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Gene Expression Profiles Related with TCDD-Induced Hepatotoxicity

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Abstract

Toxicological studies have an object of detecting adverse effects of a chemical on an organism based on observed toxicity marker (i.e., serum biochemical markers and chemical-specific gene expression) or phenotypic outcome. To date, most toxicogenomic studies concentrated on hepatic toxicity. cDNA microarray analysis enable discrimination of the responses in animals exposed to different classes of hepatotoxicants.

In an effort to further characterize the mechanisms of 2, 3, 7, 8,-Tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin)-mediated toxicity, comprehensive temporal-responsive microarray analyses were performed on hepatic tissue from Sprague-Dawley rats treated with TCDD. Hepatic gene expression profiles were monitored using custom DNA chip containing 490 cDNA clones related with toxicology. Gene expression analysis identified 26 features which exhibited a significant change. In this study, we observed that the genes related with oxidative stress in rats exposed to Dioxin, such as CYPIIA3 and glutathione S-transferase, were up-regulated at 24hr after exposure. In this study, we carried out to discover novel evidence for previously unknown gene expression patterns related to mechanism of hepatic toxicity in rats exposed to dioxin, and to elucidate the effects of dioxin on the gene expression after exposure to dioxin.

Keywords: 2, 3, 7, 8,-Tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin), hepatotoxicity, cDNA microarray, gene expression

Toxicological studies have an object of detecting adverse effects of a chemical on an organism based on observed toxicity marker (i.e., serum biochemical markers and chemical-specific gene expression) or phenotypic outcome[1]. Previous toxicogenomic studies have shown that both large-scale measurement of gene expression (transcriptomics) and metabolite levels (metabolomics) complement the current methods to identify and discriminate different types of toxicity[2]. Transcriptomics using DNA-microarrays are highly efficient technology for identifying the gene expression regulated by mutagenic, carcinogenic and toxic xenobiotics.

Liver is one of the first organs to be exposed to be oral administered chemicals via the portal vein. Also, the major site for xenobiotic metabolism, and various chemicals can lead to the formation of active metabolites with toxic effects. The high concentration exposure and metabolic activity make the liver one of the primary targets for various types of chemical-induced toxicity[1]. To date, most toxicogenomic studies concentrated on hepatic toxicity. cDNA microarray analysis enable discrimination of the responses in animals exposed to different classes of hepatotoxicants[3-5].

TCDD is well known as one of the most potent toxicants[6]. 2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin is the prototype for a class of halogenated aromatic hydrocarbons that are common environmental contaminants and carcinogens[7]. The environmental pollutant, TCDD, is an aromatic hydrocarbon that has a long biologic half-life and accumulates in animals, especially those higher in the food chain[8]. TCDD and related compounds are legacy environmental contaminants that cause controversial human health effects at environmental levels[9]. Many of epidemiological studies have supported the increased risk for specific types of cancer including hepatocellular carcinoma. Couture *et al.*[10] emonstrated that adult rats exposed to TCDD had an elevated incidence of hepatic carcinoma. Several researchers reported that TCDD causes various biological and toxic responses in experimental animals[11]. This chemical elicits a broad spectrum of toxic and biochemical responses in a tissue-, sex-, age- and species-specific manner that include a wasting syndrome, carcinogenicity, teratogenesis, hepatotoxicity, modulation of endocrine systems, immunotoxicity and enzyme induction[7,12,13] Recent

studies proposed a possible mechanism for TCDD-mediated hepatotoxicity, including aryl hydrocarbon receptor (AhR) signaling pathway[14], and inappropriate increases in hepatic uptake of fatty acids[12]. However, the toxic effects of TCDD at the cellular level are just as diverse.

In this study, we carried out to discover novel evidence for previously unknown gene expression patterns related to mechanism of hepatic toxicity in rats exposed to dioxin, and to elucidate the effects of dioxin on the gene expression after exposure to dioxin.

