Environ Health Prev Med (2010) 15:105–114
DOI 10.1007/s12199-009-0117-6

REGULAR ARTICLE

Carbon tetrachloride affects inflammation-related biochemical networks in the mouse liver as identified by a customized cDNA microarray system

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Received: 26 July 2009 / Accepted: 15 October 2009 / Published online: 13 November 2009

Abstract
Objectives We have attempted to upgrade and validate an in-house cDNA microarray system developed by our group for the evaluation of chemical toxicity.
Methods To establish an in-house microarray, we selected genes that play pivotal roles in detoxifying exogenous substances and maintaining homeostasis in the liver. To validate the system, we examined gene expression profiles in mouse liver following treatment with different doses of carbon tetrachloride (CCl_4). The data were also analyzed by pathway analysis tools.
Results We upgraded our array system by collecting genes that are responsive to xenobiotic receptors, apoptosis-related genes, and stress-responsive genes. The acute toxicity of CCl_4 was confirmed by elevated levels of serum transaminase and histopathological findings. The microarray data showed the CCl_4 treatment induced significant changes in gene expression in the mouse liver, and the ingenuity pathways analysis revealed alterations in gene expression in inflammation-related networks.
Conclusions We have established a focused microarray system that may be useful for use in toxicogenomics studies. Using this array system, we gained insight into the mechanisms by which CCl_4 exerts its toxic effects. The results of our study also indicate that the combination of focused arrays and bioinformatics tools is helpful in the mechanistic analysis of chemical toxicity.

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Keywords Bioinformatics · Carbon tetrachloride · Microarray · Pathway analysis · Toxicogenomics

Abbreviations

AhR	Aryl hydrocarbon receptor
CAR	Constitutive androstane receptor
PPAR	Peroxisome proliferator-activated receptor
PXR	Pregnane X receptor
SAGE	Serial analysis of gene expression

Introduction

A considerable body of evidence supports the ability of DNA microarrays to monitor simultaneously the expression of thousands of genes [1]. One of the advantages of microarrays is that they enable a comprehensive analysis of gene responses under specific conditions, thereby revealing gene networks and providing useful clues on their mechanisms of interest [2]. Although large-scale and whole transcriptome analyses are initially necessary to identify the diagnostic gene set, once these diagnostic sets of indicator transcripts have been identified, focused array systems may enable detailed analyses [3]. Thus, gene expression analysis using focused microarrays is attracting increasing attention in the toxicology field.

Carbon tetrachloride (CCl_4) is a highly toxic chemical agent, and the hepatotoxicity of CCl_4 has been a long-term focus of toxicological studies [4, 5]. Acute toxicity by CCl_4 results in liver steatosis and centrilobular necrosis, while the chronic effects of CCl_4 exposure include cirrhosis, fibrosis, and carcinogenicity [6, 7]. To date, CCl_4-induced hepatotoxicity has been extensively studied in animal

models, and changes in biochemical pathways and gene expression analysis during toxic insults have been explored [8, 9].

The liver is the first significant site of chemical exposure and the major site of xenobiotic metabolism. In an earlier publication, we reported our development of a customized DNA microarray of 355 transcripts, which we designated the mouse Liver Stress Array ver. 1.0 [10]. To establish this array system, we selected liver-related genes based on the liver transcriptome as revealed by serial analysis of gene expression (SAGE) in order to eliminate liver-unrelated genes and facilitate the efficient detection of the signals [10, 11]. In the study reported here, we upgraded this array system by including genes important in detoxifying exogenous substances and sensing homeostasis in the liver. Using our upgraded focused array system, we have investigated the mechanism by which CCl_4 may exert its toxic effects. In particular, using bioinformatics tools, our aim was to clarify the biochemical networks that are affected by CCl_4 intoxication.

Materials and methods

Reagents

CCl_4 was purchased from Wako Pure Chemicals (Osaka, Japan). The RNAlater solution, Oligotex-dT30 mRNA Purification kit, and SYBR Green PCR Master Mix were obtained from Takara (Osaka, Japan). The Trizol reagent was purchased from Invitrogen (Carlsbad, CA), the Superscript III Reverse Transcriptase (RT) and mouse Cot-1 DNA were from Life Technologies (Rockville, MD), the fluorescent nucleotide Cy5- and Cy3-deoxyuridine triphosphates (dUTPs) were from PerkinElmer (Boston, MA), the QIAquick Purification kit was from Qiagen (Valencia, CA), and the yeast tRNA and poly(dA) polymer were from Sigma–Aldrich (St. Louis, MO).

cDNA microarray system

Our upgraded cDNA microarray, the mouse Liver Stress Array ver. 2.0, contains 399 cDNAs and was constructed in collaboration with Kaken Geneqs (Chiba, Japan). The mouse Liver Stress Array ver. 1.0 comprised a total of 355 individual transcripts and contained major liver-related proteins based on the liver transcriptome, cDNAs encoding drug metabolism, and inflammation-related proteins [10, 11]. To upgrade our microarray system, we selected genes whose expressions are reported to be affected by known toxicants, namely, responsive-genes of the xenobiotic receptors, stress-responsive genes, and apoptosis-related genes. The list of genes selected to upgrade ver. 1.0 is presented in Table 1. The complete list of the transcripts in ver. 2.0 is available at http://www.med.u-toyama.ac.jp/pubhlth/index.html. The PCR products confirmed by sequencing were purified by ethanol precipitation, re-suspended in spotting buffer, and spotted onto amino silane-coated glass slides using a modified, robotic DNA arrayer (Kaken Geneqs). Each cDNA clone was spotted four or five times onto each slide.

