Gene expression profile analyses of mice livers injured by Leigongteng

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Abstract

AIM: To analyze the gene expression profiles of mice livers injured by Leigongteng and explore the relationship between the differentially expressed genes and liver damage.

METHODS: The experimental mice were randomly divided into a control group and a liver-injured group in which the mice were administrated 33 $\mu\gamma$ of triptolide/kg per day for 30 d. Liver mRNAs were extracted from animals in both groups and were reverse-transcribed to cDNA with dUTP labeled by different fluorescence (Cy3, Cy5) as hybridization probes. The mixed probes were hybridized with oligonucleotide microarray chips. The fluorescent signal results were acquired by scanner and analyzed with software.

RESULTS: Among the 35 852 target genes, 29 genes were found to be significantly differentially expressed, with 20 genes up-regulated and 9 genes down-regulated. The reliability of the differentially expressed genes was validated by RT-PCR experiments of 5 randomly selected differentially expressed genes.

CONCLUSION: Based on the biological functions of the differentially expressed genes, it is obvious that the occurrence and development of liver damage induced by Leigongteng in mice are highly associated with immune response, metabolism, apoptosis and the cell skeleton of liver cells. This might be important for elucidating the regulatory network of gene expression associated with liver damage and it may also be important for discovering the pathogenic mechanisms of liver damage induced by Leigongteng.

Chen Y, Zhang XM, Han FM, Du P, Xia QS. Gene expression profile analyses of injured mice livers by Leigongteng. *World J Gastroenterol* 2007; 13(26): 3619-3624

http://www.wjgnet.com/1007-9327/13/3619.asp

INTRODUCTION

Tripterygium wilfordii Hook.f., which is called Leigongteng in Chinese traditional medicine, is a type of euonymus plant and is widely used to treat autoimmune diseases, such as rheumatoid arthritis, chronic nephritis, ankylosing spondylitis and various dermatoses. It also has other functions, such as anti-inflammatory, anti-tumor, and anti-fertility functions[1,2], and has special efficacy in ocular diseases, such as White Behcet's disease, Raiter syndrome, scleritis and severe uveitis. Additionally, Leigongteng is promising as an anti-rejection application after organ transplantation.

Leigongteng plays a role in the treatment, to some extent, by inhibiting cell-mediated immune responses.

Triptolide is the main active ingredient of Leigongteng, and also the major toxic component. During the past 50 years, the poisoning incidents caused by Leigongteng and its related drug were reported frequently[3], and the adverse effects were mainly damage to the reproductive system, blood circulation system, digestive system and urinary system. Liver function abnormalities, liver intumescence and an increase in alanine transaminase (ALT) activity were often seen in the poisoned patients. Therefore, Leigongteng's clinical application is limited due to low safety. Liver toxicity associated with Leigongteng has been verified by conventional pharmacological experiments and clinical observations, however, it is still necessary to search for the mechanism of liver injury on a molecular level.

Microarray techniques developed in the last decade with properties of high throughput, minisize and automation, are ideal technical platforms for toxicological research. Microarray technology has been widely used for assessment of environmental harm and drug safety[4-7]. Our group has used microarray techniques to study the gene expression of some typical synthetic chemicals and Chinese traditional drugs, and we have analyzed the cor-

relation between liver injury and differentially expressed genes[8-10]. In the present work, complete mouse genome oligonucleotide microarrays were used to compare the gene expression profiles of mice livers between control and Leigongteng-treated groups. We also analyzed the mechanisms for key differentially expressed genes and liver injury induced by Leigongteng.

MATERIALS AND METHODS

Apparatus and chemicals

Crystal core® CAP-F0006 mice genome oligonucleotide microarrays (35 852 Oligo DNA of 70 mer in length, which come from the mice Oligo library of Operon Corporation) and a LuxScan 10KA dual-channel laser scanning device were purchased from CapitalBio Corporation, Beijing. GenePix Pro 4.0 image processing software was purchased from Axon Instruments Corporation. Tripterygium tablets were purchased from 999 Huangshi Pharmaceutical Corporation, China (batch number: 050 704, 33 μγ of triptolide in each pill). Assay reagents for determining ALT, aspartate aminotransferase (AST), alkaline phosphatase (AKP), and total protein (TP) were all purchased from Nanjing Jiancheng Bioengineering Institute, China. The Experimental Animal Center of Hubei University permit number for using experimental animals was SYXK 2005-0035.