Analysis of Scanned Image in Rats Exposed to Dioxin

Fluorescent intensity was visualized by phosphoimager technologies. The scanning image, which is the result of primary capture by the phosphoimager, is shown in Fig. 1. Visual inspection of the hybridization patterns readily identified a number of signals differentially expressed between control and rats exposed to dioxin. Fig. 1 is a scaned image in which the red color represents up-regulation, the green represents down-regulation in rats exposed to dioxin and the yellow represents genes of higher expression in rats exposed to dioxin and the controls, such as housekeeping genes. Analysis of the median densitometric signal intensity revealed that regulated genes compared to control were 26 genes in dioxin-exposed rats by a Z-ratio of 2 at the descriptive $P \leq 0.05$.

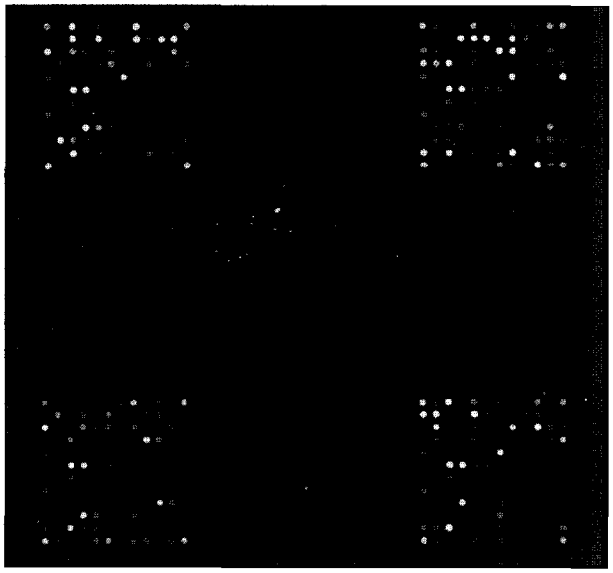

Fig. 1. Scanned image. Representative cDNA microarrays of two independent hybridization experiments comparing cDNA generated from controls or from rats exposed to dioxin.

Gene Expression Profiles in Rats Exposed to Dioxin

Using the cDNA expression array, we found that among 490 genes on the array chip, 20% of total genes presented a quantifiable expression in rats exposed to dioxin. Gene expression profiles of interest were significantly up-regulated or down-regulated in rats exposed to dioxin when compared with control. The up- and down-regulated genes are listed in Table 1. Gene expression profiles showed that 13 genes were up-regulated in rats exposed to dioxin. (Table

Table 1. Gene expression in rats exposed to dioxin.

Gene name	
Up-regulation	Z-ratio
Transcription_factor_12	7.18
'Rat_messenger_encoding_alpha-1-acid _glycoprotein	4.83
'Metallothionein	4.44
'Cytochrome_P450,_subfamily_I_ (aromatic_compound-inducible), _member_A2_(Q42,_form_d)	3.67
'Diaphorase_(NADH/NADPH)	3.20
'Cytochrome_P450,_subfamily_IIA_ (phenobarbital-inducble)/ _(Cytochrome_P450_IIA3)	3.16
Early_growth_response_1	2.48
'Rat_carnitine_palmitoyltransferase_I_mRNA,_ complete_cds	2.40
'E._coli_ompA	2.35
Glutathione-S-transferase,_alpha_type_(Yc?)	2.27
Glucose-6-phosphate_dehydrogenase	2.19
'Rat_mRNA_for_contrapsin-like_protease_ inhibitor_related_protein (CPi-26)	2.07
'Glucose-6-phosphate_dehydrogenase	2.01
Down-regulation	Z-ratio
'Bone_morphogenetic_protein_6	−5.77
'Rat_alpha-crystallin_B_chain_mRNA, _complete_cds	−5.63
'Rattus_norvegicus_adipocyte_lipid-binding _protein_(ALBP)_mRNA,_complete_cds	−4.03
R.norvegicus_mRNA_for_collagen_alpha1 _type_I	−3.63
'Retinoblastoma_1_(including_osteosarcoma)	−2.84
Actin,_gamma_2,_smooth_muscle,_enteric	−2.73
'RT1_class_Ib_gene	−2.58
'Rattus_norvegicus_FAT_mRNA,_complete_cds	−2.51
'Cytochrom_P450_ (cholesterol_hydroxylase_7_alpha)	−2.29
'Matrix_Gla_protein	−2.28
'Rattus_norvegicus_mRNA_for_collagen_alpha _2_type_V,_partial_CDS	−2.12
'Rat_S-100_related_protein_mRNA, _complete_cds,_clone_42C	−2.01
'Lactate_dehydrogenease_B	−2.01