Animals and treatment procedures

Six-week-old male C57BL/6J mice (Sakyo Laboratory, Toyama, Japan) were used throughout this study. The mice were housed in the animal center at University of Toyama under specific pathogen-free conditions, at a constant temperature and under a 12/12-h light/dark cycle. All animal procedures were carried out in accordance with our institutional guidelines. The mice were acclimated for 1 week prior to use. After acclimation, animals were randomly assigned to five groups ($n = 5$ each): a control group and four treatment groups. The indicated doses of CCl_4 dissolved in corn oil (2% v/v) were administered into the peritoneal cavity of the mice. Control animals received an equal volume of corn oil only. At 8 h post-injection, the mice were killed and their livers removed for pathological examination and gene expression analysis.

Measurement of serum transaminases

Blood samples were immediately collected in tubes, kept at room temperature for 1 h, and centrifuged at 1000 *g* for 10 min to obtain serum. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured using a Transaminase CII-Test kit (Wako Pure Chemicals) according to the manufacturer's protocol.

Histopathological examination

The liver was immediately removed and cross-sectioned into small pieces. Each piece was fixed in 10% buffered formalin, dehydrated through a graded ethyl alcohol series, and embedded in paraffin. Four micrometer-thick sections were stained with hematoxylin/eosin (H/E). Histopathological assessment of the H/E-stained sections was performed under a light microscope.

RNA isolation and microarray hybridization

RNA isolation and microarray hybridization were performed as described previously [10]. Briefly, the dissected liver was immediately soaked in RNAlater solution to prevent RNA degradation. Total RNA was isolated from the liver using Trizol reagent, from which mRNA was enriched using the

Table 1 List of the selected genes used to upgrade the mouse Liver Stress Array ver. 1.0

Gene	Description	Full name	Accession no.	Forward primer 5→3	Reverse primer 5→3	Size (bp)
Gsta2	CAR-responsive	Glutathione S-transferase, alpha 2	NM_008182	GCAAAAGACAGGACCAAAA	GCACAATAGCCAGAATCAAC	384
SLC21A6	CAR-responsive	Liver-specific transporter-1/ organic anion transporter	AF250912	TTTTTCACTGCACTGGGAAG	TACAGGTTCTGGGTTTCCTT	387
CAR	PXR-responsive	Constitutive androstane receptor	NM_009803	CATCTCCAGGAGCCTGAGTA	CAGCATTTCATTGCCACT	410
MRP2	CAR, PXR-responsive	Multidrug resistance protein	NM_013806	GATTGTTGAATACGGCAGTC	GGGGGTTTTAGGGATCA	413
Sod1	PPARalpha-responsive	Superoxide dismutase 1, soluble	NM_011434	ACAGGATTAACTGAAGGCCAGC	ATGGTTTGAGGGTAGCAGATGAG	430
Sucla1	PPARalpha-responsive	Succinyl-CoA synthetase	NM_019879	AGTCTTCCTGAATGATCCAGCC	TCCCACATACTGAGAGACCAACC	450
Casp3	Apoptosis	Caspase 3, apoptosis related cysteine protease	NM_009810	GTCTTTGTACGCTACCACTGCCT	ATGGTGGTCCAACTCTTCACTTC	418
Bax	Apoptosis	Bcl2-associated X protein	NM_007527	TGACATGTTTGCTGATGGCA	TCCCACCCCTCCCAATAATT	425
Cryz	AhR-responsive	Crystallin, zeta/quinone reductase	NM_009968	AGTGATGCACACTACCACACACAG	GACCATACTGAAGACCACAGACCC	470
PPARalpha	Nuclear receptor	Peroxisome proliferators-activated receptor alpha	NM_011144	ACGAGGGTGTGATTTCATCCA	CAGGAACCAAGCCCCTCC	417
Cpt2	PPARalpha-responsive	Carnitine palmitoyltransferase 2	NM_009949	CAGTGAGCCTCGGTGGCTT	TCCAACCCGATCTCCTTAAGAA	405
CEBPdelta	Transcription factor	CCAAT/enhancer binding protein, delta	NM_007679	GCCTCTACTTTCCTCAATCT	GCACTGTCACCCATACAAT	437
CyE1	Cell cycle	Cyclin E1	NM_007633	GCAGATGCTGTGCTCTATGGAG	CATGAAGGCAGGACATGCTG	401
Por	PXR-responsive	NADPH-cytochrome P450 oxidoreductase	NM_008898	GCCCACAAGGTCTATGTTCA	CTAGGCTGCCTGGTACAATG	422
Bak	Apoptosis	BCL2-antagonist/killer 1	NM_007523	TCTGAACACATCCATCAGGCTC	CCCTATGTTGTGCAAGACAGAGA	388
Fah	Metabolizing enzyme	Fumarylacetoacetate hydrolase	NM010176	AGAAGGAATGAGCCAGGCG	ATAAGGCAAGGGTGTGTCTTGTG	403
AhR repressor	AhR-responsive	Aryl-hydrocarbon receptor repressor	NM_009644	AGTGGCATGAATCCCAGCA	GAATGAGCAAAAGTGCAAGTTGG	456
Ddit3	Stress-responsive	DNA-damage inducible transcript 3/CHOP10/gadd153	NM_007837	GCAAGGAAGAACTAGGAAACGGA	CAATGTACCGTCTATGTGCAAGC	394
Jun	Transcription factor	Jun oncogene	BC021888	TGCCCCAACAGATCCCG	GCCCTGACAGTCTGTTCTCAAA	403
m36B	Housekeeping gene	Acidic ribosomal phosphoprotein P0 (Arbp)	NM_007475	CAACGGCAGCATTTATAA	CCCATTGATGATGGAGTGTGG	171
Nrf2	Transcription factor	Nuclear factor, erythroid-derived 2, like 2	NM_010902	GAAAAGGGAGAAAACGACAG	CCCAAATGGTGCCTAAGA	429
Ccng2	Cell cycle	CyclinG2	NM_007635	GGTGGTGTATTGTCCGATA	CATCACCACACAGAATTGCT	438
MT1	Stress-responsive	Metallothionein 1	BC027262	ACGACTTCAACGTCCTGAGTACCT	CACGTACTCGGTAGAAAACGGG	382
MT2	Stress-responsive	Metallothionein 2	NM_008630	AAACCGATCTCTCGTCGATCTTC	CAAGTCAACGGCTTTTATTGTCAG	384
Gadd45a	DNA-damage-inducible	Growth arrest and DNA-damage-inducible 45 alpha	NM_007836	CGGGAAAGTCGCTAC	AAAAATACCCAAACTATTGC	421
Bad	Apoptosis	Bcl-associated death promoter	NM_007522	CTGGACGCGCATTATCCAGT	GACCTCGGGTCCCCAGTTAT	420

