



## Transcriptome Analysis of the Effects of Gomisin A on the Recovery of Carbon Tetrachloride-Induced Damage in Rat Liver

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Gomisin A possesses a hepatic function-facilitating property in liver-injured rats. Its preventive action on carbon tetrachloride-induced cholestasis is due to maintenance of the function of the bile acids-independent fraction. To investigate alterations in gene expression after gomisin A treatment on injured rat liver, DNA microarray analyses were performed on a Rat 44K 4-Plex Gene Expression platform with duplicated reactions after gomisin A treatment. We identified 255 up-regulated and 230 down-regulated genes due to the effects of gomisin A on recovery of carbon tetrachloride-induced rat liver damage. For functional characterization of these genes, Gene Ontology and Kyoto Encyclopedia of Genes and Genomes biochemical pathways analyses were performed. Many up-regulated or down-regulated genes were related to cell cycle or focal adhesion and cell death genes, respectively. Our microarray experiment indicated that the liver repair mechanism induced by gomisin A was strongly associated with increased gene expressions related to cell cycle and suppression of the gene expression related in cell death.

**Key words:** Gomisin A, carbon tetrachloride, hepatic necrosis, microarray, transcriptome

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*Schisandra chinensis* (Turcz.) Baill grows wild in the easternmost parts of Russia, the Kuril islands, Southern Sachalin, Northeastern China, Korea and Japan [1]. *S. chinensis* has some beneficial effects, including hepatoprotective, antioxidative, anti-inflammatory, anticancer, and anti-HIV actions. *S. chinensis* has many active ligands, including gomisins A, B, C, F, G, H, J, M and N, and schisandrin B and C [2]. These ligands isolated from *S. chinensis* showed protective effects from inflammatory infiltration and liver cell necrosis induced by carbon tetrachloride (CCl<sub>4</sub>) [3], including acetaminophen-induced hepatotoxicity and glutamate-induced oxidative neuronal damage [4,5]. In particular, gomisin A has been known to possess a liver function-facilitating property that

is related to preventive action on CCl<sub>4</sub>-induced fibrosis in liver-injured rats by maintaining the function of the bile acids-independent fraction [6]. In addition, gomisin A has been reported to be effective in improving immunologically induced acute hepatic failure [7], as well as in stimulating liver regeneration after a partial hepatectomy [8].

Liver fibrosis is characterized by excessive deposition of connective tissue and distortion. Many mediators are involved in the process of fibrogenesis. Molecular mechanisms involved in fibrogenesis revealed that transforming growth factor- $\beta$  (TGF- $\beta$ ) played a pivotal role, and depletion of fibrosis by regulating the expression of TGF genes is expected to be a new therapy for liver fibrosis. For example, taurine, heparin-

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superoxide dismutase conjugate, tetramethyl pyrazine, imatinib mesylate, perindopril, and ginkgo biloba down-regulated the TGF pathway [9-14]. These therapy molecules protect the rat liver from fibrogenesis induced by  $\text{CCl}_4$ , and the possible mechanism could involve the down-regulation of TGF- $\beta$ .

As the human genome project has been completed, attention is currently focused on understanding the gene expression profiles of disease states in cells and tissues, as well as the development of platform technology or methodology for detecting and quantitating gene expression levels. Northern blots, Southern blots, PCR, S1 nuclease protection, differential display, cDNA library sequencing, and serial analysis of gene expression (SAGE) methods have limited ability to analyze a large amount of data quantitatively. The DNA microarray system is one of the most powerful technologies for analyzing gene expression in many fields of biological research analyzing the expression profiles of thousands of genes in a wide range of biological systems [15-19]. This technology enables scientists to do a high-sensitivity parallel screening of a large number of genes with a small amount of starting material. Recently, the introduction of fluorescent probes has made it possible to array tens of thousands of short oligo-nucleotides representing the full transcriptome of a species on a miniaturized slide-glass array [20].

