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Research Paper

## Effects of apigenin on cell proliferation of human pancreatic carcinoma cell line BxPC-3 *in vitro*

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### Abstract

**Objective:** To observe the effects of apigenin on cell proliferation of human pancreatic carcinoma cell line BxPC-3 *in vitro*. **Methods:** The inhibitive effects of apigenin at different concentrations (0  $\mu\text{mol/L}$ , 100  $\mu\text{mol/L}$ , 200  $\mu\text{mol/L}$ , and 400  $\mu\text{mol/L}$ ) on human pancreatic carcinoma cell line BxPC-3 were detected by MTT assays, transmission electron microscope, agarose gel electrophoresis and flow cytometry. The immunohistochemistry was used to detect the expression of Bcl-2 and Bax gene. **Results:** Apigenin at different concentrations could inhibit the proliferation of human pancreatic carcinoma cell lines BxPC-3, and the inhibitive effect was dose-dependent. The cell cycle of pancreatic carcinoma cells was arrested at G<sub>2</sub>/M phase. The results of immunohistochemistry showed that the density of apigenin increased, and the expression of Bcl-2 gene was reduced gradually. At the same time the expression of Bax gene was enhanced. **Conclusion:** Apigenin could inhibit the proliferation of human pancreatic carcinoma cell lines BxPC-3 *in vitro*. The effect of apoptosis was accompanied with the expression of Bcl-2 decrease and Bax increase.

**Keywords:** apigenin; pancreatic carcinoma; cell cycle; gene

### INTRODUCTION

Apigenin (4', 5, 7-trihydroxyflavone) is a natural flavonoid found abundant in fruits, vegetables, beans and tea, especially in celery<sup>[1]</sup>. It showed different pharmacological effects in recent years, particularly on inhibiting the growth of some cancer cell lines and inducing apoptosis<sup>[2-7]</sup>. Wu K<sup>[8]</sup> reported that apigenin could cause concentration-dependent anti-proliferation and apoptosis in gastric cancer SGC-7901 cells *in vitro*. Chiang LC<sup>[9]</sup> found that apigenin could inhibit growth of human Hep G2, 3B, and PLC/PRF/5 cells through G(2)/M phase cell cycle arrest. Further studies found that apigenin could also induce apoptosis of these cells through p53 dependent pathway and increase the expression of p21. The research of Rithidech KN<sup>[10]</sup> improved the protective effect of apigenin on radiation-induced chromosomal damage

in human lymphocytes. However the effects of apigenin on pancreatic carcinoma have not been investigated. Therefore, we carried out studies to observe the effects of apigenin on human pancreatic carcinoma cell lines BxPC-3 *in vitro*.

### MATERIALS AND METHODS

#### Target cell

Human pancreatic carcinoma cell line BxPC-3 was bought from Shanghai Institute of Cell.

#### Reagents

Apigenin and MTT were bought from Sigma Chemical Co. USA. Propidium Iodide (PI) and Rnase was bought from Huamei Bioengineering Company.

#### MTT assay

Human pancreatic carcinoma cell line BxPC-3 at logarithmic growth phase was plated at  $0.8 \times 10^3$  cell per milliliter in 96-well micro titer plates and incubated in 37°C, 5% CO<sub>2</sub>. The culture solution was re-

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placed 24 hours later. The cells were treated with various concentrations of apigenin (0, 100, 200, 400  $\mu\text{mol/L}$ ) and detected at day 1, 3 and 5. Then the cells were determined by MTT assay. The absorbances were determined with an enzyme-linked immunoassay (East China Electronics Tube Factory). The repress rate<sup>[11]</sup> =  $(1 - A_{\text{treatment group}} / A_{\text{control group}}) \times 100\%$ .

### Cell apoptosis under transmission electron microscope

Cells were prepared for transmission electron microscopy observation. Cell morphology, cytoplasm and nucleus were observed, and then photos were taken through the transmission electron microscope.

### DNA-agarose gel electrophoresis of the apoptotic cells

Specimens were prepared for agarose gel electrophoresis. Electrophoresis was done in 1.5% agarose gel and the electrophoretograms were recorded.

