Available online at www.tox.or.kr

Differential Gene Expression Induced by Naphthalene in Two Human Cell Line, HepG2 and HL-60

Youn-Jung Kim[1], Mee Song[1], Mi-Kyung Song[1], Da-Young Youk[1], Han-Saem Choi[1], Sailendra Nath Sarma[1,2] & Jae-Chun Ryu[1,2]

[1]Cellular and Molecular Toxicology Laboratory, Korea Institute of Science and Technology, PO Box 131, Cheongryang, Seoul 130-650, Korea
[2]Department of Biopotency/ Toxicology Evaluation, University of Science & Technology, Daejeon, Korea
Correspondence and requests for materials should be addressed to J. C. Ryu (ryujc@kist.re.kr)

Accepted 11 May 2009

Abstract

Naphthalene is bicyclic aromatic compound that is widely used in various domestic and commercial applications including lavatory scent disks, soil fumigants and moth balls. Exposure to naphthalene results in the development of bronchiolar damage, cataracts and hemolytic anemia in humans and laboratory animals. However, little information is available regarding the mechanism of naphthalene toxicity. We investigated gene expression profiles and potential signature genes in human hepatocellular carcinoma HepG2 cells and human promyelocytic leukemia HL-60 cells after 3 h and 48 h incubation with the IC_{20} and IC_{50} of naphthalene by using 44 k agilent whole human genome oligomicroarray and operon human whole 35 k oligomicroarray, respectively. We identified 616 up-regulated genes and 2,088 down-regulated genes changed by more than 2-fold by naphthalene in HepG2 cells. And in HL-60, we identified 138 up-regulated genes and 182 down-regulated genes changed by more than 2-fold. This study identified several interesting targets and functions in relation to naphthalene-induced toxicity through a gene ontology analysis method. Apoptosis and cell cycle related genes are more commonly expressed than other functional genes in both cell lines. In summary, the use of *in vitro* models with global expression profiling emerges as a relevant approach toward the identification of biomarkers associated with toxicity after exposure to a variety of environmental toxicants.

Keywords: Naphthalene, HepG2, HL-60, Gene ontology, Differentially expressed gene (DEG)

Naphthalene is one of components of jet fuel, diesel fuel and cigarette smoke[1]. Naphthalene is a widespread environmental contaminant because of its generation of a byproduct of incomplete combustion[2]. Naphthalene is a bicyclic aromatic hydrocarbon that is widely used commercially in synthetic leather tanning agents, resins, dyes, surfactants, moth repellents, lavatory scent disc and soil fumigants[1,3]. Toxicity from naphthalene exposure is associated with cataract formation, the development of hemolytic anemia and respiratory tract irritation[3-5].

The toxicity of naphthalene is in part associated with free radicals and free radical-mediated oxidative stress[3]. The metabolic activation such as production of reactive metabolites has been considered a key player in the mechanism of naphthalene toxicity[1]. Naphthalene is metabolized by the cytochrome P450s (CYP450s), multiple metabolic enzymes, into naphthalene epoxide. The production of epoxides has several competing reactions: conjugation to glutathione (GSH), binding to protein, nonenzymatic rearrangement into naphthol or enzymatic conversion to dihydrodiol. Both naphthol and dihydrodiol are enzymatically converted to naphthalenediol. This metabolite is subsequently oxidized to naphthoquinone which generates reactive oxygen species (ROS). ROS leads to cell death caused by severe oxidative stress within the cells. ROS formation induces cellular GSH depletion caused by the conjugate of naphthoquinone with GSH. In addition, DNA or protein adducts formed by quinones induce DNA single strand breaks leading to cellular damage[3,6].

Naphthalene is classified as a group 2B, a possible human carcinogen, by both the International Agency for Research on Cancer (IARC) and the U.S. Environmental Protection Agency (EPA)[1,2]. Cytogenetic tests with chinese hamster ovary cells showed an increase in sister chromatid exchanges with and without metabolic activation and an increase in chromosomal aberrations with liver S9 activation[7].