Table 1 continued

Gene	Description	Full name	Accession no.	Forward primer 5→3	Reverse primer 5→3	Size (bp)
p21	Cell cycle	Cyclin-dependent kinase inhibitor 1A	NM_007669	CTTGGTGGTGAGACAGGCCT	TTATTGAGCACCAGCTTTGGG	451
MRP1	CAR, PXR-responsive	Multidrug resistance protein	AF022908	GGAGAAAAGGTGGGTATTGT	GCCTCGTCCAACACTAGAAT	410
CD36	PPARalpha-responsive	CD36 antigen	NM_007643	ACTTCAATTTCTGTAGACCC	CAGCATGGAACATGACG	461
Lfas	PPARalpha-responsive	Long chain fatty acyl CoA synthetase	U15977	GATGGCTGGTTACACACGGG	TCTTCGCCTTCAGTGTTGGA	455
PXR	PXR-responsive	Pregnane X receptor	AF031814	GTCGGCCATATCCTGCTC	GCGGGATTTGGAAGTTTATT	397
Rad23b	DNA-damage-inducible	RAD23b homolog	NM_009011	CCCATTCTTCGTGTTCA	TAAAGGCAGCAGTTTGACTC	426
STA2	CAR, PXR-responsive	Hydroxysteroid sulfotransferase (mSta2)	L27121	ATGAAGACATGAAAAAGGAT	TTTCCACAACAGGTTTTATT	413
Tnfrsf6	Apoptosis	Tumor necrosis factor receptor superfamily, member6	NM_007987	ACCTTGGAAAATCAACCC	GTCTTCAATTAACTGCGAGT	433
HSP60	Stress-responsive	Heat shock 60-kDa protein	X53584	GCGGCATGTTCTAACTCCTA	TCCACAGAAAGGCTGTCTTC	413
MnSOD	Anti-oxidant enzymes	Manganese superoxide dismutase 2	X04972	GAGTTGCTGGAGGCTATCAA	CCAGTCATAGTGCTGCAATG	423
OATP2	PXR-responsive	Organic anion transporter 2	AY007379	CTCCTGCAGGCTGTATGACT	GCATACCTTTGGAAGCAGAA	391
OSP	Stress-responsive	Oxidative stress-induced protein	U40930	GGAATGTGTGATGCCTTTTC	CCATTAGAAAGGGGGACAGT	435
p53	Stress-responsive	Cellular tumor antigen p53	X01237, K01700	CTCCAGCTACCTGAAGACCA	GGTGGATAAATGCAGACAGG	463
PCNA	Stress-responsive	Proliferating cell nuclear antigen	X53068	TTTTTCACAAAAGCCACTCC	TGCATTTAGCGTCAAGATCA	446
Rad50	Stress-responsive	DNA damage repair and recombination protein 50 homolog	U66887	AAGCCATCGGTGATCATTTA	GAGGTGACACCCAACAAATC	469
Rad51	Stress-responsive	DNA damage repair and recombination protein 51 homolog	D13473	TCACAGGCTCATGTTGTGAC	TGCAGGGATCGTAGTCTAGC	415
Tst	Metabolizing enzyme	Thiosulfate sulfurtransferase	S80191	ACCGGAGCCGGATATAGTAG	AACCTACAGCAGGTCACACC	415
Vim	Stress-responsive	Vimentin	X51438	ATTGAGATCGCCACCTACAG	TCTTTTGGGGTGTCAGTTGT	402

