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Rapamycin inhibits the proliferation of SW1990 pancreatic cancer cell

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Abstract. – OBJECTIVE: To study the effect of rapamycin on pancreatic cancer cell proliferation, we designed a serial of experiments in human pancreatic cancer cell line SW1990.

MATERIALS AND METHODS: SW1990 cells were treated with different concentrations of rapamycin. Cell proliferation was measured by CCK-8 assay and cell colony formation. Cell cycle and apoptosis was analyzed by flow cytometry. The existence of mTOR signaling pathway was demonstrated by immunocytochemistry. Western-blot and real time-PCR were used to test whether mTOR-signaling pathway was inhibited with rapamycin treatment.

RESULTS: Our results showed that rapamycin inhibited the cell colony formation and proliferation ($p < 0.05$). Rapamycin induced G1 cell cycle arrest ($p < 0.05$) but not cell apoptosis ($p > 0.05$). p-mTOR, *p*-p70S6K and p-4E-BP1 were expressed in the cytoplasm of SW1990 cells and those proteins were significantly reduced with rapamycin ($p < 0.05$).

CONCLUSIONS: Rapamycin inhibits SW1990 pancreatic cancer cell proliferation through inhibiting the activation of mTOR pathway.

Key Words:
Rapamycin, Pancreatic cancer cell, SW1990.

Introduction

Pancreatic cancer is one of the most common malignancies in digestive system with an annually increasing incidence. Mortality of pancreatic cancer ranks 4-5 among all of the carcinomas. The overall 5 year survival rate of pancreatic cancer is still less than 5%, and the prognosis is extremely bad[1,2]. Till now, it is not clear what the cause and mechanism of pancreatic cancer, so the current research about pancreatic cancer focuses on exploring the disease mechanism with cutting-edge cell biology and molecular biology technologies[3,4].

Rapamycin is an antifungal discovered in 1970s. With a structure similar to FK506, rapamycin is widely used as an immunosuppressant drug to prevent rejection in organ transplantation. Recent results showed that rapamycin and its derivatives possess anti-cancer effect[5,6]. There were also reports that rapamycin is effective to treat carcinoma of kidney, breast cancer, and non small cell lung cancer[7], through inhibiting mTOR signaling activation and angiogenesis[8,9]. Rapamycin has no effect on cell apoptosis, but arrest cells in G1, suggesting that rapamycin inhibits mTOR-signaling pathway and affects cell cycles. Furthermore, activation of AKT-mTOR signaling pathway was identified in human pancreatic cancer cells although the activation mechanism is largely unknown[10,11].

In this study, we explored the effect of rapamycin on human pancreatic cancer cell SW1990 and mTOR signaling pathway, providing more evidence to decipher the molecular mechanism of pancreatic cancers.

Materials and Methods

Cell Culture

SW1990 cancer cells was grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), ampicillin (100 kU/L), and streptomycin (100 g/L) at 37°C with 5% CO_2. Cells were digested with 0.25% Trypsin and transplanted every 2-3 days. All the cells used for the experiment were at exponential stage.

Colony Formation Assay

SW1990 cancer cells were planted in a 6-well plate at density of 750/well and cells were gently shaken to evenly distribute. After the cells attached to plate well, cancer cells were treated with rapamycin at different concentrations (0, 0.2, 2, 20, 200 ng/ml respectively and each concentration point was triplicate). The medium was

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changed every other day for 2-3 weeks. Then, the cells were washed with phosphate buffered saline (PBS) twice and fixed with 2 ml 4% formaldehyde for 10 min. The fixation medium was discarded and cells were washed twice with PBS and stained with hematoxylin 30s. Stained cells were washed with running water, dried in air, and the colony number was counted.

Cell Proliferation Test

Cancer cells at exponential stage were plated in 96-well plates at density of 2×10^3/well in 100 µl medium. After the cells adhered, the regular growth medium was replaced with medium without serum. 24h later, cells were treated with rapamycin at different concentrations (0, 0.2, 2, 20, 200 ng/ml respectively and each concentration point was repeated in 5 wells). Cell proliferation was examined at 3 time points (24h, 48h, and 72h). At each time point, 10 µl CCK-8 was added to each well and incubated for 2h. Cell survival rate was determined with the following equation cell survival rate = (absorption A450 of experiment group – absorption A450 of blank) / (absorption A450 of control group – absorption A450 of blank).

