

Full Length Research Paper

Effects of Saikosaponin D on apoptosis genes expression profile of the colon cancer cells HT-29

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The present study aims to investigate the change of apoptosis genes expression of HT29 cells induced by Saikosaponin D (SSD), which is the major component of Xiao Chai Hu Tang, the classic Chinese medicine prescriptions. In this study, gene expression profiles of HT29 cells with or without the SSD treatment were obtained by means of using RT ProfilerTMPCR Array Human Apoptosis and quantitative real-time polymerase chain reaction (PCR) and analyzed to detect differential gene expression. According to results of 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, a final concentration of 10 mg /L of SSD for 24 h was used in the SSD group and the growth inhibition rate of HT-29 cells was about 5%. In SSD treated cells, expression levels of 14 kinds of apoptosis genes changed significantly. Compared with the control group, some apoptosis genes such as DIVA, CASP10, and TRAILR were expressed at much higher levels, while some anti-apoptosis gene such as HIAP1, NAIP, BCL2 were also expressed at much higher levels. SSD induces the apoptosis of HT29 through the apoptosis pathway of TRAIL, TRAIL-R and caspase10 and/or caspase8. And these data could provide targets for the treatment of colon cancer.

Key words: Saikosaponin D, colon carcinoma, apoptosis, gene expression.

INTRODUCTION

Xiao Chai Hu Tang first recorded in the Chinese Ancient medicine book "Febrile Diseases" is widely used in clinical treatment of inflammation, cancer prevention and control, and is a classic Chinese medicine prescription. Saikosaponin is the major component of Xiao Chai Hu Tang. There have been many studies on anti-tumor effect of the saikosaponin and its extracts by local and foreign scholars, confirming that saikosaponin D (SSD) has the strongest anti-inflammatory effect in the four kinds of saikosaponin; same as some other saponins drugs with anti-tumor effects. However, the antitumor mechanism of SSD is still unclear (Wang et al., 2006; Zu 2000). It was confirmed that in leukemia, liver cancer, lung cancer and other tumor cells, SSD could induce apoptosis through different mechanisms, such as increasing FASL, FAS, and BAX protein expression, reducing B-cell lymphoma 2 (BCL2), BCL-XL expression, activating poly (ADP) poly-

merase (PARP) shear, or cracking inhibitors of apoptosis (IAP) family members XIAP and cIAP-2 among others (Hsua et al., 2004a; 2008; Yu et al., 2008). It was reported that as one of the four kinds of saikosaponin, saikosaponin A (SSA) could induce apoptosis in colon cancer cells through activation of CASP2 and CASP8 (Kim et al., 2010), so whether SSD is able to induce apoptosis in colon cancer cells and the mechanism of SSD induced apoptosis is still unclear.

In this study, by means of using the apoptosis gene polymerase chain reaction (PCR) microarray and quantitative real-time PCR technology, we screened the SSD-induced differentially expressed apoptosis genes in the colon cancer cells. Here, we found and reported that there were 15 kinds of apoptosis genes whose expression were significantly changed, and these apoptosis genes might play a major role in SSD-induced

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Table 1. Inhibition effects of saikosaponin D on the growth of colon cancer HT-29 cell.

Group	Inhibition rate (%)		
	24 h	48 h	72 h
0 (mg/L)	0.11±0.02	0.09±0.012	0.14±0.021
5 (mg/L)	1.9±0.14	17.8±0.24	19.1±0.21
10 (mg/L)	5.0±0.17	31.2±0.12	33.4±0.28
20 (mg/L)	9.4±0.18	42.3±0.31	44.5±0.18

apoptosis of the colon cancer cells. These results laid the foundation for understanding the mechanisms of saikosaponin D-induced apoptosis of colon cancer cells, and could provide targets for the treatment of colon cancer.

MATERIALS AND METHODS

Materials

Saikosaponin D was purchased from Chinese medicine and biological products Institution (Beijing, China); Colon cancer cell line HT-29 cells were purchased from Shanghai Institute of Cell Biochemistry Life Sciences (Shanghai, China). Cell culture, RNA purification and reverse transcription reagents were mainly purchased from Invitrogen company and TakaRa company; RT Profiler™ human apoptotic gene PCR microarray (PAHS-012A) were purchased from SABiosciences company.