The complete list of the transcripts is available at http://www.med.u-toyama.ac.jp/pubhlth/index.html

Oligotex-dT30 mRNA Purification kit. Fluorescent-labeled cDNA probes were constructed from 1-μg aliquots of mRNA samples by oligo(dT)-primed reverse transcription using Superscript III RT. The fluorescent nucleotide Cy5- and Cy3-deoxyuridine triphosphates (dUTPs) were used to label the cDNA of the experimental and reference tissue, respectively. The probes were purified using the QIAquick Purification kit. The blocking reagents, 20 μg of yeast tRNA, 20 μg of poly(dA) polymer, and 20 μg of mouse Cot-1 DNA, were added to the probes. Hybridization was performed at 65°C for 16 h in a hybridization cassette (Kaken Geneqs). After washing, the slides were dried and subjected to image processing. A twofold change in gene expression was used as the cut-off threshold. The analysis was repeated in two independent experiments. The data were further analyzed using GeneSpring software (Silicon Genetics, Redwood City, CA) to extract the significant genes.

Ingenuity pathways analysis

To examine the gene ontology, including biological processes, cellular components, molecular functions, and genetic networks, we analyzed the data using Ingenuity Pathways Analysis (IPA) tools (Ingenuity Systems, Mountain View, CA; http://www.ingenuity.com/index.html), a web-delivered application that enables the discovery, visualization, and exploration of molecular interaction networks in gene-expression data. The gene lists identified by GeneSpring and natural legalism were uploaded into the IPA system. Differentially expressed genes that were associated with a canonical pathway in the Ingenuity Pathways Knowledge Base were considered for evaluation. The IPA computes a score for each network according to the fit of the user's set of significant genes of interest. The score is derived from a *p* value and indicates the likelihood of the selected genes being found together in a network because of random chance.

Statistical analysis

All comparisons were made by analysis of variance with Scheffe's post hoc test. All calculations and analyses were performed using Stat-View software (SAS Institute, Cary, NC). *P* values of less than 0.05 were considered statistically significant.

Results

Upgrading our in-house cDNA microarray system

In an earlier publication, we reported our development of an in-house cDNA microarray system, the mouse Liver Stress Array ver. 1.0, which contained 355 unique genes [10]. To upgrade this array system (ver. 2.0), we selected genes that were absent from the ver. 1.0 array but are known to be important in toxicogenomics (Table 1).

Xenobiotics may accumulate to toxic levels unless they are properly metabolized and eliminated. It is well established that xenobiotic receptors play a pivotal role in the induction of genes involved in the detoxification process. At least four receptors are involved: the peroxisome proliferator-activated receptor (PPAR) α, the aryl hydrocarbon receptor (AhR), the pregnane X receptor (PXR), and the constitutive androstane receptor (CAR) [12]. We included the genes responsive to these receptors that were absent in the ver. 1.0 microarray.

Cellular responses to stress involve a number of defensive processes aimed at minimizing cell damage and redressing the balance of homeostasis [3]. In addition, toxicity is commonly manifested as apoptosis [3]. We selected known stress-responsive genes and apoptosis-related genes. Several chemicals can affect cell growth and induce oxidative stress and DNA damage [13]. Therefore, we included cell cycle-related genes, oxidative stress-responsive genes, and DNA damage-inducible genes. The final array contained 399 individual transcripts. The complete list of the transcripts in ver. 2.0 is available at http://www.med.u-toyama.ac.jp/pubhlth/index.html.

Clinical chemistry and histopathology

CCl_4 has been widely used in animal models to investigate chemical toxin-induced liver damage [5, 6, 9]. To validate our microarray system, we used CCl_4 as a typical hepatotoxicant. CCl_4 was administered intra-peritoneally to the mice in the treatment groups at a dose of 1, 10, 100, or 1000 μl/kg (corresponding to 1.5, 15, 150 or 1500 mg/kg, respectively). Mice were killed 8 h post-injection. The acute toxicity of CCl_4 was first evaluated by serum AST and ALT activities. Treatment with 1 or 10 μl/kg CCl_4 was found not to affect serum transaminase activities, but that with 100 or 1000 μl/kg CCl_4 significantly elevated the activities compared with vehicle control (Fig. 1a).

The livers of vehicle-treated and CCl_4-treated mice were examined histopathologically (Fig. 1b). Histological analysis principally corroborated the biochemical findings. In general, liver sections from vehicle- and 1 μl/kg CCl_4-treated mice showed normal morphology, those from 10 μl/kg CCl_4-treated mice showed minimal fatty change, and those from mice treated with 100 or 1000 μl/kg CCl_4 showed fatty degeneration and some degree of necrosis (Fig. 1b). In particular, the livers of 1000 μl/kg CCl_4-treated mice showed prominent necrotizing damage.

