行政院國家科學委員會專題研究計畫成果報告

探討中草藥萃出物處理對人類肝癌細胞之效應並建立中草藥抗癌功效性之表現基因分子圖譜（Studying the effects of Chinese herbal extracts on human hepatoma cells and establishing the molecular map of expressed genes for evaluating anticancer activity of Chinese herbs）

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主持人：林志生 國立交通大學 生物科技學系

一、中文摘要

我們製備紫蘇、霍香、防風、刺五加葉、石蓮花及蘆薈等六種中草藥萃出物來處理人類肝癌細胞 HepG2，以分別檢測其抑制癌症細胞生長的效應。在所受測的中草藥中，紫蘇萃出物顯示具有較高抗癌的潛能。以紫蘇萃出物處理 HepG2 細胞 72 小時顯示出甚高的細胞毒性 (IC_{50} = 105 ± 8 μg/ml)，然而該萃出物對於正常的肝臟細胞株 WRL 的細胞毒性很低 (IC_{50} > 2,500 μg/ml)，我們由以 DNA 片斷化與流式細胞儀分析結果中得知紫蘇萃出物處理會誘發 HepG2 細胞凋亡。我們以 cDNA microarray 被用來探討以 105 μg/ml 紫蘇萃出物分別處理 HepG2 細胞 24、48 與 72 小時後，HepG2 細胞的基因表現態樣，晶片雜交結果經 cluster 軟體分析後顯示許多與細胞凋亡路徑有關的基因，在本實驗有顯著差異性。本研究結果顯示紫蘇萃出物具有可被純化出抗肝癌成份的價值。

關鍵詞：中草藥、細胞凋亡、HepG2 細胞、微陣列基因晶片

Abstract

Six herbal extracts, *Perilla frutescens, Agastache rugosa, Saposhnikovia divaricatae, Acanthopanax senticosus L., Echeveria elegans* and *Aloe Vera*, were prepared and used to evaluate the effect of growth inhibition on human hepatoma HepG2 cells. Among the herbs studied, the extract of *P. frutescens* exhibits the high capability of anticancer activities. The water extract of *P. frutescens* exhibited potently cytotoxic activity in HepG2 cells (IC_{50} = 105 ± 8 μg/ml) that were treated with *P. frutescens* extract for 72 h. However, the extract did not have any significant cytotoxic effect on normal liver cell line WRL (IC_{50} > 2,500 μg/ml). Significant induction of apoptosis was observed in the HepG2 cells treated with the extract by DNA fragmentation assay and flow cytometric analysis. cDNA Microarray slides were used to evaluate the gene expression profiling of HepG2 cells treated with *P. frutescens* extract at the dose of 105 μg/ml for 24, 48, and 72 h. The clustering results of cDNA microarrays indicated that numerous genes associated with the apoptosis pathways show significant difference on the expression. These findings revealed that *P. frutescens* extract is a material with the potential to be purified as an agent for treating hepatocellular carcinoma.

Keywords: Chinese herb; Apoptosis; HepG2 cells; cDNA microarray

二、前言、研究目的及文獻探討

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors

worldwide, and HCC may be the most common fatal cancer [1]. Surgical resection is potentially curative in cases of localized HCC. However, many patients with HCC have metastatic diseases; curative surgical resection is frequently not an option due to cirrhosis and other pathologic changes in the liver parenchyma [2]. Therefore, the development of a new therapeutic approach to HCC remains one of the most challenging areas in cancer research. Many Chinese herbs have been reported to suppress the proliferation of hepatoma cell lines. For example, *Sedum sarmentosum* extract may inhibit the proliferation of human hepatoma HepG2 cells [3]. Extract of *Paeoniae radix* may suppress the growth of HepG2 cells by inducing apoptosis [4]. The root of *Stephania tetrandra* S. Moore inhibits the growth of HepG2 cells, and induced apoptosis in the cells [5].

