MOLECULAR & CELLULAR TOXICOLOGY, Vol. 2, No. 2, 120-125, June 2006

Whole Genomic Expression Analysis of Rat Liver Epithelial Cells in Response to Phenytoin

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Accepted 13 May 2006

Abstract

Phenytoin is an anti-epileptic. It works by slowing down impulses in the brain that cause seizures. The recent microarray technology enables us to understand possible mechanisms of genes related to compounds which have toxicity in biological system. We have studied that the effect of a compound related to hepatotoxin *in vitro* system using a rat whole genome microarray. In this study, we have used a rat liver epithelial cell line WB-F344 and phenytoin as a hepatotoxin. WB-F344 was treated with phenytoin for 1 to 24 hours. Total RNA was isolated at times 1, 6 and 24h following treatment of phenytoin, and hybridized to the microarray containing about 22,000 rat genes. After analysis with clustering methods, we have identified a total of 1,455 differentially expressed genes during the time course. Interestingly, about 1,049 genes exhibited differential expression pattern in response to phenytoin in early time. Therefore, the identification of genes associated with phenytoin in early response may give important insights into various toxicogenomic studies *in vitro* system.

Keywords: Phenytoin, toxicogenomics, DNA chip, gene expression, rat liver epithelial cell

Microarray technology permits the comparison of thousands of genes in different biological system. This system has been used for the prediction of toxicity through gene expression induced toxicant and has shown that compounds with similar toxic mechanism produce similar changes in gene expression *in vivo* and *in vitro* system[1,2].

Phenytoin (PT) is an anticonvulsant drug which can be useful in the treatment of epilepsy[3,4]. The primary site of action appears to be the motor cortex where spread of seizure activity is inhibited. Possibly by promoting sodium efflux from neurons, PT tends to stabilize the threshold against hyperexcitability[5] caused by excessive stimulation or environmental changes capable of reducing membrane sodium gradient. This includes the reduction of posttetanic potentiation at synapses[6]. Loss of posttetanic potentiation prevents cortical seizure foci from detonating adjacent cortical areas. PT reduces the maximal activity of brain stem centers responsible for the tonic phase of tonic-clonic (grand mal) seizures[7-9].

In this study we carried out microarray technology for toxicogenomics study using PT. In the experiment by treatment PT compound, we show many early response genes in 1 hr and 6 hr. The most of genes were specially decreased in 24 hr. The specific gene pattern included genes related molecular signaling. Therefore the results show that PT regulates expression of numerous genes via molecular signaling mechanisms *in vitro*.

Cytotoxicity Assay

WB-F344 cells were exposed concentration of PT ranging from 0 to 500 μM. As shown in Fig. 1, cell survival decreases with increasing doses of PT. Following 24 h of treatment with PT, 20% cell death was observed with 500 μM PT (Fig. 1).

Analysis of Microarray Expression Data with Hierarchical Clustering

In order to identify genes that are related to time point about toxic compound, hierarchical clustering was performed based on the expression profiles about triplicate hybridization in each time point (Fig. 2). In this experiment, signals lower than background signals and signal spot differ from proper signal spot except from analysis doing cut off. Selected genes have signals over two times among triplicate hybridization, the signals is calculated to mean value. The expression pattern is analyzed by mean intensity.

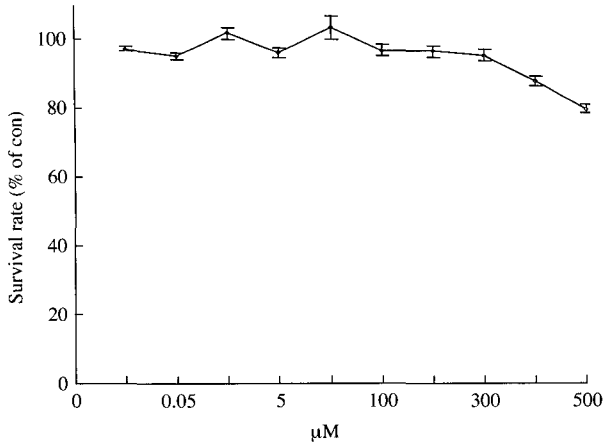

Fig. 1. Cytotoxicity assay data from PT treatment over a period of 24 h.