In the present work, we investigated the transcriptome profile related to the hepatoprotective effects of gomisin A on  $\text{CCl}_4$ -induced rat liver damage. Using microarray technology, we screened for genes differentially expressed after treatment of gomisin A on rat livers that were damaged by  $\text{CCl}_4$ . DNA microarray-based gene profiling identified 255 up-regulated genes and 230 down-regulated genes, and their specific metabolic pathways were described.

## Materials and Methods

### Extraction and elucidation of gomisin A

The fruits of *S. chinensis* used in this study were collected from Moongyeong, Korea in September, 2005. A voucher specimen (accession No. SC-PNUNPRL-1) was deposited in the Herbarium of Pusan National University. Pure gomisin A was identified by high performance liquid chromatography on a Phenomenex Luna C18 column (150×4.6 mm internal diameter, 5- $\mu\text{m}$  particle size; Phenomenex, Torrance, CA, USA) [21]. The chemical structure of gomisin A used in this study was verified by liquid chromatography-mass spectrometry (LC-MS; Bruker BioApex FT Mass Spectrometer, Billerica, MA, USA) and nuclear magnetic resonance (NMR) analysis (Varian

Inova 500 Spectrometer, Vernon Hills, IL, USA). Optical rotations were recorded on a Jasco DIP-370 Digital polarimeter, Essex, UK). IR spectra were recorded on an ATI Mattson Genesis Series FTIR, Golden Valley, MN, USA). NMR spectra ( $^1\text{H}$ ,  $^{13}\text{C}$ ) were recorded in  $\text{CDCl}_3$  on a Varian Inova 500 Spectrometer operating at 500 MHz for  $^1\text{H}$  and 125 MHz for  $^{13}\text{C}$ , running gradients and using residual solvent peaks as internal references. High-resolution mass spectra were recorded on a Bruker BioApex FT Mass Spectrometer.

### Maintenance of rats

All subjects were female Sprague-Dawley (SD) rats, purchased from Samtaco Biokorea (Osan, Korea). These rats were kept in Pusan National University/Laboratory Animal Resources Center in accordance with the US National Institute of Health (NIH) Animal Care policies. The rats were given a standard irradiated chow diet (Purina Mills, St. Louis, MO, USA) *ad libitum* and were maintained in a specified pathogen-free state under a strict light cycle (light on at 06:00 and off at 18:00). Furthermore, the animal protocol used in this study has been reviewed and approved by the Pusan National University Institutional Animal Care and Use Committee (PNU-IACUC).

### Experimental design for gomisin A treatment

Eight-week-old SD rats were randomly divided into four groups with five rats per group. The first group of SD rats was not treated by any compounds as an untreated control group (UT group), while the second group received 100 mg/kg body weight per day of gomisin A via oral administration for 4 days (gomisin A-treated group, GT group). The third group received a comparable volume of olive oil via oral gavage daily, while the fourth group received 100 mg/kg body weight per day of gomisin A via oral administration for 4 days. At the fifth day, all animals in the first and second groups were immediately euthanized using  $\text{CO}_2$  gas and the liver samples collected. Meanwhile, the third ( $\text{CCl}_4$ -treated group, CT group) and fourth groups (gomisin A and  $\text{CCl}_4$ -treated group, GCT group) received 0.1 mL of  $\text{CCl}_4$  (Sigma-Aldrich, St. Louis, MO, USA) solution via intraperitoneal injection. After 24 hours post- $\text{CCl}_4$  injection, these animals were immediately euthanized using  $\text{CO}_2$  gas. Liver samples were collected and stored in Eppendorf tubes at  $-70^\circ\text{C}$  until assayed.

### Histological analysis

Liver tissues collected from rats were fixed with 10% formalin for 12 hours, embedded in paraffin wax, and then sectioned into 5  $\mu\text{m}$ -thick slices. The liver sections were stained with

hematoxylin and eosin (H&E, Sigma-Aldrich) and morphological features were observed under light microscopy (Leica Microsystems, Heerbrugg, Switzerland).