Cell Cycle Examination

Cancer cells at exponential stage were planted in 6-well plates at density of 105/well. After the cells adhered, the regular growth medium was replaced with medium without serum and the cells grew for another 24h. The cells were treated with 0 ng/ml or 20 ng/ml of rapamycin (each concentration point was triplicate). After 24h and 48h, cells were harvested and re-suspended with cold PBS at the density of 106/ ml. 2 x volume of precooled 100% ethanol was used to fix the cells for 1h. Cells were washed with PBS, and incubated with DNase-free PBS containing 50 µg/ml propidium iodide (PI) and 100 µg/ml RNase in dark at 4°C for 1h. Cell cytometry was utilized to determine the cell cycle distribution.

Cell Apoptosis Examination

Cancer cells at exponential stage were planted in 6-well plates at density of 105/well. After the cells adhered, the regular growth medium was replaced with medium without serum and the cells grew for another 24h. The cells were treated with 0 ng/ml or 20 ng/ml of rapamycin (each concentration point was triplicate). After 24h and 48h, cells were harvested and washed with pre-cooled PBS. Cells were re-suspended in hybridization buffer at the density of $2\text{-}5 \times 10^5$/ml. 195 µl of the cell suspension was taken and added with 10 ul Annexin V-FITC. Cells were gently shaken for 3 min, and added with 5 µl 20 µg/ml propidium iodide (PI), and then incubated in dark at room temperature for 10 min after fully mixed. 300 µl hybridization buffer was added into the cell suspension and mixed well. FACS (Fluorescence-activated cell sorting) was used to examine apoptosis. Annexin V-/PI- is normal cell, Annexin V+/PI- is apoptotic cell, Annexin V+/PI+ is necrotic cell.

Western Blot

Cells were treated with rapamycin at different concentrations (0, 0.2, 2, 20 ng/ml) and harvested after 48h, washed with PBS twice, added with lysis buffer, and put on ice for 30 min, then centrifuged at 15,000 g for 30 min. The supernatant was kept and the protein concentration was determined with bicinehoninic (BCA) kit. The protein samples were run in 10%-15% SDS-PAGE electrophoresis, transferred to Nitro cellules membrane, blocked in 5% fat-free milk for 1h at room temperature and incubated in primary antibodies (1:1000 dilution for p-mTOR, p- p70S6K and p-4E-BP1 primary antibodies) over night at 4°C. After washed 4 times with TBST (Tris buffered saline with tween), the membranes were incubated with horseradish peroxide (HRP) goat anti rabbit IgG for 30 min at room temperature. Wash with TBST and illuminated with electrochemiluminescence (ECL), and analyzed with GIS image analysis system.

Real-time PCR

Cells were treated with rapamycin at different concentrations (0, 0.2, 2, 20 ng/ml) and harvested after 48h. Total RNA was extracted with Trizol and reverse-transcribed into cDNA. Then real-time PCR was performed as previously described and SYBR Green I was used to label the synthesized DNA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control. Primers were designed with Primer Permier 5. Experiments were triplicates. Primer sequences are shown in Table I.

Statistical Analysis

SPSS13.0 (SPSS Inc., Chicago, IL, USA) was used for all the statistics analysis. All the data was average of 3 independent experiments. If $p < 0.05$ in variance analysis, the result is considered statistically significant.

Table I. Real-time PCR primers.

Target genes	Primer sequence
CyclinD1	5′-GATGCCAACCTCCTCAACGAC3′
	5′-CTCCTCGCACTTCTGTTCCTC-3′
VEGF	5′-TCACCAAGGCCAGCACATAG-3′
	5′-GGGAACGCTCCAGGACTTAT-3′
c-myc	5′-TGCCAAGAGGGTCAAGT-3′
	5′- CAATCAGCCAAGGTTGTG-3′
GAPDH	5′-CCACAGTCCATGCCATCACT-3′
	5′-TCCACCACCCTGTTGCTGTAG-3′

Results

Rapamycin Treatment Inhibits SW1990 Cancer Cell Proliferation

SW1990 cancer cells can form cell colony after one-week growth and the colony grows bigger afterwards. The colony formation was inhibited 2 weeks after rapamycin treatment, and the inhibition effect was dosage-dependent. The variation among different rapamycin doses was statistically significant ($p < 0.05$), except that between 20 ng/ml group and 200 ng/ml group ($p > 0.05$) (Figure 1 and Table II). CCK-8 test showed that rapamycin drastically inhibits the proliferation of SW1990, and the inhibition depends on treatment time and dosage of rapamycin. Statistical analysis revealed that it was not significantly different between 20 ng/ml and 200 ng/ml ($p > 0.05$), suggesting that the inhibition effect (31%) probably saturated at 20 ng/ml (Figure 2).