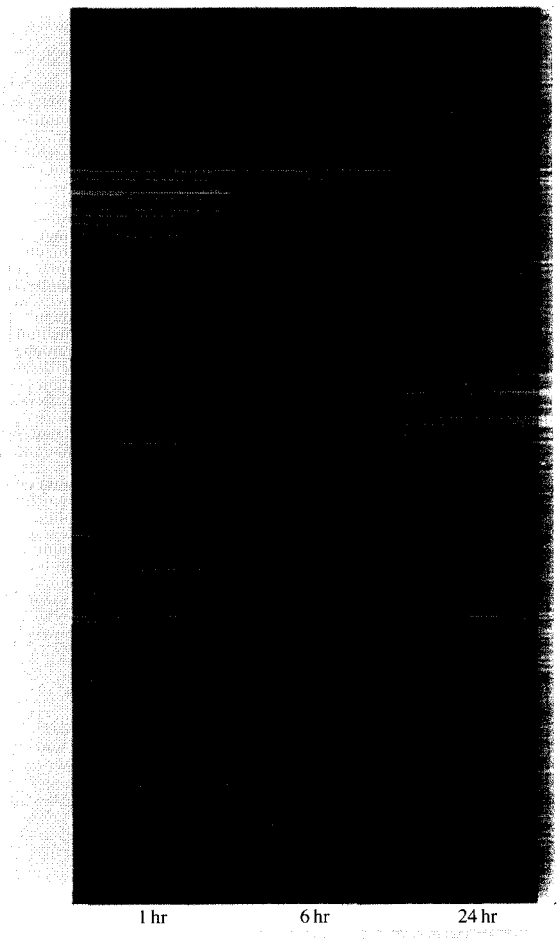

Fig. 2. Hierarchical clustering of gene expression profiling with Phenytoin treatment.

Table 1. Number of genes related to each expression pattern 3 and 4.

Ontology	Phenytoin	
	Pattern 3	Pattern 4
G-protein coupled receptor protein signaling pathway	42	42
Cell surface receptor linked signal transduction	48	36
Cell death	14	8
Detection of chemical stimulus	40	29

Expression Pattern Analysis Based on the Time After PT Administration

In order to identify the major gene expression profiles, expression pattern analyses were performed at 1, 6 and 24 hr after treatment with PT. The similar six expression patterns have increased in gene expression and decreased in gene expression by time points (Fig. 3).

Gene expression patterns treated with PT in 1 and 6 hr were not shown big differences. But gene expression patterns between 6 and 24 hr (pattern 3 and 4) were quite different. Gene expression variances were identified by comparative analyses on gene expression pattern on each time points. We have classified the genes whose expressions were significantly changed in pattern 3 and 4. From the results, classified the genes included G-protein coupled receptor protein signaling pathway, cell surface receptor linked signal transduction, cell death and detection of chemical stimulus ontology (Table 1).

Classification of Pathway Related Genes in PT-Induced *in vitro*

We analyzed specific 2 fold changed gene expression pathways in each time phase. From the results, numerous genes were distributed into adherens/tight junction, MAPK signaling pathway, cell communication and leukocyte transendothelial migration pathway category (Table 2). Also most of gene expression variance related to each pathway category was decreased after 24 h treatment. These analyses also indicated that the identification of genes associated with PT in early response may give important insights into various toxicogenomic studies *in vitro* system.

