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Original Article

Two Chinese Herbal Regimens Safe for the Elderly on Inhibiting Liver and Bladder Tumor Cell Growth and Regulating Gene Expression[†]

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S U M M A R Y

Background: Cancers have become one of the most lethal diseases in elders. In traditional Chinese medicine, Tan-Chih-Hsiao-Yao-San (TCHYS) and Long-Daan-Shiah-Gan-Tang (LDSGT) are used to treat cancers. However, the growth-inhibitory effects and gene expression profiles of these drugs on cancer cells are still unclear.
Methods: This study assessed the effects of TCHYS and LDSGT on viability of liver and bladder tumor cells, and bladder TCCSUP cells were further subjected to profile gene expression patterns with microarray technology for identifying gene candidates that may be involved in the tumorigenesis.
Results: The results revealed that both drugs significantly eliminated the growth of Chang liver and three hepatoma cells. On the contrary, the embryonic liver WRL68 cells showed less response to the treatments, whereas the control agent genistein had much higher inhibitory effect in WRL68 cells than in the other hepatoma cells. Both TCHYS and LDSGT, as well as cisplatin and paclitaxel, exhibited dose-dependent suppression on the viability of all bladder cancer cells. To characterize the possible regulation for such effects, the profiling of gene expression was performed with complementary DNA chips. When bladder transitional cell carcinoma (TCC) TCCSUP cells were treated with TCHYS, 29 upregulated and 28 downregulated genes were detected; whereas 54 genes were upregulated in the same cells treated with LDSGT. Moreover, the detected gene expression patterns were also confirmed by using the reverse transcription-polymerase chain reaction assay.
Conclusion: This study initiates the evaluations of drug efficacies and gene expression profiles of traditional Chinese medicines, which may provide important information and identify useful biomarkers for treating cancers.
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1. Introduction

Aging involves a complex physiologic process that may lead to increased susceptibility to cancers and other diseases[1,2]. An article investigated the male-to-female incidence rate ratios for specific cancer sites and histologies for the period from 1975 to 2004 and found that urinary bladder cancer and hepatocellular carcinoma (HCC) (aged 40–59 years) are two of the largest increases of cancers[3]. Although surgical and chemotherapeutic procedures are the routine treatment approaches, many cancer patients in different stages of disease have selected treatment with alternative medicines, because there are numerous successful cases of prescribing traditional Chinese medicine (TCM) to treat cancer patients that result in substantial health improvements[4,5]. The clinical practice and therapeutic theory of TCM are completely different from those of Western medicine. Accordingly, the doctors of Chinese medicine select the appropriate herbal formulas for treating patients, after considering holistically patients' constitution, predisposition,

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stamina, as well as their physical and mental strengths against the illness. For hundreds of years, if not thousands, several kinds of traditional Chinese herbal formulas or TCM have been recommended for treating disease symptoms similar to cancers in China, Taiwan, and the Chinese-populated countries/regions. In particular, Tan-Chih-Hsiao-Yao-San (TCHYS) and Long-Daan-Shiah-Gan-Tang (LDSGT) are two of the most well known and popular herbal formulas used for treating cancers of liver, kidney, and bladder origins[6]. However, patients who have been taking anticancer herbal medicines for a long time may also develop some side effects with different levels of severity[7]. Moreover, the efficacies of these frequently prescribed medicines like TCHYS and LDSGT have never been confirmed with the modern biomedical methodology, and the complexity of TCM formulas containing many constituents makes it very difficult to standardize the preparation of the herbal products.

For years, many laboratories worldwide have participated in the isolation of pure and bioactive chemical compounds from herb mixture-decocted extracts[8]. They anticipated the identification of similar or identical compounds from the functional-related herbal medicines so that the historical medicinal theory of “curing a disease with the administration of different Chinese herbal medicines” may be confirmed; however it seems not the case. We believe that the investigation of the molecular regulatory pathways in cells is the key to elucidating efficacies of herbal drugs, because different herbs blended and decocted together may produce many unaccountable bioactive compounds, but they may only exert their activity through the limited numbers of genes or molecular pathways *in vivo*. Therefore, the original intention was to use functional genomic technology to assess gene expression patterns in a variety of tumor cell lines treated with two clinical application-alike Chinese herbal drugs, TCHYS and LDSGT, both of which have been suggested to be anticancer.

Aging increases incidence of cancers and TCM is used for a long time to treat cancers. Because CHYS and LDSGT are commonly prescribed TCMs used for promoting health; their safety has been accepted for decades. Therefore, those regimens may be beneficial to the elderly with cancers who may not tolerate toxic regimens. The present study uses the cell line model to test the effects of TCHYS and LDSGT on inhibiting the growth of liver and bladder tumors, which have been reported to be susceptible to drug treatments clinically, and compare with those exerted by the modern anticancer agents such as genistein, cisplatin, and paclitaxel. Because both TCMs showed promising efficacies on all bladder transitional cell carcinoma (TCC) cell lines tested, bladder TCCSUP cell line was further subjected to profile gene expression patterns with microarray technology, which resulted in identifying many gene candidates that may be involved in the tumorigenesis of urothelial cell carcinoma. In summary, the significance of this study is to initiate the systematic and scientific evaluations of TCM, whose molecular and cellular bases of treating human diseases remain largely unknown.

2. Materials and Methods

2.1. Materials and herbal drug preparations

Molecular biological enzymes were mainly purchased from Stratagene (La Jolla, CA, USA) unless otherwise specified, whereas standard chemicals and reagents were obtained from Sigma (St. Louis, MO, USA). All reagents were used according to the recommendations of the manufacturers. Both TCHYS and LDSGT extracts were prepared and provided by Koda Pharmaceutics, Ltd (Taoyuan, Taiwan), a pharmaceutical company that conforms to Good Manufacturing Practice and is approbated by the Taiwan government. The herbal formulas and their preparation protocols are described in Table 1. When preparing LDSGT, Mutong (*Caulis akebiae*, Koda Pharmaceutics), but not Guamutong (*Aristolochia manshuriensis* Kom, Koda Pharmaceutics), was used so as to avoid possible toxicity of aristolochic acid that is produced by incorrect processing of Guamutong[9,10]. Three other anticancer compounds, genistein, cisplatin (PLATINOL-AQ, Bristol Myers Oncology, Princeton, NJ), and paclitaxel (TAXOL, Bristol Myers Oncology), were also used for comparative studies. The stock solution of genistein (Sigma, St Louis, MO, USA, 50 mM) was prepared in pure DMSO (Sigma) and was later diluted to the appropriate concentrations with Dulbecco's Modified Eagle medium (DMEM) before use. The other two drugs were produced by the same pharmaceutical company (Bristol-Myers Squibb Co, New York, NY, USA), and made as aqueous reagents. All the above drugs (TCHYS, LDSGT, genistein, cisplatin, and paclitaxel) and diluting solvents (DMSO, water, and cell culture media) were prepared and handled under the endotoxin-free environment in our laboratories.

