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Genome Wide Expression Analysis of the Effect of the Chinese Patent Medicine Zilongjin Tablet on Four Human Lung Carcinoma Cell Lines

Ping Zhang,[1] Xin Wang,[2] Songjin Xiong,[3] Shaoping Wen,[3] Song Gao,[2] Lei Wang[4,5,6,7] and Boyang Cao[4,5,6,7]*

[1]Tianjin Zhongxin Pharmaceutical Group Co., Ltd, 17# Zhongxin Building, Baidi Road, Nankai District, Tianjin 300193, China
[2]Tianjin Long Shun Rong Pharmaceutical Factory of Tianjin Zhongxin Pharmaceutical Group Co., Ltd, 21# the 10th Street, TEDA, Tianjin 300457, China
[3]Tianjin Biochip Corporation, 23# HongDa Street, TEDA, Tianjin 300457, China
[4]TEDA School of Biological Sciences and Biotechnology, Nankai University, 23# HongDa Street, TEDA, Tianjin 300457, China
[5]Key Laboratory of Molecular Microbiology and Technology of the Ministry of Education, College of Life Sciences, Nankai University, Tianjin 300071, China
[6]Tianjin Research Center for Functional Genomics and Biochips, TEDA College, Nankai University, Tianjin 300457, China
[7]Tianjin Key Laboratory of Microbial Functional Genomics, TEDA College, Nankai University, 23# HongDa Street, TEDA, Tianjin 300457, China

Zilongjin (ZLJ) tablet, which is a traditional Chinese medicine, has been approved as a new anti-tumor drug by the State Food and Drug Administration of China; however, its anti-cancer mechanisms remain elusive. The goal of this study was to investigate the underlying anti-cancer activities of ZLJ tablet *in vitro*. In this study, four lung cancer cell lines, A549, H446, H460 and H520, were treated with 2.2 mg/mL of ZLJ solution for 24 h at 37 °C under 5% CO_2. RNA was isolated and a microarray experiment using the Affymetrix Human Genome U133 plus 2.0 Array was employed to differentiate the expression patterns of cancer-related genes after drug treatment. Of 483 genes in 63 functional categories and 25 different pathways that showed at least a 2-fold change of expression level in the four cancer cell lines, 170 genes were upregulated, and 313 genes were downregulated. Eleven of the 483 genes were cancer-related and belong to the three known pathways: apoptosis, cell cycle regulation and mitogen-activated protein kinase (MAPK) cascade. The microarray data were validated by real-time RT-PCR. The results of this investigation suggest possible anti-cancer mechanisms of the ZLJ tablet, and lay a foundation to further analyse its therapeutic roles. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: Zilongjin tablet; traditional Chinese medicine; lung cancer; gene expression; microarray.

INTRODUCTION

Lung cancer is the most deadly cancer in both men and women throughout the world. The American Cancer Society reports that approximately 219000 people, or 103000 women and nearly 116000 men, are diagnosed with lung cancer in the USA each year. The U.S. National Cancer Institute calculates that approximately one of every 14 men and women in the USA will be diagnosed with cancer of the lung or airways during their lifetime. The National Cancer Institute's statistics indicate that lung cancer is predominantly a disease of the elderly; almost 70% of the patients are over 65 years of age, and less than 3% of the cases occur in people under the age of 45 years. There are two main types of lung cancer: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) (Watanabe *et al.*, 2010). About 8–9 of 10 cases of lung cancer are NSCLC. NSCLC is further divided into the following three subtypes on the basis of the size, shape and chemical make-up of the cancerous cells: adenocarcinoma, squamous cell carcinoma and large-cell (undifferentiated) carcinoma (Stinchcombe and Socinski, 2009).

