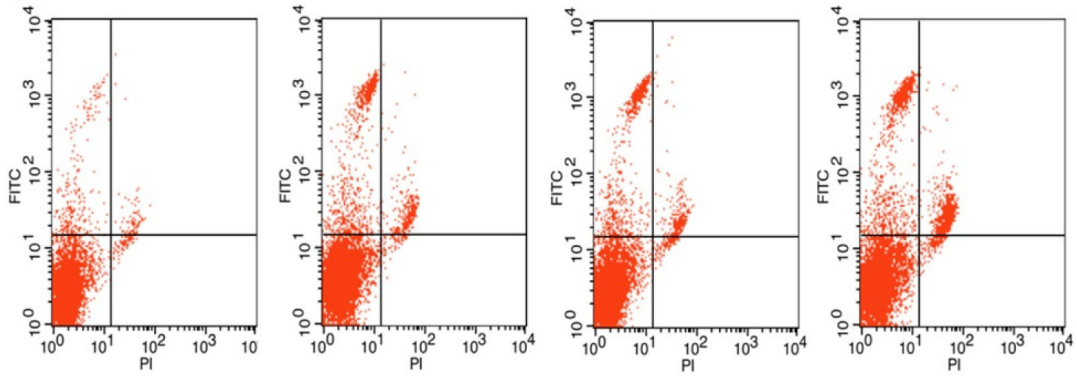

Figure 1. Effects of administration of mTOR alone, cisplatin alone or both drugs for 24 h on the apoptosis rate of human pancreatic cancer SW1990 cells by flow cytometry. From left to right: DMSO control group, mTOR group, cisplatin group and combined administration group.

Table 2. Effects of mTOR alone, cisplatin alone or combined administration of both drugs on the apoptosis rate of pancreatic cancer SW1990 cells

Time	*Group*	*Early apoptosis rate of cells (%) mean±SD*	*Late apoptosis rate of cells (%) mean±SD*
24 h			
	Rapamycin group	5.45±0.39#	2.63±0.21*#
	Cisplatin group	7.09±0.89*#	2.74±0.18*#
	Combined administration group	10.15±0.97*	5.01±0.32*
	DMSO group	4.83±0.43	1.42±0.19
48 h			
	Rapamycin group	6.12±0.58*#	3.54±0.41*#
	Cisplatin group	8.09±0.76*#	4.17±0.45*#
	Combined administration group	13.24±0.98*	8.18±0.87*
	DMSO group	4.52±0.36	1.54±0.22
72 h			
	Rapamycin group	8.16±0.67*#	3.79±0.45*#
	Cisplatin group	10.25±1.23*#	5.36±0.62*#
	Combined administration group	21.18±1.35*	10.24±0.9*
	DMSO group	5.01±0.42	1.39±0.13

At the same time point, * the difference is significant compared with the DMSO group ($p<0.05$), and # the difference is significant compared with the combined administration group ($p<0.05$).

Effects of single or combined administration of drugs on the expressions of related proteins in SW1990 cells

Western blotting results are shown in Figure 2 and Table 4. Compared with DMSO group, mTOR alone could induce downregulation of p-mTOR protein expression. With the increase of administration time, the phosphorylation levels and activities of mTOR and AKT declined continuously, and the activity of PI3K was also continuously decreased, while the expression of PTEN was continuously increased, showing significant differences ($p<0.05$). The differences found in the phosphorylation

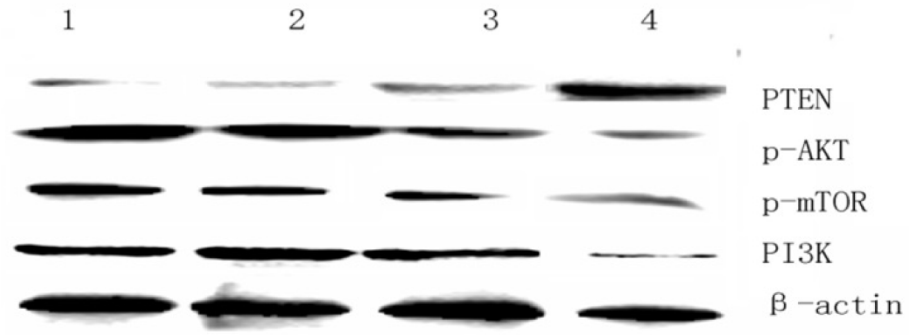

Figure 2. Effects of administration of mTOR alone, cisplatin alone or both drugs for 48 h on the protein expressions in SW1990 cells (western blotting). 1: DMSO group, 2: cisplatin group, 3: mTOR group, and 4: combined administration group.