A broader understanding of the impact of exposure to toxicants in specific cells can be mechanistically

informative through a number of differentially expressed genes (DEGs) contributing to variations in individual susceptibility to toxicants. DNA microarray offers a powerful technology that enables the examination of the expression of thousands of genes simultaneously and may give key insights into exposure to toxicants[8,9]. In particular, there has been interested in using arrays in toxicology to discriminate and classify toxicants on the basis of DEGs profiles induced by putative toxic actions[9]. The Gene Ontology (GO) analysis of microarray data provides structured and controlled vocabularies and classifications for several domains of molecular and cellular biology[10]. GO is generally used to annotate the functions of genes and is composed of three domains, biological process, cellular components and molecular functions. Biological process can be prospected according to combination of microarray results biological pathway information[11].

The aim of this study is the identification of potential gene-based markers and of biological processes induced by naphthalene in independent of cell type. In this study, we have analysed the mRNA levels of two distinct human cell lines, HepG2 derived from a hepatocellular carcinoma and HL-60 originating from a promyelocytic leukemia, after exposure to naphthalene. Profiles of transcription signatures generated for HepG2 and HL-60 cells were used to compare overall patterns of gene expression and to identify DEGs. And also response of these DEGs to naphthalene can give greater understanding of molecular mechanisms.

Cytotoxicity of Naphthalene in HepG2 and HL-60 Cells

Relative survival of HepG2 and HL-60 cells following exposure to a range of concentrations of naphthalene was determined by MTT assay. The survival percentage relative to solvent control (DMSO) was determined as a percentage of optical density value measured after treatment. Based on the results of MTT assay, 20% and 50% cell viability inhibitory concentration (IC_{20} and IC_{50}) of each cells were calculated, respectively. Dose dependent cell viability curves were obtained after 48 h of exposure to naphthalene in HepG2 cells as shown in Figure 1. The IC_{20} value for naphthalene was 1,772.23 μM. And the IC_{50} value after 3 h exposure to naphthalene in HL-60 cells was 773 μM.

Analysis of Genes with Altered Expression in HepG2 Cells Treated to Naphthalene

HepG2 cells were treated with 1,772.23 μM naphthalene for 48 h, and the total RNA was subjected to microarray analysis. The changes of gene expression were analyzed by comparing with treated group and

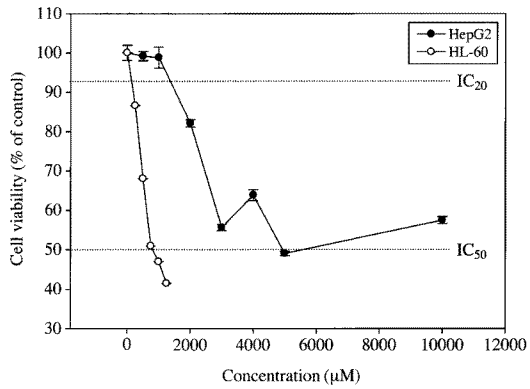

Figure 1. Cell viability of HepG2 and HL-60 cells treated to naphthalene. Dose-response curve assessed after treatment. HepG2 and HL-60 cells were treated with naphthalene at the indicated concentration in 24-well plate for 3 h and 48 h, respectively. MTT (4 or 5 mg/mL in PBS) solution was added to each well and incubated for 3 h. DMSO solution was added to each well and transfer in 96 well plates. The optical density (O.D.) of the purple formazan product was measured at a wavelength of 540 nm. The IC_{20} value of naphthalene in HepG2 was calculated to 1,772.23 μM after 48 h treatment. And the IC_{50} value of naphthalene in HL-60 was calculated to 773 μM after 3 h treatment. Values are expressed as percentage of corresponding controls and standard deviation of data from triplicate experiments.

control group using a statistical criteria of $\geq$2-fold changes with a significant t-test P-value<0.01. From this analysis, 616 genes were up-regulated and 2,088 genes were down-regulated. These genes were classified according to GO biological process to analyze molecular mechanism to naphthalene. EASE analysis was performed on genes to find biological processes significantly over represented in these gene lists in order to identify any biological terms that occur in response to naphthalene using program in http://david.abcc.ncifcrf.gov/. And then the biological process terms were condensed to the most common parent term without going higher than the fourth GO level below biological process. Biological processes significantly affected (Fisher exact test, P-value<0.05) by naphthalene are listed in Table 1. As shown in Table 1,397 up-regulated genes could be annotated and 52 biological processes containing at least two gene hits were found. Differentially up-regulated genes involved in cell development, apoptosis, phosphate metabolic process, regulation of cell cycle, generation of neurons, signal transduction, hemopoiesis, angiogenesis, Wnt receptor regulation of transcription and cell migration.

