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Toxicogenomics Study on Carbon Tetrachloride-induced Hepatotoxicity in Mice

Sun-Young Jeong[3,4], Jung-Sun Lim[4], Ji-Yoon Hwang[4], Han-Jin Park[4], Jae-Woo Cho[4], Chang-Woo Song[4], Yang-Seok Kim[1,2], Wan-Seon Lee[1], Jin-Hee Moon[1], Sang-Seop Han[4] & Seokjoo Yoon[4]

[1]Bioinformatics Division, ISTECH Inc., Goyang, Gyeonggido, Korea, [2]Cancer Metastasis Research Center, Yonsei University College of Medicine, Seuol, Korea
[3]Department of Biopotency/Toxicology Evaluation, University of Science & Technology, Daejeon, Korea
[4]Korea Institute of Toxicology, Korea Research Institute of Chemical Technology, Daejeon, Korea
Correspondence and requests for materials should be addressed to S.-j. Yoon (sjyoon@kitox.re.kr)

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Abstract

Carbon tetrachloride (CCl_4) is well known hepatotoxicant. Its overdose cause severe centrilobular hepatic necrosis in human and experimental animals. We administered CCl_4 at low (0.2 mL/kg p.o.) and high (2 mL/kg p.o.) doses to mice. Mice were sacrificed at 24 h after administration. We evaluated liver toxicity by serum AST and ALT level and by microscopic observation. Using cDNA chip, we conducted gene expression analysis in liver. Mean serum activities of the hepatocellular leakage enzymes, ALT and AST, were significantly increased compare to control, respectively, in the low and high dose groups. H&E evaluation of stained liver sections revealed CCl_4-related histopathological findings in mice. Moderate centrilobular hepatocellular necrosis was present in all CCl_4 treated mice. We found that gene expression pattern was very similar between low and high dose group. However, some stress related genes were differently expressed. These results could be a molecular signature for the degree of liver injury. Our data suggest that the degree of severity could be figure out by gene expression profiling.

Keywords: Carbon tetrachloride, hepatotoxicity, cDNA microarray, gene expression

Carbon tetrachloride (CCl_4), a well known hepatotoxicant, has been used widely for decades to induce liver injury in various experimental models to elucidate the mechanisms on hepatotoxicity[1-3]. CCl_4 is a colorless, non-inflammable volatile liquid with an odor and immiscible in water, is produced by chlorination of methane, ethane, propane, or propene. Numerous studies have shown that the toxicity of CCl_4 is mediated by normal metabolic processes (via Cytochrome P450 system) generating the highly reactive trichloromethyl radical[4] which leads to peroxidation of cellular lipids, proteins and DNA[5,6]. It is generally accepted that CCl_4 toxicity is due to a reactive intermediate, which is generated by its reductive metabolism by hepatic Cytochrome P450[7,8]. This ultimately causes the body to experience oxidative stress and seems to play a major role in the pathogenesis of both acute and chronic liver damage[9-11]. Experimentally induced cirrhotic response in the rat by CCl_4 is shown to be superficially similar to human cirrhosis of the liver[12]. Another liver injury following CCl_4 administration is supposed to be caused by inflammatory processes from products of activated Kupffer cells (resident macrophage of liver). Thus, the observed toxicity is due to direct action of the free radicals and to indirect action mediated by cytokines such as TNF -α[13]. Toxicogenomics approaches are considered to be useful tools for the identification of predictive toxicity marker for future use an toxicological and clinical studies. In this paper we investigated the effect of the well-known hepatotoxicant CCl_4 in the gene expression and histopathological change in mouse liver.

