

A cDNA MICROARRAY STUDY OF THE EFFECT OF KANGLAITE INJECTION IN INDUCING APOPTOSIS OF PATU-8988 HUMAN PANCREATIC CANCER CELLS

Yuan Yaozong, Bao Ying, Xia Lu, Zhang Yongping, and Zhang Xuejun

Department of Gastroenterology

Ruijin Hospital, Shanghai Second Medical University

Shanghai 200025, China

ABSTRACT

OBJECTIVES: To study the expression patterns of apoptosis-related genes in Patu-8988 human pancreatic cancer cells induced by Kanglaite Injection(KLT).

METHODS: Apoptosis of the Patu-8988 cells induced by Kanglaite Injection was determined by flow cytometry with Annexin V-FITC and propidium iodide staining. The different expression patterns of apoptosis-related genes before and after KLT treatment were detected by a human apoptosis microarray containing 96 cDNA fragments. Then changes in the expression of selected gene products were confirmed by Western blot analysis.

RESULTS: KLT induced apoptosis of Patu-8988 cells in a time-dependent manner. cDNA microarray analysis identified significant changes (more than 3 folds) in the expression of 17 genes among the 96 apoptosis-related target genes in the first 24 hours as a consequence of KLT treatment. Among the 17 genes, the expressions of 12 were up-regulated and 5 were down-regulated. Western blot performed for protein expression changes of P53, Bcl-2, Bax and caspase-3 were consistent with the microarray results. The enhanced activity of caspase-3 was evidently verified by the cleavage of the substrate of caspase-3, 89x10³ form of poly (ADP ribose) polymerase.

CONCLUSIONS: KLT can alter the expression profile of multiple apoptosis-related genes in Patu-8988 human pancreatic cancer cells, which further reveals its mechanism of action in inducing the apoptosis of human pancreatic cancer cells.

KEY WORDS:Kanglaite Injection, Pancreatic cancer, Apoptosis, cDNA microarray

KLT is a state class II new anti-cancer preparation, which has been used in clinics extensively in recent years. The drug is prepared by extracting the natural anti-cancer components from Coix seed, a traditional Chinese medicine, and formulated into a dosage form of emulsion by advanced technology¹. Previous studies have demonstrated that KLT is effective in inhibiting the

growth of human pancreatic cancer cells in vitro and inducing apoptosis. Cell apoptosis is a complicated molecular process involving multiple apoptosis-related genes (such as the caspase family and the apoptosis inhibiting gene Bcl-2 family) and signal transduction pathways (such as the ATM, Fas signal pathways)². The microarray technology is able to conduct parallel analysis of large amount of signals in gene expression and has become an important tool for molecular biology research. We are reporting in this paper a cDNA microarray study of the expression patterns of apoptosis-related genes in pancreatic cancer cells induced in vitro by KLT.

MATERIALS AND METHODS

1 Experimental Materials

1.1 Cell Culture and Treatment: Patu-8988 human pancreatic cancer cells were provided by Chang Hai Hospital affiliated to the Second Military Medical University. Patu-8988 cells were routinely cultivated in an incubator with DMEM culture medium (Gibco) containing 10% calf serum at 37°C with 5% CO₂. Our previous studies have demonstrated that as Patu-8988 cells were treated with 20µl/ml KLT for 24 hr, the growth inhibition effect is most suitable for microarray analysis because RNA would rapidly degraded during the death process of cells. Therefore, the treatment group was treated with 20µl/ml KLT after inoculation for 24 hr, while the control group was not treated with any drug in this study.

1.2 Drugs and Reagents: KLT was provided by Zhejiang Kanglaite Pharmaceutical Co., Ltd. Annexin V apoptosis kit was purchased from BD. GEArray^{TMQ} apoptosis specific microarray assay kit were purchased from Super Array. P53, Bcl-2, Bax and Caspase 3 and poly (ADP ribose) polymerase (PARP) monoclonal antibody were purchased from Cell Signaling Technology.

2 Apoptosis determined by Annexin V/Propidium Iodide (PI) Double Staining Method

Cells were collected respectively from the control group and the treatment group (treated by 20µl/ml KLT for 12, 24, 48 and 72 hours). Concentration of the cells to be analyzed was adjusted to 5x10⁵ - 1x10⁶/ml. The samples were centrifuged with 600 g at 4C° for 5 minutes. The supernatant was discarded. 1 ml pre-cooled phosphate buffered saline (PBS) was then added. The cells were washed once and suspended in 150 µl Annexin-binding buffer. 5 µl Annexin V Fluorescein isothiocyanate (FITC) and 5 µl Propidium Iodide (PI) were added, mixed well and kept from light, after reacted at room temperature for 15 min another 200 µl Annexin-binding buffer was added. A flow cytometric scan (Becton Dickinson) analysis was immediately performed. The experiment was repeated 3 times. The rate of apoptosis was shown in the form of mean ± standard deviation. T test was performed for group comparisons. Differences were significant only when p<0.05.