### RNA preparation and microarray procedure

Total liver RNA was isolated from rat liver tissue using Trizol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. The total RNA was dissolved in diethyl pyrocarbonate-treated distilled water, and stored at  $-70^{\circ}\text{C}$ . DNA microarray analyses were performed according to the manufacturer's standard protocol. Briefly, Cy5-dUTP-labeled cRNA were prepared from 200  $\mu\text{g}$  of total RNA isolated from a liver of each group. Complementary RNA was labeled using a fluorescent cRNA labeling kit (Agilent, Delaware, DE, USA) according to the manufacturer's instructions. Then, 10X blocking agent and  $\times 25$  fragmentation buffer were added to the labeled cRNAs by following the instructions from the hybridization kit (Agilent). For hybridization, total cRNAs were applied to a microarray (Rat 44K 4-Plex Gene Expression platform; Agilent) followed by incubation at  $65^{\circ}\text{C}$  overnight under humidified conditions. To control the labeling differences, technical replication was conducted in which duplicated reactions were carried out by switching over the fluorescent dyes. Microarray analysis was performed on using total cRNA hybridization images captured by a microarray scanner system (G2545CA; Agilent) using feature extraction (FE) 9.5.3 software at the scanning resolution of 5 mm.

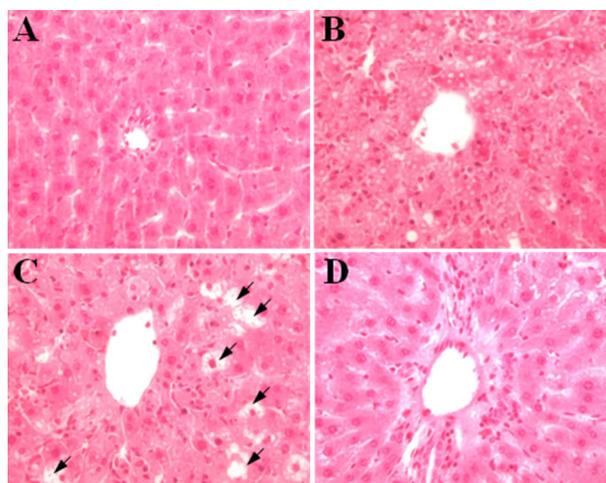
### Analysis of microarray data

Analysis of feature extraction text files produced by the Agilent scanner and further data evaluations were performed by using the Genespring GX7.3.1 software package (Agilent Technologies, Santa Clara, CA, USA). Relative expression levels of genes were calculated after normalization of the signal intensity values and outputted with the unigene and genebank descriptors to a Microsoft Excel data spreadsheet. Gene ontology (GO) analysis based on biological process and molecular function ontology analysis was performed using the web tool Database for Annotation, Visualization, and Integrated Discovery (DAVID). Kyoto Encyclopedia of Genes and Genomes (KEGG) biochemical pathways analysis was also performed by the web tool KEGG (<http://www.genome.jp/kegg/pathway.html>).

## Results and Discussion

### Histological analysis of the rat liver

To investigate the protective effects of gomisin A on  $\text{CCl}_4$ -



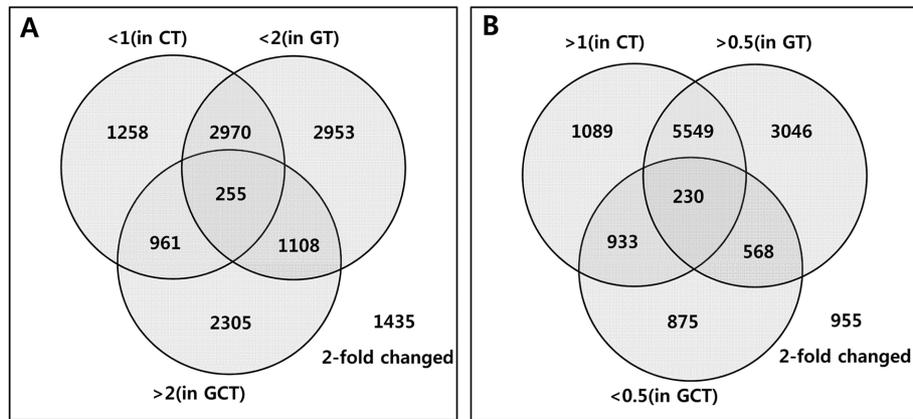
**Figure 1.** Histology of liver tissue. A, vehicle; B, gomisin A alone; C, carbon tetrachloride ( $\text{CCl}_4$ ) alone; D,  $\text{CCl}_4$ +gomisin A. The hepatocellular morphology on the liver section stained with hematoxylin and eosin was observed with light microscope at  $\times 400$ . Arrows indicate the necrotic hepatocytes.