Blood Biochemical Analysis & Histopathological

The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the serum are good indexes of the liver damage caused by CCl_4. While these activities have been shown to reflect the observed histopathology at a single time point, after 24 h after administration. Mean serum activities of the hepatocellular leakage enzymes, ALT and AST, were significantly increased compare to control, respectively, in the low and high dose groups (Fig. 1). Especially, ALT level in High dose group was increased 2 fold above to control group. Mice were treated with CCl_4 showed significant increases in serum ALT

and AST activities relative to control. H&E evaluation of stained liver sections revealed CCl_4-related histopathological findings in mice[14]. Moderate centrilobular hepatocellular necrosis was present in all CCl_4 treated mice (Fig. 2). In this study, hepatotoxic endpoint induced by CCl_4, were measured across two dose points in single time point in order to identify expression changes as they correlated with the onset and progression of histopathologic findings. Doses and duration exposure were selected from preliminary study. Histopathology and blood biochemistry confirmed that the study reproduced typical CCl_4 induced endpoint.

Gene Expression Analysis

RNA was isolated from the livers of CCl_4 treated mice. The RNA integrity of the RNA was determined using an Agilent 2100 Bioanalyzer. DNA chip analysis was done to determine differences in hepatic gene expression between CCl_4- and vehicle-treated rats. For each of the approximately 7400 genes present on TwinChip Mouse 7.4 K (Digital Genomics, Korea). Gene expression profiles of interest were significantly up- or down- regulated in mice treated with CCl_4 when compared to control. The up- and down-regulated genes are listed in Table 1, 2. Gene expression profiles showed that top 10 genes were up-regulated in mice treated low dose of CCl_4 (Table 1: upper panel); e.g. plexin B1, neoplastic progression3, glutamate-cysteine ligase, small proline-rich protein 1A, CD 68 antigen, transmembrane 4 super family member

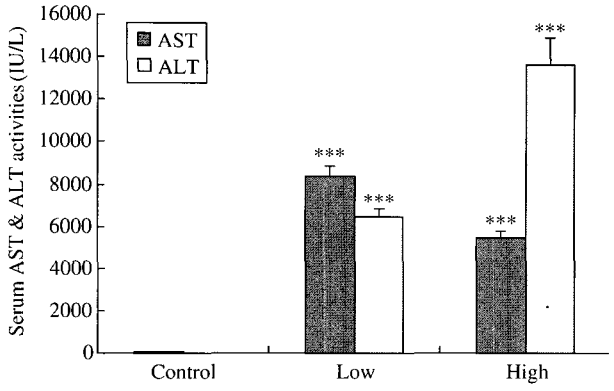

Fig. 1. AST and ALT activities in the sera of CCl_4 treated mice. Each value represents the mean±SD. ***$p<0.001$, vs. control.

Table 1. Significantly changed hepatic gene expressions at high dose of CCl_4 treatment

Gene name	
Up regulation	Fold change
Plexin B1	3.71
Neoplastic progression 3	3.03
Glutamate-cysteine ligase, catalytic subunit	2.44
Small proline-rich protein 1A	2.43
CD68 antigen	2.33
Transmembrane 4 superfamily member 4	2.31
Cytochrome P450, family 2, subfamily a, polypeptide 4	2.24
Ceroid-lipofuscinosis, neuronal 2	2.21
Apolipoprotein A-IV	2.21
IQ motif containing GTPase activating protein 1	2.14
Down regulation	Fold change
Hydroxysteroid dehydrogenase-4, d	−5.13
Carbonic anhydrase 3	−4.83
Down syndrome critical region homolog 2 (human)	−4.22
Nudix (nucleoside diphosphate link moiety X)-type motif 7	−4.16
Betaine-homocysteine methyltransfer	−3.65
Purine rich element binding protein B	−3.46
Zinc finger protein 291	−3.15
4-aminobutyrate aminotransferase	−3.12
Mus musculus transcribed sequence wit	−2.84
Aldo-keto reductase family 1, me	−2.77