3 Microarray Test and Analysis

3.1 RNA Extraction: Cells were collected respectively from the control group and the treatment group (treated by 20µl/ml KLT for 24 hr). Total RNA of the cell was extracted using TRIzol reagent (Gibco) following its instructions. Its concentration and purity were determined by spectrophotometry.

3.2 Preparation of the Probe: Total RNA was used as the framework to synthesize the biotin labeling probe using GEArray apoptosis microarray assay kit according to the methods discussed in the literature [3] of this report. Total RNA 5 µg was added to primer miscible liquid for 2 minutes at 70°C and another 2 minutes at 42°C for preparation of annealing mixtures. Non-rad GEArray labeled buffer solution, biotin-16-dUTP, RNase inhibitor and reverse transcriptase were then added for incubation at 42°C for 90 min. The labeled reaction liquid would be used directly for hybridization after the reaction stopped.

3.3 Hybridization and Washing: The fluorescence labeled Probe was put into a 94°C water bath for denaturation for 5 min. The probes were then put onto pre-hybridized microarray for hybridization at 60°C for 12 hr. The microarray was then cleaned with 20x standard sodium citrate (SSC) and 20% sodium dodecyl sulfate (SDS). After the microarray was blocked by blocking solution, alkaline phosphatase coupled chain affinant was added, mildly shaken for 10 min and the membrane was repeatedly washed with buffer solution.

3.4 Chemiluminescence Test: CDP-Star® chemiluminescence substrate was added and kept still at room temperature for 2 min, the Microarray was then placed on clean filter papers to get rid of residual CDP-Star® solution and exposed with X ray. The experiment results were scanned. Gene expressions were determined by microarray analysis software of the kit.

Steps 1-4 were repeated and the results from both were comprehensively analyzed.

4 Western Blot Analysis of Protein Expression

Cells were collected respectively from the control group and the treatment group (treated by 20µl/ml KLT for 6, 12, 24, 48, 72 and 96 hr). After cleaving solution (20 mmol/L Tris-HCl pH 7.4, 200 mmol/L NaCl, 1% Triton x 100, 0.1% SDS, 0.2% deoxysodium cholate, 5 mmol/L EDTA) was added and then put on ice for 10 minutes. After centrifugation the supernatant and SDS loading buffer were mixed and boiled for 10 min, the protein was quantitated with a Bradford method. The protein was separated with a 10% SDS-PAGE gel electrophoresis. After being transferred to cellulose nitrate membrane it was blocked for 2 hr and then cultivated with a blocking liquid containing the antibody I at room temperature for 3 hr. The membrane was washed and then

cultivated with a blocking liquid containing antibody II at room temperature for 2 hr. The membrane was finally washed and put into a Super Signal West reaction liquid for cultivation at room temperature for 5 min and then processed with X ray film exposure.

RESULTS

1 Apoptosis of Patu-8988 cells Induced by Kanglaite Injection

It was demonstrated by flow cytometry with Annexin V / propidium iodide double staining that the apoptosis rate for Patu-8988 cells not being treated with KLT was (2.47±0.36)% and that the apoptosis rate for the cells treated with 20µl/ml KLT for 24 hr was markedly increased in a time dependent manner. The apoptosis rate reached the highest level at (13.17±0.32)% when the cells were treated for 72 hr. The differences between the apoptosis rate for control group and those for the 24, 48 and 72 hr treatment groups were all statistically significant (p<0.01). It has been verified by this study that KLT has the action of inducing apoptosis in Patu-8988 cells in a time dependent manner. See Fig. 1, 2.

2 Effect of Kanglaite Injection on Apoptosis-Related Gene Transcription

Among the 96 apoptosis-related genes on the microarray, the expression of 17 genes changed between before and after KLT treatment at a ratio larger than 3 or smaller than 0.33. Among them 12 genes were up-regulated and 5 genes down-regulated. Most of these genes with transcription differences belong to the Bcl-2 family, caspase family, P53 family, and tumor necrosis factor (TNF) receptor super family.

Table 1 Genes with Transcription Differences after KLT Treatment

Pool Number	Symbol	Name	Treatment Group/Control Group
Upregulated Genes			
U20537	CASP6	Apoptosis-related cysteine protease 6	84.849
U66879	BAD	Bcl-2 /Bcl-xl associated death promoter	77.858
U67320	CASP7	Apoptosis-related cysteine protease 7	14.373
L22474	BAX	Bcl2-associated X protein	10.952
AF016582	CHEK1	Cell cycle checkpoint protein kinase 1	9.479
M14694	TP53	Onco-inhibiting gene p53	5.157
NM.004346	CASP3	Apoptosis-related cysteine protease 3	5.024
AA923639	BAK1	BCL-2 antagonist gene 1	4.913
U13021	CASP2	Apoptosis-related cysteine protease 2	3.913
NM.004402	DFFB	DNA fragmentation factor, beta subunit	3.551