### Flow cytometry

Logarithmic growth phase human pancreatic carcinoma cells were inoculated into some 6 orifice laminas with cell concentration of  $1.0 \times 10^4 \text{ml}^{-1}$ . The cultures were incubated in box at  $37^\circ\text{C}$ , 95% humidity and 5%  $\text{CO}_2$ . 24 hours later culture solution was replaced. The cells were cultured with different concentrations of apigenin (0  $\mu\text{mol/L}$ , 100  $\mu\text{mol/L}$ , 200  $\mu\text{mol/L}$ , 400  $\mu\text{mol/L}$ ) for 1, 3 or 5 days individually, and then cells were gathered. Meanwhile the gathered cells were agitated into single cell suspension, fixed with 95% alcohol, washed twice with PBS fluid, centrifuged  $1000 \times g$  for 5 minutes. The cleaning fluid was removed and RNase (500  $\mu\text{g/ml}$ ) 100  $\mu\text{l}$  was added to each centrifuge tube and cells were incubated at  $37^\circ\text{C}$  for 30 minutes. Finally, PI 300  $\mu\text{l}$  was added to stain for 30 minutes. Then the cells were analysed by flow cytometry (BD Company in the U. S). **The expression of Bcl-2 and Bax**

The expressions of Bcl-2 and Bax were detected by immunohistochemistry. The gray scale was assayed with Qwin550CW image processing and analysis systems (Leica Company in Germany).

### Statistical analysis

All data were processed by statistical software SPSS11.0 and one-way ANOVA was used.  $P < 0.05$  was considered statistically significant.

## RESULTS

### The inhibitory effects of apigenin at different

### concentrations on human pancreatic carcinoma cell lines BxPC-3

Compared with the control group, it was obvious that the cultures were different each time. For 1 day, the repress rates were 8.0%, 19.1% and 32.6%. For 3 days, then were 15.7%, 30.9% and 49.1%. For 5 days, they were 24.4%, 41.0% and 59.0% (**Tab 1**). Shown by analysis in statistics compared 100  $\mu\text{mol/L}$  group with the control group about the inhibitory effects on the cultures for one day, they were of no significance ( $P > 0.05$ ), but there was significant difference between 100  $\mu\text{mol/L}$  and 200  $\mu\text{mol/L}$  group ( $P < 0.05$ ). Compared 100  $\mu\text{mol/L}$  and 200  $\mu\text{mol/L}$  group with 400  $\mu\text{mol/L}$  group, the difference was markedly significant. As for 3 and 5 days, the difference between test group and control group was also markedly significant ( $P < 0.01$ ). We could see that the inhibitory effects were improved as the concentration increased.

Compared with the control group, the repress rates of the cultures were different in each treatment group after 1, 3 or 5 days. 100  $\mu\text{mol/L}$  group was 8.0%, 15.7% and 24.4%. 200  $\mu\text{mol/L}$  was 19.1%, 30.9% and 41.0%. For 400  $\mu\text{mol/L}$  was 32.6%, 49.1% and 59.0%. Shown by disposal in statistics; after 1, 3 or 5 days, the difference was markedly significant to the inhibitory effects of apigenin at different concentrations (0  $\mu\text{mol/L}$ , 100  $\mu\text{mol/L}$ , 200  $\mu\text{mol/L}$ , and 400  $\mu\text{mol/L}$ ) ( $P < 0.01$ ). Compared 100  $\mu\text{mol/L}$  group with control group about the inhibitory effects on the cultures for one day, it was of no significance ( $P > 0.05$ ), but there was significant difference between 100  $\mu\text{mol/L}$  and 200  $\mu\text{mol/L}$  group ( $P < 0.05$ ). Compared 100  $\mu\text{mol/L}$  and 200  $\mu\text{mol/L}$  groups with 400  $\mu\text{mol/L}$  group, the difference was significant. As for 1, 3 and 5 days, the cell cycle of pancreatic carcinoma cells was of no significance ( $P > 0.05$ ). We could see that the inhibitory effects increased as time prolonged.

### The result of transmission electron microscope experiment

Pancreatic carcinoma cell; the cell presented round, and the cell membrane was integrated with a few cytodendrites on the surface. The organelles inside cytoplasm were abundant, such as rough endoplasmic reticulum, lysosome and mitochondrion. The cell nucleus was large and the shape was irregular. The nucleolus was big in the center, and the caryotin was abundant. The heterochromatin was aggregate around the sides (**Fig 1**).