Rapamycin Treatment Arrests SW1990 Cancer Cells

After 24h treatment of rapamycin, G0-G1 phase portion significantly increased compared with dimethyl sulfoxide (DMSO) control (from 40.34% ± 1.67% to 62.95% ± 1.31%), while the portion of S phase and G2-M phase markedly decreased (decreased to 16.28% ± 1.63% and 20.78% ± 1.22% respectively) ($p < 0.05$). Similar results were obtained after 48h treatment (G0-G1 phase portion increased from 46.52% ± 2.96% to 56.76% ± 2.59% while S phase and G2-M phase decreased to 26.82% ± 1.23% and 16.42% ±

Table II. Effect of rapamycin treatment on the colony formation of human pancreatic cancer cell line SW1990.

Concentration of rapamycin (ng/ml)	Colony number
0	230.67 ± 13.61
0.2	195.00 ± 11.36
2	166.00 ± 9.17
20	146.67 ± 7.02
200	145.33 ± 7.02

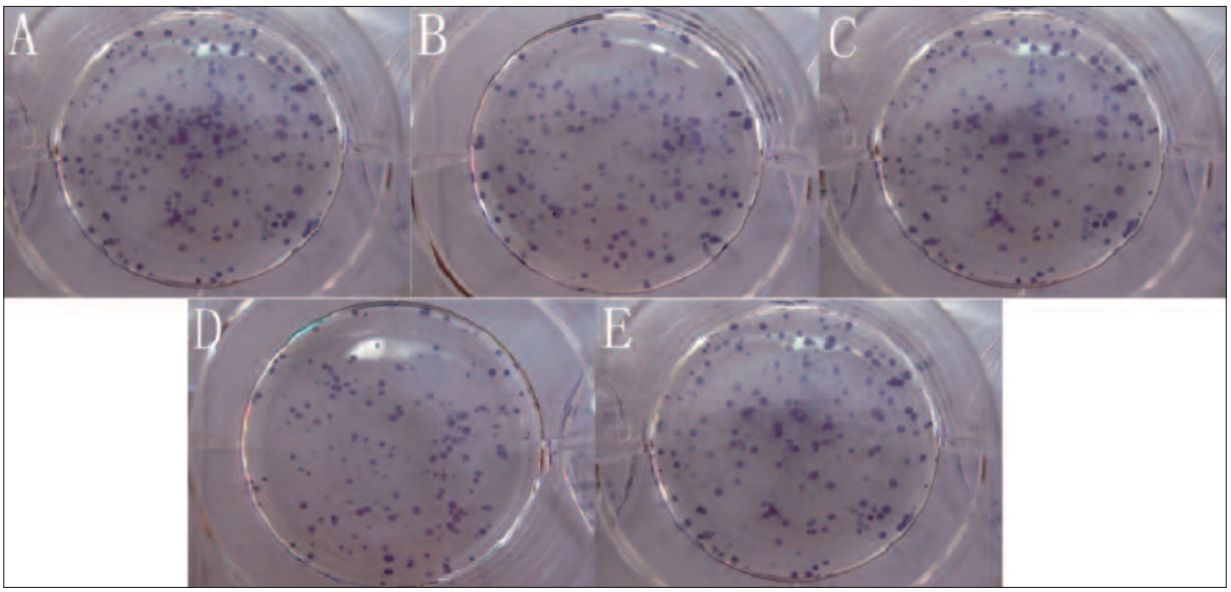

Figure 1. Effect of rapamycin treatment on the colony formation of human pancreatic cancer cell line SW1990. ***A,*** DMSO (dimethil sulfoxide). ***B,*** 0.2 ng/ml rapamycin. ***C,*** 2 ng/ml rapamycin. ***D,*** 20 ng/ml rapamycin. ***E,*** 200 ng/ml rapamycin.