Emerging evidence has suggested that traditional Chinese medicine is effective for cancer treatment (Ji *et al.*, 2004; Moalic *et al.*, 2001; Mujoo *et al.*, 2001; Parton *et al.*, 2001; Wang *et al.*, 2001; Wu *et al.*, 2002; Yang *et al.*, 2010; Tian *et al.*, 2010). Zilongjin (ZLJ) tablet, which is a traditional Chinese medicine containing the herbs *Radix Astragali*, *Radix Angelicae sinensis*, *Solanum lyratum* and *Solanum nigrum*, has been approved as a new antitumor drug by the State Food and Drug Administration of China (License No. Z20010064). As a supplementary drug for lung cancer patients undergoing actinotherapy and chemotherapy, the tablet helps to increase the assessment score of the patient's clinical symptoms and physical strength, improves the immunity index of the NK and CD4 cells, and minimizes the side effects of chemotherapy such as liver and kidney injury (Li *et al.*, 2004; Wang and Yan, 2008). Conventional expression profiling methods such as northern blot, reverse transcription-polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA), *in situ* hybridization and immunohistochemistry are useful for the analysis of one or a few

* Correspondence to: Boyang Cao, TEDA School of Biological Sciences and Biotechnology, Nankai University, 23# HongDa Street, TEDA, Tianjin 300457, China.
E-mail: boyangcao@nankai.edu.cn

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genes. However, DNA microarrays have been developed in response to the need for a high-throughput, efficient and comprehensive detection of the expression of all genes (Schena *et al.*, 1995, 1996), and, therefore, are particularly suitable for determining the effects of herbal drugs and extracts since they modulate multiple targets simultaneously in a complex system (Chavan *et al.*, 2006). The GeneChip Human Genome U133 plus 2.0 Array (Affymetrix, Santa Clara, CA, USA) covers over 47000 transcripts and is the first and most comprehensive whole human genome expression array.

To investigate the anticancer activities and the underlying mechanisms of ZLJ, four human lung cancer cell lines (A549, H446, H460 and H520) were treated with ZLJ, and analysed for their gene expression profile by using Affymetrix Human Genome U133 plus 2.0 Array. The results suggest that the drug significantly inhibited cancer cell growth and induced cancer cell apoptosis.

MATERIALS AND METHODS

Cells and culture media. The human lung adenocarcinoma A549 cell line, the squamous cell carcinoma H520 cell line, the NSCLC H460 cell line and the SCLC H446 cell line were obtained from the Chinese Academy of Medical Sciences. A549 was grown in Ham's F-12 medium, and H460, H520 and H446 were grown in RPMI-1640 medium. Both media were supplemented with 10% fetal bovine serum (FBS) and 100 units/mL penicillin at 37 °C in an incubator containing 5% CO_2.

Preparation and sterilization of ZLJ solution. The ZLJ tablet, which was provided by Tianjin Long Shun Rong Pharmaceutical Factory of Tianjin Zhongxin Pharmaceutical Group Co., Ltd (Tianjin, China), was dissolved in the cell culture media at a concentration of 66.0 mg/mL. Aliquots of 1.2 mL were placed in 1.5 mL Eppendorf tubes and sterilized by a Co60-γ ray for 4 h at a dosage of 4 kGy.

MTT assay. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich Corp., St Louis, MO, USA) assay was performed as described previously (Wang *et al.*, 2001). Briefly, 10^6 cells were seeded in a 100 mm diameter petri dish with serial concentrations of 0, 1.65, 2.2, 3.3 or 6.6 mg/mL ZLJ solution), 2% FBS and 100 units/mL penicillin. The cells were incubated at 37 °C for 24 h in a humidified atmosphere with 5% CO_2. The solutions were then removed, and 9 mL of the media and 1 mL of MTT (250 mg/mL) were added to the cells and incubated at 37 °C for 4 h. Each experiment was repeated with each concentration six times. The medium was removed and 4 mL of DMSO was added. Then 100 μL of the solution was used for the optical density (OD) measurements at 550 nm by using an Ultramark microplate imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The growth inhibition was determined using the following formula: Growth inhibition = (Control OD – Sample OD)/Control OD (Yin *et al.*, 2004).