Comparison of Real-Time PCR (Q-PCR) and Microarray Data

Genes were tested by Q-PCR, including Rasgrp 1 and GAPDH which was used as a housekeeping control. Similar differences in RNA levels were identified by Q-PCR. As predicted by Q-PCR results, Rasgrp 1 is highly expressed in 24 hr. The expression profiles

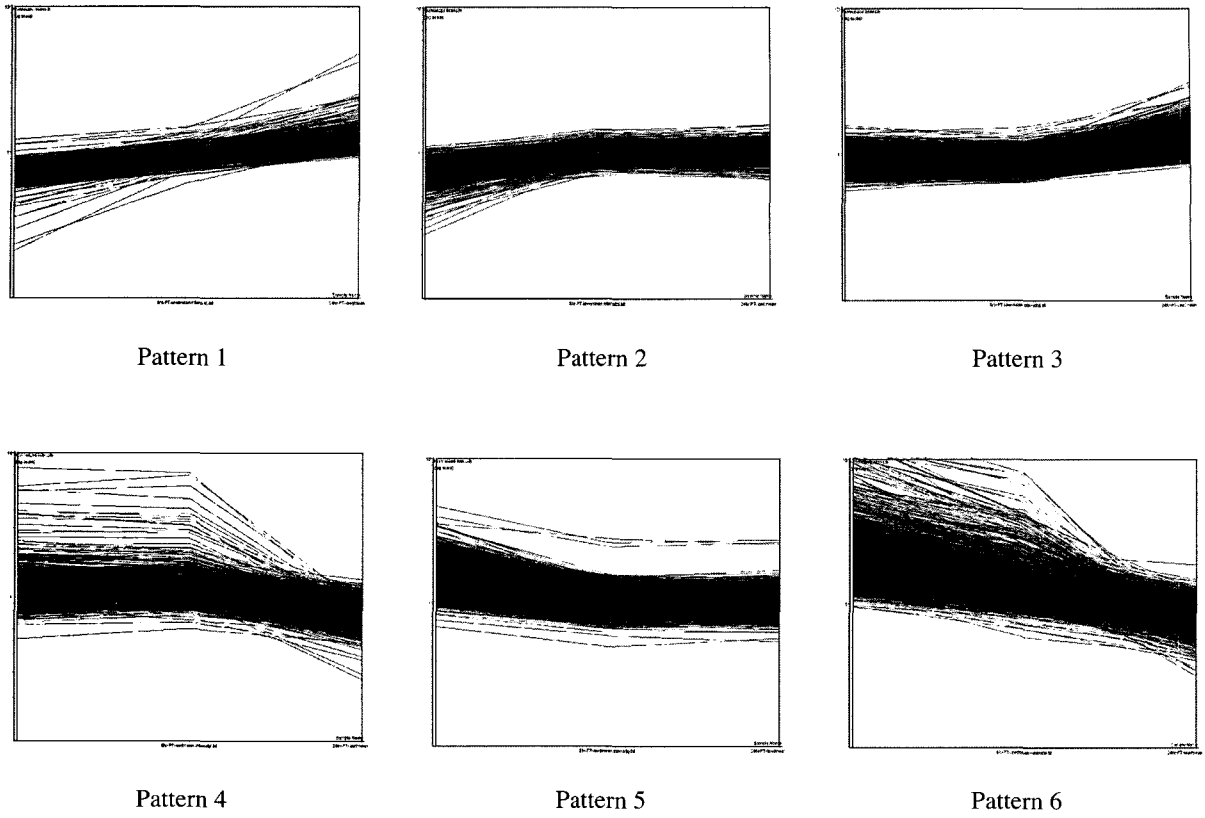

Fig. 3. Expression Pattern analysis based on the time after PT administration.

Table 2. Pathway classification of 2-fold changed genes.

Pathway	Phenytoin		
	1 hr	6 hr	24 hr
Glycolysis/Gluconeogenesis	11	1	3
Oxidation phosphorylation	7	2	–
Cell Communication	13	5	1
Ribosome	9	6	2
MAPK signaling pathway	14	1	3
Neuroactive ligand-receptor interaction	7	3	1
Cell cycle	6	2	–
Apoptosis	5	–	1
Adherens/Tight junction	28	5	–
T cell/B cell receptor signaling pathway	13	2	1
Leukocyte transendothelial migration	11	2	
Alzheimer's disease	3	2	

and extent of Rasgrp 1 gene expressions were almost the same as those of the DNA microarray with time course (Fig. 4).