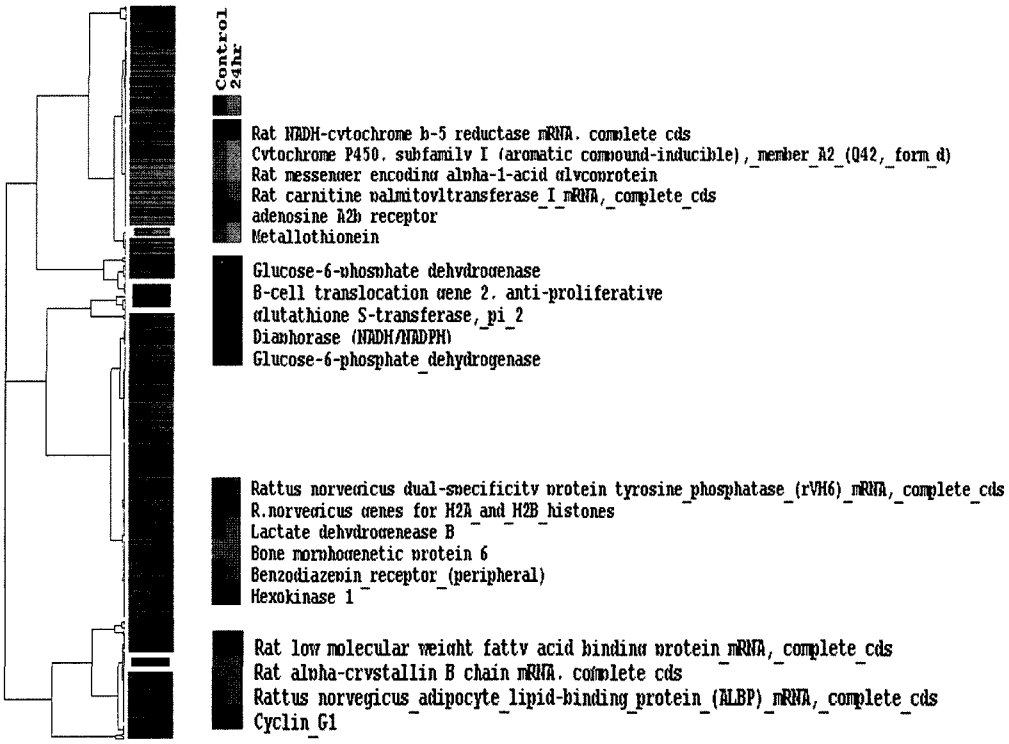

Fig. 2. Clustergram of gene expression in rats exposed to dioxin. Microarray data from controls and rats exposed to dioxin were combined and clustered. Cluster analysis was performed on Z-transformed microarray data using two separate programs available as shareware from Michael Eisen's lab. Each gene is represented by a single row of colored boxes; each experimental sample is represented by a single column. The entire clustered image is shown on the left. These clusters contain uncharacterized genes and genes not involved in these processes.