And, functional classification of the annotatable 1,042 genes repressed by naphthalene exposure reveal-

Table 1. Biological processes significantly affected (Fisher exact test, P-value < 0.05) by naphthalene in HepG2 cells as determined by EASE.

Up-regulated genes

Biological process	Probability	Biological process	Probability	Biological process	Probability
cellular component organization and biogenesis	6.72E-07	regulation of neurogenesis	5.28E-03	protein import	2.37E-02
cell morphogenesis	5.93E-06	signal transduction	5.99E-03	actin filament organization	2.43E-02
cell development	1.44E-05	wound healing	6.22E-03	negative regulation of growth	2.60E-02
negative regulation of cellular process	3.29E-05	regulation of cell growth	6.64E-03	protein metabolic process	2.73E-02
phosphate metabolic process	1.10E-04	nuclear import	8.09E-03	hemopoiesis	2.74E-02
apoptosis	1.39E-04	regulation of cell differentiation	8.25E-03	mitochondrion organization and biogenesis	3.16E-02
regulation of cell proliferation	5.59E-04	negative regulation of developmental process	8.58E-03	angiogenesis	3.20E-02
protein kinase cascade	5.72E-04	intracellular protein transport	9.05E-03	nuclear transport	3.43E-02
regulation of cell cycle	6.02E-04	glucose catabolic process	1.06E-02	negative regulation of metabolic process	3.47E-02
cell growth	1.45E-03	regulation of gene expression, epigenetic	1.07E-02	regulation of protein kinase activity	3.65E-02
positive regulation of cellular process	2.03E-03	vasculature development	1.08E-02	regulation of MAP kinase activity	4.11E-02
cellular carbohydrate catabolic process	2.16E-03	regulation of apoptosis	1.25E-02	blood coagulation	4.28E-02
enzyme linked receptor protein signaling pathway	3.05E-03	neuron differentiation	1.31E-02	organ development	4.41E-02
protein modification process	3.18E-03	dephosphorylation	1.64E-02	Wnt receptor signaling pathway	4.47E-02
phosphorylation	3.26E-03	protein complex assembly	2.12E-02	mRNA catabolic process	4.49E-02
generation of neurons	3.52E-03	negative regulation of cellular metabolic process	2.33E-02	negative regulation of transcription	4.78E-02
cytoskeleton organization and biogenesis	5.12E-03	alcohol catabolic process	2.36E-02	nervous system development	4.84E-02
				cell migration	4.89E-02

Down-regulated genes

Biological process	Probability	Biological process	Probability	Biological process	Probability
regulation of cellular metabolic process	2.89E-07	ethanol metabolic process	5.32E-03	cell cycle arrest	2.60E-02
transcription	1.74E-06	protein ubiquitination	6.01E-03	microtubule polymerization	2.60E-02
regulation of transcription	2.17E-06	negative regulation of transcription	7.15E-03	forebrain development	3.09E-02
RNA splicing	4.01E-06	negative regulation of metabolic process	8.29E-03	positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	3.17E-02
mRNA processing	1.41E-05	methionine metabolic process	9.15E-03	cholesterol metabolic process	3.19E-02
RNA processing	2.22E-05	Wnt receptor signaling pathway	1.11E-02	pancreas development	3.40E-02
cellular component organization and biogenesis	1.15E-04	protein-RNA complex assembly	1.17E-02	nervous system development	3.48E-02
regulation of cell cycle	1.82E-04	nucleic acid transport	1.41E-02	intracellular protein transport	3.84E-02
M phase	1.03E-03	RNA transport	1.41E-02	chromosome organization and biogenesis	4.05E-02
cell cycle phase	2.01E-03	apoptosis	1.48E-02	neuron migration	4.16E-02
amino acid biosynthetic process	3.50E-03	negative regulation of cellular process	1.64E-02	genitalia development	4.31E-02
cell development	3.90E-03	regulation of cellular component organization and biogenesis	1.67E-02	positive regulation of cellular process	4.79E-02
spliceosome assembly	4.02E-03	protein kinase cascade	2.07E-02		

Table 2. Biological processes significantly affected (Fisher exact test, P-value < 0.05) by naphthalene in HL-60 cells as determined by EASE.