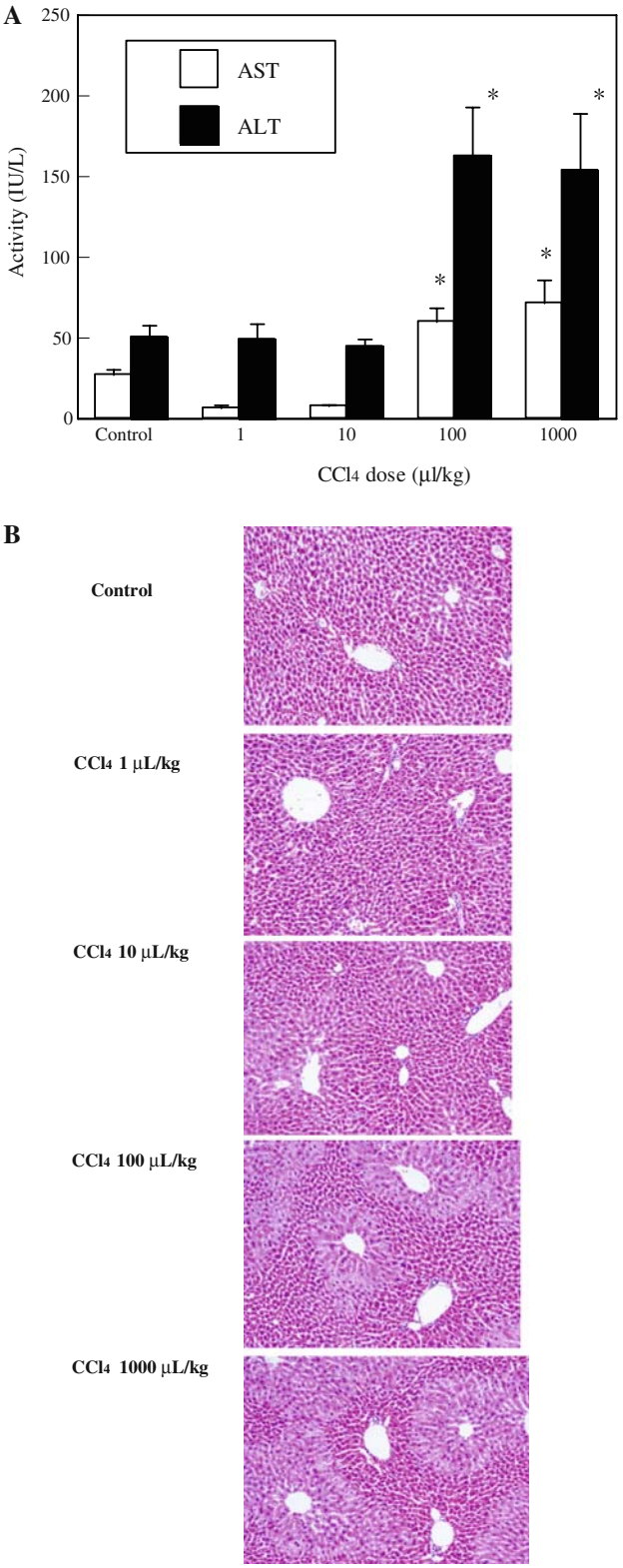

Fig. 1 **a** Effect of carbon tetrachloride (CCl_4) treatment on serum aspartate aminotransferase (*AST*) and alanine aminotransferase (*ALT*) levels. Serum AST (*white bars*) and ALT (*black bars*) were determined as described in the Materials and methods. Values represent the mean ± standard error of the mean (SEM). *$p < 0.05$ versus vehicle control. **b** Paraffin-embedded liver sections were stained with hematoxylin–eosin. Original magnification 100×

Gene expression changes revealed by an in-house cDNA microarray

The list of genes that showed at least twofold expression changes at any given dose is available at http://www.med.u-toyama.ac.jp/pubhlth/index.html. Although the 1 μl/kg CCl_4 treatment did not affect serum transaminase levels and the histological findings, the expressions of several genes were changed even at this low dose. Hierarchical cluster analysis was employed to cluster the responses to each dose, and the gene responses were clustered according to the fold change (Fig. 2).

Figure 3 presents the dose–response changes of the certain up-regulated genes. The dose–response changes of each gene differed from each other. The most dramatically up-regulated gene was oxidative stress-induced protein (*OSP*; 27.2-fold up-regulation after 1000 μl/kg CCl_4 treatment) followed by DNA-damage inducible transcript 3 (*Ddit3*; 15.4-fold up-regulation after 100 μl/kg CCl_4 treatment).

Ingenuity pathways analysis

Pathway analysis technology enables the mapping of gene expression data into relevant pathway maps based on their functional annotation and known molecular interactions. After the complete analysis set had been imported into IPA for canonical pathway analysis, we were able to obtain useful information on the molecular interaction networks. Figure 4a shows the most significant network constructed from genes that were up-regulated after the CCl_4 treatment. The results show that tumor necrosis factor alpha (TNF-α) plays a central role in related pathway alterations and that these pathways are highly activated after CCl_4 treatment (Fig. 4a). The pathway that showed the second highest IPA score was the transforming growth factor beta (TGF-β)-related network (Fig. 4b).