Total RNA isolation. Treated and untreated cells were collected after 24 h incubation. RNA extraction was performed with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions with minor modifications. In brief, the cells were lysed directly by adding 3 mL of TRIzol to the 10 cm diameter dish. Then, 1 mL of the solution was transferred into a 1.5 mL tube, and 200 μL of chloroform was added. The upper aqueous phase was removed to a fresh tube, 0.5 mL of isopropyl alcohol was added, and the solution was incubated at room temperature for 10 min and centrifuged at 12000 × **g** for 10 min in order to precipitate the total RNA. The RNA pellet was washed with 75% ethanol, air-dried, redissolved in 30 μL of diethylpyrocarbonate (DEPC)-treated water, and separated by denaturing agarose gel electrophoresis with formamide and MOPS. The OD_{260}/OD_{280} ratio was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) for quality control. The RNA was purified using a Qiagen RNeasy total RNA cleanup protocol [Qiagen China (Shanghai) Co., Ltd, Shanghai, China] and quantitated using the spectrophotometer.

Microarray GeneChip expression. The microarray experiment was performed according to the GeneChip® 3′ IVT Express Kit protocol (Affymetrix Inc., Santa Clara, CA, USA). For each sample, 100 ng of purified RNA was reverse transcribed using T7-(dT) 24 primers containing a T7 RNA polymerase promoter to generate the first-strand complementary DNA (cDNA), which was then converted into a double-stranded cDNA template. The DNA template was used to transcript cRNA and incorporates a biotin-conjugated nucleotide. Then, 10 μg of the cRNA of each sample was hybridized with the Affymetrix Human Genome U133 plus 2.0 Array for 16 h at 45 °C with constant rotation at 60 rpm according to the Affymetrix protocol. After hybridization, the genechip was washed and stained in an automated fluidics station (Affymetrix). The arrays were then transferred to a confocal scanner (Affymetrix) and scanned twice at an emission wavelength of 570 nm and a resolution of 2.5 μm. The intensity of the hybridization for each probe pair was computed by the Affymetrix GeneChip Operating Software (GCOS) Version 1.4.

Data processing and analysis. Data analysis was performed using the DNA-Chip Analyser (dChip) with a model-based computation (Li and Wong, 2001) (version 2009). All arrays were normalized to a common baseline array having a median overall brightness. After the normalization, the expression level of each gene in all of the samples was computed using the direct average of the Perfect Match-MisMatch (PM-MM) differences of all of the probes in a probe set. The expression profile of each individual cancer cell line was analysed separately. *E* stands for the expression value of the gene in the drug-treated experimental group, and *B* stands for the expression value of the gene in the non-drug-treated control group. A combined analysis was also performed in order to identify the genes that showed the same expression patterns in all four cancer cell lines. The genes with significant expression were identified after the following steps: (a) genes with absent calls were filtered out; (b) *E*/*B* was >2 or *B*/*E* was >2; (c) two group *t*-test results had *p* values less than 0.05; and, (d) when the fold change

varied among the four cancer lines, the average value was used.

Real-time RT-PCR reaction. Twelve pairs of primers were designed for RT-PCR quantitation, and these included 11 pairs that targeted the 11 differentially expressed genes in the microarray or cyclin E2 (CCNE2), cyclin G2 (CCNG2), cyclin-dependent kinase inhibitor 1A (CDKN1A), cyclin-dependent kinase inhibitor 2C (CDKN2C), growth arrest and DNA-damage-inducible, alpha (GADD45A), helicase, lymphoid-specific (HELLS), hematological and neurological expressed 1-like (HN1L), jun oncogene (JUN), minichromosome maintenance complex component 6 (MCM6), transcription factor Dp-2 (TFDP2), and X-linked inhibitor of apoptosis (XIAP). In addition, one gene pair of β-actin was used as an internal control (Table 1). The reverse transcription was performed with a PrimeScript RT reagent Kit (Takara Bio Inc., Shiga, Japan) in 20 μL of reaction mixture consisting of 0.8 μg of RNA, 1× PrimeScript buffer, 20 μM random 6-mers, 2.5 μM oligo dT primer and 1 μL of PrimeScript RT Enzyme Mix I. The reaction parameters included 37 °C for 15 min and 85 °C for 5 s. The quantitative real-time PCR experiment was conducted with Power SYBR® Green PCR Master Mix (Applied Biosystems., Warrington, UK) in 20 μL of reaction mixture that consisted of 1 μL of cDNA, 10 μL of Sybr mix, 0.5 μL of primer F and 0.5 μL of primer R. The PCR cycling conditions included 40 cycles of 95 °C for 10 s and 60 °C for 30 s using the ABI7300 Real-Time PCR System. Fold changes were calculated using the formula $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct$ is ΔCt (drug treatment) − ΔCt (background), ΔCt is Ct (target gene) − Ct (β-actin), and Ct is the threshold cycle (Whyte *et al.*, 2007).