Apoptosis, also called programmed cell death, is characterized by profound morphological alterations to the cell and, specifically, the nucleus. Typical features of apoptosis are the sequential occurrence of cell shrinkage, loss of cell-cell contact, membrane blabbing, and chromatin condensation. Apoptosis appears to be implicated in the pathogenesis and therapeutic applications of cancer [6]. Apoptosis-inducing substances have been usually screened and used as anti-tumor agents *in vitro* [7, 8], *in vivo* [9], and in clinical research [10].

This investigation elucidated the effects of *P. frutescens* extract on the inhibition of proliferation and the induction of apoptosis in human hepatoma HepG2 cells by apoptosis assay and cDNA microarray technology.

三、研究方法

1. Cell culture and Cytotoxicity assay

HepG2 and normal liver cell line (WRL) were obtained from the American Type Culture Collection (ATCC). Cytotoxicity was determined by 3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

2. Apoptosis assay

The ApopLadder Ex^{TM} (TaKaRa Bio Inc., Japan) was utilized for DNA fragmentation assay (Takagi et al., 2002). In the flow cytometry analysis, the Annexin V Apoptosis Kit (Gene Research Lab. Co. Ltd., Taiwan) was used for apoptosis assay. Apoptosis Kit.

3. cDNA microarray analysis

HepG2 cells were cultured for 24, 48 and 72 h with 105 μg/ml of P. frutescens extract or with PBS. Total cellular RNAs following each treatment were extracted using Trizol Reagent (GIBCO) according to the instructions by the manufacturer. RNAs from the PBS-treated cells were labeled with cyanine 3-dUTP (Cy3-dUTP) and RNAs from the P. frutescens extract treated cells were labeled separately with cyanine 5-dUTP (Cy5-dUTP) (Cy3- and Cy5-dUTP: Amersham Pharmacia Biotech, Piscataway, NJ).

The procedure of competitive hybridization of cDNA microarray was performed following the authors' previous report. A 24 mm x 50 mm cover slip was used gently to cover the slide with 7,680 spots (UniversoChip 8K-1, Asia-Bioinnovations Co., Newark, CA).

After hybridization, the Axon GenePix 4,000A scanner (Axon Instruments Inc., Foster City, CA) that excites cyanine dyes at appropriate wavelengths (635 nm for Cy5 and 532 nm for Cy3) in high-resolution (10 micron pixel) photo multiplier tubes (PMT) was used for scanning the hybridized DNA microarray.

四、結果與討論

The cytotoxicity of the *P. frutescens* extract was evaluated from the inhibition of growth in hepatoma HepG2 cells. The growth of HepG2 cells in the presence of various concentrations of *P. frutescens* extract for 72 h was determined. The growth inhibitions of HepG2 cells were 12 ± 8%, 53 ± 12%, 78 ± 20%, 89 ± 12%, 95 ± 4% and 96 ± 2% following treatment with doses of 50, 100, 200, 300, 400 and 500 μg/ml of *P. frutescens* extract, respectively. The IC_{50} (50% growth inhibition) was 105 ± 8 μg/ml. Therefore, 105 μg/ml of *P. frutescens* extract was used in the further apoptosis assay and in the cDNA microarray study. The cytotoxicity of *P. frutescens* extract in a normal liver cell line WRL was also evaluated. However, WRL cells were much less susceptible to the cytotoxic effect of *P. frutescens* extract. The inhibition of growth of WRL cells reached only 4 ± 1% at 500 μg/ml of the extract treatment, and the IC_{50} was > 2,500 μg/ml (data not shown).

The cultivated HepG2 cells were incubated with FITC-labeled Annexin V and harvested by mechanical scraping to enable the frequency of apoptotic cells to be estimated. The cell suspensions were subsequently analyzed by flow cytometry. We plotted the Annexin V-FITC/PI analysis of such cell suspension, either not subjected to *P. frutescens* extract (control) or cultured with 105 μg/ml of *P. frutescens* extract for 24, 48 and 72 h. Apoptotic cells appeared in the Annexin V^+/PI^- fraction, whereas cells damaged by scraping appeared in the annexin V^-/PI^+ fraction. Late apoptosis or necrosis cells were evident in the Annexin V^+/PI^+ fraction. Undamaged cells remained negative for both parameters. The three independent measurements made after 24, 48 and 72 h of exposure to 105 μg/ml of *P. frutescens* extract revealed that the apoptotic cell percentages reached 18 ± 4%, 20 ± 3% and 33 ± 4%, respectively. The DNA fragment also presented evidence of apoptosis. In the DNA fragmentation assay, the isolated DNA of HepG2 cells exposed to 105 μg/ml of *P. frutescens* extract for 72 h showed the formation of the DNA ladder.