The apoptotic cell; the cell was atrophic. The frag-

mented nucleus was enveloped by a thin layer of cytoplasm named apoptotic body(**Fig. 2**).

### The result of DNA agarose gel electrophoresis

It appeared only in one band in the control group and typically ladder-shaped bands in the intervened group(**Fig. 3**).

**Tab 1** The inhibitory effects at different concentrations

( $\bar{x} \pm s, n = 6$ )

concentration	1 day		3 days		5 days	
	OD	Repress rate(%)	OD	Repress rate(%)	OD	Repress rate(%)
0 $\mu\text{M}$	0.23 $\pm$ 0.023	0	0.375 $\pm$ 0.020	0	0.512 $\pm$ 0.027	0
100 $\mu\text{M}$	0.21 $\pm$ 0.016	8.0 $\pm$ 0.4	0.316 $\pm$ 0.032	15.7 $\pm$ 0.4	0.387 $\pm$ 0.035	24.4 $\pm$ 0.5
200 $\mu\text{M}$	0.19 $\pm$ 0.010	19.1 $\pm$ 0.7	0.259 $\pm$ 0.024	30.9 $\pm$ 0.6	0.302 $\pm$ 0.021	41.0 $\pm$ 0.5
400 $\mu\text{M}$	0.15 $\pm$ 0.017	32.6 $\pm$ 0.2	0.191 $\pm$ 0.018	49.1 $\pm$ 0.5	0.210 $\pm$ 0.015	59.0 $\pm$ 0.6

**Tab 2** The result of flow cytometry at different concentrations

( $\bar{x} \pm s, n = 6$ )

concentration	1 day			3 days			5 days		
	G <sub>0</sub> /G <sub>1</sub>	S	G <sub>2</sub> /M	G <sub>0</sub> /G <sub>1</sub>	S	G <sub>2</sub> /M	G <sub>0</sub> /G <sub>1</sub>	S	G <sub>2</sub> /M
0 $\mu\text{M}$	50.62 $\pm$ 3.12	42.75 $\pm$ 2.24	7.26 $\pm$ 3.26	50.62 $\pm$ 5.12	42.75 $\pm$ 2.24	7.26 $\pm$ 1.26	50.62 $\pm$ 5.12	42.75 $\pm$ 2.24	7.26 $\pm$ 1.26
100 $\mu\text{M}$	49.27 $\pm$ 3.25	39.34 $\pm$ 2.83	10.35 $\pm$ 2.02	51.67 $\pm$ 9.13	35.12 $\pm$ 2.98	13.86 $\pm$ 2.36	51.42 $\pm$ 6.73	30.26 $\pm$ 3.68	18.03 $\pm$ 2.83
200 $\mu\text{M}$	51.69 $\pm$ 4.29	36.23 $\pm$ 2.57	13.21 $\pm$ 3.19	52.18 $\pm$ 7.06	30.31 $\pm$ 3.12	18.87 $\pm$ 3.04	50.59 $\pm$ 3.05	25.51 $\pm$ 2.96	24.37 $\pm$ 3.01
400 $\mu\text{M}$	50.67 $\pm$ 2.96	32.41 $\pm$ 3.33	17.42 $\pm$ 2.51	50.36 $\pm$ 7.76	26.32 $\pm$ 2.65	23.21 $\pm$ 2.16	51.79 $\pm$ 4.27	20.45 $\pm$ 3.57	29.79 $\pm$ 2.37

### The result of flow cytometry

Apigenin at different concentrations could inhibit the proliferation of human pancreatic carcinoma cell line BxPC-3 cells, and the inhibitory effect was increased with the concentration of apigenin increased and the time prolonged. The cell cycle of pancreatic carcinoma cells was arrested at G<sub>2</sub>/M phase (**Tab 2, Fig 4 and 5**).