1); e.g., Transcription factor 12, Metallothionein, Cytochrome P450 IA2, Cytochrome p450 IIA3, Glutathione-S-transferase, Carnitine palmitoyl transferase, NADPH, and Glucose-6-phosphate dehydrgenase. Gene expression profiles showed that 13 genes were down-regulated in rats exposed to dioxin.

Fig. 3 Scatter plot for comparison of expression profile between controls and rats exposed to dioxin.is a scatter plot for comparing of expression profiles between controls and rats exposed to dioxin. Regression analysis of Z scores from two independent samples of dioxin and control were performed and Z scores of individual genes were plotted.

To obtain a molecular portrait of the mechanism associated with rats exposed to dioxin, we used a hierarchical clustering algorithm to group genes on the basis of similar expression patterns[17] and the data is presented in a matrix format (Fig. 2). Each row of Fig. 2 represents the hybridization results for a single DNA element of the array and each column represents the expression levels for all genes in a single hybridization sample. The expression level of each

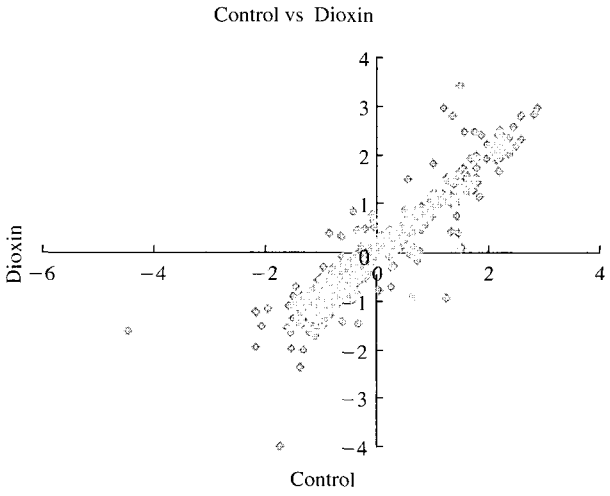

Fig. 3. Scatter plot for comparison of expression profile between controls and rats exposed to dioxin. Expression profiles of controls and rats exposed to dioxin are shown as bivariated scatter plot of 490 genes from the microarray. The values are corrected intensities relative to control, representing levels of expression for the cDNA elements of the microarrays.

gene was visualized in color, relative to its median expression level across all samples. Red represented expression greater than the mean, green represents expression less than the mean and color intensity denotes the degree of deviation from the mean. Gray represented the median expression level. Distinct samples representing similar gene patterns from control cells were aligned in adjacent rows. The clustergram revealed that clusters of genes related to progression were up- and down-regulated in dioxin-exposed rats, as compared to controls (Fig. 2).

Discussion

TCDD induces a broad spectrum of biological responses, including disruption of normal hormone signaling pathways, reproductive and developmental defects, immunotoxicity, liver damage, wasting syndrome, and cancer[18]. Although various mechanisms for toxicity of dioxin were suggested by many researchers, the accurate mechanism was not known. In this study, we carried out the evaluation of the differences in gene expression profiles between dioxin exposed groups and control according to time span after exposure.

We observed that gene expression of CYP450, GST, Diaphorase, CPT, and G6PD were up-regulated by dioxin exposure. Fisher *et al.*[7] reported a similar results previously, which shown to induce the expression of several genes through the TCDD-exposure mechanism, including cytochrome P4501A1/2, cytochrome P4501B1, glutathione S-transferase Ya, aldehyde dehydrogenase 3 and UDP-glucuronosyl transferase 1, which were called "AhR gene battery"[19].