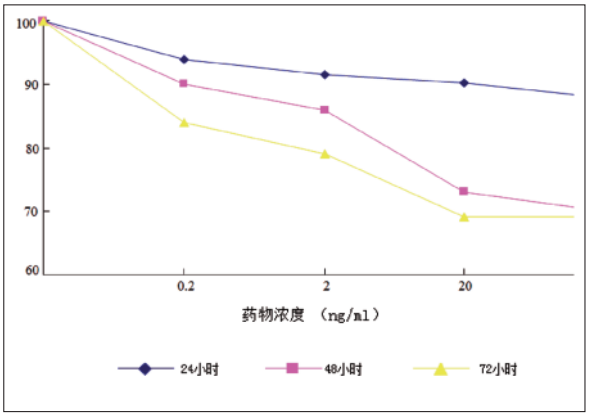

Figure 2. CCK-8 test revealed inhibition of rapamycin on SW1990 proliferation.

1.36% respectively) ($p < 0.05$). These results suggested that rapamycin arrested cells at G0-G1phase and inhibited them from entering S phase (Figure 3 and Table III).

Rapamycin Treatment Does not Affect Apoptosis of SW1990 Cancer Cells

Apoptosis among rapamycin treated group and control group was not significantly different ($p > 0.05$), suggesting that rapamycin affects SW1990 cell cycle through cell cycle arrest but not through apoptosis (Figure 4 and Table IV).

Rapamycin Treatment Inhibits mTOR-signaling Pathway in SW1990 Cancer Cells

Immumo-biochemistry assay was used to detect the expression of p-mTOR, p-p70S6K and p-4E-BP1 in SW1990 cancer cells after ra-