Discussion

The recent DNA microarray technology enables us to understand a large number of gene expression profiling. The technology has potential possibility to comprehend mechanism of multiple genes are related to toxicants which have toxicity in biological system[10]. Therefore, the toxicogenomics through this technology may be very powerful for understanding the effect of unknown toxic mechanism in biological system. In this study, we have examined gene expression profiling after PT treatment *in vitro* using whole genomic microarray.

Phenytoin is an antiepileptic drug which can be useful in the treatment of epilepsy. However, phenytoin was also found to have side effects as liver injury[11], leukocytosis and eosinophilia. In this study, our purpose was to identify genetic biomarkers for toxicity by measurements of gene expression levels of PT-induced hepatotoxicity and analysis of pathways using whole genomic microarray.

The gene expression patterns in 1 and 6 hr treatments showed similar patterns but differ from 24 hr treatment (Fig. 3). In these early responsed genes, G-

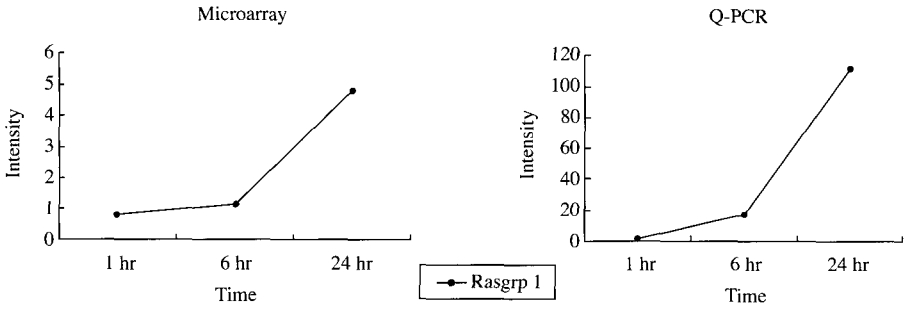

Fig. 4. Comparison of Real-time PCR (Q-PCR) and microarray data on Rasgrp 1.

protein coupled receptor protein signaling pathway[12], cell surface receptor linked signal transduction, cell death, detection of chemical stimulus were included.

We also identified side effect-related genes of PT. PT was reported side effects of leukocytosis and eosinophilia, and we have identified genes related to the condition. Because Flt3 (FMS-like tyrosine kinase 3) and Il4rα (Interleukin 4 receptor α) that related in differentiation of hematopoietic stem cell[13] to leukocyte and eosinophil were up-regulated. We assumed that they will increase leukocyte and eosinophil production. In addition, PT was reported to reduce convulsions by blocking incoming of sodium into the cell, Scn7α (sodium channel voltage-gated type VII α) was down-regulated in every time point. So we could identify the action of PT as the antiepileptic drug.

We also observed significant up-regulated genes related to liver injury. Cacnb2 (calcium channel voltage-dependent β2), Ras, Map2k1 (mitogen-activated protein kinase 1) and Atf4 (activating transcription factor 4) were up-regulated which may related to abnormal proliferation[14].

To validate Microarray data, we performed real-time PCR (Q-PCR) on the same RNA sample that we were used for the microarray experiments[15,16]. RasGRP is a Ras activator that contains a DAG-binding C1 domain. RasGRP expression has only been documented in some hematopoietic lineages, including T cells and thymocytes, as well as in a subset of brain neurons and cells of the kidney[17]. Q-PCR result of Rasgrp 1 gene was shown same pattern as the microarray data.

Our results suggest that the identification of genes associated with phenytoin in early response may give important insights into various toxicogenomic studies *in vitro* system.