2.2. Cells and cell culture

Five human liver cell lines, including one Chang Liver cell line, three HCC (equal to hepatoma) HA22T/VGH, Hep3B and HepG2 cell

Table 1
Component herbs of TCHYS and LDSGT

Drugs	Component herbs/amounts	Extraction processes
TCHYS	Baishaoyao (*Paeoniae lactiflora*)/4 g Baizhu (*Atractylodis macrocephalae*)/4 g Chaihu (*Bupleurum chinense*)/4 g Danggui (*Angelicae sinensis*)/4 g Fuling (*Poria cocoa*)/4 g Ginger root (*Zingiber officinale*)/4 g Mudanpi (*Moutan radicis*)/2.5 g Peppermin (*Mentha haplocalyx*)/2 g Shanzhi (*Gardenia jasminoides*)/2.5 g Zhigancao (*Glycyrrhizae praeparatae*)/2 g	The herb mixtures (total weight of 33 g) were decocted under the endotoxin-free environment with 1.5 L of water to produce 7 g of extract paste containing 57.3% moisture.
LDSGT	Chaihu (*B chinense*)/4 g Cheqianzi (*Semen plantaginis*)/2 g Danggui (*A sinensis*)/2 g Huangqin (*Scutellaria baicalensis*)/2 g Longdancao (*Gentiana scabra*)/4 g Mutong (*Caulis akebiae*)/2 g Shanzhi (*G jasminoides*)/2 g Shengdihuang (*Rehmania glutinosa*)/2 g Shenggancao (*Glycyrrhiza uralensis*)/2 g Zexie (*Rhizoma alismatis*)/4 g	The herb mixtures (total weight of 26 g) were decocted under the endotoxin-free environment with 1.5 L of water to produce 5 g of extract paste containing 66.1% moisture.

Table 2
Alternations in gene expression levels in TCCSUP cells treated with TCHYS

	Genes for	Gene products	Hs.ID	Acc. No	Biological functions
Upregulated genes					
1.	AHCYL1	S-Adenosylhomocysteine hydrolase-like 1	Hs.4113	AA179513	One-carbon compound metabolic process
2.	AKAP1	A kinase (Adenosine 5′ monophosphate-activated protein kinase, PRKA) anchor protein 1	Hs.78921	R37961	RNA/protein binding
3.	AOP2	Antioxidant protein 2	Hs.120	T65419	(1) Lipid catabolism (2) Oxidative stress
4.	BCR	Breakpoint cluster region	Hs.234799	R51875	(1) Signal transduction (2) Protein phosphorylation
5.	BRCA2 homolog	EST1669, highly similar to BRCA2	NA	NA	NA
6.	CCNE	Cyclin E	NA	NA	(1) Cell cycle (2) Cell division
7.	CEBPG	CCAAT/enhancer binding protein (C/EBP), gamma	Hs.2227	AA179189	(1) Differentiation (2) Transcription
8.	E2F4	E2F transcription factor 4, p107/p130-binding	Hs.108371	R59900	Transcription
9.	EIF4B	Eukaryotic translation initiation factor 4B	Hs.93379	AA173296	Regulation of translation
10.	ELK1	ELK1	NA	NA	Regulation of transcription
11.	EPRS	Glutamyl-prolyl-tRNA synthetase	Hs.55921	T47357	tRNA aminoacylation
12.	ESTs 4002	NA	Hs.79308	R51324	NA
13.	ESTs	Weakly similar to alternatively spliced product using exon 13A	NA	NA	NA
14.	ESTs 6051	NA	Hs.164352	T57042	NA
15.	HERC1	HECT and RCC1 (CHC1)-like domain 1	Hs.76127	AA181680	(1) Protein modification (2) Transport
16.	HMGCR	3-Hydroxy-3-methylglutaryl-coenzyme A reductase	Hs.11899	R51541	(1) Biosynthetic process (2) Development (3) Oxidation reduction
17.	IRF-6	IRF-6	Hs.11801	NA	NA
18.	MLN62	MLN62	NA	NA	(1) Apoptosis (2) Development (3) Signal transduction
19.	NA6106	NA6106	NA	AA063310	NA
20.	NUP210	KIAA0906 protein	Hs.56966	R37724	(1) Transport (2) Translocation
21.	PKIA homolog	ESTs, highly similar to PKIA	Hs.3407	NA	NA
22.	RALBP1	RalA binding protein 1	Hs.75447	AA085619	GTPase activator
23.	RCN1	Reticulocalbin 1	Hs.167791	AA114081	Calcium ion binding
24.	RPL27	Ribosomal protein L27	Hs.111611	AA190881	Translational elongation
25.	SLC16A1	Solute carrier family 16 (monocarboxylic acid transporters), member 1	Hs.75231	R49543	Transport
26.	SOX22	SRY (sex-determining region Y)-box 22	Hs.43627	AA283123	Transcription
27.	TNNI2	Troponin I, skeletal, fast	Hs.83760	AA181380	Transcription
28.	UBE2H	Ubiquitin-conjugating enzyme E2H	Hs.28505	N35796	Protein ubiquitination
29.	UBXD2	KIAA0242 protein	Hs.77495	H10657	Response to unfolded protein
Downregulated genes					
30.	C4A	Complement component 4A	Hs.170250	R37128	Complement activation
31.	COH1	KIAA0532 protein	Hs.21077	R37311	NA
32.	CRABP1	Cellular retinoic acid-binding protein 1	Hs.7678	W95693	(1) Development (2) Signal transduction (3) Transport
33.	DLX4	Distal-less homeobox 4	Hs.172648	R32170	(1) Development (2) Transcription
34.	DTNA	Dystrobrevin, alpha	Hs.54435	H09172	Signal transduction
35.	ESTs 3978	NA	Hs.6262	T89093	NA
36.	EST	Homo sapiens mRNA full length insert cDNA clone EUROIMAGE 248114	Hs.8963	R33043	NA
37.	ESTs	Weakly similar to C44C1.2	Hs.10463	AA088448	NA
38.	EST	*Homo sapiens* clone 23808 mRNA sequence	Hs.12537	R39535	NA
39.	ESTs 5577	NA	Hs.21124	R20560	NA
40.	EST	*Homo sapiens* cDNA FLJ20562 fis, clone KAT11992	Hs.107444	H09790	NA
41.	ESTs 5606	NA	Hs.113137	R60530	NA
42.	FEZ1	Fasciculation and elongation protein zeta 1 (zygin I)	Hs.79226	H09157	(1) Axon guidance (2) Cell adhesion
43.	FGB	Fibrinogen, B beta polypeptide	Hs.7645	W94873	(1) Signal transduction (2) Protein polymerization
44.	HPCL2	2-Hydroxyphytanoyl-CoA lyase	Hs.63290	R41792	Fatty acid alpha-oxidation
45.	MKP-1	MAP kinase phosphatase-1	Hs.109895	NA	(1) Protein dephosphorylation (2) Cell cycle (3) Oxidative stress
46	MPI	Mannose phosphate isomerase	Hs.75694	H15442	Carbohydrate metabolic process
47.	MXI1	MAX-interacting protein 1	Hs.118630	AA115514	(1) Transcription (2) Cell proliferation

Table 2 (*continued*)