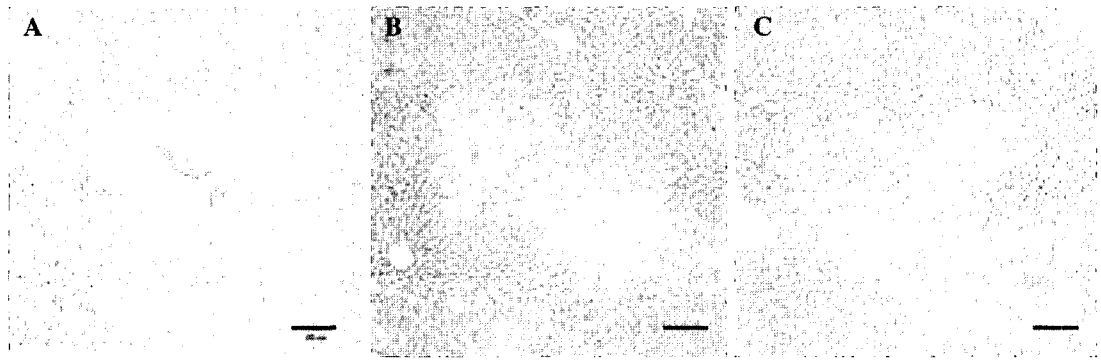

Fig. 2. H & E stained sections from mice liver after 24 h CCl_4 treatment. (A) Control (B) Low dose (C) High dose (× 100).

4, cytochrome p450 2A, ceroid-lipofuscinosis, apolipoprotein A-IV, IQ motif containing GTPase activating protein1. Also gene expression profiles showed that top 10 genes were down-regulated in mice treated low dose of CCl_4 (Table 1: lower panel). In high dose treated group, gene expression profiles that top 10 genes were up- and down-regulated in mice treated high dose of CCl_4 (Table 2). According to histopathology and blood biochemistry results, we could observe similar phenotype in low and high dose groups. We found commonly up-regulated genes in the low and high dose groups; e.g. plexin B1, small praline-rich protein 1A, neoplastic progression 3, insulin-like growth factor binding protein 1, CD68 antigen, N-myc downstream regulated 1. Also, we found commonly down-regulated genes in the both group; e.g. carbonic anhydrase 3, hydroxysteroid dehydrogenase-4, Down syndrome critical region homolog 2, zinc finger protein 291, nudix (nucleoside diphosphate linked moiety X)-type motif 7. To obtain a molecular signature of the mechanism associated with mice exposed to CCl_4, we used a hierarchical