Methods

Cell growth inhibition assay

Cells were seeded into 96-well plates (5×10^3 cells/well) for 12 h, followed by treatment with various concentrations of saikosaponin D. Cell viability was determined using the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. In brief, 15 μ l (5 mg/ml) MTT working solution was added to per well and then incubated at 37°C for 4 h. Then the supernatant was removed and 150 μ l of dimethylsulphoxide (DMSO) was added to each well to dissolve the crystals. The absorbance of each well at 570 nm was measured under an ELx 808 Universal Microplate Reader (BIO-TEK, INC). Data were expressed as the percentage of cellular viability relative to the control culture (assuming 100%) in the present study.

Cells drug treatment and RNA purification identification

The same number of HT-29 cells were inoculated in the cell culture dish with an equivalent diameter of 10 cm. After 24 h, cells were treated with 0 mg / L SSD (control group) or 10 mg / L (experimental group), and for another 24 h, the cells were collected. Total RNA of the cells sample was extracted with Trizol reagent (Gibco, USA) as described by the manufacturer. RNA was purified using RNeasy® MinElute™ purification kit (Qiagen); quality, concentration and purity of RNA were measured using the agarose gel electrophoresis method and the UV absorption. RT-PCR was performed using SuperScript. III Reverse Transcriptase (Invitrogen). PCR was performed for 30 cycles in 25 μ l of reaction mixture.

Apoptosis gene PCR microarray assay

10 μ l cDNA and SuperArray PCR master mix (Cat. No. PA-112) premixed solution was added to each well of PCR microarray. Real-time PCR procedure was set as follows: Denaturation at 95°C for 10 min, amplification of 40 cycles at 95°C for 15 s and 60°C for 1 min. After collecting the fluorescence, the melting curve was analysed.

Data were analyzed by $\Delta\Delta$ Ct method, and the fluorescence threshold was set to 0.15. Δ Ct was the mean number of the threshold cycle number of each gene subtracting the threshold cycle of housekeeping gene B2M, HPRT1, GAPDH, ACTB. Gene $\Delta\Delta$ Ct was the Δ Ct of the SSD group minus the control group Δ Ct. The differences of each gene expression were calculated with 2- $\Delta\Delta$ Ct calculation of DHA and control groups (Livak and Schmittgen, 2001). PCR microarray experiments technical services were provided by the Kangcheng Sheng Bio-Engineering Co., Ltd. (Shanghai, China).

RESULTS AND DISCUSSION

Identification of RNA samples from Saikosaponin D-treated Colon cancer cells

According to results of MTT assay (Table 1), a final concentration of 10 mg / L of SSD for 24 h was used in the SSD group, and the growth inhibition rate of HT-29 cells was about 5%, consistent with the results reported by Hsua et al. (2004b) in non-small lung cancer cells. The UV absorption ratio Abs260/Abs280 reflecting the purity of RNA samples from SSD and control groups were 2.00 and 2.02, respectively. As shown in Figure 1, two clear 28S, 18S ribosomal RNA bands and one RNA band with a little spread and low molecular weight were observed in both the SSD and control groups. And above the 28S ribosomal RNA band, the diffusion of high molecular weight substances or belt usually resulting from DNA contamination was not observed.

The analyses of SSD-induced apoptosis genes differential expression profile in HT-29 cells

As shown in Figure 2a, the column height of three-dimensional histogram meant multiples of differentially expressed genes, $2^{-\Delta\Delta$ Ct ratio of gene mRNA expression from the SSD group to that from the control group. The measured genes distribution is shown in Figure 2b.