In recent years, genome-wide expression analysis has been exploited in vitro and in vivo to uncover additional AhR-dependent or -independent TCDD-responsive genes[20]. It is well known that many effects of dioxin are due to regulation of gene expression mediated via activating the aryl hydrocarbon receptor (AhR)[21]. The AhR is one of the best-studied cellular targets of TCDD and other dioxin-like environmental contaminants[22]. TCDD is a potent hepatotoxin that exerts its toxicity through binding to the aryl hydrocarbon receptor (AhR) and the subsequent induction or repression of gene transcription[13]. Many of molecular biology studies show that the aryl hydrocarbon receptor (AhR) acts as a nuclear ligand-induced transcription factor that interacts with xenobiotics such as TCDD. Therefore, TCDD has the potential to directly alter the expression of a large number of genes[7]. The AhR gene also displays affinity for structurally related xenobiotics, including polychlorinated dibenzo-p-dioxin (PCDDs), polychlorinated dibenzofurans (PCDFs), and coplanar polychlorinated bipenylas (PBCs)[23]. The initial step in the mechanism of TCDD-toxicity involves binding to the AhR followed by a subsequent increase or decrease in the transcription of AhR-regulated genes[13,24]. Dioxin-AhR complex triggers the dissociation of interacting proteins and results in the subsequent translocation of the ligand-bound AhR to the nucleus, where it heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT)[19,25]. Thus, the formed AhR /Arnt heterodimer recognizes an enhancer DNA element designated xenobiotic responsive element (XRE) sequence located in promoter regions of target genes, which causes sustained inhibition of intercellular communication in mouse hepatoma cells and induces extensive oxidative damage in cultured cells, in mice, and in female rats.

We showed that the genes of CYP450 family were up-regulated in dioxin injected rats. The CYP gene transcript cytochrome P450 family is responsible for phase II detoxification of dioxin, which contains multiple copies of the DRE sequence[13]. Furthermore, DRE elements were well conserved with respect to location within the CYP1A1 gene for mice, rats, and humans.

Also, we observed that the genes related with oxidative stress, detoxification or antioxidant system including Cytochrome P450 family (CYP450), Metallothionein (MT), and Glutathione-S-transferase (GSH) were up-regulated. CYP450 IA2 and MT were up-regulated at all exposure time, however CYP450 subfamily IIA and GST were up-regulated at 24 and 48 hours after dioxin exposure. CYP450 is a member of a multigene family of xenobiotic metabolizing enzymes (XMEs). Beside its physiological role in the detoxification of polycyclic aromatic compounds, the activity of this enzyme can be deleterious since it generates mutagenic metabolites and active oxygen[25]. TCDD is a particularly potent mediator of ROS formation as a result of its pronounced induction of CYP450 enzymes such as Cyp1a1[26], whereas Cyp1a2 is considered only a minor contributor[11,27]. A novel P450 form CYP450 subfamily IIA was recently identified and also found to be controlled by AHR[28]. Rivera *et al.*[29] suggested that certain CYP2A enzymes can be up-regulated by ligands of AHR. In this study, we observed some differences in the time of gene expression according to CYP450 subfamily, but we failed to find some the reason of these differences. Although their induction serves an important role in detoxification, their activity also contributes to the formation of ROS, which can lead to cellular oxidative stress, lipid peroxidation, and DNA fragmen-

tation[11,26,30]. Oxidative stress refers to the generation of ROS, such as superoxide, hydroxy radicals and hydrogen peroxide and to the adaptive cellular responses to ROS (e.g., depletion of anti-oxidant stores and activation and induction of protective and repair enzymes)[31]. Oxidative stress is a biochemical modification of cells, tissues and lipids due to interactions with free radicals. Free radicals are highly unstable molecules that interact quickly and aggressively with other molecules in our bodies to create abnormal cells.

Pons *et al.*[32] reported that the rate of oxidation of TCDD shown by induced microsomes in presence of NADPH alone could be attributed to CYP1A2; whereas the stimulation of these rates caused by adding PCBs appeared to depend on CYP1A1. In this study, the gene of diaphorase related with NADPH synthesis was up-regulated at short span after dioxin exposure, and seemed to be depleted at long span after dioxin exposure.