Up-regulated genes

Biological process	Probability	Biological process	Probability	Biological process	Probability
apoptosis	6.03E-05	myeloid leukocyte activation	1.38E-02	regulation of transcription factor activity	3.61E-02
regulation of apoptosis	9.47E-05	negative regulation of transport	1.57E-02	regulation of transport	3.61E-02
I-kappaB kinase/NF-kappaB cascade	1.72E-04	nuclear import	1.63E-02	regulation of cell cycle	3.83E-02
protein kinase cascade	5.73E-04	nucleosome assembly	1.63E-02	peptidyl-amino acid modification	3.84E-02
negative regulation of cytokine production	1.24E-03	production of molecular mediator of immune response	1.66E-02	positive regulation of cellular metabolic process	3.87E-02
positive regulation of cellular process	2.33E-03	transcription	1.86E-02	mast cell degranulation	4.12E-02
cell development	2.77E-03	anti-apoptosis	1.87E-02	mast cell mediated immunity	4.12E-02
negative regulation of cellular process	2.85E-03	signal transduction	2.15E-02	negative regulation of DNA binding	4.12E-02
response to hypoxia	2.96E-03	cell migration	2.34E-02	immune system development	4.19E-02
positive regulation of programmed cell death	4.37E-03	mast cell cytokine production	2.37E-02	amino acid transport	4.29E-02
positive regulation of metabolic process	5.19E-03	negative regulation of immune effector process	2.37E-02	regulation of amino acid metabolic process	4.43E-02
induction of apoptosis	9.08E-03	protein import	2.83E-02	peptidyl-tyrosine phosphorylation	4.58E-02
inflammatory response	9.40E-03	T cell activation	3.32E-02	negative regulation of binding	4.69E-02
regulation of phosphorylation	9.92E-03	hemopoiesis	3.52E-02		

Down-regulated genes

Biological process	Probability	Biological process	Probability	Biological process	Probability
cellular component organization and biogenesis	3.40E-04	biopolymer methylation	3.12E-02	negative regulation of cellular component organization and biogenesis	4.43E-02
cell cycle phase	1.18E-03	protein depolymerization	3.43E-02	negative regulation of signal transduction	4.44E-02
M phase	5.05E-03	cytoskeleton organization and biogenesis	3.85E-02	cation transport	4.87E-02
negative regulation of protein metabolic process	5.72E-03	negative regulation of metabolic process	4.04E-02	RNA processing	4.98E-02
regulation of protein metabolic process	2.49E-02	receptor-mediated endocytosis	4.26E-02		

ed 38 biological processes including transcription, RNA splicing, M phase, cell development, protein ubiquitination, Wnt receptor signalling pathway, apoptosis, cell cycle arrest and nervous system development.

Analysis of Genes with Altered Expression in HL-60 Cells Treated to Naphthalene

HL-60 cells were treated with 773 μM naphthalene for 3 h, and the total RNA was subjected to microarray analysis. The changes of gene expression were analyzed by comparing with treated group and control group using a statistical criteria of ≥2-fold changes with a significant t-test *P*-value < 0.01. In this analysis, 138 genes were up-regulated and 182 genes were down-regulated.

As shown in Table 2, these genes were classified into a variety of biological processes by functional classification. Annotatable 93 up-regulated genes were classified into 41 biological processes. Differentially up-regulated genes involved in apoptosis, I-kappaB kinase/NF-kappaB cascade, protein kinase cascade, cell development, response to hypoxia, inflammatory responses, transcription, anti-apoptosis, signal transduction, cell migration, hemopoiesis and regulation of cell cycle. And of the down-regulated 182 genes, annotatable 113 genes were classified into 14 biological processes. Distinctively down-regulated genes by naphthalene were involved in mitosis, protein depolymerisation, receptor-mediated endocytosis, negative regulation of signal transduction and RNA processing.

Commonly Induced Functions and Genes among Two Cell Lines - HepG2 and HL-60 - by Naphthalene

In both cell lines, naphthalene was up-regulated a number of processes related to apoptosis, cell development, cell migration, hemopoiesis, nuclear import, protein import and signal transduction. And down-regulated genes in both cell lines included M phase in cell cycle phase and RNA processing (Table 3).