induced hepatocellular damage, we examined the histopathology of livers from all four groups. In the GCT group rat liver, histological alteration of liver tissue was observed. In both the UT group (Figure 1A) and GT group (Figure 1B), the histopathology showed a normal distribution of hepatocytes with clear visible nuclei, portal triads, and central veins. After  $\text{CCl}_4$  treatment, extensive centrolobular necrosis was observed in and around the central vein of liver (Figure 1C). However, necrosis was significantly decreased in the liver section of the group pretreated with gomisin A for 4 days (Figure 1D). These results suggest that pretreatment with gomisin A may protect against hepatocellular damage induced by  $\text{CCl}_4$  treatment.

### RNA preparation and microarray analysis

Preparation of total RNA with high purity is essential for a successful microarray experiment. The quality of RNA was measured by Agilent 2100 Bioanalyzer (Agilent). The Agilent 2100 Bioanalyzer provides a platform for the electrophoresis of nucleic acids on a disposable chip [22,23]. Each RNA sample (1 mL) was loaded into individual wells on a chip and electrophoresed in the Bioanalyzer. 18s and 28s ribosomal peaks were present in all samples by giving RNA Integrity Numbers (RINs) from 8.0 to 10.0, which indicated the verification of a good quality of RNA samples for microarray analysis.

Liver RNA samples from three individual rats were pooled for each experimental group. Each RNA sample was hybridized with 41,012 probes on Rat 44K 4-Plex Gene Expression platform. Systematic errors across all variables in an experiment



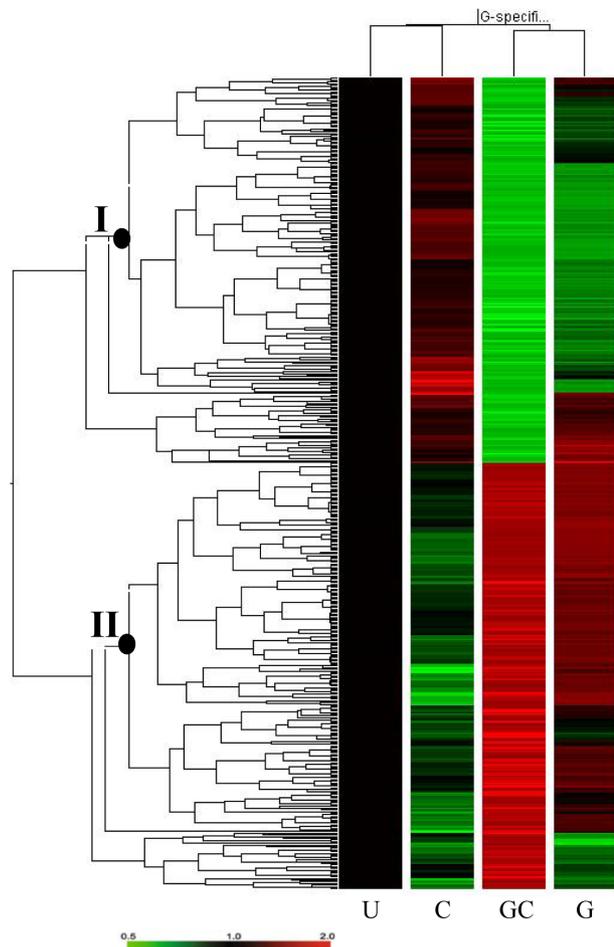
**Figure 2.** Dendrograms showing strategies for identifying specifically regulated genes in the process of gomisin A-induced recovery of rat liver tissues; 255 up-regulated (A) and 230 down-regulated (B) genes were identified. In A, <1 (in CT) and <2 (in GT) indicate the number of genes that did not showed significant up-regulation in CT and GT groups, respectively, and >2 (in GCT) indicates the number of genes that showed significant up-regulation in GCT group. In B, >1 (in CT) and >0.5 (in GT) indicate the number of genes that did not showed significant down-regulation in CT and GT treated groups, respectively, and <0.5 (in GCT) indicates the number of genes that showed significant down-regulation in GCT group.