MTs, syteine-rich metal binding proteins plays a detoxifying role against hamful heavy metals, and has been thought to be involved as a free radical scavenger in cooperation with established biomolecules that act as antioxidants[33]. The results from this study are consistent with other previous study about an antioxidant role for MT. Bauman *et al.*[34] demonstrated that liver MT levels were significantly increased in mice with oxidative stress induced by injection of a variety of chemicals. Increase in metallothionein produced by chemicals that induce oxidative stress. Nishimura *et al.*[33] suggested that MT levels are increased in the liver upon exposure to TCDD, perhaps by TCDD-generated ROS, and that it may play a protective role in TCDD-induced oxidative stress responses as an antioxidant.

In addition, binding of AhR/Arnt heterodimer to DRE drives activation of the GST gene expression[19,35]. In this study, GST was up-regulated only at the short span time after dioxin exposure. It is known to catalyze the conjugation of reduced glutathione (GSH) to electrophiles and products of oxidative stress Boverhof *et al.*[12] demonstrated that cellular GSH levels were depleted by TCDD induced ROS and it caused to leave cells susceptible to oxidative damage. Consistent with this finding, the two GSH-synthesis enzymes, glutamate-cysteine ligase, which catalyzes the first and rate-limiting step, and glutathione synthetase, which catalyzes the second step, were induced by TCDD. These secondary AhR independent hepatic damages might be mediated via AhR-independent mechanism by ROS and fat accumulation.

In this study, the genes of glucose-6-phosphate dehydrogenase (G6PD) and carnitine palmitoyl transferase (CPT1) were up-regulated 3.3-fold and 2.4-fold, respectively. CPT1, which is localized in the outer mitochondrial membrane and is generally considered to be the rate limiting enzyme for oxidation of long chain fatty acids in the liver[14].

G6pd, the key regulatory enzyme of the pentose phosphate pathway, was increased in dioxin injected rats. In addition to hormonal regulation, it has been suggested that G6pd could be responsive to oxidative stress with the ability to rapidly meet the need to maintain cellular redox state[36]. For example, hepatic G6pd has also been shown to be induced by chemicals that induce oxidative stress, including diquat and thioacetamide[37] as well as common substances such as alcohol. Likewise, Hori *et al.*[38] showed that G6pd activity was increased in mice and rats following PCB126 exposure. In addition, the expression of NADPH generating enzyme, diaphorase was increased. Boverhof *et al.*[12] reported expression of mRNA for malic enzyme, another NADPH generating enzyme was markedly increased at 24 h and 7 days in TCDD-treated rats.

Currently, there has been interest in using array in toxicology to quickly classify toxicants based on characteristic expression profiles and to use these profiles as means of identifying putative mechanisms of action. The expression profiles associated with dioxin could be useful in monitoring human populations in the environment for potential point sources of environmental contamination[39]. The microarray-based genomic survey is a high-throughput approach that allows parallel study on expression patterns of numerous of genes[40]. This technique can identify the correlation of gene expression pattern and environmental contamination by performing comparison-rank analysis of genes expressed by the transcription of DNAs into RNAs.

In summary, we discovered evidence for gene expression patterns in TCDD injected rats. The genes of CYP450 family, GST and MT were up-regulated in its early exposure to dioxin. It is suggested that dioxin exposure caused oxidative stress related with hepatic damage, and enable to inhibit the communication between cells via AhR at long time. cDNA microarray is highly likely to be an efficient technology for evaluating the gene regulation by dioxin exposure.

Methods

Animals and Drugs

Adult male Sprague-Dawley rats (Hallim Laboratories, Seoul, Korea) weighing 250 to 310 g were hou-

sed in cages in a temperature- and light-controlled environment and allowed free access to the standard laboratory chow and tap water. For 1 week before treatment and during the entire period of treatment, TCDD (Cerilliant; Cambridge Isotope Laboratories, Wo-burn, MA, USA) was administered and the rats were weighed and randomly assigned to study groups. All animals were treated according to the principles outlined in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (Bethesda, MD). Experimental animals were randomly divided into two groups (6 rats each), intraperitoneal treated with TCDD at a dosage of 40 μg/kg bw, and sacrificed after 24 hr.