Animal experiments

36 SPF level Kunming mice (male, weight 20 ± 2 g, provided by Hubei Laboratory Research Animal Center, permit number: SCXK 2003-0005) were randomly divided into a Leigongteng-treated group and a control group, each containing 18 mice. The mice of the Leigongteng-treated group were given, by intragastric infusion, the suspended liquid of tripterygium tablet every morning in a dosage of 0.2 mL/10 g body weight (equal to 10 times the adult dose). Each milliliter of the above suspended liquid contained 0.33 μγ triptolide. The mice of the control group were given, by intragastric infusion, the same volume of saline daily. After 30-d administration, 12 mice were randomly selected from each group to withdraw blood samples from the inner palpebrale angle, with the eyeball removed for determining serum ALT, AST and AKP. The liver and spleen indices were calculated, some liver tissues from the left section were taken and fixed in 10% formaldehyde solution for pathological observation, and 1% homogenate of the remaining liver tissues was taken for testing total protein. The other six mice in each group were used for microarray experiments.

Liver gene expression profiles

RNA extraction and probe preparation: For each of the remaining six mice in each group, 100 mg of liver tissue from the left section was taken to extract total RNA by a modified one-step method[11]. The quality of total RNA was checked by denaturing agarose gel electrophoresis in formaldehyde. The total RNAs were taken equally from each sample and mixed, then reversely transcribed to cDNA, followed by labeling according to the method reported by Schena[12,13]. The labeled cDNAs were used as probes to hybrid with Crystal core® CAP-F0006 mice genome oligonucleotide microarrays. The microarray experiment was an obversely labeled experiment if the probes of the Leigongteng-treating group and control group were labeled with Cy5-dUTP and Cy3-dUTP, respectively.

Otherwise, the microarray experiment was a reversely labeled experiment. Two obversely labeled chips were used for a repeat experiment to avoid false positive results. One reversely labeled chip was used to avoid the deflection of the fluorescence label of Cy5 and Cy3[14].

Microarray hybridization: Labeled probes were isometrically dissolved in 30 μL of hybridization solution (3 × SSC, 0.2% SDS, 5 × Denhardt's solution, 25% formamide), then hybridized with an oligonucleotide microarray chip at 42℃ overnight. After hybridization, the oligonucleotide microarray chips were washed first in 0.2% SDS and 2 × SSC at 42℃ for 5 m, then in 0.2 × SSC for 5 m at room temperature.

Hybridization signal analysis: GenePix Pro 4.0 software was used to transform the image signals of the chip into digital signals, and then the digital signals were normalized by using the Lowess method[15]. The genes that were compatible with the following three conditions were judged to be differentially expressed genes: (1) signal value $\geq$ 800, (2) Ratio $\geq$ 2.0 or $\leq$ 0.5, and (3) the tendency of gene expression was consistent on the above three chips.

Experimental verification of differentially expressed genes: To verify the authenticity of the differentially expressed genes mentioned above, five of them, such as Sult5a1, Lcn2, Mt1.Clrfa and Hamp, were randomly selected for RT-PCR verification by using GAPDH as a control. The RT-PCR product abundance of each of the mentioned 5 genes was determined by agarose gel electrophoresis.

RESULTS

Animal experiments

General Observation: Compared with the control group, the mice of the Leigongteng-treated group were observed to have worse appetites, weaker activities, dark hair, and slower increases in body weight.

Liver index and biochemical assays: Compared with the control group, the liver index, spleen index, and serum ALT, AST, and AKP activities of the mice in the Leigongteng-treated group were significantly increased, and liver TP levels were significantly lower (Table 1).

Histopathological observation: Compared with the control group, it was observed in the Leigongteng-treated group that liver cells were extremely swollen, cytoplasts were loosened, cytosolics were colored lightly, the hepatic plates were fractured, hepatic cords were disordered, hepatic cells were fused and dissolved, karyon were condensed, obvious vacuolar and balloon-like changes were observed, and there was also considerable inflammation-related cell infiltration (Figure 1).