Comparison of the gene lists in HepG2 and HL-60 induced by naphthalene identified 11 genes - 6 up-regulated genes and 5 down-regulated genes - as being modulated in both cell lines (Table 4). Up-regulated genes in both cell lines were NCF2, OKL38, MGC16-635, H2B, RPS2 and FOSL1. And also down-regulated genes were ITGB2, ATP13A3, DAZAP1, SPATA5L1 and HNRPA0.

Table 3. Biological processes significantly affected (Fisher exact test, *P*-value < 0.05) by naphthalene in both HepG2 and HL-60 cells.

Up-regulated genes
apoptosis
cell development
cell migration
hemopoiesis
negative regulation of cellular process
nuclear import
positive regulation of cellular process
protein import
protein kinase cascade
regulation of apoptosis
regulation of cell cycle
signal transduction
Down-regulated genes
cell cycle phase
cellular component organization and biogenesis
M phase
negative regulation of metabolic process
RNA processing

Discussion

Microarray technology is a powerful tool for identifying gene expression patterns which response to toxicant exposure and can give insight into mechanism of action. The aim of this study was to identify genes involved in the biological process and to elucidate the cellular response to naphthalene, in human cells, using this technology. HepG2 and HL-60 cells have been used in this study for toxicity studies and gene expression studies. Although these cell lines are not normal cells, they have the advantage of being easy to maintain. And HepG2 cell line is a suitable tool to study gene regulation in liver cells because regulation of xenobiotic metabolising genes is similar between in HepG2 and in primary human hepatocytes[8].

Several studies have reported gene expression changes after naphthalene exposure. Naphthalene results in decreased expression of FUS and increased expression of BCL-2, TCF7, IL-8, c-FOS, c-JUN and RAF-1 in the regulation of cell growth, differentiation and development in human mononuclear cord blood cells[12]. And naphthalene inhibits the activity of CYP1A2[13]. Carlson reported that the expression of CYP450 might be important in carcinogen metabolism[14]. CYP enzymes are a family of heme proteins involved in the metabolism of various toxicants[15]. CYP enzymes are xenobiotic metabolizing enzymes responsible for the oxidation of many xenobiotics such as naphthalene[16]. In our microarray data, naphthalene changed the mRNA levels of CYP isoforms in both cell lines. Naphthalene induced up-regulation of CYP51A1 and CYP24A1 and down-regulation of CYP2U1, CYP4B1, CYP3A4 and CYP26B1 in HepG2. And also, naphthalene in-

Table 4. Genes that were significantly altered by at least 2.0-fold in response to naphthalene in both HepG2 and HL-60 cells.

Genbank accession number	Gene symbol	Gene title	Biological process	Intensity of expression alteration	
				HepG2	HL-60
AB209647	NCF2	neutrophil cytosolic factor 2	Cellular defense response; Respiratory burst; Innate immune response; Superoxide release	4.00	2.45
AY258066	OKL38	pregnancy-induced growth inhibitor	Regulation of growth; Multicellular organismal development; Negative regulation of cell growth; Cell differentiation	2.18	2.63
BC009980	MGC16635	kelch domain containing 7B		2.84	2.79
BC069193	H2B	histone 2, H2be	Nucleosome assembly; Defense response to bacterium	2.75	2.50
BM467031	RPS2	Ribosomal protein S2	Translational elongation	2.39	2.34
NM_005438	FOSL1	FOS-like antigen 1	Positive regulation of transcription from RNA polymerase II promoter, mitotic; Cellular defense response; Positive regulation of cell proliferation; Chemotaxis	2.37	3.70
AB208909	ITGB2	Integrin, beta 2 (antigen CD18 (p95)	apoptosis; cell adhesion; development; inflammatory response	0.41	0.39
AJ306929	ATP13A3	ATPase type 13A3	cation transport; metabolism	0.40	0.49
AK124583	DAZAP1	DAZ associated protein 1	cell differentiation	0.34	0.46
BC051861	SPATA5L1	Spermatogenesis associated 5-like 1	cell division	0.48	0.46
NM_006805	HNRPA0	Heterogeneous nuclear ribonucleoprotein A0	Nuclear mRNA splicing, via spliceosome	0.39	0.41

duced the down-regulation of CYP1B1 in HL-60.