Genes for		Gene products	Hs.ID	Acc. No	Biological functions
48.	NA1337	NA1337	NA	R98174	NA
49.	NCL	Nucleolin	Hs.79110	H05666	Angiogenesis
50.	NELL1	NEL (chicken)-like 1	Hs.21602	H10041	Cell adhesion
51.	OAZIN	Antizyme inhibitor	Hs.223014	R32814	Polyamine biosynthetic process
52.	PR48	Protein phosphatase 2A 48 kDa regulatory subunit	Hs.124942	W88679	NA
53.	PSG7	Pregnancy-specific beta-1-glycoprotein 7	Hs.173609	R35240	Female pregnancy
54.	PSMA6	Proteasome (prosome, macropain) subunit, alpha type 6	Hs.74077	H06487	Ubiquitin-protein activity
55.	ROR1	Receptor tyrosine kinase-like orphan receptor 1	Hs.274243	H10667	(1) Protein phosphorylation (2) Transmembrane receptor protein tyrosine kinase activity
56.	RPS9	Ribosomal protein S9	Hs.180920	H22514	(1) Cell proliferation (2) Translational elongation
57.	TAF1B	TATA box binding protein-associated factor, RNA polymerase I, B, 63 kDa	Hs.121044	R32478	Regulation of transcription

The Hs. ID numbers identify the genes sorted in the NCBI UniGene database in NIH, USA, whereas the Acc. No are the official identification numbers for the genes stored in databases. Gene numbers 5 (BRCA homolog), 7 (CEBPG), and 8 (E2F4) were chosen for the semiquantitative reverse transcription-polymerase chain reaction confirmation on the gene expression patterns (Fig. 4).
Acc. No = accession numbers; cDNA = complementary DNA; NA = nonavailable.

lines, one embryonic WRL68 cell line, and three bladder TCC T24, TSGH-8301 and TCCSUP cell lines, were obtained from Bioresources Collection and Research Center (Hsinchu, Taiwan). All of the cells were routinely cultured in Dulbecco's-modified Eagle's medium, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (these tissue cultural reagents were from Gibco, Grand Island, NY, USA), under 5% CO_2 at 37°C.

2.3. *Cell viability assay and microarray hybridization*

The growth rates of tumor and normal cell lines were determined by cell proliferation assay using a tetrazolium compound and phenazine ethosulfate provided in a commercial reagent package (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Kit, Promega; Madison, WI, USA). When performing assays, approximately 5,000 cells were seeded into the wells of 96-well culture plates, followed by the addition of 100 μL of culture medium containing various concentrations of TCHYS, LDSGT, genistein, cisplatin, or paclitaxel, as indicated in the figures. The cells were incubated for 72 hours before being applied to the cell proliferation assaying reagents according to the protocol of the Kit. The production of complementary DNA (cDNA) microarray (cDNA chip) containing 6,371 human genes/expression sequence tags followed the protocols described previously[11–13]. The experiments of profiling gene expression patterns were performed at least three times with mRNA samples derived from bladder TCC TCCSUP cells treated with 1.0 mg/mL of TCHYS or LDSGT extract individually for 0, 1, 2, 6, 12 and 24 hours. In every experiment, 4 μg of mRNA were subjected to one-step cDNA conversion and biotin labeling, followed by hybridizing to cDNA chips, as described previously[11–13]. The colorimetric detection procedure produces a blue image when the biotin-labeled cDNA targets hybridize to human gene probes spotted on cDNA chips. The darker the color, the higher the transcriptional levels of the target genes in the cells are. The cDNA-chip image can be computerized using a high-resolution scanner (PowerLook 3000, UMAX; Taipei, Taiwan) and then digitized using a Web site available (www.microarrays.org/) program ScanAlyze[14].

2.4. *Reverse transcription-polymerase chain reaction verification of gene expression patterns*

Although all cDNA microarray hybridization experiments had been performed three times to obtain the consensus lists of genes with altered gene expression patterns (Tables 2 and 3), the expression levels for the genes of interest were subjected to experimental verification by a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) method described earlier[11,15]. In the present study, the genes for BRCA2 homolog, CEBPG (accession number AA179189) and E2F4 (accession number R59900), which show altered expression patterns at various time points during the treatment of TCCSUP cells with TCHYS, are selected for the confirmative experiments. The amplification primer sets used were (1) the BRCA2 homolog gene: 5′-GTG GCACCAAATACGAAACA-3′ and 5′-CCCGATACACAAACGCTGAG-3′; (2) the CEBPG gene: 5′-TCGGGAATTGTGAGGTGACT-3′ and 5′-AACA GCTGCAGAATGGACTT-3′; and (3) the E2F4 gene: 5′-GCAGGGGAA CAGGACTCAGC-3′ and 5′-AGGTGGGGCCAATCATCAGG-3′. In all experiments, the amplification of the β-Actin gene was used to normalize the amount of RNA used. RT-PCR of these genes was performed at the cycles when the detected amplicon molecules were in the exponential increasing stage. After performing many tests to correlate amounts of the PCR product with number of thermocycles (data not shown), we used 20 amplification cycles for the β-Actin gene and 27 cycles for other three genes. The RT-PCR products of the target genes were then visualized by agarose gel electrophoresis in the presence of ethidium bromide, followed by fluorescent intensity quantifications and comparisons.

2.5. *Statistical analysis*

The results are expressed as the mean ± standard derivations from at least three experiments. Statistical comparisons were based on the Student t test or analysis of variance. Differences were considered significantly at $p < 0.05$. All statistical analyses were carried out using SigmaStat software (Jandel Scientific, San Rafael, CA, USA).

3. Results

3.1. *Inhibitory effects of TCHYS and LDSGT on hepatoma cell lines*

The components and the extraction procedures of TCHYS and LDSGT were listed in Table 1 and the yield of these two drugs were about 21.6% and 19.2%, respectively. TCHYS and LDSGT extracts were first tested for their growth inhibitory capability on nonmalignant liver epithelial (Chang Liver) and liver embryonic (WRL68), hepatoma (HA22T/VGH, Hep3B, and HepG2) cell lines. The results

Table 3
Alternations in gene expression levels in TCCSUP cells treated with LDSGT