RESULTS

ZLJ inhibited A549 growth

The effect of ZLJ solution on cancer cell growth was examined in the A549 human lung cancer line. Under the experimental conditions used in the 24 h treatment, ZLJ exhibited a marked inhibitory and dose-dependent effect on the growth of A549 cells (Fig. 1). The growth inhibition of A549 cells by 0.8, 1.6, 2.2, 3.3 and 6.6 mg/mL of ZLJ was 22.35% (SD, 5.27%), 37.26% (3.48%), 53.10% (1.94%), 56.05% (5.47%) and 87.16% (1.21%), respectively. A ZLJ concentration of 2.2 mg/mL was selected and applied to the other three cancer cell lines, H460, H446 and H520, for treatment.

ZLJ treatment stimulated the expression of a large number of genes in cancer cells

Four RNA samples were obtained from the human lung cancer cell lines (A549, H520, H446 and H460) after

Table 1. Microarray data validation by real-time PCR

Pathway	Gene		Primer[a] (5′ - 3′)	Average fold change[b] RT-PCR	Average fold change[b] Microarray
Apoptosis	HELLS	F	AATATGAAGTGCCGTCTAATCAGG	0.32	−3.69
		R	GAAGTCATCAAATACATCTGGCAAC		
	JUN	F	CGGACCTCCTCACCTCGC	17.54	11.76
		R	CGAACCCCTCCTGCTCATC		
	XIAP	F	TGCTAATTTTCCAAGTGGTAGTCC	1.72	5.85
		R	AACTGCTGAGTCTCCATATTGCC		
Cell_cycle	MCM6	F	AGCTCAGAGATGAGGAACAGACAG	0.27	−4.05
		R	GCCATGTATAGTAGGGAACAGGC		
	CDKN2C	F	GATTTGGAAGGACTGCGCTG	0.13	−11.71
		R	TCTGGCCGCATCATGAATG		
	CCNE2	F	CATCTATGCTCCTAAACTCCAAGAG	0.14	−5.17
		R	ACCAGGAGATGATTGTTACAGGAC		
	HN1L	F	ACCTCAGAACATACCCAAGAGGA	0.36	−3.67
		R	AGAGACCCAAAAATGTCGCTG		
	TFDP2	F	AGATAGAGAAGCAGAGGCGGATAG	0.35	−3.92
		R	ATTATGAATGGCAGCTGAATGGTA		
	CCNG2	F	TGCCAAAGTTGAAGATTTAAGGAG	2.26	2.95
		R	CAGCCAGCAAAAAAGAACAGAC		
	GADD45A	F	GATCACTGTCGGGGTGTACGA	25.82	9.79
		R	GGATCAGGGTGAAGTGGATCTG		
	CDKN1A	F	TCCTGCCCAAGCTCTACCTTC	7.07	4.35
		R	GACAGTGACAGGTCCACATGGTC		
MAPK_Cascade	JUN	F	CGGACCTCCTCACCTCGC	17.54	11.76
		R	CGAACCCCTCCTGCTCATC		
Control	β-actin	F	ACATTGGCAATGAGCGGTTC		
		R	CGGATGTCCACGTCACACTTC		

[a]F, forward primer; R, reverse primer.

[b]For microarray, '+' indicates gene expression level was upregulated; '−' indicates gene expression was downregulated. For real-time RT-PCR, >1 indicates gene expression was upregulated; <1 indicates that gene expression was downregulated.

