Apoptosis of human pancreatic carcinoma cell-1 cells induced by Yin Chen Hao Decoction

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Abstract

AIM: To evaluate human pancreatic carcinoma cell line (PANC-1) cells apoptosis and Bcl-2 and Bax expression induced by Yin Chen Hao Decoction (YCHD).

METHODS: The cell growth inhibitory rate was determined by MTT assay. Apoptosis of PANC-1 cells before and after treatment with YCHD was determined by TUNEL staining. Expression of the apoptosis-associated genes, Bcl-2 and Bax, was detected by immunohistochemical staining and reverse transcription-PCR.

RESULTS: YCHD inhibited the growth of PANC-1 cells. Following treatment with YCHD for 24-96 h, the apoptotic rate of PANC-1 cells increased with time. In addition, the positive rate of Bcl-2 protein expression decreased in a time-dependent manner, whereas the positive rate of Bax protein expression increased in a time-dependent manner. Following treatment of with YCHD for 24-96h, expression of BAX mRNA increased gradually and BCL-2 mRNA reduced gradually with time.

CONCLUSION: YCHD induces apoptosis of PANC-1 cells mediated in part via up-regulation of BAX and down-regulation of BCL-2.

Key words: Apoptosis; Pancreatic carcinoma; Yin Chen Hao Decoction

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Core tip: Yin Chen Hao Decoction (YCHD) inhibits the growth of pancreatic carcinoma cells. Following treatment with YCHD for 24-96 h, the apoptotic rates in these cells is increased by YCHD, which is accompanied by time-dependent increased expression of Bax and...
decreased expression of Bcl-2. YCHD-induced apoptosis of these cells may be mediated by upregulation of BAX and downregulation of BCL-2.

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INTRODUCTION

Yin Chen Hao Decoction (YCHD) is a classic Chinese medicine formula consisting of three herbal drugs Rheum officinale Baill, Artemisia capillaries Thunb and Gardenia iasminoides Ellis, and has long been used to treat cholestasis, hepatitis C11, primary biliary cirrhosis7-10, and liver fibrosis11-20. Previous research reported that YCHD is effective for choleresis and has anti-inflammatory and anti-asthmatic activity to relax bronchial smooth muscle4,5. Moreover, YCHD is a potent inhibitor of carcinoma6. The anti-cancer activity of YCHD may induce apoptosis of carcinoma cells.

The Bcl-2 family plays a key role in the apoptosis. The Bcl-2 family includes proapoptotic members, such as Bax and Bad, and antiapoptotic members such as Bcl-2 and Bcl-xL7-10, whereas overexpression of Bax can quicken cell death11-20, whereas overexpression of Bcl-2 can delay cell death20-29. The critical determinant of cell apoptosis is the ratio of Bcl-2/Bax20.

In this study, the cell growth inhibitory rate was determined by MTT assay. The apoptosis status in pancreatic carcinoma PANC-1 cells after YCHD treatment was determined by the TUNEL staining method. Gene and protein expression of Bcl-2 and Bax was detected by immunohistochemical staining and reverse transcription (RT)-PCR, respectively.

MATERIALS AND METHODS

Materials

Rheum officinale Baill, Artemisia capillaries Thunb and Gardenia iasminoides Ellis were purchased from the Second Affiliated Hospital of Zhejiang University, China. MT was obtained from Sigma-Aldrich (St. Louis, MO, Unite States). The in situ cell detection kit and anti-Bcl-2 and anti-Bax monoclonal antibodies were purchased from Beijing Zhongshan Biotechnology Co, Ltd., (Beijing, China). Human pancreatic carcinoma PANC-1 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China).

Methods

For the preparation of YCHD, Artemisia capillaries Thunb (200 g), Gardenia iasminoides Ellis (100 g) and Rheum officinale Baill (60 g) were placed in a round-bottomed flask and boiled for 1 h in distilled water was added and then boiled for 1 h. The decoction was then percolated to obtain the filtrate and the residue was reboiled twice. The collected filtrates were then added together and concentrated under reduced pressure. The concentrated liquid was then vacuum dried. The yield of YCHD extract was 28.6% and the concentration was 40 μg/mL. The extract was stored in a desiccator until use.

Cell culture

The PANC-1 cells were incubated in Dulbecco’s modified Eagle’s medium (HyClone of Thermo Fisher Scientific, Waltham, MA, United States) supplemented with 2 mmol/L glutamine, 0.05 g/L penicillin, 0.1 g/L streptomycin, and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO2. The culture medium was replaced every two days.

MTT assay

Cells were seeded at 1 × 105 cells/well in 96-well plate overnight and treated with various concentrations of YCHD (5 μg/mL, 10 μg/mL, 20 μg/mL and 40 μg/mL) for different times for 24 h, 48 h and 72 h. After treatment, an MTT assay was used to determine cell densities. All experiments were performed in triplicate three separate times.