	Genes for	Gene products	Hs. ID	Acc. No	Biological functions
Upregulated genes					
1.	AKT2 homolog	Highly similar to Rac-beta serine/threonine kinase	NA	NA	NA
2.	BIP	BiP	Hs.95008	X87949	NA
3.	CAPN2	Calpain, large polypeptide L2	Hs.76288	R39609	Proteolysis
4.	CCT2	Chaperonin containing TCP1, subunit 2 (beta)	Hs.6456	R12806	Protein folding
5.	CD138	CD138	Hs.82109	NA	NA
6.	CDC37	CDC37 (cell division cycle 37, *Saccharomyces cerevisiae*, homolog)	Hs.160958	R17767	(1) Protein targeting (2) Cyclin-dependent protein kinase (3) IFN-mediated signaling
7.	CFTR	Cystic fibrosis transmembrane conductance regulator, ATP-binding cassette (subfamily C, member 7)	Hs.663	AA515982	Respiratory gaseous exchange
8.	CGI-119	CGI-119	Hs.25615	AA133459	Protein binding
9.	CHI3L2	Chitinase 3-like 2	Hs.154138	T65854	Chitin catabolic process
10.	CKS2	CDC28 protein kinase 2 (CKS2) mRNA	NA	NA	(1) Cell division (2) Cell proliferation (3) Signal transduction (4) Spindal organization
11.	CLTA	Clathrin, light polypeptide (Lca)	Hs.104143	T65963	Transport
12.	COL4A4	Collagen, type IV, alpha 4	Hs.180828	H68555	Strengthening of neuromuscular junction
13.	CPG2 homolog	EST, highly similar to CPG2 protein	Hs.43398	N23629	NA
14.	CRABP1	Cellular retinoic acid-binding protein 1	Hs.7678	H92182	(1) Development (2) Signal transduction (3) Transport
15.	CYP4F3	Cytochrome P450, subfamily IVF, polypeptide 3 (leukotriene B4 omega hydroxylase)	Hs.106242	AA099667	(1) Leukotriene metabolic process (2) Oxidation reduction
16.	DF	D component of complement (adipsin)	Hs.155597	AA037782	NA
17.	EST	NA	NA	AA070188	NA
18.	EST	NA	Hs.8469	T40769	NA
19.	EST	Weakly similar to hypothetical protein	Hs.31848	AA069144	NA
20.	EST	Weakly similar to RCN1_human reticulocalbin 1 precursor	Hs.39619	H73236	NA
21.	EST	Weakly similar to T20B12.3	Hs.95196	H61179	NA
22.	EST	KIAA1285	Hs.119175	AA031699	NA
23.	EST	NA	Hs.123069	AA043364	NA
24.	EST	NA	Hs.193771	T95064	NA
25.	EXT2	Exostoses (multiple) 2	Hs.75334	R39966	(1) Cell cycle (2) Signal transduction (3) Biosynthesis (4) Ossification
26.	FKBP4	FK506-binding protein 4 (59 kDa)	Hs.848	H21077	Protein folding
27.	HLA-DPB1	Major histocompatibility complex, class II, DP β1	Hs.814	N23695	Antigen processing and presentation, MHC II
28.	HSD11B2	Hydroxysteroid (11-beta) dehydrogenase 2	Hs.1376	T49293	(1) Glucocorticoid biosynthesis (2) Oxidation reduction
29.	IGF2R	Insulin-like growth factor 2 receptor	Hs.76473	R40651	(1) Transport (2) Signal transduction
30.	ITGB1	Integrin beta-1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12) mRNA	Hs.166373	NM_002211	(1) Adhesion (2) Integrin-mediated signaling pathway
31.	IVD	Isovaleryl-coenzyme A dehydrogenase	Hs.77510	R38739	Oxidation reduction
32.	KCNJ13	Potassium inwardly rectifying channel, subfamily J, member 13	Hs.11364	H97186	Potassium ion transport
33.	MARCKS	Myristoylated alanine-rich protein kinase C substrate	Hs.75607	AA031774	(1) Actin filament binding (2) Calmodulin binding
34.	MDH2	Malate dehydrogenase 2, NAD (mitochondrial)	Hs.111076	H19304	(1) Glycolysis (2) TCA cycle (3) Oxidation reduction
35.	METTL1	Methyltransferase-like 1	Hs.42957	R17664	tRNA modification
36.	MMP7	Matrix metalloproteinase 7 (matrilysin, uterine)	Hs.2256	AA031514	(1) Collagen catabolism (2) Proteolysis
37.	NPM1	Nucleophosmin (nucleolar phosphoprotein B23, numatrin)	Hs.173205	R37231	(1) Antiapoptosis (2) Transport (3) Signal transduction (4) Stress (5) Cell proliferation
38.	OLFR91 homolog	ESTs, moderately similar to olfactory receptor-like protein	Hs.269151	H69005	NA
39.	PFKP	Phosphofructokinase, platelet	Hs.99910	NA	Glycolysis
40.	POLR2E	Polymerase (RNA) II (DNA directed) polypeptide E (25 kD)	Hs.24301	AA031493	(1) Transcription (2) mRNA splicing (3) Interspecies interaction
41.	PPA2267 homolog	ESTs, highly similar to 6-phosphofructokinase, type C	Hs.99910	NA	NA

Table 3 (*continued*)

Genes for		Gene products	Hs. ID	Acc. No	Biological functions
42.	PRKAA2 homolog	ESTs, highly similar to 5′-AMP-activated protein kinase, catalytic α2 chain	NA	NA	NA
43.	QDPR	Quinoid dihydropteridine reductase	Hs.75438	R38198	(1) Metabolism (2) Oxidation reduction
44.	RAC1	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	Hs.173737	R37122	(1) Structure morphogenesis (2) Cell adhesion (3) Cell motion (4) Signal transduction (5) Hydrogen peroxide metabolism
45.	RB1CC1	KIAA0203 gene product	Hs.50421	R38102	(1) Cell cycle (2) Transcription
46.	RPL28	Ribosomal protein L28	Hs.4437	AA035400	Translational elongation
47.	RPS15A	Ribosomal protein S15a	Hs.2953	R33435	(1) Cell cycle (2) Cell proliferation (3) Translational elongation
48.	SERPINBS	Cyclin H assembly factor	NA	NA	Cell motion
49.	STAT1	Signal transducer and activator of transcription 1, 91 kDa	Hs.21486	AA035362	(1) IKK/NF-kB cascade (2) Caspase activity (3) Interspecies interaction (4) Transcription (5) Tyrosine phosphorylation
50.	TIAF1	TGFB1-induced antiapoptotic factor 1	Hs.75822	AA035188	(1) IKK/NF-KB cascade (2) Apoptosis
51.	TPP2	Tripeptidyl peptidase II	Hs.1117	R39682	Proteolysis
52.	VIL2	Villin 2 (ezrin)	Hs.155191	AA068995	(1) Cytoskeletal anchoring (2) Adhesion (3) Cell shape
53.	XRCC5	X-ray repair complement defective repair in Chinese hamster cells 5 (Ku autoantigen, 80 kDa)	Hs.84981	H17933	(1) Double-strand break repair (2) Viral infection (3) Telomere maintenance
54.	YWHAQ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation	Hs.74405	H11374	Transcription

The Hs. ID numbers identify the genes sorted in the NCBI UniGene database in NIH, USA, whereas the Acc. No are the official identification numbers for the genes stored in databases.
Acc. No = accession numbers; NA = nonavailable; NAD = nicotine adenine dinucleotide; TCA = tricarboxylic acid.

revealed that the viability of WRL68 cells remained unchanged when being treated with 1.5 mg/mL or less of either drug, whereas the cell viability just reduced slightly (to about 80% growth rate of mock-treated cells) when the maximal concentration of 2.0 mg/mL was used (Fig. 1A). On the other hand, the growths of all other liver cell lines were mildly stimulated by TCHYS and LDSGT at low concentrations (1.0 mg/mL or less of either drug), ranging from 110 to 130% of the mock-treated cells (Fig. 1B–E). When being treated with drug of higher concentrations (greater than 1.5 mg/mL TCHYS or at 2.0 mg/mL LDSGT), the growth rate of Chang Liver cells reduced dramatically to 10–20% (Fig. 1B). In the malignant hepatoma or HCC cells, high concentration (2.0 mg/mL) of these two drugs significantly decreases the cell viability (Fig. 1C–E).