Microarray tests

The quality of total RNA: The ratios of A260/A280 in the total RNA ranged from 1.8 to 2.2, indicating the RNA was quite pure. The results of gel electrophoresis are

Table 1 Physiological and biochemical indices (mean ± SD, *n* = 12)

	Liver index	Spleen index	ALT (U/L)	AST (U/L)	AKP (U/L)	TP (g/L)
Control group	4.31 ± 0.14	0.27 ± 0.01	35.35 ± 4.22	39.29 ± 4.33	295.31 ± 43.84	1.42 ± 0.10
Leigongteng group	5.30 ± 0.20[b]	0.33 ± 0.04[b]	48.71 ± 11.57[b]	48.25 ± 12.27[a]	367.42 ± 32.84[b]	1.09 ± 0.08[b]

liver index: 100 × weight of live/weight of body, spleen index: 100 × weight of spleen/weight of body. [a]$P < 0.05$, [b]$P < 0.01$.
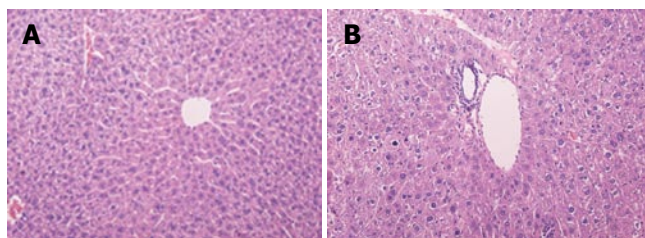

Figure 1 Microscope graphs of mice liver slices. **A**: control group; **B**: Leigongteng group (HE, × 200).
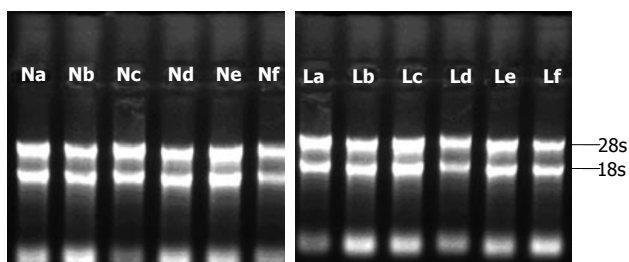

Figure 2 Total RNA showed by agarose gel electrophoresis. Na-Nf: control group, La-Lf: Leigongteng-treating group.

shown in Figure 2. There were two clear bands standing for 28S rRNA and 18S rRNA, which indicated that the extracted mRNA was integral, since the 28S rRNA band was almost two times brighter than the 18S rRNA band.

Differentially expressed genes: Figure 3 shows the schematic plots for signals of the chip hybridization experiment. The horizontal and vertical axes represent the fluorescence signals of the control group and the Leigongteng group, respectively. Each spot stands for hybridization signal for each gene in the microarray. The red and green spots distributed in the ranges of slope > 2.0 and of slope < 0.5, representing differentially expressed genes. The black spots distributed in the range of slope 0.5-2, representing genes that were not differentially expressed.

Compared with the control group, there were 29 differentially expressed genes in the Leigongteng-treated group. Among them, 20 genes were up-regulated and 9 genes were down-regulated, and 27 genes were function-known genes, which could be divided into groups according to their biological functions (Table 2).