As shown in Table 3, DEGs in naphthalene-exposed HepG2 and HL-60 cells were related to functions such as apoptosis, hemopoiesis and cell cycle. Naphthalene induces cell death[17-19]. Several reports have suggested that apoptosis is in part mediated by an oxidative stress from the down-regulation of key antioxidant defense systems of cells[20]. Daily treatment of 1 g/kg doses of naphthalene to rats increases levels of serum and liver lipid peroxides and induces lipid peroxidation[21,22]. Enhancement of peroxidation induces the formation of naphthalene metabolites which may directly inhibit mitochondrial respiration or divert electron flow, leading to inhibition of ATP synthesis and depolarization of the membrane potential[20]. Naphthalene exposure has a membrane-damaging effect, especially to hemopoietic organs such as kidney[23]. In addition, metabolites of naphthalene such as 1-naphthol and 2-naphthol block M phase and induce mitotic chromosomal aberrations in chinese hamster cells[13]. In this study, down-regulated genes included CCNG2, NUDC, CDC2, FBXO5 and CENPF in HepG2 and CDC25A2, TARDBP, STAG2, PPP1R9B, PES1, PRMT5 and SPAG5 in HL-60 are associated with the progression of M phase such as G2/M cell cycle arrest.

Although the majority of expression changes identified was cell type specific, 11 genes were identified as altered in both cell clines (Table 4), indicating that naphthalene induces a general gene expression response in different cell types. These common genes had functions that reactive oxygen species metabolic process (NCF2), cell growth (OKL38), nucleosome assembly (H2B), gene expression (RPS2, FOSL1, HNRPA0), apoptosis (ITGB2), metabolism (ATP13A3) and cell division (SPATA5L1). These common gene expression changes may represent potential biomarkers of naphthalene exposure.

In conclusion, our results showed that the changes of gene expression patterns were associated with naphthalene toxicity. And it is suggested that DNA microar-

ray analysis using oligomicroarray is an efficient technology for evaluation the gene regulation, the possibility to identify the molecular markers and of potential hazards of environmental samples including a variety of chemicals. Also, GO analysis is good for a prediction of the mechanism associated with DEGs in cells or organisms influenced by chemicals.

Materials & Methods

Materials

Naphthalene, sodium bicarbonate and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Phosphate buffer saline (PBS), 0.5% trypsin-EDTA, Dulbecco's Modified Eagle Medium (DMEM), RPMI-1640, fetal bovine serum (FBS), sodium pyruvate, penicillin and streptomycin were the products of Gibco™ (Carlsbad, CA, USA). Trizol reagent was produced by Invitrogen (Carlsbad, CA, USA) and RNeasy mini kit and RNase-free DNase set were purchased from Qiagen (Valencia, CA, USA). All other chemicals used were of analytical grade or the highest grade available.

Cell Lines and Culture

Human hepatocellular carcinoma cell line (HepG2) and human promyelocytic leukemia cell line (HL-60) were used throughout the study, purchased from Korean Cell Line Bank (KCLB, Seoul, Korea) and ATCC (CCL-240, USA), respectively. HepG2 cells were grown in DMEM medium supplemented with 10% inactivated FBS plus 0.044 M sodium bicarbonate, 10 mM sodium pyruvate and 1% penicillin and HL-60 cells were grown in RPMI-1640 medium supplemented with 10% inactivated FBS plus 1 mM sodium pyruvate, 100 U/mL penicillin and 100 μg/mL streptomycin, and 10 mM HEPES at 37°C in 5% CO_2 atmosphere. For cell growth, the medium was renewed every two or three days. HepG2 and HL-60 cells were approximately 80% confluence achieved by plating 6×10^6 cells/mL in 100 mm culture dish and density of 5×10^5 cells/mL in T75 tissue culture flask.