Duplicative detections of cDNA microarray were made for each treatment of HepG2 cells with 105 μg/ml of *P. frutescens* extract for 24, 48, and 72 h. A total of 7,680 genes and EST sequences were printed on the microarray (Fig. 1). After hybridization, an average of 4,800 sequences (around 70%) had signal intensities that were greater (2 folds) than background in both Cy3 and Cy5 channels in this investigation. In this study, a difference of at least a factor of two in the normalized intensity ratio (Cy5/Cy3) was regarded as significant. These genes whose differential up-regulation induced by the extract were involved in a large variety of apoptosis-related functions (Table 1). Furthermore, the clustering result indicated some up-regulated genes associated with apoptosis pathways. One of the clusters shown apoptosis-related genes, including caspase 8-apoptosis-related cysteine protease (CASP8), oncogene JUN-B (JUNB), tumor necrosis factor ligand superfamily-member 9 (TNFSF9) and FBJ murine osteosarcoma viral oncogene homolog B (FOSB), revealed up-regulated expression in a time-dependent manner (Fig. 2).

We describe the time-course studies of seven apoptosis related genes, including CASP8, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor- alpha (NFKBIA), TNFSF9, v-jun sarcoma virus 17 oncogene (JUN), JUNB, FOSB and B-cell CLL/lymphoma 2 (BCL2). The gene expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also shown as a reference. These expressed genes profiles exhibited significant up-regulation (CASP8, NFKBIA, TNFSF9, JUN, JUNB and FOSB) and down-regulation (BCL2) in the different period of HepG2 cells treated with the *P. frutescens* extract.

Many active extracts or isolated compounds from Chinese herbs, such as solamargine from *Solanum incanum* [7],

tetrandrine from *Stephania tetrandra* S. Moore [5], *Ginseng saponins* [11], Salvia Miltiorrhiza [12], *Hemsleya amabilis* [13] and *Paeonia radix* [8], have been evaluated for apoptosis induction in cancer cells. In this study, a high-density cDNA microarray was selected to differentiate the expressed genes in response to treatment with *P. frutescens* extract in HepG2 cells. From the microarray results, we found the interesting genes associated with the process of apoptosis in HepG2 cell line mediated by *P. frutescens* extract, such as CASP8, NFKBIA, TNFSF9, JUN, JUNB, FOSB and BCL2.

In the experiment herein on cDNA microarrays, CASP8, NFKBIA, TNFSF9, JUN, JUNB and FOSB were found to be up-regulated and BCL2 were discovered to be down-regulated in the HepG2 cells treated with *P. frutescens* extract. The finding supported the idea that the anti-proliferation effect of *P. frutescens* on HepG2 cells may relate to the gene-mediated apoptosis mechanism. However, the results (down-regulation of BCL2, activation of caspase, and increase of apoptotic signaling) also revealed that apoptosis induced by *P. frutescens* extract may be mediated through multiple pathways. Moreover, it is suggested that various compounds in the *P. frutescens* extract may act to induce apoptosis in HepG2 cells. In fact, the compounds such as luteolin and tormentic acid isolated from *P. frutescens* have been reported to exhibit anti-tumor promoting activity [14, 15]. However, their involvement in *P. frutescens* extract-induced apoptosis in human hepatoma HepG2 cells remains to be explored.

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六、計畫成果自評

1. The water extract of *P. frutescens* exhibits the high capability of anticancer activities by the cytotoxicity and microarray results.