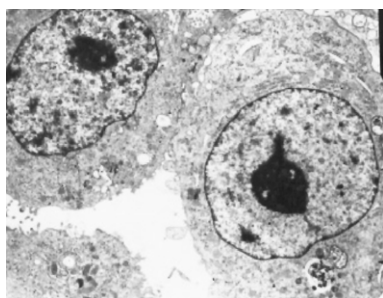
### The result of the immunohistochemistry

With the concentration of apigenin increasing, the gray scale value of protein Bcl-2 descended from 138.93 to 129.26, Bax rose from 129.97 to 142.04, and Bcl-2/Bax descended from 1.07 to 0.91. The statistical analysis showed that there was obvious difference between the control and each intervened group and there was obvious difference even in every two invented groups at different dosages ( $P < 0.05$ ). It proved that the expression of the Bcl-2 gene was

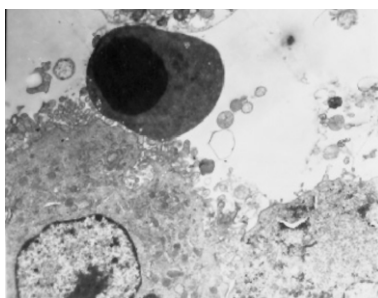
attenuated gradually with apigenin increase, but the expression of Bax gene was enhanced(**Fig 6, 7, 8 and 9**).

## DISCUSSION

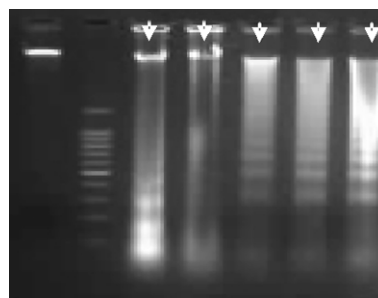
Pancreatic carcinoma is one of the malignant tumors that endanger humans health seriously. The cases of pancreatic carcinoma are mostly in advanced stage and unresectable. Traditional chemotherapy didn't ameliorate the sufferer's long-term survival rate. Therefore, it is of great importance to explore a curative method for this disease. With fast development of modern molecular biology and oncology, people have known that tumor cell cycle is disordered. It is characterized as growth out of control in a manner of excessive proliferation and little apoptosis. Lots of factors are involved in cell cycle destruction, including abnormality in the beginning, circulation, and termination of cell cycle. The



**Fig 1** Human pancreatic carcinoma cell line BxPC-3 by the transmission electron microscope( $\times 4000$ )



**Fig 2** Apoptotic bodies by the electron microscope( $\times 5000$ )



**Fig 3** The result of apoptotic cell DNA agarose gel electrophoresis

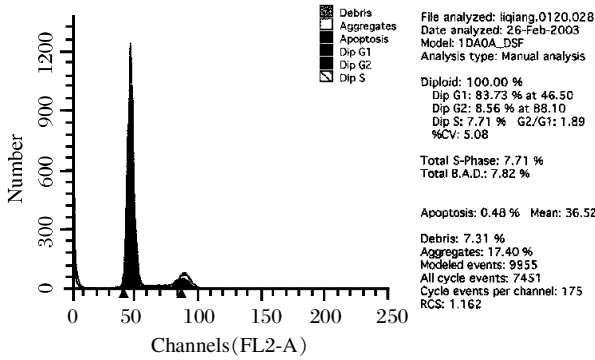


Fig 4 The result of flow cytometry without Apigenin

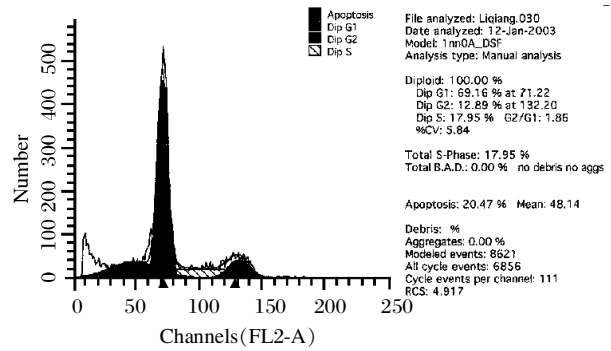


Fig 5 The result of flow cytometry with apigenin

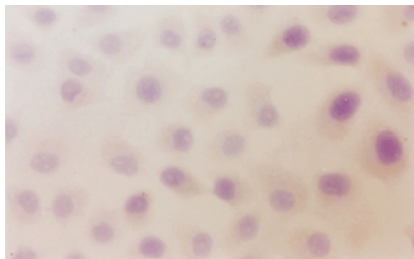


Fig 6 The expression of Bcl-2 in BxPC-3 without apigenin( × 40)