RNA Isolation and Probe Labeling

Total RNA from liver samples was isolated using TRIzol reagent™ (Invitrogen) following the manufactrer's instructions. All procedures were performed according to the instructions of Genomic Tree, Inc. (Daejon, South Korea). tRNA from dioxin exposed animals (6 rats a group) was reverse transcribed in the presence of Cy3-dCTP. Pooled RNA from controls (6 rats) was reverse transcribed in the presence of Cy5-dCTP and used as a reference for the experimental animals. After tRNA (20 μg per sample) was primed with oligo d (T) at 70°C for 10 min, reaction mix containing dNTP mix, 0.1 M dithiothreitol, and first strand reaction buffer was added. For experimental RNA, dNTP mix constituted 0.5 mM dATP/dGTP/dTTP, 0.125 mM dCTP and 0.125 mM Cy3-dCTP. For reference RNA, dNTP mix contained 0.5 mM dATP/dGTP/dTTP, 0.15 mM dCTP and 0.1 mM Cy5-dCTP. Superscript II Rnase H-reverse transcriptase (Life Technologies) was added and the reaction was carried out at 45°C for 2 h. Labeled cDNA probes were purified using Wizard® Series 9600TM DNA Purification Resin (Promega Co., Madison, WI). Briefly, labeled cDNA was added to resin in 96 well filter plate, washed twice with 80% isopropanol, and eluted with water. Fluorescence intensity of Cy3 and Cy5 was determined using a Wallac Victor2 1420 Plate Reader (Perkin-Elmer Wallac Inc., Geithersburg, MD). Acceptable fluorescence intensities for each channel were 250,000-700,000 units for Cy3 and 100,000-250,000 units for Cy5 .

Hybridization, Scanning and Quantification

Cy5- and Cy3-labeled probes were combined and hybridized to microarrays (684 genes or ESTs) overnight at 42°C. Due to the presence of both biological replicates (multiple animals/group) and spot replicates (all genes represented in four replicates across the array), each liver RNA sample was hybridized to only one array. Microarrays were washed, dried, and scanned using an 428™Array Scanner (Affymetrix Inc.). Fluorescence intensities of the Cy3 and Cy5 channels were quantitated using Imagene software.

Data Analysis

The data were normalized with Z scores by subtracting each average of gene intensity and dividing with each standard deviation. A Z score represents the variability from the average intensity, expressed in units of standard deviation, for each of the 490 genes. A Z score provides flexibility to compare different sets of microarray experiments by adjusting differences in hybridization intensities. Gene expression differences between microarray experiments were calculated by comparing Z score differences for the same genes in different microarrays, which allowed gene expression to be compared in different samples. Z differences were calculated by subtracting the Z score of the control from each Z score of the control sample. These Z differences were further normalized to distribute their position by dividing with the average z difference and dividing with the standard deviation of the z differences. These distributions represented Z ratio values and allowed efficient comparisons to be made between microarray experiments[15]. Scatter plots of intensity values were produced in Spotfire (Spotfire, Inc., Cambridge, MA)[16]. Color overlay images were produced in Adobe Photoshop 5.0 (Adobe Systems, Inc., San Jose, CA). Cluster analysis was performed on Z-transformed microarray data by using two programs available as shareware from Michael Eisen's laboratory (http://rana.lbl.gov). Clusterings of changes in gene expression were determined by using public domain Cluster based on pair wise complete-linkage cluster analysis[17]. Raw gene expression, log values, and Z scores were averaged and are expressed as means ± standard deviation. Raw gene expression, log values and Z scores were averaged and are expressed as means ± standard deviation.

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