RT-PCR results

According to the results of RT-PCR experiments, the abundances of five randomly selected differentially expressed genes (*Sult5a1, Lcn2, Mt1, Clrf, Hamp*) are shown in Figure 4. Based on the results of the chip experiment,
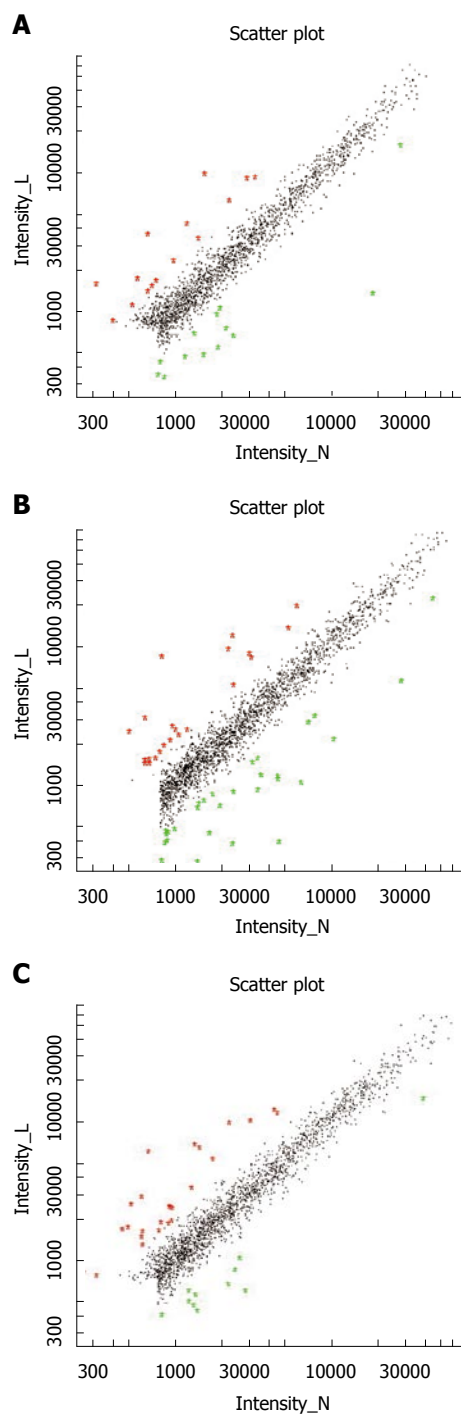

Figure 3 Scatter plot analysis of expression ratio. **A** and **B**: obversely labeled microarray; **C**: reversely labeled microarray.

Lcn2, Mt1, and *Clrf* were significantly up-regulated, while *Sult5a1* and *Hamp* were significantly down-regulated in the Leigongteng-treated group. The RT-PCR experiment

Table 2 Differentially expressed genes of liver tissue in the Leigongteng group

Catalog	GenBank access number	Names of the genes	Cy5/Cy3 (average)
Metabolism	AB059565	aldo-keto reductase family 1, member C18 (*Akr1c18*)	0.226
	NM_032541	hepcidin antimicrobial peptide (*Hamp*)	0.354
	L38990	glucokinase (*Gck*)	0.412
	AY273812	3-ketoacyl-CoA thiolase B (MGC29978)	0.445
	NM_013786	hydroxysteroid (17-beta) dehydrogenase 9 (*Hsd17b9*)	2.277
	BC010769	apolipoprotein A-IV (Apoa4)	2.759
	BC013476	cytochrome P450, family 4, subfamily a, polypeptide 10 (*Cyp4a10*)	2.948
	BC018263	cis-retinol/3alpha hydroxysterol short-chain dehydrogenase-like (*CRAD-L*)	2.988
	CA478631	metallothionein 1 (*Mt1*)	4.404
	BC050123	asparagine synthetase (*Asns*)	5.031
	AK005018	H-2 class II histocompatibility antigen, E-B beta chain precursor	0.243
	BC061489	Ia-associated invariant chain (*Ii*)	0.311
	NM_010738	lymphocyte antigen 6 complex, locus A (*Ly6a*)	2.257
Immune response	AA285471	Lymphocyte antigen Ly-6F.1 precursor	2.318
	NM_011318	serum amyloid P-component (*Apcs*)	2.657
	M31418	interferon activated gene 202B (*Ifi202b*)	2.823
	NM_009117	serum amyloid A 1 (*Saa1*)	3.763
	BC052495	Serum amyloid A-1 protein precursor	5.467
	BG917714	lipocalin 2 (*Lcn2*)	8.587
	NM_011315	serum amyloid A 3 (*Saa3*)	13.1
Cell skeleton	BI247008	orosomucoid 2 (*Orm2*)	2.132
	NM_145594	fibrinogen-like protein 1 (*Fgl1*)	2.823
Receptors	BC054522	transferrin receptor (*Tfrc*)	2.817
Apoptosis	AK051011	DNA fragmentation factor alpha subunit (DNA fragmentation factor 45 kDa subunit) (*DFF-45*)	4.042
	U44088	pleckstrin homology-like domain, family A, member 1 (*Phlda1*)	0.297
	NM_011817	growth arrest and DNA-damage-inducible 45 gamma (*Gadd45g*)	0.214
Cancer related genes	BC050858	RasGEF domain family, member 1B (*Rasgef1b*)	2.311
Unknow	NM_183257	RIKEN cDNA 1810073K19 gene (*1810073K19Rik*)	0.368
	NM_025326	RIKEN cDNA 0610011I04 gene (*0610011I04Rik*)	2.421