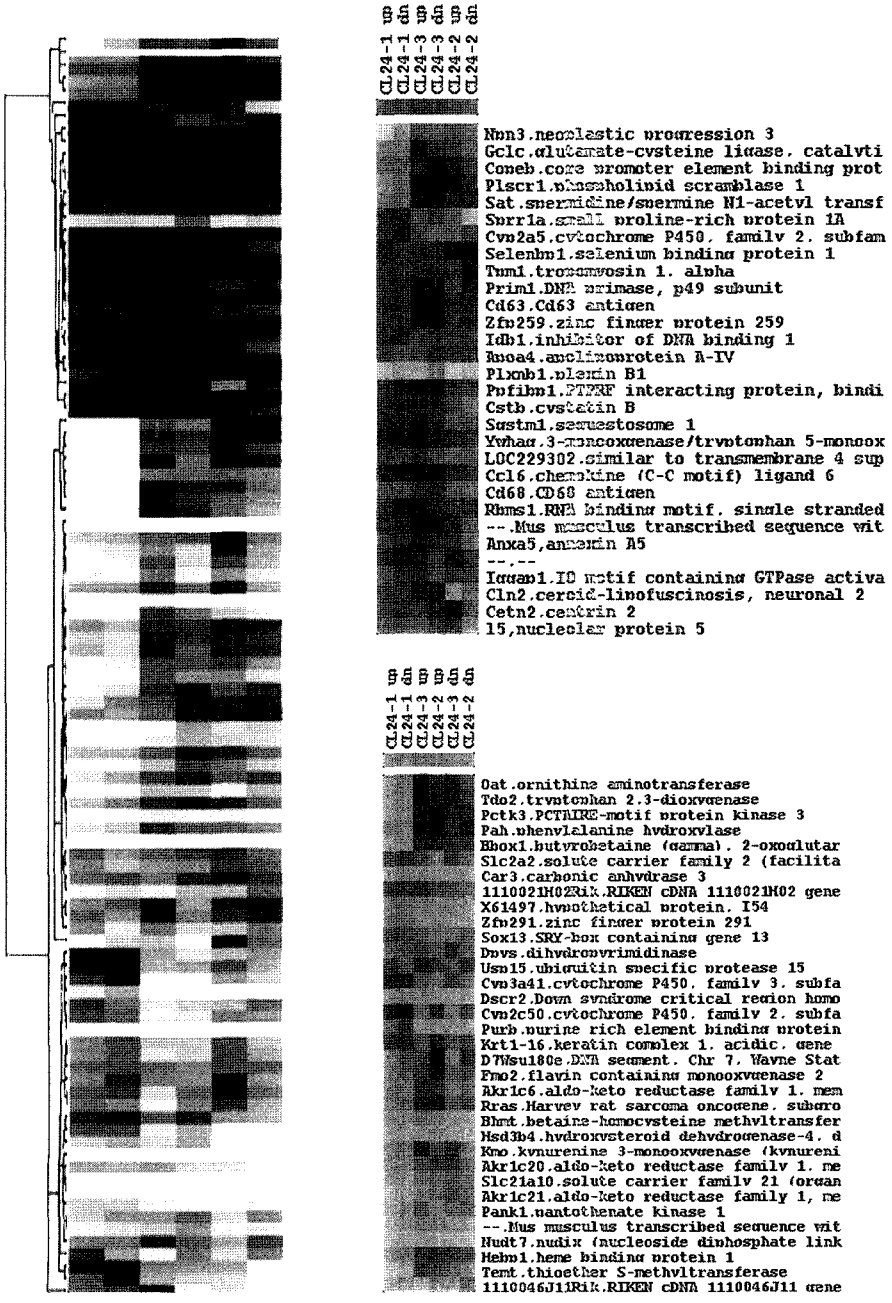

Fig. 3. Clustergram of up or down regulated genes (>3 folds) at. low dose of CCl_4 treatment. Each experimental sample is represented by a single column

Table 2. Significantly changed hepatic gene expressions at high dose of CCl_4 treatment

Gene name	
Up regulation	Fold change
Plexin B1	4.17
Small proline-rich protein 1A	3.95
Neoplastic progression 3	3.48
Insulin-like growth factor binding protein 1	3.30
CD68 antigen	3.09
Phosphomannomutase 1	2.97
Cystatin B	2.96
Core promoter element binding protein 1	2.93
N-myc downstream regulated 1	2.52
Apolipoprotein A-IV	2.46
Down regulation	Fold change
Carbonic anhydrase 3	−4.24
Hydroxysteroid dehydrogenase-4, d	−3.68
Down syndrome critical region homolog 2 (human)	−3.63
Ornithine aminotransferase	−3.14
Zinc finger protein 291	−3.07
Stearoyl-Coenzyme A desaturase 1	−2.98
Nudix (nucleoside diphosphate linked moiety X)-type motif 7	−2.68
Flavin containing monooxygenase 2	−2.65
Purine rich element binding protein B	−2.61
Cyp7b1, cytochrome P450, family 7, subfam	−2.52

clustering analysis to group genes on the basis of expressed patterns (Figs. 3, 4).

Discussion

The goal of this study was to determine whether generation of chemically associated gene expression profiles, using microarray technology, would permit classification of chemical associated signatures. Treatment with CCl_4 caused the statistically significant, at least two fold up- or down regulation of many probe set on the DNA chip. A Selection of regulated genes (Top 10) which are discussed further is displayed in Table 1, 2. The number of affected genes changed with the administered amount of CCl_4 (ranging from 158 (up: 48, down: 110) for the low dose to 200 (up: 99, down: 101) for the high dose), thus reflecting the severity of the toxic injury. While some genes appeared transiently regulated, others showed an induction or repression which was maintained throughout both dose groups. Increased levels of enzymes were observed in the mice administered low and high dose of CCl_4. At high dose, the ALT level was significantly increased (two folds) compare to low dose. Histopathological observation of the livers of the mice treated with CCl_4 at low dose (0.2 mL/kg) or high dose (2 mL/kg) showed moderate centrilobular necrosis 24 after treatment.