In parallel, an isoflavone and tyrosine protein kinase inhibitor, genistein, which had been reported to be effective in treating bladder tumor cells[11,12,15], was used as the control agent. The data showed inhibitory effect on WRL68 cell growth, as the cell viability could readily be suppressed to 40% of the mock-treated cells at the concentration greater than 20 μM (Fig. 1A). For the other liver and hepatoma cells, Chang Liver, HA22T, and Hep3B cells, genistein at high concentration of 80 μM has only less than 25% inhibitory effect (Fig. 1B–D). Furthermore, there was almost no response with genistein treatment in hepatoma HepG2 cells (Fig. 1E).

3.2. *Inhibitory effects of TCHYS and LDSGT on human bladder TCC cell lines*

In contrast to human liver and hepatoma cell lines, both TCHYS and LDSGT had marked inhibitory effects on growth of all bladder TCC cell lines tested in this study (Fig. 2A and B). All T24, TCCSUP, and TSGH-8301 cell lines exhibited dose-dependent reductions in growth rates responding to the treatments of increasing concentrations of TCHYS or LDSGT (from 0.6 to 2.0 mg/mL), and the levels could be reduced to less than 20% growth rate of untreated cells. The positive control drug cisplatin can also dramatically reduce the growth rates of all bladder TCC cell lines to less than 10% at the concentrations of as low as 10 μg/mL (Fig. 2C). However, T24, TCCSUP, and TSGH-8301 cells exhibited somewhat dose-dependent growth inhibitory patterns at lower concentrations of paclitaxel, from 0.001 to 0.1 μM. With greater amounts (>0.1 μM) added to the culture medium, their growth rates reduced to different levels (Fig. 2D), with the TSGH-8301 cell line being most susceptible to the inhibition (to less than 30% growth rate of mock-treated cells), followed by TCCSUP cells (to about 50%) and the least responsive T24 cells (only to 80%).

3.3. *Profiling of gene expression patterns in TCCSUP cells treated with TCHYS or LDSGT*

Among all cell lines treated with TCHYS and LDSGT, the bladder tumor TCCSUP cell line exhibited relatively the best, most similar growth inhibitory responses (down to about 30%) to both drugs (at 1.0 mg/mL concentrations), and was thus chosen for further profiling of gene expression patterns by cDNA-chip hybridization/colorimetric detection. Approximately 0.9% (57 genes selected in TCHYS experiments, Table 2) and 0.8% (54 genes in LDSGT group, Table 3) of the total 6,371 genes spotted on cDNA chip exhibited substantial altered gene expression levels. There are 29 and 28 genes whose expression levels were induced and repressed, respectively, when TCCSUP cells were treated with TCHYS (Fig. 3A and B); whereas only gene expression inductions were detected, 54 of them, in LDSGT-treated cells (Fig. 3C and D). The genes identified here may be involved in inhibiting bladder tumor cell growth that warrants further investigation.

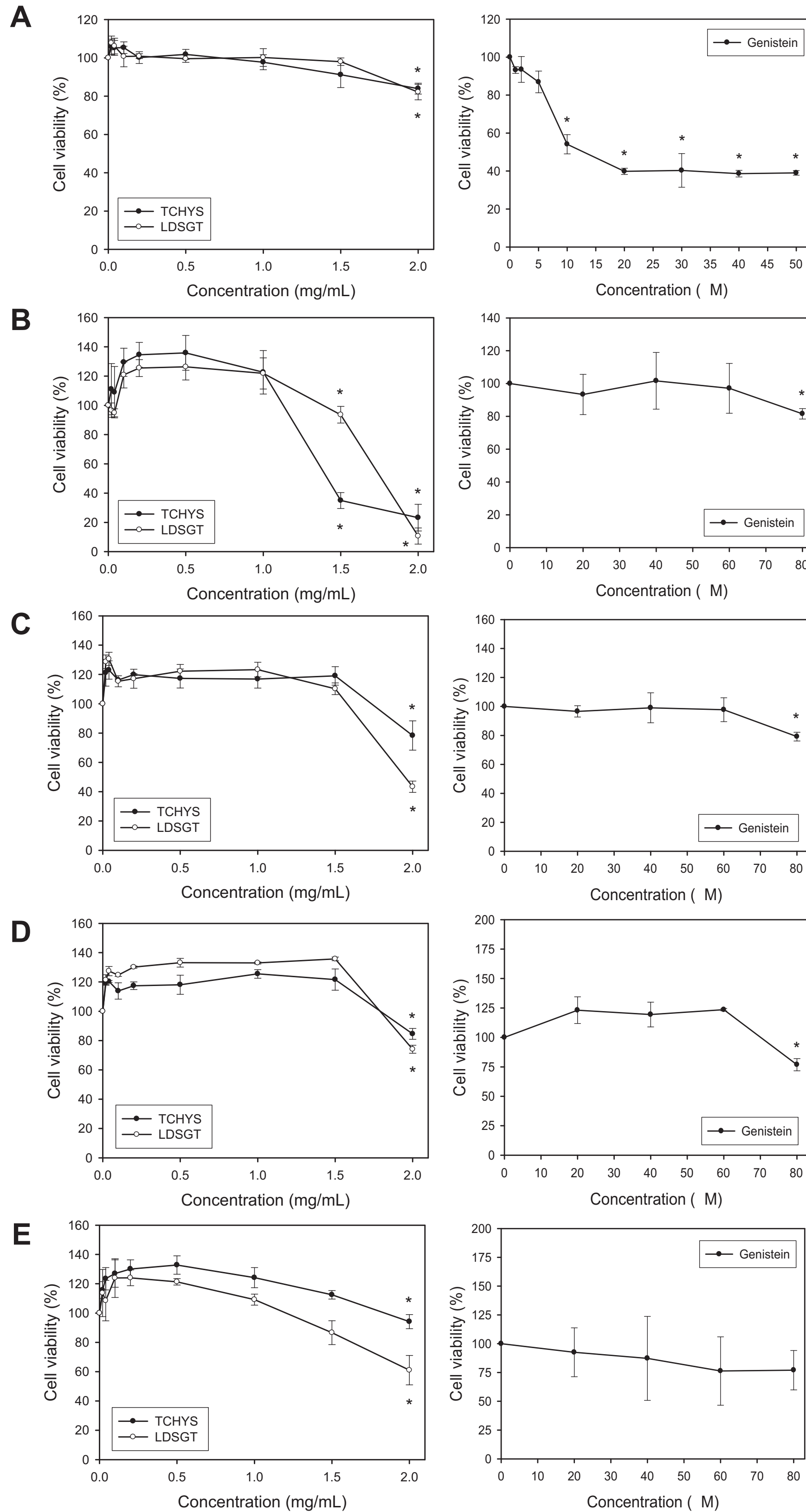

Fig. 1. Growth inhibitory effects of TCHYS and LDSGT extracts on liver cell lines. Viabilities of (A) WRL68, (B) Chang Liver, (C) HA22T/VGH, (D) Hep3B, and (E) HepG2 liver cells treated with TCHYS, LDSGT, or genistein are shown. Approximately 5,000 cells were treated with the indicated concentrations of the TCHYS or LDSGT extract (0–2.0 mg/mL) or genistein (0–50 μM for WRL68 cells or 0–80 μM for other cell lines), followed by assaying cell growth rates. Experiments in the present study are performed at least three times, and the results are shown as means ± standard deviations. *Significance ($p < 0.05$ by the *t* test), as the tested values are statistically compared with those of mock-treated cells for the two drugs or cells treated with 1% DMSO, the solvent to dissolve genistein. LDSGT = Long-Daan-Shiah-Gan-Tang; TCHYS = Tan-Chih-Hsiao-Yao-San; DMSO.