2. A manuscript entitled ”Inhibiting proliferation and inducing apoptosis by *Perilla frutescens* extract in hepatoma HepG2 cells” is preparing for paper submission.

3. The result may improve our understanding of the actions of herbs with anti-tumor activities. Our ongoing work is to identify and purify the component(s) in the extract of *P. frutescens* that inhibit the growth of hepatoma HepG2 cells and to elucidate the apoptosis-inducing mechanism of the herbal medicine.

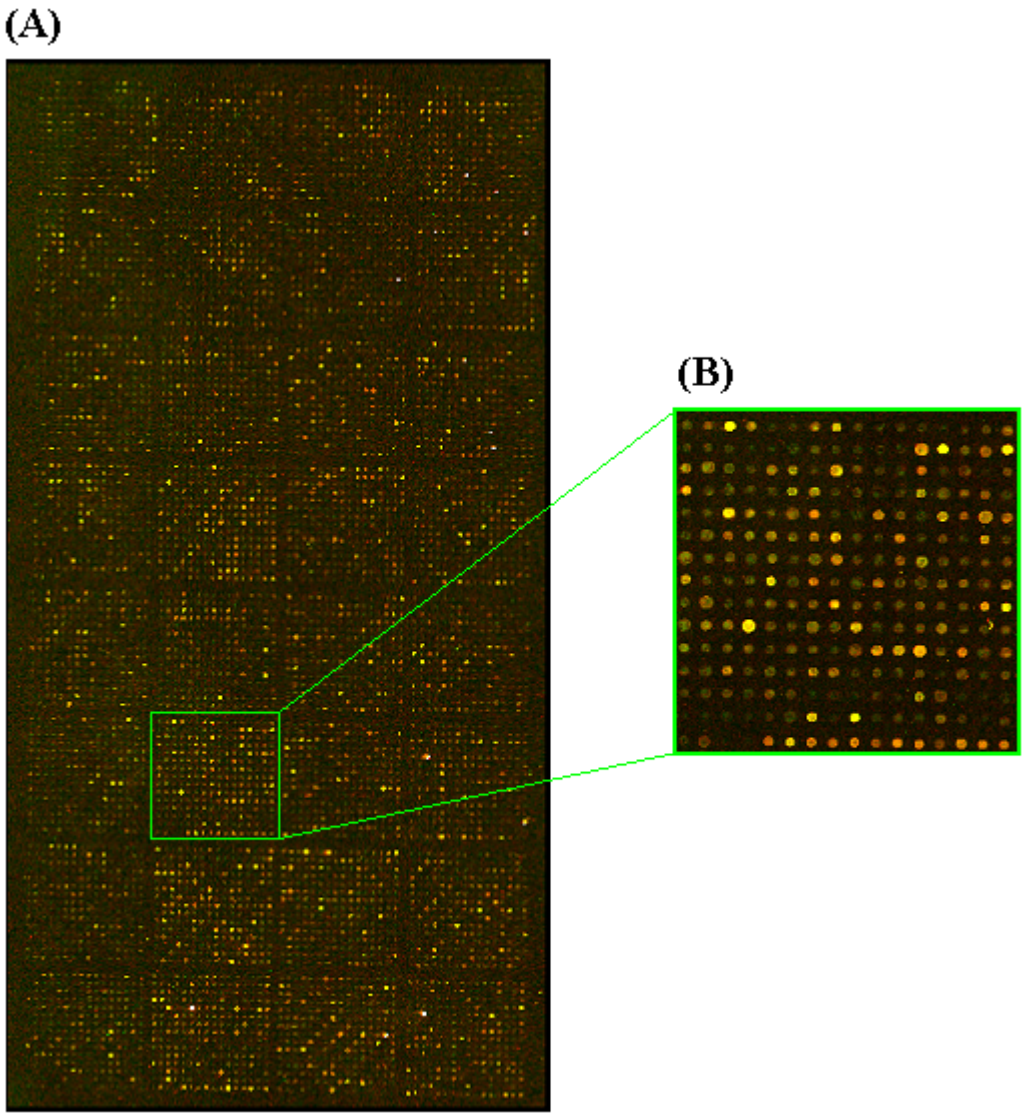

Figure 1. One of cDNA microarray data after competitive hybridization and fluorescent scanning **(A)**, and the partially raw images in the microarray **(B)**. Total cellular RNAs of the HepG2 cells treated with *P. frutescens* extract and PBS for 24 h were labeled with cyanine 5-dUTP (Cy5-dUTP) and cyanine 3 (Cy3-dUTP) by reverse transcription, respectively. The labeled cDNAs were applied on the cDNA microarray for competitive hybridization.