Methods

Cell Line Treatment

WB-F344 (WB) cell was cultured in D-media (Formula No. 78-5470EF, Gibco BRL, Grand Island, NY, USA) containing 3 mL/L Penicillin-Streptomycin-Neomycin solution (Invitrogen, USA) mixture in the presence of 5% fetal bovine serum. Cells were in a 37°C humidified incubator containing 5% CO_2 and 95% air. Cells were grown in 175 cm^2 flask and the culture medium was changed every other day. The cytotoxic effects of phenytoin in WB cells were measured by MTT assay, based on the ability of live cells to convert tetrazolium salt into purple formazan. In brief, the cells were seeded at a density of 2×10^5 cells/mL in 24-well microplates and incubated overnight at 37°C, 5% CO_2. Then the cells were treated with different concentrations of chemicals or its vehicle, deionized water for 12 hr. Following treatment, the cells were rinsed three times with PBS and then incubated with addition of phenytoin (10 mM) stock for the last 4 hr of incubation. After media was removed, added 200 μL of dimethyl sulfoxide (Sigma, USA) to each well and then pipetted up and down to dissolve the crystals. WB cells were seed at a density 2×10^5 cells/mL in 10 cm dishes and incubated overnight at 37°C, 5% CO_2.

RNA Isolation

Following the appropriate incubation period, WB cells were washed three times in PBS and the cells scraped into 1 mL TRIzol Reagent™ (Invitrogen, Carlsbad, CA) per dish. RNA was extracted from four individual culture incubations and equal amount of RNA from each conditions were pooled. The extracted RNA was dissolved in RNase-free water, and its concentration and purity was determined from absorbance measurements at 260 and 280 nm using a spectrophotometer.

Microarray Hybridization

In this experiment, we used OpArray Rat genome 27 K oligonucleotide chip (Operon Biotechnologies GmbH) consisting of 26,962 oligos representing about 22,000 genes including ESTs. Microarray experiments were performed using the manufacturer's

protocol. For microarray hybridization, total RNA of untreated WB control RNA was pooled and used for hybridization and each total RNA sample (30 μg) was labeled with Cyanine (Cy3) or Cyanine (Cy5) conjugated dCTP (Amersharm, Piscataway, NJ) by a reverse transcription reaction using reverse transcriptase, SuperScrip II (Invitrogen, Carlsbad, California). The fluorescently labeled cDNAs were mixed and hybridized simultaneously to the OpArray rat 27 K oligo microarray. We have hybridized TA treated samples three times per each time points (1 hr, 6 hr and 24 hrs). Processed slides were scanned with an Axon 4000B Scanner (Axon Instruments, Foster City CA) using laser excitation of the 2 fluor at 532 and 635 nm wave length for the Cy3 and Cy5 labels, respectively. The scanned images for each slide were analyzed using the GenPix pro 5.1 Software (Axon instruments).

Data Management

The raw intensity data was global normalized by intensity dependent normalization in lowess method, then was normalized by with-print-tip group normalization method for each print-tip, 48 tips were used for making OpArray Rat genome 27 K microarray. Statistical software was used to determine the mean for triplicate experiment data. Microarray data management was performed with GeneSpring 7.2 software (Agilent Technologies). Comparison of present genes, fold change determinations and various clustering analyses were performed. The gene expression values for each array were normalized to their respective median value. All clustering analyses were performed using standard correlations. Fold change filters included the requirement that the genes be present in at least 200% of controls for up-regulated genes and lower than 50% of controls for down-regulated genes.

Real-Time Quantitative PCR Analysis

Real-time quantitative PCR was performed in triplicate in 384-well plates; each 20 μL reaction consisted of 10 μL of Universal TaqMan Probe Master Mix (Applied Biosystems), 0.8 μL of 10 pM forward and reverse primers of Rasgrp 1 and GAPDH. 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 30 s and 60°C for 30 s, 72°C for 30 s. The sequences of the primers designed to span within Rasgrp 1 gene are as follows: 5′-tca aat acc agc aag aag cg-3′ (forward primer), 5′-ata tcc gtc gga agg act tg-3′ (reverse primer). Each of the 384-well real-time quantitative PCR plates included serial dilutions (1, 1/2 and 1/4, 1/8, 1/16) of cDNA, which were used to generate relative standard curves for Rasgrp 1 and GAPDH. We then converted real-time PCR cycle numbers to gene amounts (ng) on the basis of the equation. The real-time PCR analysis was performed on an Applied Biosystems Prism 7900 Sequence Detection System (Applied Biosystems).

Acknowledgements

This study was supported by a grant of the Korea Health 21 R & D Project, Ministry of Health & Welfare, Republic of Korea (02-PJ10-PG4-PT02-0015).

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