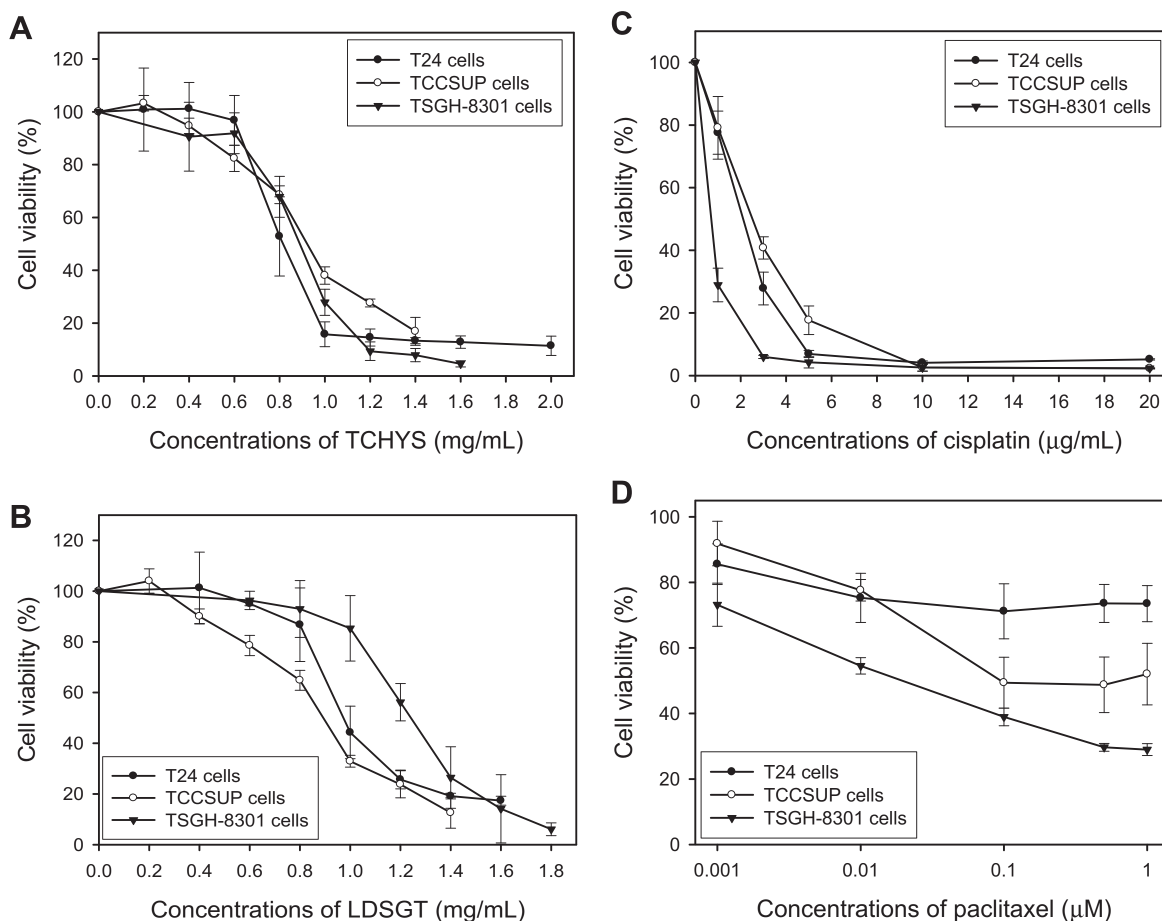

Fig. 2. Growth inhibitory effects of TCHYS and LDSGT extracts on bladder cell lines. Viabilities of T24, TCCSUP, and TSGH-830 bladder transitional cell carcinoma (TCC) lines treated with (A) TCHYS, (B) LDSGT, (C) cisplatin, and (D) paclitaxel were assessed. Approximately 5,000 cells were treated with different concentrations of TCHYS, LDSGT, and the positive control drugs cisplatin and paclitaxel, followed by cell viability assays to reveal the growth inhibitory effects. The concentrations used here are: 0–2.0 or 1.8 mg/mL with increasing intervals of 0.2 mg/mL for the TCHYS or LDSGT extract, respectively; 0–20 μg/mL with increasing intervals of 2.0 μg/mL for cisplatin; and 0–1 μM for paclitaxel. Experiments in the present study are performed at least three times, and the results are shown as means ± standard deviations. *Significance ($p < 0.05$ by the t test), as the tested values are statistically compared with those of mock-treated cells. LDSGT = Long-Daan-Shiah-Gan-Tang; TCHYS = Tan-Chih-Hsiao-Yao-San.

3.4. Confirmation of gene expression patterns by semiquantitative RT-PCR assay

To verify the detected gene expression patterns, semiquantitative RT-PCR was used to check the transcription levels of three genes of interest identified previously. Fig. 4A shows that, basically, the expression levels for the BRAC2 homolog, CEBPG, and E2F4 genes (Gene Nos. 5, 7, and 8, respectively) are all upregulated in TCCSUP cells by the TCHYS treatment of 0–2 hours, and then decrease. Thus, the time-course changes detected by RT-PCR are similar to those detected by cDNA chips (Table 2 and Fig. 4B–D), especially at the early time points (0–6 hours posttreatment) of drug treatment to TCCSUP cells. These data suggest that the results obtained from cDNA microarray profiling gene expression patterns are faithful.

4. Discussion

In Taiwan, the prevalence of cancers, including hepatoma and bladder TCC, also increases rapidly, and it has become one of the most lethal diseases in elders[1]. In this study, we tested the inhibitory effect of TCHYS and LDSGT, two common clinically prescribed TCM for promoting health, on the growth rates of four human liver and three bladder tumor cell lines (Figs. 1 and 2). The purpose of this study was to (1) examine the efficacies of herbal medicines with the current bioassay technique using the cell line model and (2) profile the gene expression patterns that may lead to the discovery of the molecular pathway responsible for the effects of these herbal drugs. We detected that the liver cell lines did not respond well to the treatment of either herbal extracts at the concentration ranges tested, whereas both drugs suppressed the growth of all three bladder TCC cell lines in a dose-dependent manner. In parallel, this study used several control compounds in the same assaying methods, and one of the results indicates that genistein and the tested TCM extracts exert their anticancer functions on different regulatory pathways because both TCHYS and LDSGT inhibited efficiently the growth of bladder TCC T24 cell line, which overexpressed the oncogenic H-Ras that rendered the resistance to the treatment of genistein[15]. Furthermore, the present study also tested two other control anticancer drugs cisplatin, which causes cross-linking of nucleic acid molecules *in vivo*, and paclitaxel, which inhibits microtubules assembling, that were manufactured to treat patients with bladder, breast, and/or other tumors in clinics[16,17]. These two drugs were found to inhibit the growth rates of the tested bladder TCC cell lines to different levels.