Table 3. Effects of mTOR alone, cisplatin alone or combined administration of both drugs on the growth inhibition of human pancreatic cancer SW1990 cells

Group	*Growth inhibition rate (mean±SD)*		
	24 h	*48 h*	*72 h*
Rapamycin group	6.39±0.58	8.14±0.95	12.37±0.86
Cisplatin group	11.25±0.95	22.06±1.78	29.48±3.12
Combined administration group	19.36±2.17	46.43±3.55	62.26±6.24

1: In different groups, the differences are significant at the same time ($p<0.05$). 2: In the same group, there are significant differences among different time points ($p<0.05$).

Table 4. Effects of mTOR alone, cisplatin alone or combined administration of both drugs on the expressions of related proteins in SW1990 cells (mean±SD).

Time	*Group*	*PTEN*	*p-AKT*	*p-mTOR*	*PI3K*
24 h					
	Rapamycin group	0.19±0.09	0.59±0.23	0.44±0.12	0.46±0.25
	Cisplatin group	0.20±0.15	0.69±0.28	0.45±0.18	0.49±0.13
	Combined administration group	0.42±0.28*	0.48±0.26	0.39±0.12	0.37±0.26
	DMSO group	0.13±0.12	0.74±0.38	0.60±0.15	0.57±0.28
48 h					
	Rapamycin group	0.26±0.13*	0.46±0.21	0.37±0.16	0.38±0.15
	Cisplatin group	0.15±0.06	0.76±0.42	0.46±0.14	0.51±0.27
	Combined administration group	0.62±0.39*	0.33±0.17	0.26±0.13*	0.25±0.14*
	DMSO group	0.10±0.09	0.87±0.42	0.52±0.15	0.64±0.18
72 h					
	Rapamycin group	0.34±0.17	0.35±0.20*	0.23±0.17	0.29±0.16
	Cisplatin group	0.13±0.02#	0.91±0.20#	0.58±0.15*#	0.50±0.32*
	Combined administration group	0.88±0.36*	0.16±0.19*	0.13±0.06*	0.15±0.09*
	DMSO group	0.12±0.08	0.86±0.19	0.54±0.26	0.52±0.19

At the same time point, * the difference is significant compared with the control group ($p<0.05$), and # the difference is significant compared with the combined administration group ($p<0.05$).

Table 5. Effects of mTOR alone, cisplatin alone or combined administration of both drugs on the mRNA expressions in SW1990 cells (mean±SD)

Time	*Group*	*PTEN*	*AKT*	*mTOR*	*PI3K*
24 h					
	Rapamycin group	0.21±0.17	0.29±0.24	0.51±0.31	0.50±0.22
	Cisplatin group	0.16±0.12	0.31±0.26	0.47±0.28	0.61±0.39
	Combined administration group	0.28±0.23	0.33±0.27	0.49±0.22	0.33±0.24
	DMSO group	0.17±0.14	0.35±0.30	0.50±0.26	0.64±0.37
48 h					
	Rapamycin group	0.35±0.22	0.31±0.24	0.47±0.28	0.36±0.24
	Cisplatin group	0.21±0.18	0.34±0.27	0.46±0.25	0.64±0.26
	Combined administration group	0.43±0.24	0.35±0.26	0.51±0.34	0.21±0.16
	DMSO group	0.17±0.15	0.27±0.22	0.55±0.27	0.66±0.33
72 h					
	Rapamycin group	0.51±0.26*	0.30±0.24	0.49±0.28	0.26±0.19*
	Cisplatin group	0.19±0.20*	0.29±0.26	0.56±0.37	0.57±0.20*#
	Combined administration group	0.62±0.28*	0.33±0.18	0.53±0.25	0.09±0.08*
	DMSO group	0.12±0.08	0.26±0.25	0.51±0.23	0.69±0.35

At the same time point, * the difference is significant compared with the DMSO group ($p<0.05$), and # there is a significant difference between the other groups and the combined administration group ($p<0.05$).

levels of mTOR and AKT and the activity of PI3K between cisplatin alone group and DMSO control group were not evident, but there were obvious differences in the expressions of the above proteins between the combined administration group and the mTOR alone group ($p<0.05$).