Discussion

Expression profiling is one of the most advanced transcriptomics techniques to monitor and assess chemical toxicity. Toxicogenomics has its origins in the technologies related to transcriptional profiling, and an essential tool in this field is DNA microarray technology. Promising developments in this technique include tailor-made microarray systems specifically addressing the endpoints

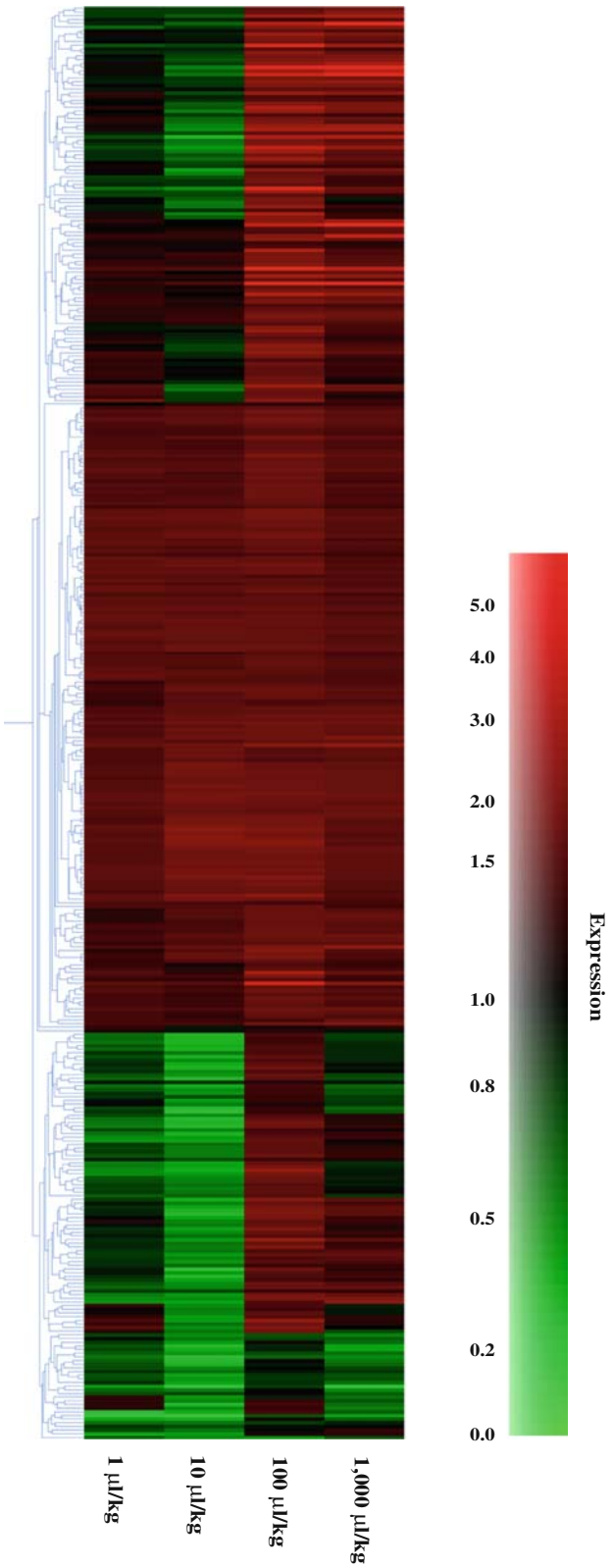

Fig. 2 Cluster analysis of gene expression profiles after treatment with CCl_4. Gene expression profiles represent up-regulated genes (*red*) and down-regulated genes (*green*) after treatment with CCl_4. Increasing *red intensity* denotes genes whose expression increased with respect to vehicle, and *green intensity* denotes genes that decreased in expression with respect to vehicle. Each *row* denotes a single gene and each *column* represents an individual dose. The *color scale* shown indicates the ratios of the signal for CCl_4 (Cy5 signal, *red*) to that for the vehicle (Cy3 signal, *green*) (color figure online)

and mechanisms of interest. We report here the results of our analysis of gene expression profiles based on an in-house cDNA microarray system that contains genes selected to facilitate a toxicogenomics approach. Using bioinformatics tools, we also attempted to identify the pathways affected by CCl_4 toxicity.

Our results show that gene expression analysis is sensitive enough to detect early organ damage, even though there were no remarkable changes in the serum chemistry parameters or histopathological findings. We found significant differences in AST and ALT activities between the 1 or 10 μl/kg CCl_4 treatment and the 100 or 1000 μl/kg CCl_4 treatment (Fig. 1). Consistent with this observation, the up-regulation of several genes, including *OSP* (oxidative stress-induced protein), *Ddit 3* (DNA-damage inducible transcript 3), *Gadd45a* (growth arrest and DNA-damage-inducible 45 alpha), and *Fth* (ferritin heavy chain), were observed only following the 100 and 1000 μl/kg CCl_4 treatments (Fig. 3). We found no significant differences in serum transaminase activities between the groups of mice treated with 100 and 1000 μl/kg CCl_4 (Fig. 1a). Although the overall changes in gene expressions paralleled each other at these two doses (Fig. 2), the expression levels of several genes, such as fatty acid binding protein 1 (*Fabp1*) and aldehyde dehydrogenase family 3, subfamily A2 (*Aldh3a2*), were significantly down-regulated at 1000 μl/kg, suggesting that the repression of these genes may reflect severe liver damage.