TUNEL assay

A MEBSTAIN Apoptosis Kit Direct (MBL International, Woburn, MA, United States) was used to perform TUNEL staining according to the manufacturer’s protocol. Nucleosome-sized DNA fragments are detected by tailing their 3’-OH ends with digoxigenin nucleotides using terminal deoxynucleotidyl transferase (TdT). After treatment, the cells were combined with POD-horseradish peroxidase and incubated with the TdT buffer. The numbers of TUNEL-positive cells was counted under a light microscope.

Immunohistochemical staining

Pancreatic carcinoma PANC-1 cells were exposed to YCHD (20 μg/mL) for various times (24 h, 48 h, 72 h and 96 h). After treatment, the cells were grown on six-well glass plates and were fixed with acetone. After washing in PBS, The cells were washed with PBS and incubated in 3 mL/L H2O2 solution at room temperature for 5 min. Then, anti-Bcl-2 or anti-Bax monoclonal antibodies at a 1:300 dilution were added and incubated at 4°C overnight. The cells were washed with PBS and incubated with the secondary antibody (biotinylated anti-rat IgG) at room temperature for 1 h. Then the cells were washed with PBS and incubated with ABC compound (Vector Laboratories Inc., Burlingame, CA, United States) at room temperature for 10 min. DAB was used as the chromagen. After 10 min, the brown color signifying

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the presence of antigen bound to antibodies was detected by light microscopy and photographed at magnification ×200.

**RT-PCR**
PANC-1 cells were exposed to YCHD (20 μg/mL) for various times (24 h, 48 h, 72 h and 96 h) and total RNA was extracted. The primers for Bcl-2, Bax, and β-actin in were as follows: β-actin (500 bp): 5’-GTGGGGCCGCCCCACCA-3‘ and 5’-CTCCTTAATGTCACGCACGATTTC-3’; Bcl-2 (716 bp): 5’-GGAAATATGGCGCACGCT-3’ and 5’-TCACTTGTGGCCCAGAT-3’; Bax (508 bp): 5’-CCAGCTCTGAGCAGATCAT-3’ and 5’-TATCAGCCTTCC-3’. PCR was performed using the following procedures: 94 ℃ for 7 min followed 35 (Bax) or 30 (Bcl-2 and β-actin) cycles of 94 ℃ for 1 min, 60 ℃ for 45 s, and 72 ℃ for 45 s, and completed with one cycle at 72 ℃ for 7 min. For visualization, 10 μL of PCR product was loaded onto a 15 g/L agarose gel.

**Statistical analysis**
Statistical differences of data were analyzed by the paired two-tailed Student’s t-test. P < 0.05 was considered significant.

**RESULTS**

**MTT assay**
PANC-1 cells were exposed to various concentrations of YCHD (5 μg/mL, 10 μg/mL, 20 μg/mL, and 40 μg/mL) for 24 h, 48 h, and 72 h. Apoptosis of PANC-1 cells was increased with dose and time (Table 1).

**TUNEL assay**
A TUNEL assay detected apoptotic cells according to the manufacturer’s instructions. Positive staining was located in the nucleus (Figure 1). The apoptotic index of PANC-1 cells was increased with time (Table 2).

**Expression of Bcl-2 proteins**
Positive staining was located in the cytoplasm (Figure 2A). PANC-1 cells were exposed to YCHD (20 μg/mL) for various times (24 h, 48 h, 72 h, and 96 h). The positive rate of Bcl-2 expression in PANC-1 cells was reduced with time (P < 0.05) (Table 3).

**Expression of Bax proteins**
Positive staining was located in the cytoplasm (Figure 2B). PANC-1 cells were exposed to YCHD (20 μg/mL) for various times (24 h, 48 h, 72 h, and 96 h). The positive rate of Bax expression increased with time (P < 0.05) (Table 4).

**RT-PCR**
When PANC-1 cells were exposed to YCHD (20 μg/mL) for various times (24 h, 48 h, 72 h, and 96 h), the expression of Bcl-2 mRNA decreased and Bax mRNA increased with time.

**DISCUSSION**
Cancer involves dysregulation of the cell cycle and uncontrolled growth due to the combined effects of hereditary and environmental factors. The mechanism of cell cycle dysregulation is an important cause of cell proliferation, which leads to cancer. Normally, each period of cell division and proliferation are strictly regulated by a variety of specific proteins.