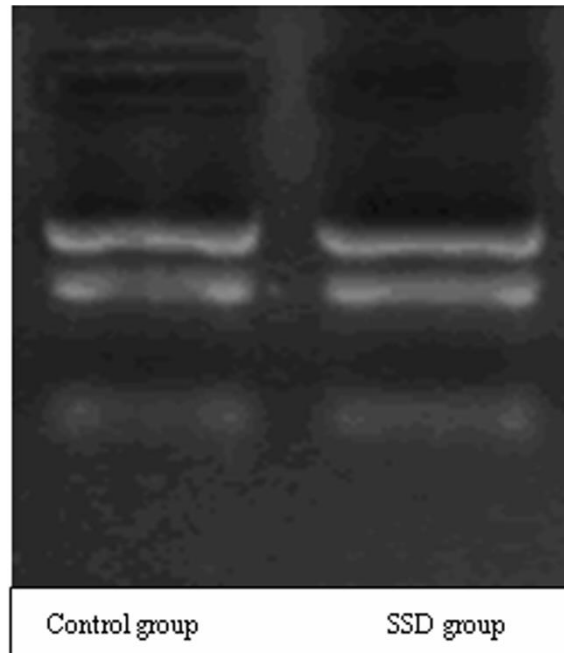
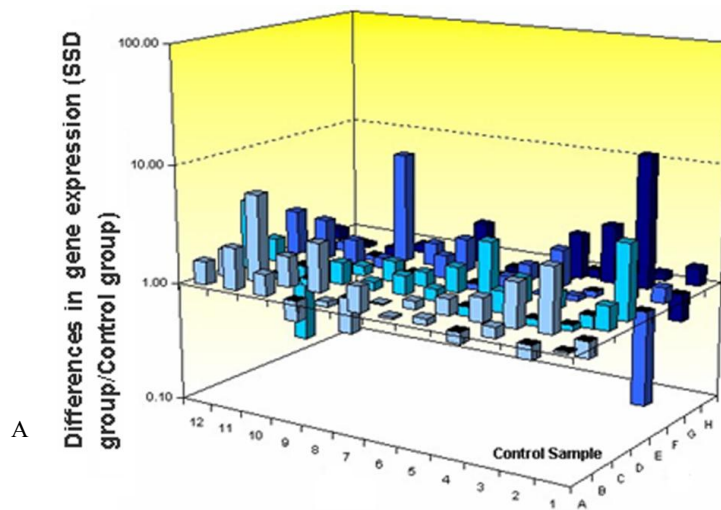


Figure 1. Purity identification of RNA samples by denatured agarose gel electrophoresis (left: control group; right: SSD group).



ABL1	AKT1	APAF1	BAD	BAG1	BAG3	BAG4	BAK1	BAX	BCL10	BCL2	BCL2A1
A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12
BCL2L1	BCL2L10	BCL2L11	BCL2L2	BCLAF1	BFAR	BID	BIK	BIRC1	BIRC2	BIRC3	BIRC4
B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
BIRC6	BIRC8	BNIP1	BNIP2	BNIP3	BNIP3L	BRAF	CARD4	CARD6	CARD8	CASP1	CASP10
C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12
CASP14	CASP2	CASP3	CASP4	CASP5	CASP6	CASP7	CASP8	CASP9	CD40	CD40LG	CFLAR
D01	D02	D03	D04	D05	D06	D07	D08	D09	D10	D11	D12
CIDEA	CIDEB	CRADD	DAPK1	DFFA	FADD	FAS	FASLG	GADD45A	HRK	IGF1R	LTA

Figure 2. The SSD-induced apoptosis genes differential expression profiles in colon cancer cell line HT-29 cells. A, SSD-induced apoptosis genes differential expression; b, PCR microarray gene distribution.

Table 2. Part of significantly up-regulated apoptosis-related genes induced by SSD in HT-29 cells.