can be attributed to well-known sources including biases linked to the different dyes used at the labeling step. Possible systemic bias in the data was removed by signal dependent LOWESS normalization of the MA-plot [24]. This normalized data were tested for significant gene expression fold changes between control and samples by a *t*-test.

### Distribution of regulated genes

We identified a number of differentially expressed genes in the GCT group. In total, the expression of 2,305 genes was up-regulated more than two-fold (>2), while the expression of 875 genes was down-regulated more than two-fold (<0.5). To narrow down the number of genes that are possibly involved in gomisin A-induced recovery from CCl<sub>4</sub>-induced injury, a selection strategy (Figure 2) was employed, and finally, 255 up-regulated and 230 down-regulated genes were selected. The up-regulated 255 genes were the genes that showed increased expression level (>2) after the treatments in the GCT group, but showed a decreased expression level in both the GT group (<2) and CT group (<1). The down-regulated 230 genes were the genes for which expression level were decreased (<0.5) in GCT group but were not significantly decreased in either the GT group (>0.5) or the CT group (>1) compared to the UT group (Figure 2).

Hierarchical cluster analysis of 488 selected genes resulted in Clusters I and II, which comprise 255 and 230 genes that were up- or down-regulated, respectively, by the treatment of gomisin A on the rat liver damaged by CCl<sub>4</sub>. A closer relation was observed between the GT group and GCT group, indicating that many genes influenced by gomisin A treatment



**Figure 3.** Hierarchical clustering of 488 genes selected as shown in Figure 1. Y-axis shows the list of genes and X-axis shows control and samples. Red fields show un-regulation of genes (absolute difference) and green fields show down-regulation of genes. U, UT group; C, CT group; G, GT group; GC, GCT group.

**Table 1.** The list of genes identified by the analysis shown in Figure 2. Only the 26 genes that were high ranked in the fold change and/or assigned in the pathway based on KEGG are shown (Figure 4). The genes involved in the cell cycle pathway or TGF- $\beta$  signaling pathway were indicated by <sup>c</sup>, or <sup>T</sup>, respectively