Figure 4 RT-PCR analyses of 5 differentially expressed genes.

showed that the change tendencies were consistent in the 5 genes. These results verified that the genes identified in the chip experiments were differentially expressed.

DISCUSSION

Assessing the pre-clinical safety of Chinese traditional medicine is an important topic that needs to be resolved in modern Chinese medicine. The liver is the most importa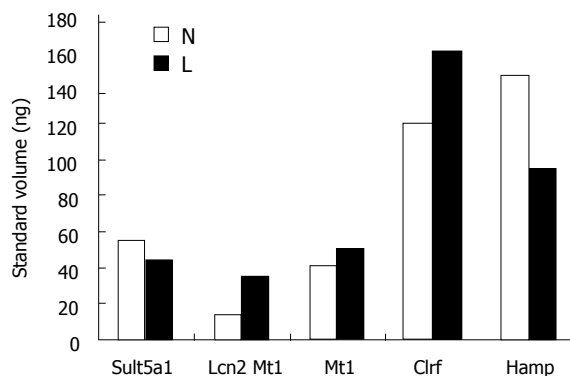nt organ for the metabolism of drugs and xenobiote, and also is an important target organ affected by toxicants. Hence, the toxicity of Chinese traditional medicine on the liver has significant meaning in pre-clinical safety assessments. The efficacy and toxic side effects of Chinese traditional medicines are inevitably related to key gene expression or gene network regulation either directly or indirectly. Exceptional changes in mRNA levels of the related functional genes in specific cells should be very valuable in assessing cell toxicity and toxic mechanisms associated with Chinese traditional medicine.

This study indicated that Leigongteng has an important impact on functional gene expression in mice liver. Here we focused on the correlation between differentially expressed genes and liver injury associated with Leigongteng.

Compared to the control group, there were 10 differentially expressed genes related to metabolism in the Leigongteng-treated group. Among them, genes AB059565, L38990, AY273812 and NM_032541 were down-regulated, while genes NM_013768, BC010769, BC013476, BC018263, CA478631 and BC050123 were up-regulated. According to the literature, exogenous drugs or toxic injury can lead to lipid peroxidation (LPO) of liver cells and damage to mitochondrial function, ultimately leading to cell swelling, degeneration and necrosis. Metallothionein protein is mainly synthesized in the liver and has very high clearance ability on O_2 radicals and H radicals, and stress reaction is the key factor in controlling the expression of metallothionein protein[16]. The up-regulated expression of the metallothionein protein gene (*Mt1*), labeled CA478631, indicated that there was a resistance response to oxidative damage in injured liver cells. Glucokinase is a type of speed limit enzyme regulating glucose metabolism to maintain the steady-state of blood glucose. The glucokinase gene (*Gck*), labeled L38990, was down-regulated. 3-ketoacyl-CoA thiolase B is a type of mitochondrial enzyme that regulates the metabolism of fatty acid and the oxidation of glucose. The gene of 3-ketoacyl-CoA thiolase B (*MGC29978*), labeled AY273812, was also down-regulated. The down-regulated expression of these genes that are related to glucose metabolism may be one of the reasons for mice growth slowness induced by administrating Leigongteng. Apolipoprotein A-IV, encoded by gene Apoa4 labeled BC010769, is the ligand of a high-density lipoprotein receptor and it plays an important role in maintaining the structure of lipoprotein and