The number of genes whose expression levels were altered at least twofold by the CCl_4 treatment did not depend on the strength of the injection doses. One reason for this effect may be that our system is a focused array but not one aimed at genome-wide transcript profiling. We especially selected genes to detect early cellular responses to stress, such as those involving a number of defensive processes to protect the cells against deleterious effects. We chose a twofold change to be the cut-off for differential expression, and approximately 48% of spotted genes reached this threshold. If we had chosen a 1.5-fold change as the cut-off, as in previous studies [14, 15], approximately 95% of the spotted genes would have been differentially expressed. Thus, the focused array system presented in this study efficiently detected gene expression changes and also evolved into a technically simple, low-cost assay.

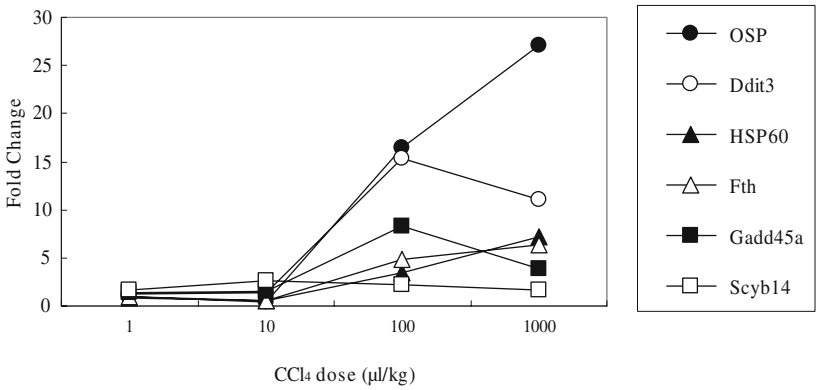

Fig. 3 Dose–response changes of the certain genes that showed at least a two-fold up-regulation at any given dose. *OSP* oxidative stress-induced protein, *Ddit3* DNA-damage inducible transcript 3, *HSP60* heat shock 60-kDa protein, *Fth* ferritin heavy chain, *Gadd45a* growth arrest and DNA-damage-inducible 45 alpha, *Scyb14* small inducible cytokine subfamily B member 14

In addition to identifying target genes, transcriptional profiling can also provide additional mechanistic information on the molecular responses of cells to chemical exposure. An important potential of molecular profiling is the development of fingerprints of cellular responses. The process of overlaying microarray data onto biological networks is commonly known as pathway mapping. These networks describe the functional relationships between gene products based on known interactions reported in the literature. Our pathway analysis showed that acute CCl_4 toxicity significantly activated gene expression in TNF-α-related networks (Fig. 4a). The pathway that obtained the second highest score was that involving TGF-β-related networks (Fig. 4b).

Cells are activated and secrete inflammatory products in response to tissue damage. In addition to its metabolic functions, the liver is known to be involved in inflammation and immune responses [16]. Although hepatocytes comprise approximately two-thirds of liver cells, other cell types are present, such as biliary epithelial cells, sinusoidal endothelial cells, Kupffer cells, stellate cells, dendritic cells, and lymphocytes. Kupffer cells and lymphocytes are particularly involved in the hepatic immune response. Kupffer cell-derived cytokines, such as interleukin (IL)-1β, IL-6, TNF-α, and leukotrienes, promote infiltration and the antimicrobial activity of neutrophils [17]. TNF-α is known to be a central regulator that facilitates tissue repair by stimulating apoptosis and cell proliferation; it also exacerbates cell damage by initiating an inflammatory process [18]. TNF-α is responsible for the activation of nuclear transcription factors, including activator protein (AP)-1, nuclear factor-kappaB (NF-κB), and signal transducer and activator of transcription (STAT)-3 [19]. As a result, the induction of inflammatory cytokines and of the C-C and C-X-C families of chemokines occurs sequentially through a complex series of events. This results in the recruitment and activation of neutrophils and monocytes into the damaged site. A previous study showed that the level of TNF-α rises consistently during CCl_4-induced liver toxicity [20], which provides additional validation of our IPA data showing that a TNF-α-related pathway was activated by exposure to CCl_4.

The toxic effect of CCl_4 on hepatocytes is due to its metabolic conversion by the NADPH-cytochrome P450 metabolizing enzyme system to the highly reactive free radical CCl_3 [7]. Free radicals cause the peroxidation of polyenoic lipids and the generation of secondary free radicals derived from these lipids. This destructive lipid peroxidation leads to a breakdown of membrane structure and function [21–23]. Thus, treatment with CCl_4 provokes oxidative stress, which is a known inducer of the pro-fibrogenic cytokine TGF-β [24, 25]. TGF-β is known to play a key role in transforming quiescent hepatic stellate cells into activated cells to produce collagen, which eventually results in hepatic fibrosis [26]. Interestingly, TNF-α is also an important activator of hepatic stellate cells in the course of hepatic fibrogenesis [26, 27]. Thus, the overall effect of this complex series of events would be additional cellular damage that would eventually lead to liver fibrosis.