Determination of Cell Viability

MTT assay was performed for the detection of cell viability[24]. In case of HepG2, 24-well plate was used for cytotoxicity assay. Cells were seeded at a seeding density of 80×10^4 cells/mL on a well in 500 μL of media. And in case of HL-60 cells, 15 mL tube was used for cytotoxicity assay. 3 mL of HL-60 cells were seeded at a seeding density of 1×10^5 cells/mL. Cells were exposed to various concentrations of naphthalene in culture medium at 37°C for 48 h and 3 h exposure times respectively. In HepG2 cells, 75 μL of MTT (4 mg/mL in PBS) solution was added to each well and in HL-60 cells, 200 μL of MTT (5 mg/mL in PBS) solution was added to each tube and incubated for 3 h. DMSO solution was added to each tube and transferred to 96 well plate. The optimal density (OD) of the purple formazan product was measured at a wavelength of 540 nm. The 20% inhibitory concentration (IC_{20}) and 50% inhibitory concentration (IC_{50}) of cell proliferation in a particular chemical was defined as the concentration that causes a 20% and 50% reduction in the cell viability versus the solvent treated control. The IC_{20} and IC_{50} values were directly determined from the linear dose-response curves.

RNA Extraction

Total RNA was extracted from the HepG2 and HL-60 cells treated with 773 μM and 1,772.23 μM naphthalene, respectively, for 48 h and 3 h using Trizol reagent and purified using RNeasy mini kit according to the manufacturer's instructions. Genomic DNA was removed using RNase-free DNase set during RNA purification. The amount of each total RNA sample was quantified using NanoDrop (NanoDrop Technologies, Wilmington, DE, USA) and its quality was checked by Experion™ (Bio-Rad Laboratories, Hercules, CA, USA).

Oligonucleotide Microarray Hybridization

Gene expression analysis was conducted on the RNA samples from HepG2 and HL-60 cells treated to naphthalene using human whole genome 44 k microarray (Agilent Technologies, Palo Alto, CA, USA) and 35 K whole human genome microarray (Operon Biotechnologies, Inc. Germany), respectively. Labeling and hybridization were followed by the coupling of the Cy3 dye for the controls or Cy5 dye for the treated samples. Hybridization was performed in a hybridization oven at 62°C for 12 h. After washing (2 × SSC/0.1% SDS for 2 min at 58°C, 1 × SSC for 3 min at RT, 0.2 × SSC for 2 min at RT), the slide was dried by centrifugation at 800 rpm for 3 min at RT. Hybridization images on the slides were scanned by GenePix 4000B (Axon Instruments, Union City, CA, USA). Scanned images were analyzed with GenePix 4.1 software (Axon Instruments, Union City, CA, USA) to obtain gene expression ratios.

Data Analysis

The fluorescent intensity of each spot was calculated by local median background subtraction. The robust scatter-plot smoother LOWESS function to perform intensity dependent normalization for the gene expres-

sion. Scatter plot analysis was made by Microsoft Excel 2000 (Microsoft, WA, USA). Significance Analysis of Microarray (SAM) was performed for the selection of the genes with significant gene expression changes[25]. The statistical significance of the differentially expressed of genes was assessed by computing a q-value for each gene. To determine the q-value, a permutation procedure was used, and for each permutation, two-sample t statistics were computed for each gene. Genes were differentially expressed when logarithmic gene expression ratios in three independent hybridizations were more than 0.5 or less than -0.5, i.e., 2.0-fold difference in expression level, and when the q-values were < 1.

Functional Analysis

In order to classify the selected genes into groups with a similar pattern of expression, each gene was assigned to an appropriate category according to its main cellular function. The necessary information to categorize each gene was obtained from several databases particularly the database located at http://david.abcc.ncifcrf.gov/home.jsp.