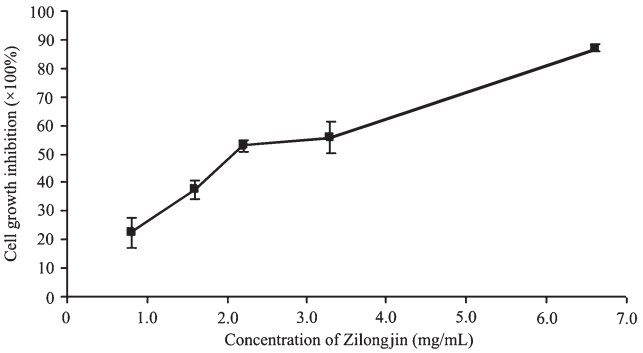

Figure 1. Growth inhibitory effect of ZLJ on A549 human lung cancer cells. Cells were treated with indicated concentrations of ZLJ for 24 h and the growth inhibition was determined by MTT assay.

treatment with 2.2 mg/mL of drug for 24 h. The samples were separated by denaturing agarose gel electrophoresis, which exhibited two sharp and distinct bands at 18S and 28S. The OD_{260}/OD_{280} ratios of the RNAs had a range of 1.9–2.1. The gene expression profile data of the four human lung cancer cell lines treated with and without ZLJ were acquired using Affymetrix Human Genome U133 plus 2.0 Array. Genes with at least a 2-fold change in their expression level were analysed in each cell line. Among the total 54600 genes spotted in the microarray, the number of differentially expressed genes in each cell line were 3724 (6.8%) for the A549 cell line, 5562 (10.2%) for the H520 cell line, 3521 (5.1%) for the H446 cell line and 2765 (6.4%) for the H460 cell line.

Identification of the differentially expressed cancer-related genes after drug treatment

The gene expression profiles of the four cancer cell lines after drug treatment were generated using the Affymetrix Human Genome U133 plus 2.0 Array and examined using dChip (version 2009). A total of 483 genes was found to be changed significantly in all four of the treated cancer cell lines. Among them, 313 genes were downregulated and 170 genes were upregulated. Using a hierarchical clustering analysis, the expression pattern of the 483 differentially expressed genes were compared and classified into 63 functional categories utilizing the biological annotation database Gene Ontology (GO), as shown in Table 2. Most of the 483 genes were related to cellular physiology (44.5%), metabolism (43.6%), intracellular organelles (30.2%), membrane-bound organelles (28.4%), nucleus (21.9%), regulation of biological process (19.2%), regulation of cellular process (18.8%) or regulation of physiological process (18.2%). Further analysis showed that these genes participated in 25 different pathways, and among them, 13 of the genes belonged to three known cancer-related pathways: apoptosis, cell cycle regulation, and wnt signaling, as shown in Table 3. Six of the 11 genes, HELLS of the apoptosis pathway, CCNE2, CDKN2C, HN1L, MCM6 and TFDP2 of the cell cycle pathway, were downregulated, and the remaining five, JUN and XIAP of the apoptosis pathway, CCNG2, CDKN1A and GADD45A of the cell cycle pathway, and JUN of the mitogen-activated protein kinase (MAPK) cascade pathway, were upregulated.

Validation of microarray data by real-time RT-PCR

Real-time quantitative RT-PCR was conducted to validate the microarray data. Eleven differentially expressed genes were selected (i.e. CCNE2, CCNG2, CDKN1A, CDKN2C, GADD45A, HELLS, HN1L, JUN, MCM6, TFDP2 and XIAP). In general, there was a good correlation between the microarray data and the real-time RT-PCR data (Table 1), RT-PCR, JUN, XIAP, CCNG2, GADD45A and CDKN1A were consistently upregulated, and the rest, HELLS, MCM6, CDKN2C, CCNE2, HN1L and TFDP2, were consistently downregulated.