lipid transfer in the process of lipid metabolism. The up-regulated expression of BC010 769 may be responsible for stress and regulatory feedback in the injured liver. Aldo-keto reductase family 1, number C18, encoded by gene AB059 565, is an important enzyme in lipid metabolism. The down-regulated expression of AB059 565 indicates that there was metabolic inhibition of fatty acid in the injured liver, and this may be closely related to the fatty degeneration shown in the liver biopsy. The up-regulated expression of hydroxysteroid (17-beta) dehydrogenase 9 gene (*Hsd17b9*), labeled NM_013 786, is commonly found in liver cancer and is caused by a variety of toxicants[17]. Its up-regulated expression in the Leigongteng-treated group indicates that there was risk of carcinogenesis in the liver. Hepcidin antimicrobial peptide is an extremely important iron-regulating hormone that controls intestinal iron absorption and iron homeostasis *in vivo*. The down-regulated expression of hepcidin antimicrobial peptide gene (*Hamp*), labeled NM_032 541, suggested that the liver injury may be associated with a metabolic disorder of iron. In addition, the cytochrome P450 gene (BC013 476), short-chain dehydrogenase gene (BC018 263), metallothionein gene (CA478 631) and asparagine synthetase gene (BC050 123) were all up-regulated. The abnormal expression of the above 10 genes indicates that metabolism in liver cells was disturbed in the Leigongteng-treated group.

There are also 10 differentially expressed genes associated with immune processes. Among them, the genes of lymphocyte antigen 6 complex, locus A (NM_010 738), lymphocyte antigen Ly-6F.1 precursor (AA285 471), interferon activated gene 202B (M31 418), like protein amyloid P component (NM_011 318, NM_009 117, NM_011 315, BC052 495), lipocalin 2 (BG917 714) were all up-regulated, while the genes of Ia-associated invariant chain (*Ii*), labeled BC061 489, and H-2 class II histocompatibility antigen, labeled AK005 018, were down-regulated. The up-regulated expression of the above 8 genes is associated with inflammatory reactions in liver tissue, which is also the detoxic emergency response induced by the administration of Leigongteng. Therefore, the inflammation of liver cells may be an important cause of the damage induced by Leigongteng in mice liver[18]. The down-regulated expression of *Ii* and H-2 genes was related to the injury effect of immune mechanisms caused by drugs. The major histocompatibility complex of mice (MHC), encoded by the H-2 gene and expressed by antigen presenting cells[19], can activate the immune response by presenting antigen to T helper cells and this plays an important role in the identification and removal of the external and internal antigen[20]. The down-regulated expression of MHC class I antigen genes is a common mechanism that allows tumor cells to escape from the destruction of anti-tumor T cells. Therefore, the down-regulated expression of H-2 genes indicated that there were T cell-mediated immunological abnormalities in the Leigongteng-treated group.

Both cytoskeleton-associated genes, BI247 008 and NM_145 594, were up-regulated in the Leigongteng-treated group, and were considered to be an important cause of liver cell degeneration, osteoporosis, cytoplasm and cytoskeletal structure damage. The transferrin receptor, as a membrane protein associated with iron transfer, is widely distributed on the cell membrane. The up-regulated expression of Tfrc, labeled BC0 545 22, may be responsible for the external toxic invasion of liver cells caused by the administration of Leigongteng. The Ras oncogene is considered to be a normal housekeeping gene controlling cell proliferation and differentiation. The activation of this gene can lead to abnormal expression of the encoded products and can trigger malignant transformation of normal cells, which ultimately leads to the occurrence of malignant tumors. The RasGEF domain family member 1B (Rasgef1b), labeled BC050 858, is a cancer gene, and excessive expression of BC050 858 indicated that there was a high risk of liver cancer in the Leigongteng-treated group. DNA fragmentation factor (*DEF*), as a main apoptotic signaling pathway, plays an important role in apoptosis (Liu, 2003). The markedly excessive expression of *DFF45* (AK051 011) in the Leigongteng-treated group showed that *DEF45* created an active defense reaction by removing injured cells to maintain the tissue stability. In the formation of liver tumors, the lack of apoptosis of malignant cells is considered to be a decisive factor in tumor development. The deficient expression of apoptosis related genes, such as the nucleoprotein gene Phlda1(U44 088) and the growth arrest and DNA-damage-inducible 45 gamma gene (NM_011 817), indicate that there was potential risk of cancer for the mice treated with Leigongteng.

In addition to the above function-known genes, there were two function-unknown differentially expressed genes in the Leigongteng-treated group. The relationship between these genes and liver injury is unknown.

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