The results of the present study show that apoptosis of PANC-1 cells is time- and dose-dependent. This suggests that YCHD inhibits the growth of pancreatic carcinoma cells. Following treatment with 20 μg/mL YCHD, the positive rate of Bax protein expression was significantly increased, whereas the rate of Bcl-2 protein expression was significantly reduced with time. Furthermore, the expression of Bcl-2 mRNA decreased and the density of Bax mRNA

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| Concentration (μg/mL) | Treatment time (h) |
|-----------------------|-------------------|
|                       | 24                |
| Control               | 0.410 ± 0.007     |
| 5                     | 0.365 ± 0.003     |
| 10                    | 0.320 ± 0.004     |
| 20                    | 0.306 ± 0.004     |
| 40                    | 0.264 ± 0.004     |

Table 1 Effects of various concentrations of Yin Chen Hao Decoction on apoptosis of pancreatic carcinoma cell line-1 cells

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Figure 1 TUNEL assay of apoptotic pancreatic carcinoma cell line-1 cells induced by Yin Chen Hao Decoction. Magnification × 200.
Yin Chen Hao Decoction (YCHD) is a classic Chinese medicine formula consisting of three herbal drugs (Rheum officinale Baill, Artemisia capillaris Thunb, and Gardenia jasminoides Ellis), and has long been used to treat cholestasis, hepatitis C, primary biliary cirrhosis, and liver fibrosis. Previous research reported that YCHD is effective for choleresis and has anti-inflammatory and antiasthmatic activity to relax bronchial smooth muscle. Moreover, reports show that YCHD is a potent inhibitor of carcinoma. The anti-cancer activity of YCHD may induce apoptosis of carcinoma cells.

**Research frontiers**

YCHD has antitumor effects. The research hotspot related to YCHD is how it affects the progression of human cancer.

**Innovations and breakthroughs**

The present study demonstrates that YCHD is able to induce apoptosis of pancreatic carcinoma cells. Decreased expression of BCL2 and increased expression of BAX may induce apoptosis. YCHD may be a new drug for pancreatic cancer chemotherapy.

**Applications**

In understanding the role and mechanism of YCHD against pancreatic carcinoma, this study is expected to suggest a way of improving clinical.

**COMMENTS**

**Background**

Yin Chen Hao Decoction (YCHD) is a classic Chinese medicine formula consisting of three herbal drugs (Rheum officinale Baill, Artemisia capillaris Thunb, and Gardenia jasminoides Ellis), and has long been used to treat cholestasis, hepatitis C, primary biliary cirrhosis, and liver fibrosis. Previous research reported that YCHD is effective for choleresis and has anti-inflammatory and antiasthmatic activity to relax bronchial smooth muscle. Moreover, reports show that YCHD is a potent inhibitor of carcinoma. The anti-cancer activity of YCHD may induce apoptosis of carcinoma cells.

**Table 2** Apoptotic index of pancreatic carcinoma cell line-1 cells treated with Yin Chen Hao Decoction (%)

| Treatment time (h) | Control | 24  | 48  | 72  | 96  |
|--------------------|---------|-----|-----|-----|-----|
| Apoptotic index    | 1.26 ± 0.31 | 4.95 ± 0.81 | 18.63 ± 1.93 | 35.68 ± 0.37 | 43.63 ± 1.23 |
| \( t \)            | -6.12   | -13.92 | -183.17 | -53.90 |
| \( P \) value\(^1\) | \( P < 0.05 \) | \( P < 0.05 \) | \( P < 0.05 \) | \( P < 0.05 \) |

\(^1\) vs control group.

**Figure 2** Immunohistochemical staining. A: Bcl-2; B: Bax; magnification × 200.

**Table 3** Positive rate of Bcl-2 expression in pancreatic carcinoma cell line-1 cells treated with Yin Chen Hao Decoction (%)

| Treatment time (h) | Control | 24  | 48  | 72  | 96  |
|--------------------|---------|-----|-----|-----|-----|
| Positive rate      | 36.62 ± 0.69 | 21.71 ± 0.07 | 10.60 ± 0.49 | 7.21 ± 0.45 | 4.54 ± 0.36 |
| \( t \)            | 31.29   | 44.51 | 73.62 | 59.24 |
| \( P \) value\(^1\) | \( P < 0.01 \) | \( P < 0.01 \) | \( P < 0.01 \) | \( P < 0.01 \) |

\(^1\) vs control group.

**Table 4** Positive rate of Bax expression in pancreatic carcinoma cell line-1 cells treated with Yin Chen Hao Decoction (%)

| Treatment time (h) | Control | 24  | 48  | 72  | 96  |
|--------------------|---------|-----|-----|-----|-----|
| Positive rate      | 10.29 ± 0.51 | 20.14 ± 0.79 | 34.6 ± 0.86 | 45.24 ± 0.48 | 50.76 ± 1.47 |
| \( t \)            | -56.89   | -41.68 | -69.95 | -51.89 |
| \( P \) value\(^1\) | \( P < 0.001 \) | \( P < 0.001 \) | \( P < 0.001 \) | \( P < 0.001 \) |

\(^1\) vs control group.

increased with time. Thus, the ratio of Bcl-2/Bax decreased with YCHD treatment to result in apoptosis of these cells.

In conclusion, this study demonstrates that YCHD induces apoptosis of pancreatic carcinoma cells. Decreased expression of the BCL2 and increased expression of BAX may induce apoptosis. YCHD may be a new drug for pancreatic cancer chemotherapy.
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