Genbank	Description	Fold-change	t-test P-value
<b>Up-regulated genes</b>			
XM_342149 <sup>c</sup>	histone deacetylase 2	38.72	0.50
XM_222932	exonuclease 1 (predicted)	10.11	0.00
NM_001024741	chromatin assembly factor 1, subunit B (p60)	7.69	0.11
XM_218601	E2F transcription factor 8	7.12	0.03
NM_177931 <sup>c</sup>	origin recognition complex, subunit 1-like ( <i>S. cerevisiae</i> )	6.95	0.13
XM_226316 <sup>c</sup>	minichromosome maintenance deficient 5, cell division cycle 46 ( <i>S. cerevisiae</i> ) (predicted)	6.81	0.01
XM_213587 <sup>c</sup>	similar to cell division cycle 45 homolog ( <i>S. cerevisiae</i> )-like	6.15	0.03
NM_171992 <sup>c</sup>	cyclin D1	2.76	0.29
NM_133571 <sup>c</sup>	cell division cycle 25 homolog A ( <i>S. pombe</i> )	2.18	0.04
XM_216161 <sup>c</sup>	MAD2 (mitotic arrest deficient, homolog)-like 1 ( <i>S. cerevisiae</i> ) (predicted)	2.19	0.27
XM_230765 <sup>c</sup>	E2F transcription factor 1	3.59	0.34
XM_344135 <sup>c</sup>	minichromosome maintenance deficient 6 (MIS5 homolog, <i>S. pombe</i> ) ( <i>S. cerevisiae</i> )	4.62	0.17
XM_232168 <sup>c</sup>	minichromosome maintenance deficient 2 mitotin ( <i>S. cerevisiae</i> ) (predicted)	2.53	0.25
<b>Down regulated genes</b>			
NM_001017496	similar to small inducible cytokine B13 precursor (CXCL13) (B lymphocyte chemoattractant) (CXC chemokine BLC)	0.05	0.14
XM_342172 <sup>T</sup>	thrombospondin 4	0.17	0.22
AB002393	histidine ammonia lyase	0.20	0.24
NM_001013228	T-complex associated-testis-expressed 1-like (Protein 91/23)	0.22	0.26
NM_012533	carboxypeptidase B1 (tissue)	0.23	0.25
NM_001012034	ADP-ribosyltransferase 3	0.23	0.26
XM_235330	collectin sub-family member 10 (C-type lectin) (predicted)	0.24	0.26
XM_224757	similar to chondroitin beta1,4 N-acetylgalactosaminyltransferase (predicted)	0.25	0.29
NM_001012187	kelch-like 7 ( <i>Drosophila</i> )	0.26	0.27
XM_343410	PREDICTED: Rattus norvegicus similar to MEGF11 protein (LOC363079), mRNA [XM_343410]	0.26	0.27
XM_214778 <sup>T</sup>	thrombospondin 2	0.41	0.36
XM_217409 <sup>T</sup>	bone morphogenic protein receptor, type II (serine/threonine kinase)	0.44	0.50
NM_019191 <sup>T</sup>	MAD homolog 2 ( <i>Drosophila</i> )	0.46	0.58

on the normal liver may be associated with recovery from the liver damage induced by CCl<sub>4</sub> (Figure 3).

Among 488 up- or down-regulated genes, 26 genes that were high ranked in the fold change are listed in Table 1. A majority of up-regulated genes were related to cell cycle. These genes included histone deacetylase, which removes acetyl groups and leads to the compaction of chromatin and DNA silencing. Histone deacetylase has been shown to be recruited by the retinoblastoma protein (Rb) and related pocket proteins to the promoters of several cell cycle genes. Thus, the histone deacetylase gene has been implicated in controlling the transcription of core cell cycle regulators [25,26]. Up-regulated genes involved chromatin assembly factor (CAF) as well. CAF is essential for normal cell cycle maintenance. CAF1 loss of function in mammalian cells leads to the activation of a DNA-damage signaling pathway that slows

down the S-phase of cell division and arrests the cell cycle [27]. E2F is also involved in cell cycle maintenance [28]. Most of the down-regulated genes were related to focal adhesions and cell death. These genes included thrombospondin (TSP), which is a member of a group of extracellular matrix (ECM) proteins. When exposed to cells in its soluble form, thrombospondin has primarily anti-adhesive effects characterized by a reorganization of stress fibers and loss of focal adhesion plaques as ascertained by interference reflection microscopy [29-31]. Down-regulated genes also included ADP ribosyltransferase (ADPRT). ADPRT activity interferes with eukaryotic DNA synthesis and endocytosis and causes cytotoxicity and cell death of mammalian cells [32,33].