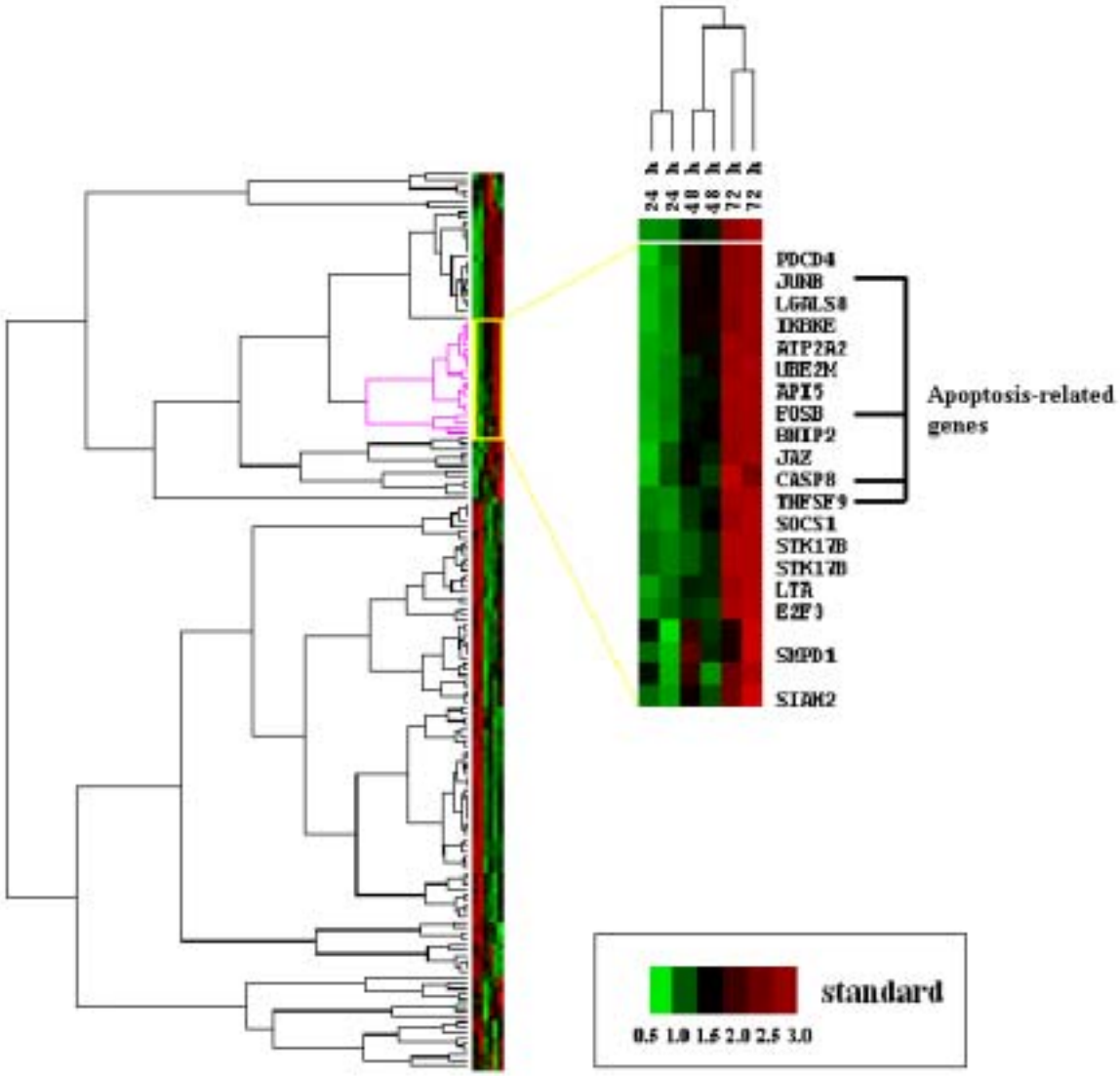

Figure 2. Partially hierarchical cluster of gene express profiles of the HepG2 cells treated with *P. frutescens* extract. The cluster was obtained using Pearson correlation and complete linkage functions provided by GeneCluster software (http://www.genome.wi.mit.edu). Cluster analysis revealed the clustering of duplicate microarray data. Parts of cluster that reveal time-dependently up-regulated genes are presented. Up-regulated CASP8, JUNB, TNFSF9 and FOSB in this cluster were associated with apoptosis. The color indicates the log of the Cy5/Cy3 ratio and the brightness increases with the magnitude the ratio. (CASP8: caspase 8- apoptosis-related cysteine protease; JUNB: oncogene JUN-B; TNFSF9: tumor necrosis factor ligand superfamily, member 9; FOSB: v-FOS FBJ murine osteosarcoma viral oncogene homolog B)

Table 1. Partial up-regulated genes related apoptosis induction in the HepG2 cells treated with the *P. frutescens* extract for 72 h.

Accession number	Gene Name	Gene Symbol	Ratio*
AA022666	Inhibitor of kappa light polypeptide gene enhancer in B-cells- kinase epsilon	IKBKE	3.41
AA282445	MAP-kinase activating death domain	MADD	3.64
AA403083	Presenilin 1 (Alzheimer disease 3)	PSEN1	2.77
AA405739	Hypothetical protein DKFZp761I2123	DKFZp761I2123	5.18
AA430573	Paxillin	PXN	4.33
AA432000	Caspase 8- apoptosis-related cysteine protease	CASP8	2.14
AA454852	Proteasome (prosome- macropain) 26S subunit-non-ATPase-2	PSMD2	3.73