The mystery of TCM is that they are usually composed of many herbal components which can only be efficacious when decocted with the appropriate amount of water or ethanol for several hours. As one may have known, TCHYS and LDSGT each contain 10 herbs

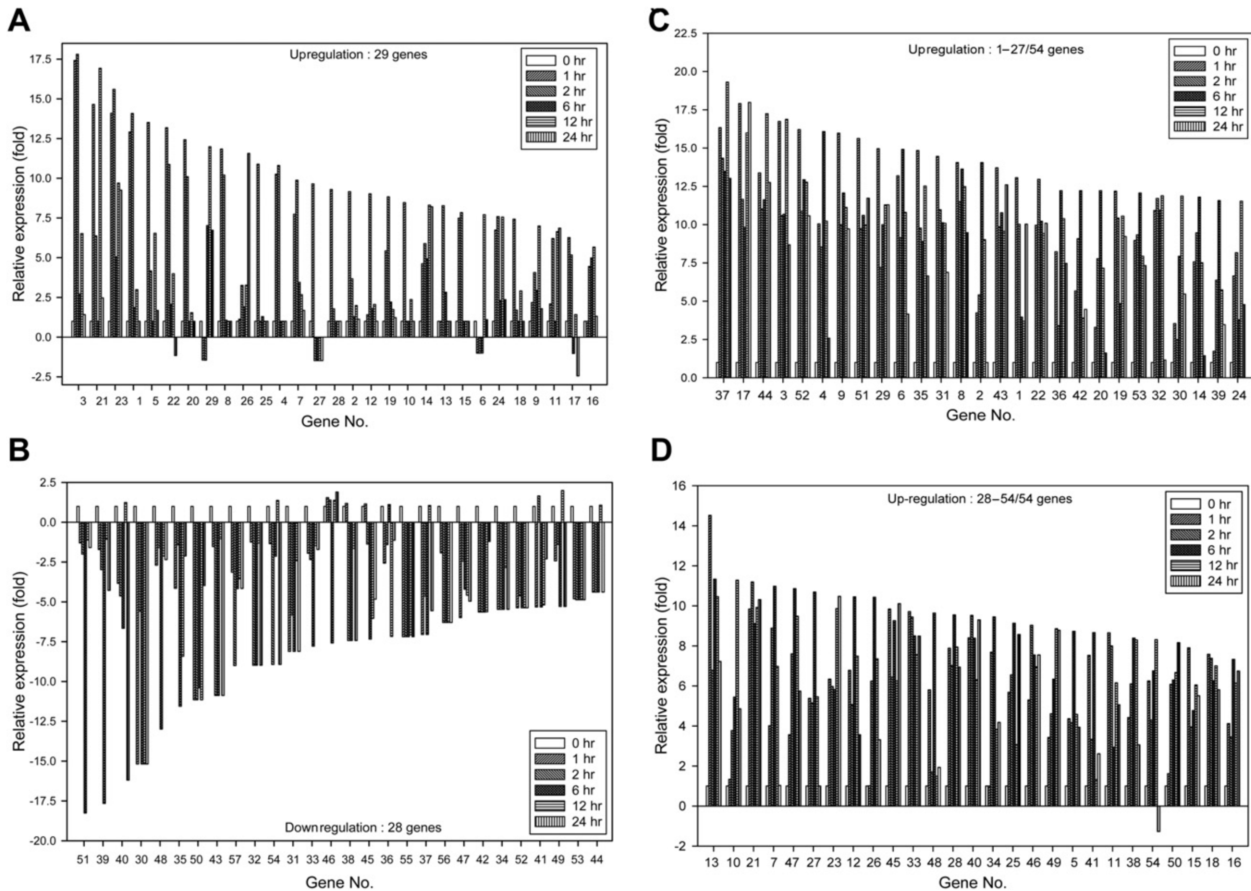

Fig. 3. Gene expression alternations in TCCSUP cells treated with TCHYS and LDSGT. The expression-level plots of (A) upregulated genes and (B) downregulated genes in TCHYS-treated cells. Briefly, the digitalized signals for all genes on complementary DNA chips detected at six time points (0–24 hr) were compared with those at time 0 to yield the fold changes at different time points. Panels (C) and (D) show the genes with induced expression patterns, during the treatment of bladder transitional cell carcinoma (TCC) TCCSUP cells with LDSGT for a period of 24 hr. The identified genes, with their Gene numbers (No.) assigned in Table 2 or 3, are sequentially arranged from the higher gene expression levels to the lower ones. LDSGT = Long-Daan-Shiah-Gan-Tang; TCHYS = Tan-Chih-Hsiao-Yao-San.

including three identical and seven specified herbs (Table 1); and yet they are used to treat different as well as similar symptoms clinically. Chaihu, Danggui, and Shanzhi are the three identical herbs used in both TCHYS and LDSGT. Extracts from Chaihu (*Bupleuri chinese*) may be effective as a therapeutic agent to treat hepatomas only, because it increased mitochondria membrane potential collapse in HepG2 cells, but not in lymphocytes[18]. Danggui (*Angelicae sinensis*) has been reported to possess immunomodulatory functions, as the treatment of Danggui-containing TCM to cancer patients, who were receiving chemotherapy and/or radiotherapy, showed improvement in their immune functions[5]. Finally, geniposide, an iridoid glucoside extracted from Shanzhi (*Gardenia jasminoides*), was an effective hypoglycemic agent, by inhibiting the hepatic glycogen phosphorylase and glucose-6-phosphatase activities, in diabetic mice[19]. The Shanzhi extract has also been reported to possess antigastritic, antioxidant, acid-neutralizing, and anti-*Helicobacter pylori* activities[20]. It is worthy to note that when we prepared LDSGT, Mutong (*Caulis akebiae*), instead of Guamutong (*Aristolochia manshuriensis* Kom), was used so as to avoid the toxic aristolochic acid produced by incorrect processing of Guamutong[11,12]. In fact, described in a recently published Chinese Internal Medicine textbook[21], TCHYS is suggested repeatedly for patients suffering from a variety of symptoms, such as abdominal/gastric distention, poor appetite, anorexia, headache, and irregular menstruation of dysmenorrhea for women; whereas LDSGT is prescribed on 10 different occasions to treat illnesses manifesting soreness of lower limbs, distending pain, insomnia, low fever, anxiety, irritability, loss of hair, and inability to have penis erection with frequent nocturnal emission for men. Therefore, it seems that these TCMs have versatile therapeutic applications and the molecular and cellular basis of such efficacies merit further investigation.