Symbol	Well	2 ^Δ -ΔCt		Fold change	Fold up- or down-regulation	Open gene table in web browser
		SSD group	Control group	SSD /control group	SSD /control group	Description
BCL2	A11	8.90E-04	3.99E-04	2.23	2.23	B-cell CLL/lymphoma 2
BCL2L10/DIVA	B02	4.35E-05	1.25E-05	3.48	3.48	BCL2-like 10 (apoptosis facilitator)
BCL2L11/BAM	B03	1.82E-02	7.82E-03	2.33	2.33	BCL2-like 11 (apoptosis facilitator)
NAIP	B09	4.13E-03	1.57E-03	2.63	2.63	NLR family, apoptosis inhibitory protein
HIAP1/BIRC3	B11	1.12E-03	2.02E-04	5.54	5.54	Baculoviral IAP repeat-containing 3
CASP10	C12	5.37E-04	1.37E-04	3.92	3.92	Caspase 10, apoptosis-related cysteine peptidase
IGF1R	E11	1.14E-02	5.25E-03	2.17	2.17	Insulin-like growth factor 1 receptor
LTA/TNFB	E12	1.01E-04	4.19E-05	2.41	2.41	Lymphotoxin alpha (TNF superfamily, member 1)
MCL1	F02	9.35E-02	4.12E-02	2.27	2.27	Myeloid cell leukemia sequence 1 (BCL2-related)
PYCARD	F04	2.71E-05	1.31E-05	2.07	2.07	PYD and CARD domain containing
TNFRSF9/CD137	G02	3.73E-05	2.87E-06	12.98	12.98	Tumor necrosis factor receptor superfamily, member 9
TNFSF10/TRAIL	G03	5.09E-05	1.62E-05	3.14	3.14	Tumor necrosis factor (ligand) superfamily, member 10
CD70	G04	8.33E-04	3.56E-04	2.34	2.34	CD70 molecule
TP53BP2	G07	6.07E-02	2.72E-02	2.23	2.23	Tumor protein p53 binding protein, 2
CIDEA	E01	1.30E-05	3.72E-05	0.35	-2.90	Cell death-inducing DFFA-like effector a

These results suggest that SSD could induce 14 kinds of significantly up-regulated apoptosis genes in colon cancer cell line HT-29, and the differences multiples of these genes were higher than 2 (Table 2). Interestingly, the gene CIDEA expression level was significantly reduced, but the difference multiple was less than 0.5, maybe because this gene expression level in both the SSD group and control group was low, the reduced significance in this experiment was not statistically significant.

SSD induced significantly up-regulated expression of 14 kinds of apoptosis genes in HT-29 cells, including pro-apoptotic genes TRAIL, CASP10, TNFB, BAM and DIVA, and anti-apoptotic genes BCL2, HIAP1, NAIP and so on. Meanwhile, there were also some genes, which were up-regulated but increased in less than 2-fold expression, including CASP8 (1.48), CASP9 (1.23), CASP3 (1.39) and TRAIL receptor gene TNFRSF10A (1.88), TNFRSF10B (1.55). These results suggest that SSD triggered apoptosis in HT-29 cells by the

TRAIL death receptor pathway. In the TRAIL-induced apoptosis, CASP10 may replace CASP8 or synergy with CASP8 from the classic death receptor pathway (Kischkel et al., 2001). CD137, which has a significant increase in SSD-treated cells, might play an anti-tumor role by regulating the immune responses of tumor cells (Ju et al., 2003).

TNFB has been found to induce the apoptosis by the mitochondrial pathway, although the molecular mechanisms were still unclear. In HT-29

cells, SSD induced increased expression of TNFB possibly resulted in the cells apoptotic by the mitochondrial pathway. And moreover, the pro-apoptotic BCL2 family members BAM and DIVA were significantly up-regulated in SSD-treated cells and this suggested that the mitochondrial pathway played an important role in SSD-treated cells apoptosis. In addition, up-regulated TNFB heterotrimer binds with receptors, and may in turn recruit TRAF2, TRAF3 and cIAP1, and the BIR1 domain of cIAP1 were removed in this process, so that CASP9, CASP7, and CASP3 activity were activated (Kuai et al., 2003). In short, these data suggest SSD induced the apoptosis of HT29 through the apoptosis pathway of TRAIL, TRAIL-R and caspase10 and/or caspase8, and could provide targets for the treatment of colon cancer.

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ABBREVIATIONS

SSD, Saikosaponin D; **PARP**, poly(ADP)polymerase; **BCL2**, B-cell lymphoma 2; **IAP**, inhibitors of apoptosis; **SSA**, saikosaponin A; **PCR**, polymerase chain reaction; **MTT**, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; **DMSO**, dimethylsulphoxide.

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