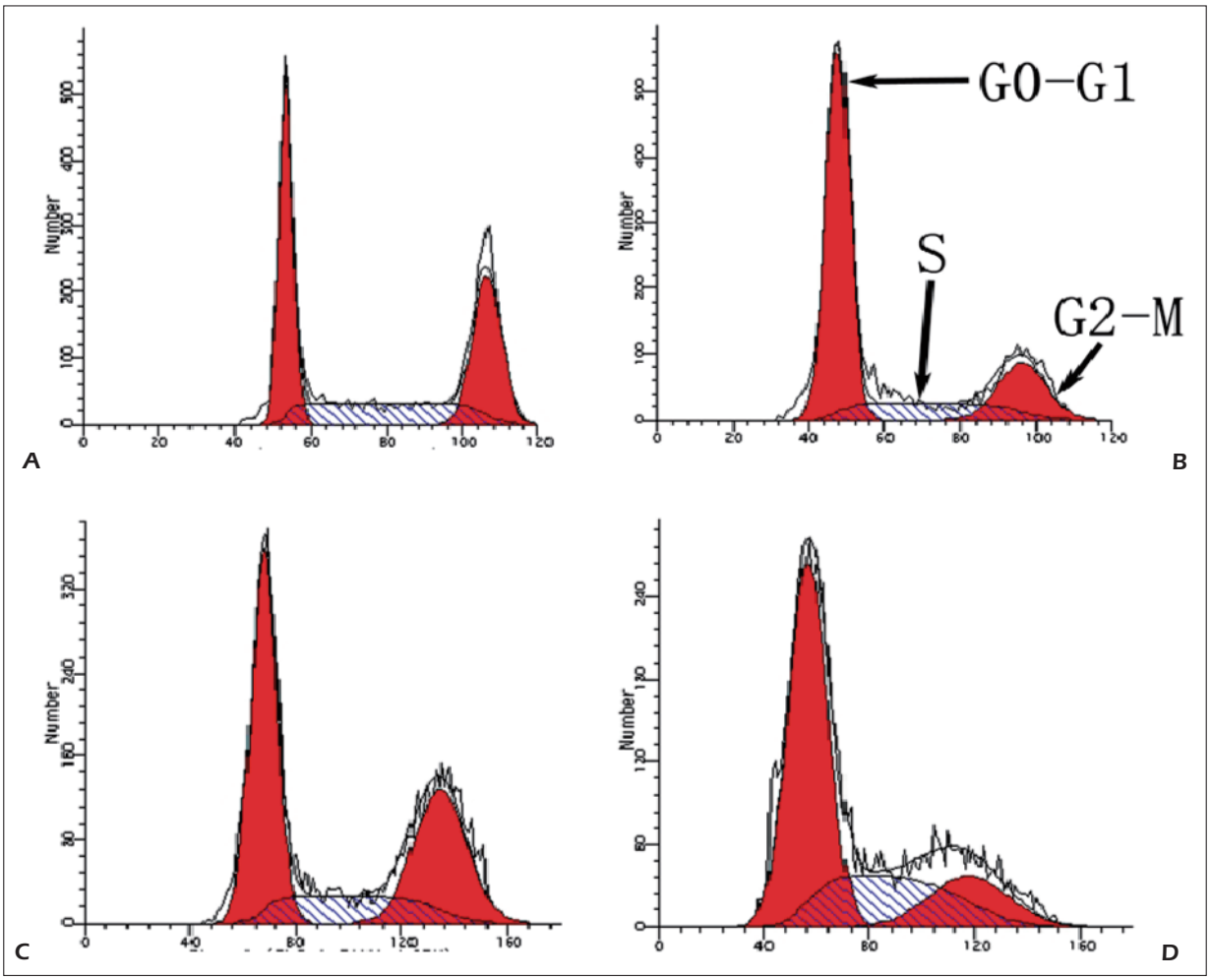

Figure 3. Rapamycin arrested SW1990 in G0-G1 phase. ***A,*** DMSO 24h. ***B,*** 20 ng/ml rapamycin 24h. ***C,*** DMSO 48h. ***D,*** 20 ng/ml rapamycin 48h.

Table III. Portions of cells in each cell cycle phase after rapamycin treatment.

	24h			48h		
	G0-G1 (%)	S (%)	G2-M (%)	G0-G1 (%)	S (%)	G2-M (%)
0 ng/ml	40.34 ± 1.67	24.52 ± 0.92	35.14 ± 1.16	46.52 ± 2.96	18.73 ± 1.05	33.71 ± 0.22
20 ng/ml	62.95 ± 1.31	16.28 ± 1.63	20.78 ± 1.22	56.76 ± 2.59	26.82 ± 1.23	16.42 ± 1.36

pamycin treatment. Comparing to the DMSO control (Figure 5 B, D and F), p-mTOR, p-p70S6K and p-4E-BP1were all expressed in SW1990 cells (Figure 5 A, C and E), suggesting the existence of mTOR signaling pathway. Western blot showed that the expressions of p-mTOR, p- p70S6K and p-4E-BP1 were significantly decreased after SW1990cancer cell were treated with different doses of rapamycin for 48h (Figure 6). Real-time PCR detection of molecules downstream of mTOR signaling pathway was used to confirm the inhibition of mTOR-signaling pathway in SW1990 cancer cells treated with rapamycin. Real-time PCR showed that rapamycin treatment reduced the expression of CyclinD1, VEGF and c-MYC and the inhibition effect depends on dosage of rapamycin ($p < 0.05$) (Figure 7).