#### GO and KEGG biological pathway analysis

Gene ontology and biological pathway analysis were

**Table 2.** Functional classification of 251 selected genes based on statistically significant represented GO terms

Category	Term	Count	%	P-value
<b>Up-regulated genes</b>				
Biological process	Nucleoside, nucleotide and nucleic acid metabolism	26	23.64	0.01
	Cell cycle	19	17.27	1.55E-06
	DNA metabolism	11	10.00	6.98E-06
	Cell proliferation and differentiation	11	10.00	0.02
	Cell cycle control	9	8.18	0.001
	DNA replication	8	7.27	5.63E-06
	Chromatin packaging and remodeling	4	3.64	0.08
<b>Down-regulated genes</b>				
Molecular function	Oxidoreductase	10	7.09	0.06
	Dehydrogenase	8	5.67	0.001
	Actin binding cytoskeletal protein	7	4.96	0.05
	Ligase	7	4.96	0.08

**Table 3.** Pathway assignment of 251 selected genes based on KEGG

Term	Count	%	P-value
<b>Up-regulated genes</b>			
Cell cycle	10	9.09	3.33E-07
DNA replication	3	2.72	0.0292932
Pancreatic cancer	3	2.72	0.0981716
<b>Down-regulated genes</b>			
Focal adhesion	8	5.67	0.001
ECM-receptor interaction	4	2.83	0.03
TGF-beta signaling pathway	4	2.83	0.04
Valine, leucine, and isoleucine degradation	3	2.12	0.06
Vascular smooth muscle contraction	4	2.83	0.08

performed with the genes selected as being specifically regulated in the process of gomisin A-induced recovery of rat liver tissues. Among 255 up-regulated and 230 down-regulated genes, 110 and 141 genes with Entrez gene IDs were matched to genes in *Rattus norvegicus*, respectively. These genes were further analyzed for functional classification by GO term and pathway assignment by KEGG.

The most statistically significantly represented GO terms are displayed in Table 2. "Nucleic acid metabolism and cell cycle" was identified as the most significantly related biological process to the up-regulated genes. For down-regulated genes, "oxidoreductase and dehydrogenase" were identified as the most significantly related molecular functions. Oxidoreductases belong to a family of genes that are involved in the regulation of programmed cell death. Oxidoreductase mediates cell death caused by the novel anti-cancer drug  $\beta$ -lapachone [34]. Also, dehydrogenase causes a programmed cell death in ERK kinase signaling events [35].

Biological pathway assignment based on KEGG was displayed in Table 3. "Cell cycle" (Figure 4A) was identified by up-regulated genes as the most statistically significant pathway, which was in accordance with the result of GO

term analysis. While the majority of up-regulated genes were related to cell cycle in the distribution of regulated genes, focal adhesion, ECM-receptor interaction, and TGF-beta signaling pathway (Figure 4B) were identified as the most statistically significant in down-regulated genes. A single CCl<sub>4</sub> administration can cause hepatic injury that is associated with ECM accumulation. During the remodeling process of the liver architecture from hepatic damage, including inflammation, fibrosis and regeneration, TGF- $\beta$ 1 plays a pivotal role in stimulating the liver [36]. Initiation of hepatic fibrosis starts with paracrine stimulation of injured hepatocytes and endothelial cells, as well as subtle changes in ECM composition [37]. They produce pro-fibrogenic cytokines such as TGF- $\beta$ . TGF- $\beta$  as a major fibrogenic cytokine is heavily involved in abnormal ECM accumulation by increasing the synthesis of ECM components [38]. ECM molecules bind to integrin cell surface receptors and activate downstream focal adhesion molecules involved in the regulation of anchorage-dependent cell growth, proliferation, survival, differentiation, morphology, migration, and death [39,40]. Especially, liver fibrosis is characterized by excessive deposition of ECM, which leads to a severe pathological disturbance in the liver. During liver



fibrogenesis, an increase in TGF-beta activity has been reported [41]. TGF-beta is an important cytokine in regulating the production of ECM for liver fibrosis [42,43].

In conclusion, our histological observation showed that gomisin A treatment could repair the rat liver with CCL<sub>4</sub>-induced damage. Further, our microarray experiment indicated that the liver repair mechanism induced by gomisin A was strongly associated with increased expression of genes related to cell cycle and suppression of the genes involved in cell death mediated by the TGF-β pathway.

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