On examining symptoms that can be released by TCHYS and LDSGT, many similarities are found. This remains a puzzle in our clinical practice of TCM; how can different herbal drugs, which comprise many different herbs, be used for treating similar illnesses. Therefore, the present study aimed to use functional genomic technology to assess gene expression patterns in a variety of liver and bladder tumor cell lines treated with TCHYS and LDSGT, which are clinical application-alike TCM and prescribed to patients suffering from similar cancers. It is hoped that similar gene expression patterns could be detected to account for their similar therapeutic effects. Studies with gene chips demonstrated that a number of genes were indeed induced to alter their gene expression levels in TCCSUP bladder tumor cells treated with either TCHYS or LDSGT (Tables 2 and 3 and Fig. 3). The results provide important information that can further be subjected to advanced functional (anticancer) analyses. However, the gene expression patterns obtained from treating TCCSUP cells with the two drugs produced totally unrelated results, indicating that the data obtained here are still insufficient for a definitive conclusion; and therefore more gene expression profiling with different TCM is required to reveal the possible common molecular pathways mediating the similar therapeutic effects. Meanwhile, the genes whose expressions were

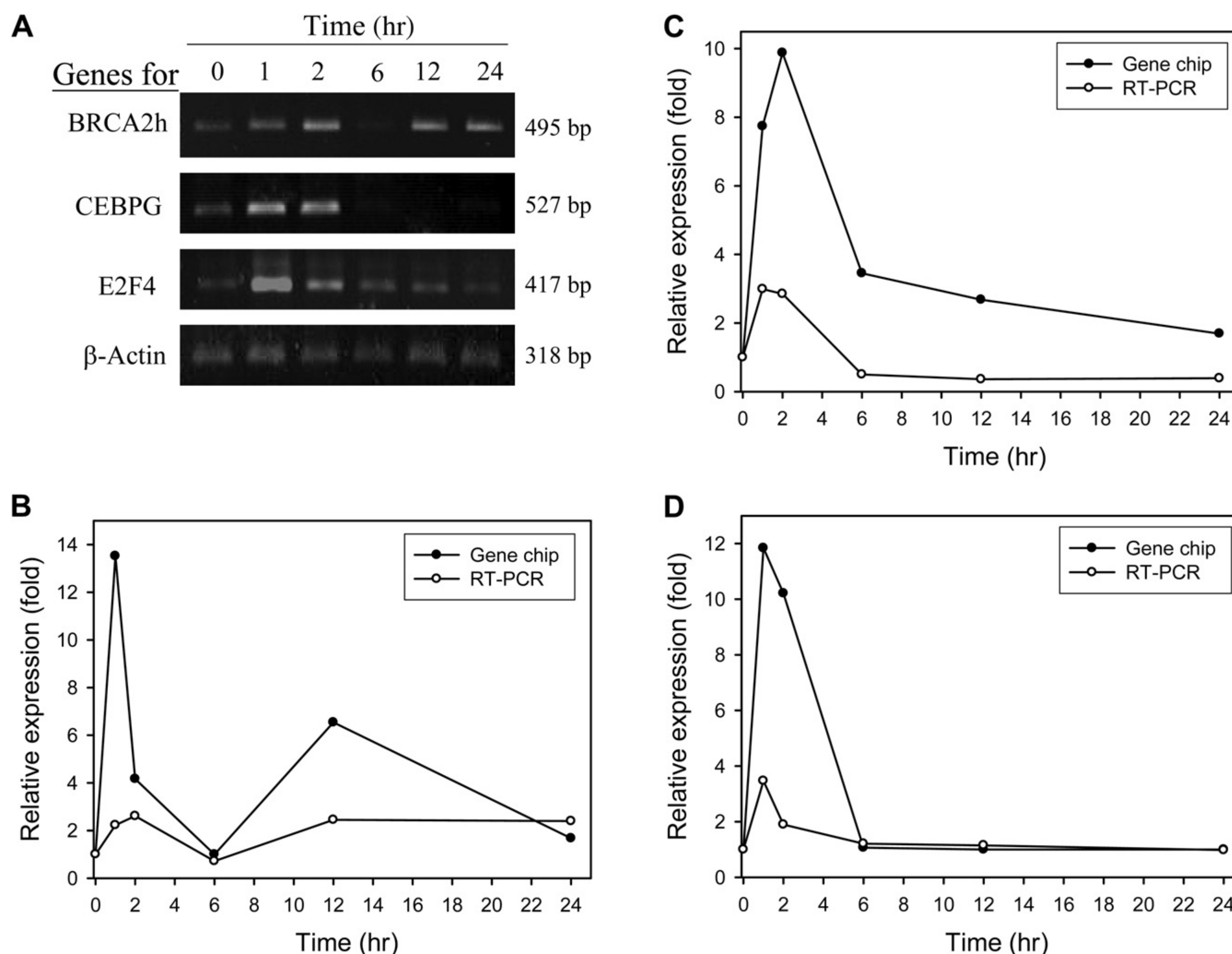

Fig. 4. Semiquantitative RT-PCR confirmation of the expression patterns detected by complementary DNA microarrays. (A) Semiquantitative RT-PCR and agarose gel electrophoretic assays on the genes for BRCA2 homolog, CEBPG, and E2F4 were performed. The comparisons of expression patterns for the (B) BRCA2 homolog, (C) CEBPG, and (D) E2F4 genes detected by RT-PCR and microarray analysis are shown. In the present study, appropriate number of TCCSUP bladder cells were treated with 1.0 mg/mL TCHYS for indicated periods of time (0–24 hr), followed by total RNA isolation. Then, 1.0 μg each of the RNA samples were reverse transcribed to become biotin-complementary DNA. The expression kinetics of the three genes was subjected to semiquantitative RT-PCR analysis, and the amplification of β-actin was used to normalize the amount of RNA used in every experiment. The RT-PCR products were visualized by 2% agarose gel electrophoresis in the presence of ethidium bromide. Experiments in this study are performed at least three times, and all experiments show similar results. RT-PCR = reverse transcription-polymerase chain reaction; TCHYS = Tan-Chih-Hsiao-Yao-San.

altered during the treatment of TCHYS and LDSGT may be useful to scientists who are currently working in the fields of identifying crucial genes involved in suppressing tumor cell growths.

To verify whether the detected gene expression patterns were reliable, semiquantitative RT-PCR was used in this study for confirming the transcription levels of three genes of interest identified in TCCSUP cells treated with TCHYS (Fig. 4). The data suggest that the results of cDNA-chip profiling gene expression patterns are faithful, and therefore microarray technology can be readily applied not only to gain insights into the molecular mechanism, but also to discover the biomarkers associated with the efficacies of TCM. Biomarkers are substances used as indicators of a biologic state in organisms, and the tumor biomarkers have now been constantly used to evaluate the clinical performances including cures and recurrences, and they have also become more and more important in cancer diagnostic and prognostic assessments[22]. For example, Elongation Factor 1 alpha, a pAkt-interacting protein, has been successfully demonstrated by Pecorari et al.[23] as a biomarker for monitoring the proliferation, survival, and invasion of breast cancer cells. Therefore, our study also initiated to set up the routine platform for identifying TCM-derived tumor biomarkers using microarray technology and the cell line model.

In summary, this report describes the testing of the efficacies of two traditional Chinese herbal medicines on liver and bladder tumor cell lines and the profiling gene expression patterns in TCCSUP cells treated with these two drugs. CHYS and LDSGT are commonly used for promoting health and may be beneficial to the elderly with cancers who may not tolerate toxic regimens. To help herbal drug-related functional genomics and bioinformatics further develop and become more useful, we appeal to all scientists currently conducting or having conducted microarray researches related to Chinese herbal medicines to join forces by comparing, combining, and posting gene expression patterns on the public domain. Not only can this accelerate the identification of tumor biomarkers, but also foster the discovery of candidate genes/pathways that mediate similar effects from different herbal drugs.

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