DISCUSSION

The gene expression patterns of the four ZLJ-treated lung cancer cells indicated that the herb medicine exhibited pharmacological effects through multiple mechanisms. JUN and XIAP, the apoptotic pathway genes, were upregulated after ZLJ treatment in all four cancer lines, which is consistent with a report that c-Jun *N*-terminal kinase (JNK) can upregulate XIAP-associated factor 1 (XAF1) and induce apoptosis or differentiation in gastrointestinal cancers (Wang *et al.*, 2009).

GADD45 genes, including GADD45A, GADD45B and GADD45G, are responsible for growth arrest and DNA damage. It has been reported that the ectopic expression of the GADD45-like proteins induced apoptotic cell death as well as activating JNK and p38 pathways (Takekawa and Saito, 1998). It was also observed that a major target of breast cancer 1 (BRCA1), a human tumor suppressor gene, is the DNA damage-responsive gene GADD45. Induction of BRCA1 triggers apoptosis through activation of c-Jun *N*-terminal kinase/stress-activated protein kinase (JNK/SAPK), a signaling pathway potentially linked to GADD45 gene family members (Harkin *et al.*, 1999). In addition, GADD45A has been implicated in several cellular functions, including MAPK signaling, cell cycle regulation, DNA repair and genomic stability, and immune responses. Defects in any one (or combination) of these processes may contribute to cancer (Hildesheim and Fornace, 2002).

The gene expression patterns of eight cell cycle pathway genes were altered in cancer cells by ZLJ with five being downregulated (CCNE2, CDKN2C, HN1L, MCM6 and TFDP2), and three being upregulated (CCNG2, CDKN1A and GADD45A). In a previous study, a similar observation of an upregulation of CCNG2 and CDKN1A and a downregulation of TFDP2 was reported in PC-3 prostate cancer cells after treatment with ovine uterine serpin (Padua and Hansen, 2009). The observation that CCNE2 was low after drug treatment but high in untreated cancer cells is consistent with a report that cyclin E2 levels were low in non-transformed cells and were increased significantly in tumor-derived cells (Gudas *et al.*, 1999).

Table 2. The 63 functional categories of the 483 genes with more than a 2-fold change of expression after ZLP treatment

No.	Function and process catalogue	Numbers of genes	*p* value
1	Cellular physiological process	215	0.000188
2	Metabolism	174	0.000096
3	Intracellular organelle	146	0.000261
4	Membrane-bound organelle	137	0.000009
5	Nucleus	106	0.000002
6	Regulation of biological process	93	0.00006
7	Regulation of cellular process	91	0.000009
8	Regulation of physiological process	88	0.000028
9	Transcription regulator activity	41	0.000479
10	Cell cycle	31	0.000036
11	Negative regulation of biological process	31	0.000009
12	Transcription, DNA-dependent	29	0.000463
13	Negative regulation of physiological process	28	0.00002
14	Negative regulation of cellular physiological process	27	0.000019
15	Negative regulation of cellular process	27	0.00006
16	Regulation of progression through cell cycle	24	0.00001
17	Transcription factor binding	21	0.000001
18	Intracellular signaling cascade	19	0.000553
19	Transcription cofactor activity	19	0.000001
20	Protein modification	18	0.00084
21	Apoptosis	12	0.000417
22	DNA binding	12	0.000665
23	Transcription	12	0.00072
24	Negative regulation of progression through cell cycle	11	0.000225
25	Protein kinase cascade	9	0.000395
26	Regulation of metabolism	9	0.000374
27	Regulation of transcription	9	0.000184
28	Biopolymer modification	8	0.000004
29	Negative regulation of transcription, DNA-dependent	8	0.000985
30	Positive regulation of cellular physiological process	8	0.000434
31	Positive regulation of physiological process	8	0.000554
32	Transcription corepressor activity	8	0.000611
33	Cell cycle arrest	7	0.000331
34	Dephosphorylation	7	0.000141
35	Negative regulation of metabolism	7	0.000913
36	Negative regulation of transcription from RNA polymerase II promoter	7	0.000479
37	Phosphoprotein phosphatase activity	7	0.000452
38	Protein amino acid dephosphorylation	7	0.000092
39	Regulation of apoptosis	7	0.000448
40	Ubiquitin cycle	7	0.000686
41	Hydrolase activity	6	0.000721
42	Negative regulation of cellular metabolism	6	0.000013
43	Negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	6	0.000006
44	Negative regulation of transcription	6	0.000004
45	Phosphoric ester hydrolase activity	6	0.000215
46	Phosphoric monoester hydrolase activity	6	0.00005
47	Protein tyrosine phosphatase activity	6	0.000051
48	Protein tyrosine/serine/threonine phosphatase activity	6	0.000258
49	Transcription factor activity	6	0.000459
50	Adenyl nucleotide binding	5	0.000819
51	ATP binding	5	0.000722
52	MAPKKK cascade	5	0.000121
53	Mitotic cell cycle	5	0.000364
54	Regulation of protein kinase activity	5	0.000414
55	Regulation of transferase activity	5	0.000414
56	ATPase activity	4	0.000033
57	Glutamine family amino acid metabolism	4	0.000364
58	Hydrolase activity, acting on acid anhydrides	4	0.000313
59	Mitochondrion	4	0.000806
60	Nucleoside-triphosphatase activity	4	0.000253
61	Purine nucleotide binding	4	0.00062