U60521	CASP9	Apoptosis-related cysteine protease 9	3.491
X63717	TNFRSF6	TNF receptor superfamily factor 6	3.283
Down-regulated Genes			
AF082185	TRAF4	TNF receptor-associated factor 4	0.003
U12597	TRAF2	TNF receptor-associated factor 2	0.006
M14745	BCL2	B-cell lymphoma/leukemia-2 gene	0.007
NM.001167	BIRC4	X-linked apoptosis protein inhibitor	0.018
Z12020	MDM2	Protooncogene MDM2	0.249

3 Protein Product Expression of Apoptosis Related Genes

Western blot analysis confirmed the changes in gene expression demonstrated in Miroarray that the Bcl-2 protein content in Patu-8988 cells was markedly decreased after cells were treated with 20µl/ml KLT for 12 hr, while almost no Bcl-2 expression on time points thereafter. The Bax protein only started to express after KLT treatment for 96 hr, the total amount of P53 protein continued to increase with the time of KLT treatment. Its activated form, phosphorylated P53 (ser 15) was markedly up-regulated at the 72nd and 96th hr time point. The total amount of the Caspase-3 protein also increased in an evident time-dependent manner. Changes in the expression level of the proteins of these genes were basically consistent with the results of the microarray study. An analysis of the PARP, a Caspase 3 substrate, also demonstrated that 89x10³ strips appeared after KLT treatment for 6 hours while no fragmentation products (89x10³ strips) appeared in the control group, indicating that Caspase 3 had certain enzyme activities.

DISCUSSION

Apoptosis is the autonomous death of cells that is controlled by genes when stimulated by physiological or pathological factors. The growth of tumor is mainly adjusted through a balance between tumor cell proliferation and apoptosis. Therefore cell apoptosis inducement is considered as a new way for treating pancreatic cancer. Previous studies have demonstrated that KLT at certain concentration is effective in markedly inhibiting the proliferation of Patu-8988 cells and that this effect might relates to its action in inducing cell apoptosis. Apoptosis of Patu-8988 human pancreatic cancer cells induced by KLT was determined by Annexin V and propidium iodide double staining in this study. There is a high specific affinity between Annexin V and phosphatidylserine (PS). Because the movement of intramembranous phosphatidylserine (PS) to membrane outside surface is an early indication of apoptosis and cell membrane is still intact at this time, propidium iodide staining is negative. Therefore Annexin V and propidium iodide double staining is able to distinguish between apoptosis cells and necrosis cells and make a precise quantitative determination of cell apoptosis³. This study has confirmed that KLT has the action of inducing apoptosis of Patu-8988 cells in a time-dependent manner.

Ever since Schena et al.⁴ published their paper in 1995 on microarray determination of gene expression patterns, the microarray technology has become a quick and effective method to study the differences in gene expressions. A specific gene probe is fixed to the microarray and the intracellular mRNA or cDNA in different cells or tissues at different development phases or under different stimulations are measured. Therefore the differences in the expressions of these genes under different conditions are analyzed with high efficiency. The theories of Traditional Chinese Medicine (TCM) are not based upon modern biomedicine. The chemical compositions of TCM drugs are very complicated and hard for quantitative determination. The research of TCM drugs, not matter at the cellular level or at the animal level, is still not an easy task. Microarray technology provides a useful tool to study the effects of TCM drugs on gene expression and to reveal their mechanisms of action⁵. We applied apoptosis specific microarray in this study to determine the expressions of 96 apoptosis-related genes affected by KLT. We then measured the protein expression levels of representative genes selected on this basis and the results were consistent with the results of the microarray study. It is obvious that microarray analysis is a quick and effective method to study the mechanisms of action of Kanglaite Injection.

This study demonstrates that when Patu-8988 cells were treated with KLT, the mRNA and protein expression levels of the bcl-2 gene in the cells were down-regulated, while the expressions of the bax gene were up-regulated, thus the lowering of the bcl-2/bax ratio might be one of the molecular pathway for KLT to induce the apoptosis of cancer cells. At the same time the expression of the p53 gene was also increased and activated through phosphorylation, indicating the p53 gene was a participant in KLT induced apoptosis as well. It has been found in recent studies that the key for cell apoptosis signal transduction is the activation of caspase. Caspase all exist in the form of inactivated prototype enzyme. Once being activated, a caspase cascade reaction will occur, that will lead to apoptosis of the cells eventually⁷. Bcl-2 and p53 all interact with the caspase family. As an important member of the caspase family, Caspase-3 is a significant effector enzyme molecule in the cell apoptosis signal transduction pathway⁸. It has the action of splitting its substrate PARP (116×10^3) into 24×10^3 and 89×10^3 polypeptide and therefore activating the endonuclease, which will lead to DNA fragmentation. We found in this study that the expression of the caspase in Patu-8988 cells increased along with the duration of time of KLT treatment. At the same time, its substrate PARP was fragmented, indicating that caspase had been activated.

In summary, the change in the expression of apoptosis-related genes is an important pathway by which apoptosis of pancreatic cancer cells is induced by KLT. The gene differentiations we found in this study have provided a concrete clue for further investigation of the molecular mechanism of actions of KLT

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