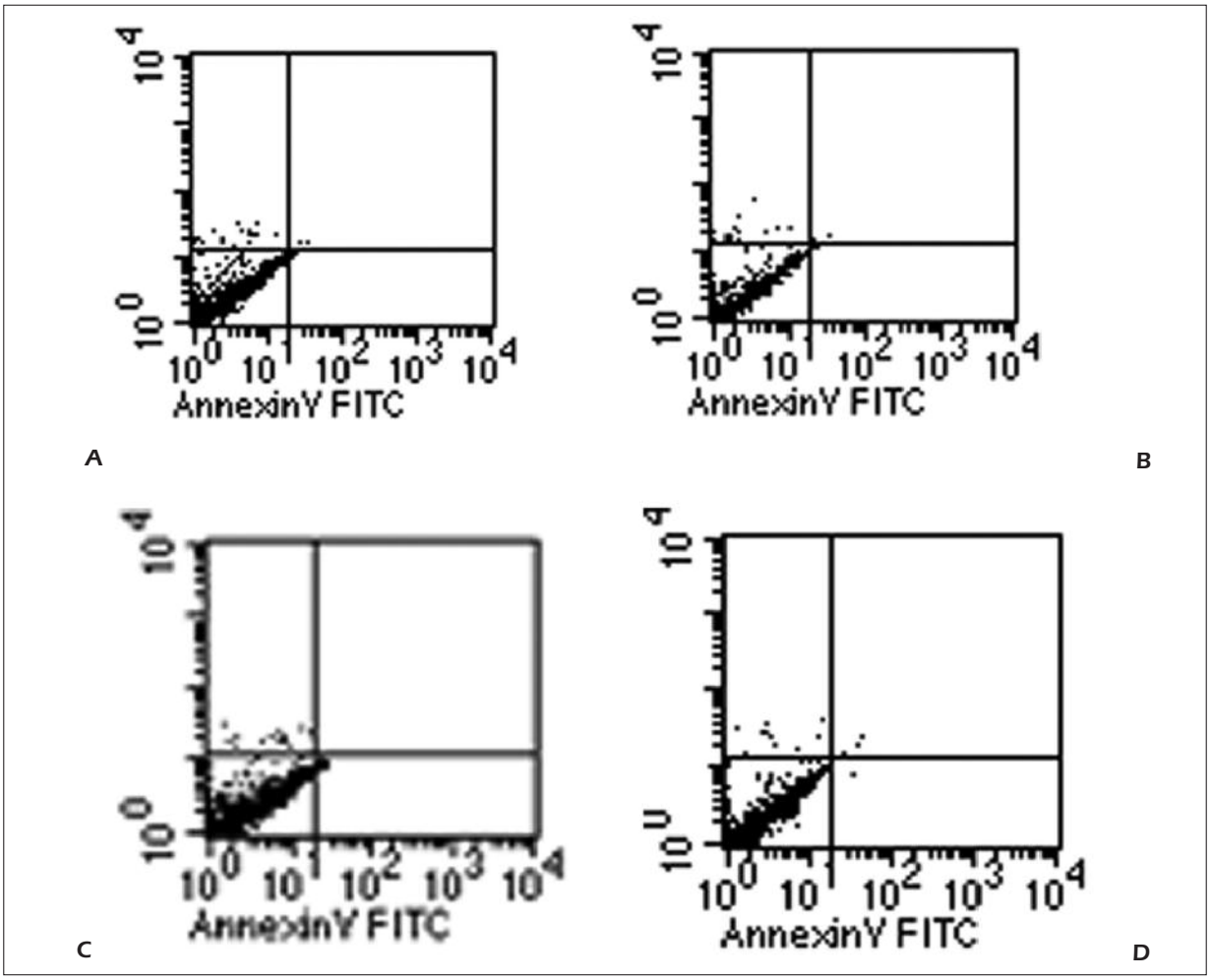

Figure 4. Rapamycin treatment did not affect apoptosis. Annexin V-/PI- is normal cell, Annexin V+/PI- is apoptotic cell, Annexin V+/PI+ is necrotic cell. ***A,*** DMSO 24h. ***B,*** 20 ng/ml rapamycin 24h. ***C,*** DMSO 48h. ***D,*** 20 ng/ml rapamycin 48h.

Table IV. Rapamycin treatment did not affect SW1990 apoptosis.

	24h			48h		
	Normal cell (%)	Necrotic cell (%)	Apoptotic cell (%)	Normal cell (%)	Necrotic cell (%)	Apoptotic cell (%)
0 ng/ml	96.3 ± 1.67	1.09 ± 0.86	2.58 ± 0.18	97.3 ± 1.02	0.64 ± 0.69	2.05 ± 0.67
20 ng/ml	95.7 ± 0.90	1.02 ± 0.78	3.07 ± 0.59	96.7 ± 0.68	1.41 ± 0.53	1.83 ± 0.16