(Continues)

Table 2. (Continued)

No.	Function and process catalogue	Numbers of genes	*p* value
62	Pyrophosphatase activity	4	0.000304
63	RNA processing	4	0.000848

Table 3. Distribution of the 25 pathways of the genes with more than a 2-fold change of expression after ZLP treatment

Pathway	Gene name	Probe set	Fold change
Apoptosis	Helicase, lymphoid-specific	227350_at	−4.83
	Helicase, lymphoid-specific	223556_at	−3.69
	jun oncogene	201466_s_at	11.76
	X-linked inhibitor of apoptosis	206536_s_at	5.85
Blood_Clotting_Cascade	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	202627_s_at	5.21
Calcium_regulation_in_cardiac_cells	ATPase, Ca^{++} transporting, cardiac muscle, slow twitch 2	212361_s_at	−3.18
	adenylate cyclase 9	204497_at	−2.71
Circadian_Exercise	Zinc finger RNA binding protein	213286_at	3.12
DNA_replication_Reactome	Minichromosome maintenance complex component 6	238977_at	−4.05
	Hematological and neurological expressed 1-like	212109_at	−3.67
	Polymerase (DNA directed), alpha 1, catalytic subunit	204835_at	−2.57
Electron_Transport_Chain	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10, 42kDa	1554719_at	3.75
Fatty_Acid_Degradation	Acyl-Coenzyme A dehydrogenase, short/branched chain	226030_at	−4.61
	Carnitine palmitoyltransferase 2	204264_at	−4.10
	Carnitine palmitoyltransferase 2	204263_s_at	−3.99
G_Protein_Signaling	Adenylate cyclase 9	204497_at	−2.71
Cell_cycle	Minichromosome maintenance complex component 6	238977_at	−4.05
	Cyclin E2	205034_at	−5.17
	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	202284_s_at	4.35
	Growth arrest and DNA-damage-inducible, alpha	203725_at	9.79
	Hematological and neurological expressed 1-like	212109_at	−3.67
	Cyclin G2	202769_at	2.95
	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	204159_at	−11.71
	Transcription factor Dp-2 (E2F dimerization partner 2)	226157_at	−3.92
G13_Signaling_Pathway	Citron (rho-interacting, serine/threonine kinase 21)	212801_at	−13.88
	SH3 domain containing ring finger 1	225589_at	3.81
Glycolysis_and_Gluconeogenesis	Glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)	208813_at	2.57
	hexokinase 2	202934_at	9.19
GPCRDB_Other	Cadherin, EGF LAG seven-pass G-type receptor 2 (flamingo homolog, Drosophila)	36499_at	−4.92
Hypertrophy_model	Interferon-related developmental regulator 1	202147_s_at	3.14
	Interferon-related developmental regulator 1	202146_at	2.69
	Vascular endothelial growth factor A	210512_s_at	3.35
	Activating transcription factor 3	202672_s_at	47.45
Integrin-mediated_cell_adhesion_KEGG	Dedicator of cytokinesis 1	203187_at	−2.75
	G protein-coupled receptor kinase interacting ArfGAP 2	225558_at	−3.34
MAPK_Cascade	jun oncogene	201466_s_at	11.76
Mitochondrial_fatty_acid_betaoxidation	Carnitine palmitoyltransferase 2	204264_at	−4.10
	Carnitine palmitoyltransferase 2	204263_s_at	−3.99
mRNA_processing_Reactome	CDC-like kinase 3	202140_s_at	3.55
	Cleavage stimulation factor, 3′ pre-RNA, subunit 2, 64kDa, tau variant	212905_at	−5.00
	Cleavage stimulation factor, 3′ pre-RNA, subunit 3, 77kDa	229666_s_at	−5.25
	Cleavage stimulation factor, 3′ pre-RNA, subunit 3, 77kDa	229665_at	−11.29
	Heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A)	235603_at	−2.87
	splicing factor, arginine/serine-rich 5	212266_s_at	−3.06
	DNA-damage-inducible transcript 3 /// nuclear receptor subfamily 1, group H, member 3	209383_at	18.76