Some of genes, such as plexin B1, small praline-rich protein 1A, neoplastic progression 3, insulin-like growth factor binding protein 1, CD68 antigen, N-myc downstream regulated 1, were commonly up regulated at both doses. Interestingly, a single high dose CCl_4 induced the regulation of known stress markers and injury inducible gene (e.g. Crystallin b). In the low dose CCl_4 induced the known xenobiotic metabolizer also known as stress marker (e.g. Cyp P450 2A4). We found that gene expression pattern was very similar between low and high dose group. However, some stress related genes were differently expressed. These results could be a molecular signa-ture for the degree of liver injury. The main goal of this study was to identify specific genes associated with different dosing induced hepatotoxicity at single time point. Our data suggest that the degree severity could be figure out by gene expression profiling.

Methods

Animal Treatment

Specific pathogen-free male C57BL/6 (12 week old) mice were obtained from Orient Co. LTD. (Korea) and were acclimated for a week to a 12 h light/dark cycle in a humidity and temperature-controlled, pathogen-free environment. All animals care was accredited by AAALAC (American Association of Laboratory Animal Care) and IACUC. For the CCl_4 study, mice were randomly assigned to 3 per group and received single dose of 0.2, 2 mL/kg CCl_4 or corn oil (for control) by p.o. injection. Mice were sacrificed by anesthetizing diethyl ester 24 hr after CCl_4 or corn oil administration. Livers were immediately dissected and submerged in an appreciate volume of RNAlater (QIAGEN). Samples overnight in the reagent at 4°C and then remove the reagent, and store at −80°C until further processing.

Assessment of Liver Injury

Hepatotoxicity was assessed using both biochemical and histological techniques. Blood samples drawn from caudal vena cava were used to measure AST (asparatate amino transferase), ALT (alanine amino transferase) activity which is indicative of parenchymal cell damage using Shimadzu Automatic Biochemistry Analyzer. Liver sections were fixed in 10% neutrally buffered formalin and paraffin embedded. Deparaffinized sections (4 μm) were stained with hematoxylin and eosin, and analyzed by light microscopy.