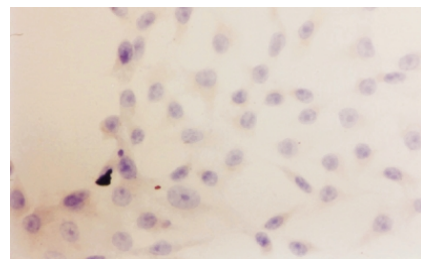


Fig 7 The expression of Bcl-2 in BxPC-3 with apigenin( × 40)

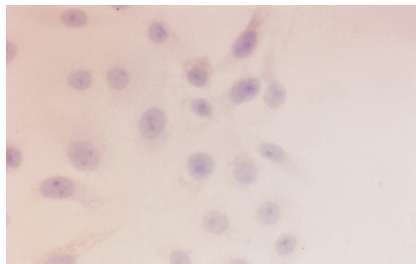


Fig 8 The expression of Bax in BxPC-3 without apigenin( × 40)

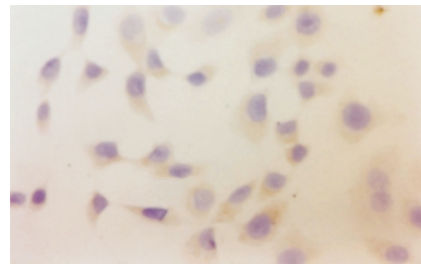


Fig 9 The expression of Bax in BxPC-3 with apigenin ( × 40)

regulatory system consisted of gene expressions that are special for regulating cell cycle in eukaryon. They induce the expression of cytokines and their receptors, cyclin and cyclin-dependent-kinase.(CDK), proto-oncogene and anti-oncogene, etc. The core is the activation of CDK. It refers to fluctuation of cyclin, phosphorylation of CDK activating kinase (CAK), phosphorylation and dephosphorylation of Weel/CDC25 and cyclin-dependent kinase inhibitor (CKI).

So does the pancreatic carcinoma. The anti-proliferation effect of apigenin on other tumors has been studied. Fabrice<sup>[12]</sup> reported apigenin blocked cell cycle at G<sub>2</sub>/M phase in melanoma OCM-1. The mechanism was that apigenin could keep tyrosine residue 15 in the N-terminal of CDK in phosphorylation via Weel protein kinase, so that further phosphorylation and activation of CDK were stopped<sup>[13]</sup>. We found that apigenin was active against pancreatic carcinoma cell line BxPC-3 Cells and blocked them at G<sub>2</sub>/M

phase, as Wang W<sup>[14]</sup> reported. To some degree, it was both time-dependent and dose-dependent.

The following pathways may play crucial roles in arresting of cell cycle by apigenin: ①apigenin inhibits cytokines (EGF, PDGF, TGF)<sup>[15-17]</sup> from connecting with their receptors, then the cells in arresting phase cannot enter into cell cycle or the cells in cell cycle cannot synthesize proteins which are correlated with regulatory system. ②apigenin inhibits cyclin and CDK. As a result, the cell cycle can't complete as usual<sup>[12,18]</sup>. Several aspects are involved, such as the activity, quantity of cyclin and CDK is inhibited directly, gurgitation of cyclin is affected, phosphorylation of CDK, phosphorylation and dephosphorylation of Weel/CDC25 and CKI(p16, p21, p24, p27, etc.)<sup>[19]</sup>, CDK can't be activated and the cell cycle can't be proceeded.③apigenin inhibits the expressions of proto-oncogene and anti-oncogene to cause retardation of the cell cycle<sup>[20]</sup>. Besides arresting cell cycle, anti-oxidase and anti-production of

tumor blood vessel may also contribute to the inhibition effect. Exact mechanism needs further investigation.

Moreover, we observed a great deal of apoptosis in our study. It may be related with the expression of Bcl-2 and Bax gene. The mechanism how apigenin causes apoptosis in human pancreatic carcinoma cell line BxPC-3 cells will be investigated in our consequent study.

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