(Continues)

Table 3. (Continued)

Pathway	Gene name	Probe set	Fold change
Nuclear_Receptors	DNA-damage-inducible transcript 3 /// nuclear receptor subfamily 1, group H, member 3	209383_at	18.76
	Nuclear receptor subfamily 2, group F, member 1	209505_at	−3.96
Nucleotide_Metabolism	polymerase (DNA directed), alpha 1, catalytic subunit	204835_at	−2.57
	dihydrofolate reductase	48808_at	−2.56
Ovarian_Infertility_Genes	Early growth response 1	201694_s_at	29.47
	Early growth response 1	201693_s_at	56.18
Smooth_muscle_contraction	jun oncogene	201466_s_at	11.76
	Adenylate cyclase 9	204497_at	−2.71
	ATPase, Ca^{++} transporting, cardiac muscle, slow twitch 2	212361_s_at	−3.18
	Activating transcription factor 3	202672_s_at	47.45
	Activating transcription factor 2	205446_s_at	4.54
	GA binding protein transcription factor, beta subunit 1	206173_x_at	2.64
	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	36711_at	10.42
Statin_Pathway_PharmGKB	Low density lipoprotein receptor	217173_s_at	3.39
TGF_Beta_Signaling_Pathway	jun oncogene	201466_s_at	11.76
	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	202627_s_at	5.21
Translation_Factors	Eukaryotic elongation factor-2 kinase	225546_at	−3.24
	Eukaryotic translation initiation factor 2-alpha kinase 1	217735_s_at	−2.65
	Eukaryotic translation initiation factor 4E binding protein 2	224653_at	−6.02
	Eukaryotic translation initiation factor 4E binding protein 2	224645_at	−5.48
Wnt_signaling	jun oncogene	201466_s_at	11.76
	Low density lipoprotein receptor	217173_s_at	3.39
	FOS-like antigen 1	204420_at	10.84

Additional studies of clinical samples from lung cancer patients will provide further evidence of whether the genes that had the changed expression profiles described in this report can serve as markers for cancer diagnosis or prognosis or as potential drug targets. The methodology described here can be applied further to determine the individual and separate roles of the four herbs in cancer gene regulation. For example, the extracts from each of the four herbs can be applied individually to the cancer cell lines and the resulting gene expression profiling can be observed and compared with that observed after ZLJ treatment. Such a comparison may reveal the exact role of each of the herbs and identify possible synergistic effects among them. Furthermore, the extract of each of the herbs can be subjected to fractionation, and the different fractions of the extract can be screened on the cancer cell lines to identify the critical compounds of traditional Chinese medicine. The information obtained can thereby be used to modify the components of the medicine. In addition, the chemical determination of these compounds may lead to the development of new anticancer medicine.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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