AA456886	Myxovirus (influenza virus) resistance 1- interferon-inducible protein p78 (mouse)	MX1	2.55
AA460768	Protein phosphatase 1- regulatory (inhibitor) subunit 15A	PPP1R15A	4.12
AA485355	Suppressor of cytokine signaling 1	SOCS1	3.34
AA496732	ATPase- Ca^{++} transporting- cardiac muscle- slow twitch 2	ATP2A2	5.42
AA778663	Tumor necrosis factor (ligand) superfamily- member 9	TNFSF9	4.86
AA806371	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor- beta	NFκBIB	2.32
AI301262	Serine/threonine kinase 17b (apoptosis-inducing)	STK17B	5.29
N62514	BCL2/adenovirus E1B 19kDa interacting protein 2	BNIP2	2.47
N71003	Programmed cell death 4 (neoplastic transformation inhibitor)	PDCD4	3.97
N92519	E2F transcription factor 3	E2F3	3.39
N94468	Jun B proto-oncogene	JUNB	5.49
R02564	Double-stranded RNA-binding zinc finger protein JAZ	JAZ	4.74
R91570	Signal transducer and activator of transcription 4	STAT4	3.99
R97308	Lectin- galactoside-binding- soluble- 8 (galectin 8)	LGALS8	3.13
T61948	FBJ murine osteosarcoma viral oncogene homolog B	FOSB	7.64
W55872	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-alpha	NFKBIA	3.04
W72329	Lymphotoxin alpha (TNF superfamily-member 1)	LTA	2.98
W96134	V-jun sarcoma virus 17 oncogene homolog	JUN	4.94

* The ratios were calculated by Cy5 intensity/Cy3 intensity, i.e., the expression level of individual mRNA in the HepG2 cells treated with 105 μg/ml of the *P. frutescens* extract for 72 h compared with that of the cells treated with PBS. The ration > 2 folds was considered significantly different.