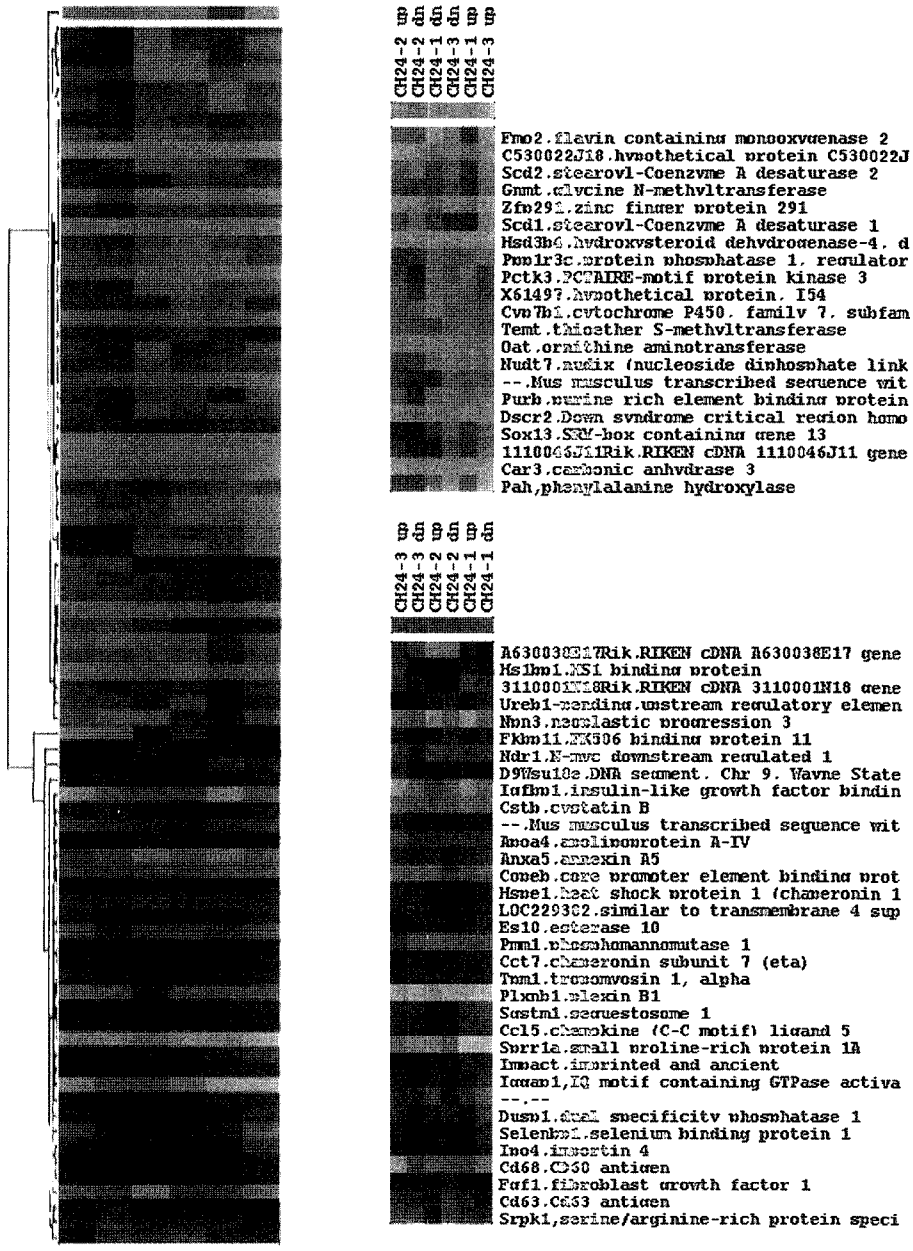

Fig. 4. Clustergram of up or down regulated genes (>3 folds) at high dose CCl_4 treatment. Each experimental sample is represented by a single column

RNA Extraction

Livers were homogenized in Trizol (InVitrogen) for RNA isolation. Total RNA was further purified using the RNeasy Mini Kit (QIAGEN). Final products yielded 260/280 nm ratios of 1.8-2.1, 230/260 nm ratio > 2.0. Quality was checked by bioanlayzer 2100 (Agilent).

cDNA Microarrays

Sample processing was performed was done according to the digital genomics Inc. Briefly, the RNA from the 3 mice in control group was pooled using equal amounts from each mouse to make a total of 30 μg of RNA and the RNA from the 3 mice in treatment group was individually prepared. These sample was labeled with Cy3 (for control) or Cy5 (for treatment) (Amersham Pharmacia, Sweden) dye coupling with aminoallyl-dUTP by a reverse-transcription reaction using reverse transcriptase, SuperScript II (InVitrogen, U.S.A.), and the oligo (dT) primer. The fluorescently labeled cDNAs were mixed from control treated sample and hybridized to the The TwinChip Mouse-7.4 K (Digital Genomics, Korea) cDNA microarray. After the washing procedure, the DNA

chips were scanned using Scanarray (Packard, U.S.A.).

Data Analysis

This microarray contains more than 7,400 distinct sequences, half of which have unknown functions. 6 replicates (duplicate × 3 biological variation) RNA samples were obtained for each experimental condition. Data analysis was performed using the GenePlex (Istech, Inc., Korea). First, all raw data were preprocessed by stages. At this stage, error flag and control spot were removed and were normalized by Block-wise centering method. The result from preprocessing could be visually expressed through MA plot, box plot and correlation plot. To find DEG (Differentially Expressed Gene), we selected up or down regulated genes (>3 folds) at 6 replicates of low and high dose. Cluster analysis and tree view were performed by using shareware from Michael Eisen's laboratory (http://rana.lbl.gov).

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