Effects of single or combined administration of drugs on the expressions of mTOR pathway levels in SW1990 cells

The results of RT-PCR are shown in Table 5. Compared with the DMSO group, mTOR alone could increase the messenger RNA (mRNA) expression of PTEN gene. With the increase of administration time, PI3K mRNA was decreased continuously, while PTEN mRNA was increased continuously until 72 h, displaying significant differences ($p<0.05$). No distinctive changes were found in the mRNA levels of mTOR and AKT ($p>0.05$). The combined administration group had significantly different mRNA levels of PI3K and PTEN from those in the other groups ($p<0.05$).

Discussion

The PI3K-AKT-mTOR signal transduction pathway plays an important role in the cell growth, information transmission and other processes. Disorders in signal transduction of this pathway may cause various diseases, including cancer. Therefore, the study of specific drugs for this signal transduction pathway has become a hot spot.

The activated PI3K and phosphatidylinositol 3,4,5-trisphosphate (PIP3), as well as the interactions of PIP3 with AKT and phosphoinositide-dependent protein kinase-1 (PDK1), promote PDK1 to phosphorylate AKT protein, producing activated p-AKT. P-AKT activates its substrate and regulates the activities of proteins that transfer key signals, so as to participate in the regulation of various physiological activities of the body. The mTOR complex 1 (mTORC1) phosphorylation target is a key component of the protein translation system, which activates the mTOR signaling pathway to regulate protein synthesis and cell growth [17]. Studies have shown that PTEN gene is a tumor suppressor that can effectively inhibit the growth and accelerate the apoptosis of tumor cells. PTEN gene reversely regulates this signal transduction pathway, and affects the phosphorylation of AKT to lead to overexpression of mTOR, a specific binding protein of rapamycin, thereby generating apoptosis-related proteins and inducing apoptosis of tumor cells. It has been found in one study that mTOR combined with cisplatin can significantly promote the apoptosis of ovarian cancer cells [18].

In this study, Western blotting results demonstrated that mTOR combined with cisplatin could induce increased expression of PTEN and down-regulation of the expressions of p-AKT, p-mTOR and PIK3. The RT-PCR results revealed that mTOR alone could cause an increase in the expression of PTEN mRNA and continuous decrease in the expression of PI3K mRNA. However, no distinctive

changes were found in the mRNA levels of mTOR and AKT. The effect of mTOR combined with cisplatin showed obvious time-dependence at different time periods used in this study, and the combined administration of the two drugs for 24 h could inhibit cell proliferation and induce cell apoptosis. Cisplatin, as a traditional cellular non-specific chemotherapeutic drug with a broad antitumor spectrum, binds DNA to form a DNA-cisplatin complex, thus inhibiting cell growth. mTOR can be involved in regulating cell growth and synthesis of related proteins via mTOR signaling pathway, and it can co-operate with cisplatin to further accelerate the apoptosis of tumor cells.

There is a close correlation between the efficacy of chemotherapeutic drugs with the intracellular and intercellular signal transduction in tumor cells. According to reports [19,20], resistance to cisplatin is often attributed to a disruption in the normal apoptotic response via aberrant activation of pathways such as the mTOR pathway. mTOR and cisplatin contribute to a synergistic effect on inhibiting the proliferation and accelerating the apoptosis of tumor cells through suppressing its activity and regulating the apoptotic signaling pathway [21,22]. Our data provide a future basis for the rational development of anti-pancreatic cancer therapy in addition to monotherapy with gemcitabine or combination with cisplatin [23].

Conclusion

mTOR can specifically act on the PETN/PI3K/AKT/mTOR signaling pathway to inhibit the phosphorylation of AKT and enhance the sensitivity of pancreatic cancer cells to cisplatin. mTOR combined with cisplatin can exert a synergistic effect on inhibiting cell growth and proliferation, and promoting cell apoptosis in a time-dependent manner. The antitumor effect of mTOR and its synergistic effect with cisplatin were also gradually enhanced with time, indicating that the combination of the two drugs has a synergistic effect in terms of time.

This study also revealed that in the treatment of SW1990 cells with mTOR alone, its ability to promote cell apoptosis was weak. The reason may be that the ability of a single target to induce cell apoptosis and inhibit cell proliferation is weak. Because the effect of single-target drug is relatively poor and tumor cells are prone to develop resistance, new target drugs combined with chemotherapeutics constitute a new research direction, which is expected to become the hope and a hot spot of tumor treatment in the foreseeable future.

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Conflict of interests

The authors declare no conflict of interests.

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