References

1. Waidyanatha, S. & Rappaport, S. M. Hemoglobin and albumin adducts of naphthalene-1,2-oxide, 1,2-naphthoquinone and 1,4-naphthoquinone in Swiss Webster mice. *Chem Biol Interact* **172**:105-114 (2008).
2. Genter, M. B. *et al.* Naphthalene toxicity in mice and aryl hydrocarbon receptor-mediated CYPs. *Biochem Biophys Res Commun* **348**:120-123 (2006).
3. Sehirli, O. *et al.* Protective effect of resveratrol against naphthalene-induced oxidative stress in mice. *Ecotoxicol Environ Saf* **71**:301-308 (2008).
4. Molloy, E. J., Doctor, B. A., Reed, M. D. & Walsh, M. C. Perinatal toxicity of domestic naphthalene exposure. *J Perinatol* **24**:792-793 (2004).
5. Kaplan, M. & Hammerman, C. Severe hemolysis and hyperbilirubinemia due to perinatal napthalene exposure. *J Perinatol* **25**:359 (2005).
6. Viravaidya, K., Sin, A. & Shuler, M. L. Development of a microscale cell culture analog to probe naphthalene toxicity. *Biotechnol Prog* **20**:316-323 (2004).
7. National Toxicology Program (2000). Toxicology and carcinogenesis studies of naphthalene (CAS No. 91-20-3) in F344/N rats (inhalation studies). U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, NIH publication 01- 4434; http://ntp.niehs.nih.gov/ntp/htdocs/LT_rpts/tr500.pdf.
8. Hockley, S. L. *et al.* Time- and concentration-dependent changes in gene expression induced by benzo(a) pyrene in two human cell lines, MCF-7 and HepG2. *BMC Genomics* **7**:260 (2006).
9. Kawata, K., Yokoo, H., Shimazaki, R. & Okabe, S. Classification of heavy-metal toxicity by human DNA microarray analysis. *Environ Sci Technol* **41**:3769-3774 (2007).
10. Ashburne, M. *et al.* Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* **25**:25-29 (2000).
11. Te Pas, M. F. *et al.* Biochemical pathways analysis of microarray results: regulation of myogenesis in pigs. *BMC Dev Biol* **7**:66-80 (2007).
12. Diodovich, C. *et al.* Naphthalene exposure: effects on gene expression and proliferation in human cord blood cells. *J Biochem Mol Toxicol* **17**:286-294 (2003).
13. Renglin, A., Olsson, A., Wachtmeister, C. A. & Onfelt, A. Mitotic disturbance by carbaryl and the metabolite 1-naphthol may involve kinase-mediated phosphorylation of 1-naphthol to the protein phosphatase inhibitor 1-naphthylphosphate. *Mutagenesis* **13**:345-352 (1998).
14. Carlson, G. P. Critical appraisal of the expression of cytochrome P450 enzymes in human lung and evaluation of the possibility that such expression provides evidence of potential styrene tumorigenicity in humans. *Toxicology* **254**:1-10 (2008).
15. Korhonen, L. E. *et al.* Predictive three-dimensional quantitative structure-activity relationship of cytochrome P450 1A2 inhibitors. *J Med Chem* **48**:3808-3815 (2005).
16. Hodgson, E. & Rose, R. L. The importance of cytochrome P450 2B6 in the human metabolism of environmental chemicals. *Pharmacol Ther* **113**:420-428 (2007).
17. Phimister, A. J., Nagasawa, H. T., Buckpitt, A. R. & Plopper, C. G. Prevention of naphthalene-induced pulmonary toxicity by glutathione prodrugs: roles for glutathione depletion in adduct formation and cell injury. *J Biochem Mol Toxicol* **19**:42-51 (2005).
18. Bagchi, M. *et al.* Protective effect of melatonin on naphthalene-induced oxidative stress and DNA damage in cultured macrophage J774A.1 cells. *Mol Cell Biochem* **221**:49-55 (2001).
19. Plopper, C. G. *et al.* Early events in naphthalene-induced acute Clara cell toxicity. II. Comparison of glutathione depletion and histopathology by airway location. *Am J Respir Cell Mol Biol* **24**:272-281 (2001).
20. Bagchi, M. *et al.* Naphthalene-induced oxidative stress and DNA damage in cultured macrophage J774A.1 cells. *Free Radic Biol Med* **25**:137-143 (1998).
21. Yamauchi, T., Komura, S. & Yagi, K. Serum lipid peroxide levels of albino rats administered naphthalene. *Biochem Int* **13**:1-6 (1986).
22. Germansky, M. & Jamall, I. S. Organ-specific effects of naphthalene on tissue peroxidation, glutathione peroxidases and superoxide dismutase in the rat. *Arch Toxicol* **61**:480-483 (1988).
23. Ahmad, I., Pacheco, M. & Santos, M. A. Naphthalene-induced differential tissue damage association with

circulating fish phagocyte induction. *Ecotoxicol Environ Saf* **54**:7-15 (2003).
24. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**:55-63 (1983).
25. Tusher, V. G., Tibshirani, R. & Chu, G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* **98**:5116-5121 (2001).