In summary, our gene expression profiling has provided insights into the mechanisms by which CCl_4 exerts its toxic effects. In response to the injury associated with CCl_4, a variety of cellular pathways is activated to maintain cellular integrity and to protect the cells against deleterious effects. In particular, CCl_4-induced liver toxicity may be provoked by the activation of proinflammatory signaling and, possibly, paracrine and/or autocrine signaling pathways involving more than one hepatic cell type may underlie its deleterious effect. Our study also suggests that selecting appropriate genes for microarrays and statistical evaluation of the selected genes related to signaling pathways can provide useful information for mechanistic analyses of chemical toxicity.

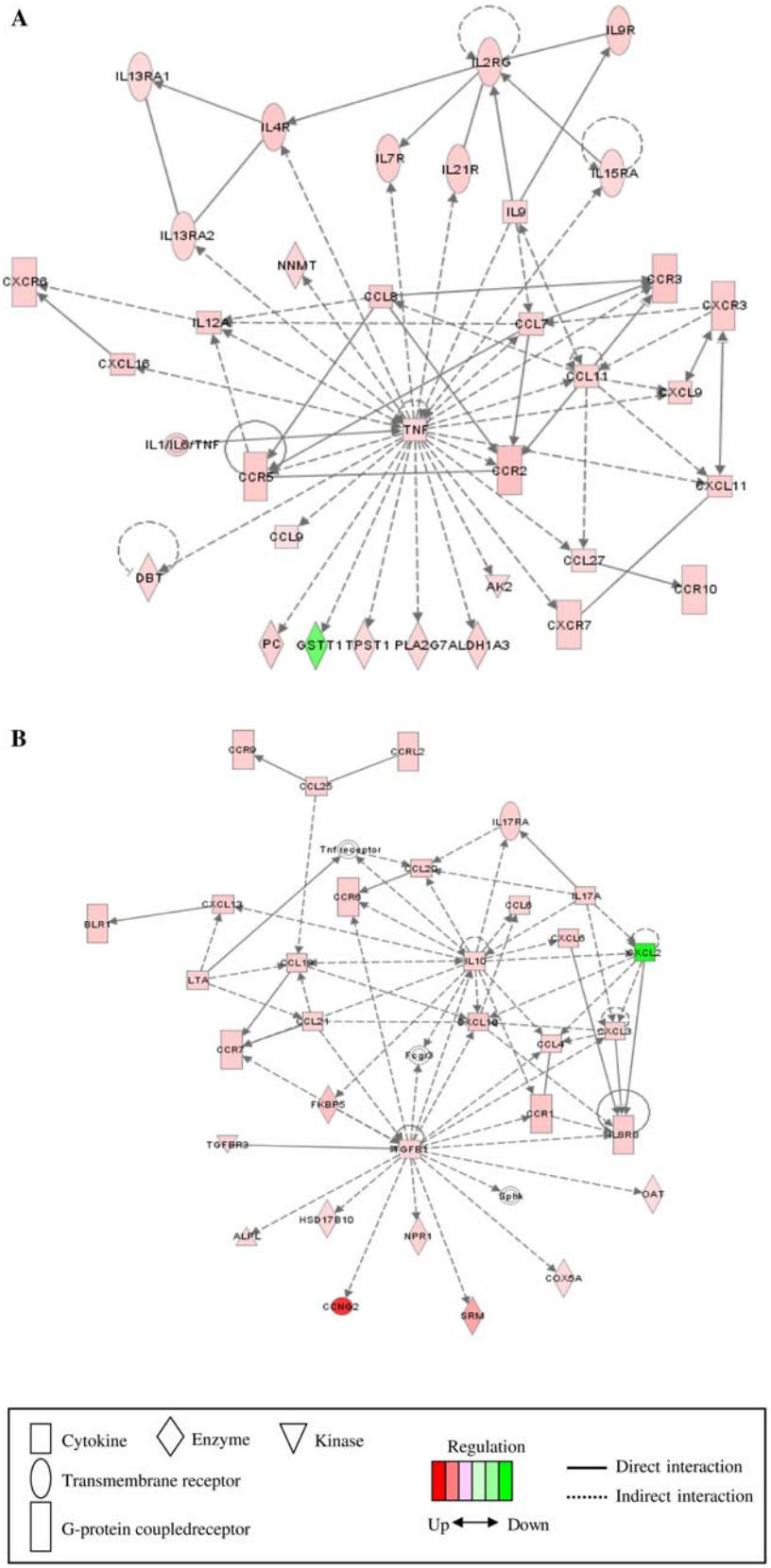

Fig. 4 Ingenuity pathway analysis of gene expression. Biological findings are assigned to each gene and network based on the information in the Ingenuity Pathways Knowledge Base, which was extracted from the scientific literature. The pathways least likely to have occurred by chance following treatment with 100 μl/kg CCl_4 are presented. **a** Tumor necrosis factor alpha (*TNF-α*)-related pathway, **b** transforming growth factor beta (*TGF-β*)-related pathway. *Red* Up-regulation, *green* down-regulation (color figure online)

Acknowledgments This study was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

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