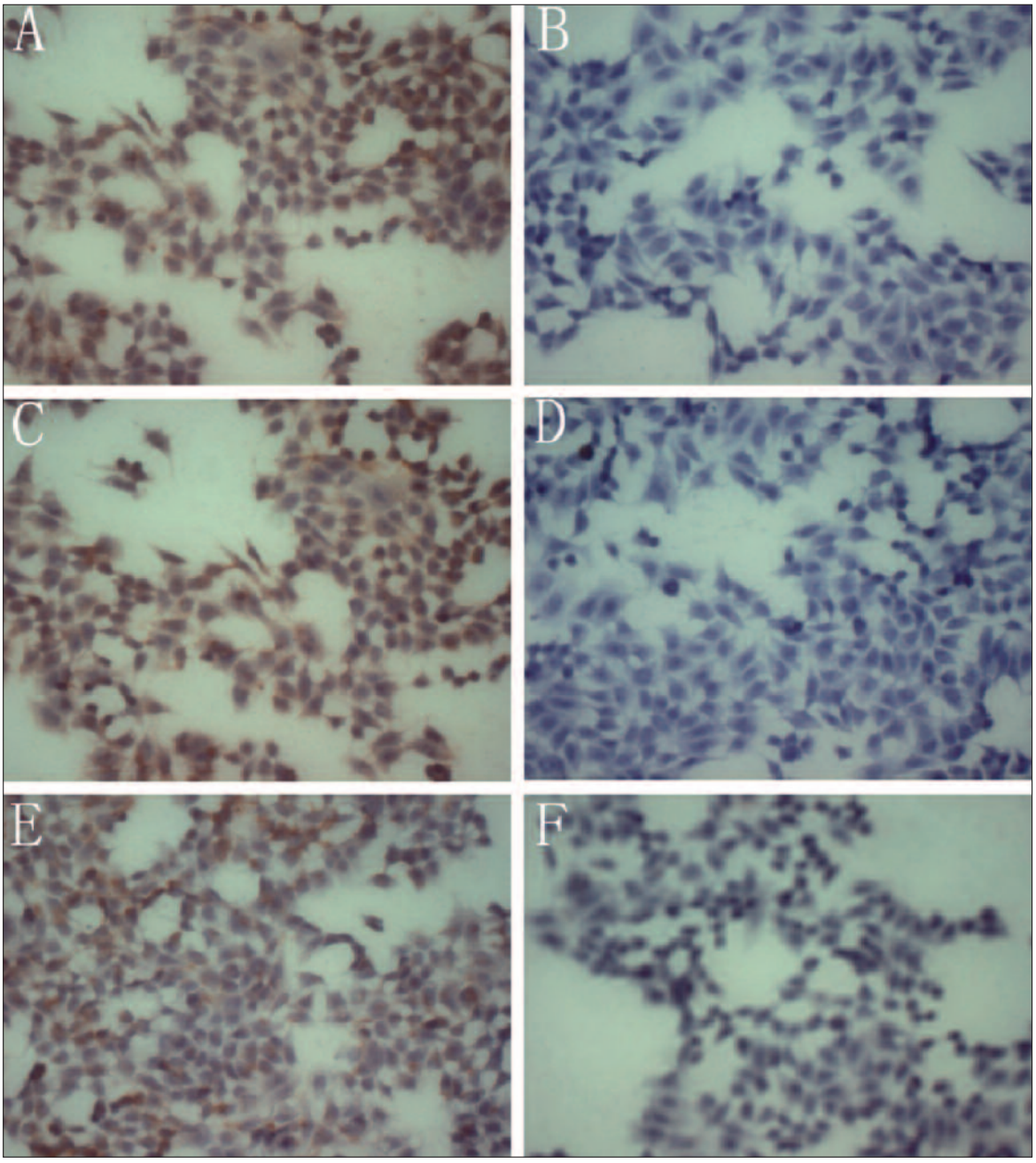

Figure 5. mTOR signaling pathway existed in SW1990 cells. ***A, C*** and ***E,*** Showed expression of p-mTOR, *p*-p70S6K and p-4E-BP1 respectively in the cytoplasm of SW1990 cells. ***B, D*** and ***F,*** Were negative controls for p-mTOR, *p*-p70S6K and p-4E-BP1 staining respectively.

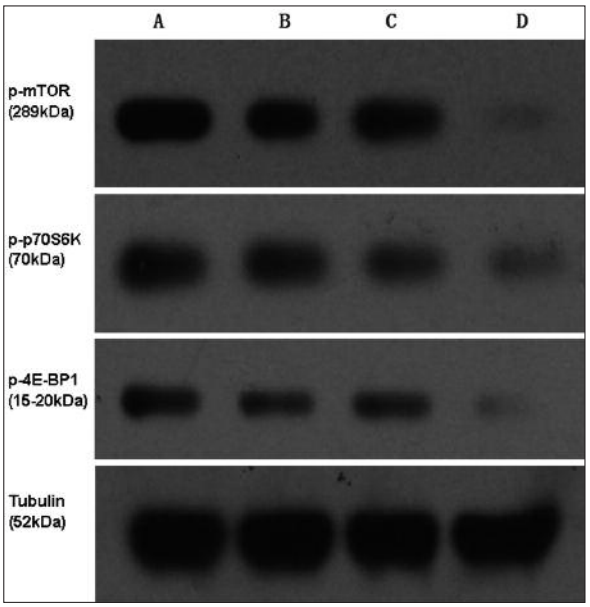

Figure 6. Expression of p-mTOR, *p*-p70S6K and p-4E-BP1were decreased with rapamycin. treatment. ***A,*** DMSO. ***B,*** 0.2 ng/ml rapamycin. ***C,*** 2 ng/ml rapamycin. ***D,*** 20 ng/ml rapamycin.

Discussion

As one of the most common malignancies in digestive system, pancreatic cancer is a big challenge because the mechanism of the disease is still largely unknown. In this study, we treated human pancreatic cancer cells line SW1990 with rapamycin, and performed a serial of experiments to evaluate the effect of rapamycin on human pancreatic cancer cells. We found that rapamycin markedly inhibited the proliferation of SW1990 cancer cells and this inhibition effect may be through G1 arrest but not cell apoptosis. Furthermore, we demonstrated that three mTOR pathway proteins including p-mTOR, p-p70S6K and p-4E-BP1were significantly decreased by rapamycin treatment, suggesting that rapamycin treatment inhibits mTOR-signaling pathway. mTOR signaling pathway related pro-growth factors: CyclinD1, VEGF and c-MYC were also negatively regulated by rapamycin treatment.

Some studies showed that rapamycin forms complex with its cytoplasmic receptor FKBP12, and this complex binds to mTOR and inhibit its activity. mTOR (mammalian target of rapamycin), also called FK506-binding protein 12 or rapamycin associated protein – FRAP, is a non-typical serine/threonine kinase and is highly evolutionally conserved. Recent research pointed out that mTOR is not only important for normal cell growth and proliferation, but is also crucial for transformation of normal cell to cancer cells. mTOR is key factor for regulation of cancer cell growth and proliferation. Activated mTOR can phosphorylate two different downstream molecules, such as eukaryote translation initiation factor 4E-BP1 and ribosome S6 kinase p70S6K. 4E-BP1 and p70S6K are key regulators of protein translation and phosphorylated 4E-BP1 enhances the translation of several growth factors including cyclin D1, c-MYC and VEGF. Phosphorylated p70S6K can increase the translation of pyrimidine-enriched mRNA translation, thus protein

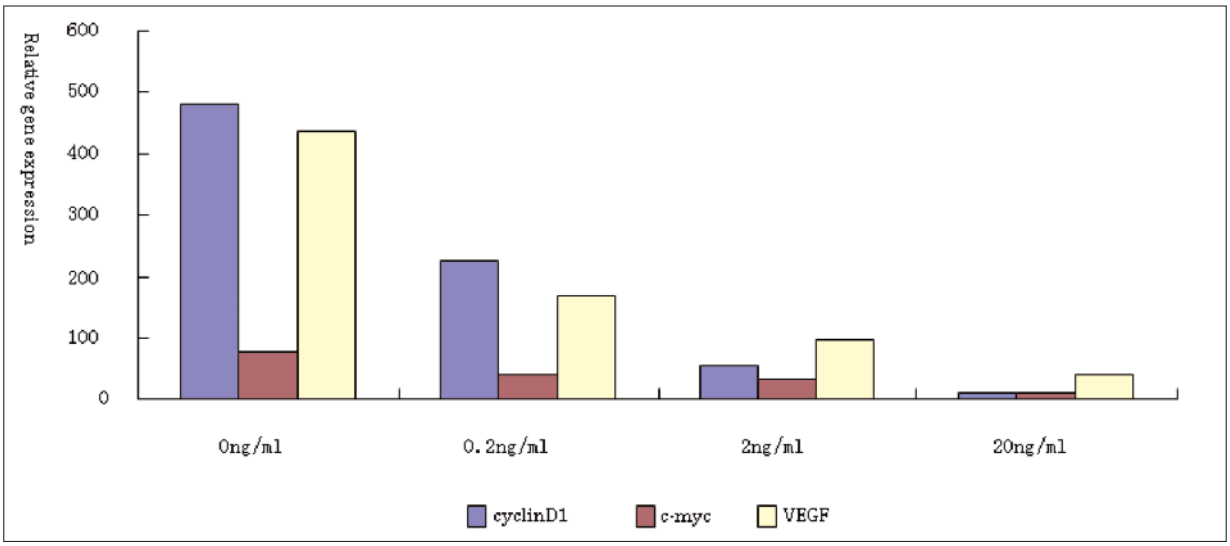

Figure 7. mRNA expression of CyclinD1, VEGF and c-MYC in SW1990 cancer cells treated with rapamycin for 48h.

synthesis[12]. Rapamycin binds mTOR and inhibits mTOR pathway, leaving phosphorylation of downstream 4E-BP1 and p70S6K decreased so that protein expression level of cyclin D1, c-Myc and VEGF decreased as well. In addition, mTOR inhibition reduces mRNA translation of cell cycle protein involved in G1-S transition, and arrests cell in G1, inhibiting cell growth and proliferation.

Conclusions

Our findings suggested that rapamycin inhibited the growth and proliferation of human pancreatic cell SW1990 through mTOR inhibition. The results provide experimental evidence to explore the mechanism of pancreatic cancer and some guidance to develop targeted drug for pancreatic cancer.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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