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Identification of Immune Responsive Genes on Benzene, Toluene and *o*-Xylene in Jurkat Cells Using 35 k Human Oligomicroarray

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Abstract

Volatile organic compounds (VOCs) are a major component of urban air pollution. It is documented that low exposure levels of VOCs induce alterations in immune reactivity resulting in a subsequent higher risk for the development of allergic reactivity and asthma. Despite these facts, there are few reports on the affected primary target and the underlying effective causal mechanisms. So in this study, to better understand the risk of BTX (benzene, toluene and *o*-xylene) which are the major VOCs and to identify novel biomarkers on immune response to these VOCs exposure in human T lymphocytes, we performed the toxicogenomic study by analyzing of gene expression profiles using 35 k human oligomicroarray. BTX generated specific gene expression patterns in Jurkat cell line. By clustering analysis, we identified some genes as potential markers on immuno-modulating effects of BTX. Four genes of these, HLA-DOA, ITGB2, HMGA2 and STAT4 were the most significantly affected by BTX exposure. Thus, this study suggests that these differentially expressed immune genes may play an important role in the pathogenesis on BTX exposure and have significant potential as novel biomarkers of exposure, susceptibility and response to BTC.

Keywords: Benzene, Toluene, *o*-xylene, BTX, Volatile organic compounds, Immune responsive gene, 35 k human oligomicroarray, Jurkat cell

Important indoor air pollutants are volatile organic compounds (VOCs). These are organic chemicals which are evaporated into the atmosphere at room temperature[1]. Paints, adhesives, building materials, cosmetics, furnishings, dry-cleaned clothes, cleaning agents, carpets, and tobacco smoke can be accounted as possible sources for VOCs exposure[1-3]. The monocyclic aromatic hydrocarbons: benzene, toluene and *o*-xylene (collectively BTX) are potential harmful VOCs commonly found in occupational or non-occupational environments.

Benzene is an important industrial chemical (>2 billion gallons produced annually in the United States) and component of gasoline[4] and is a common environmental pollutant found in automobile exhaust and cigarette smoke. It was calculated that the major source of exposure (45%) of the US population to benzene arises from cigarette smoke[5,6], which accumulates inside homes, offices and vehicles. Benzene is a well-known carcinogen that has been causally linked to leukemia[7]. Toluene, a VOC found in glues, paints and cleaning solvents, has been associated with childhood exposures[8,9]. Whereas acute exposure to toluene leads to central nervous system (CNS) effects[10,11], chronic exposures have been associated with both neurological and respiratory effects[12,13]. Xylenes are widely used as organic solvents and are present in numerous consumer products. These compounds are emitted from building materials, such as carpet adhesives, vinyl cove adhesive, latex paint and various mouldings and tobacco smoke. Furthermore, xylenes are CNS depressants while the exposure level to elicit the effect is rather high in man[14]. Therefore, it is very important to explore the influence of xylene on human health.

Microarray technologies have been widely used for comprehensive gene expression analysis as well as mutation and single nucleotide polymorphism detection[15-20]. In particular, large-scale microarray analysis of gene expression enables researchers to analyze simultaneous changes in thousands of genes and identify significant patterns.

The objective of this study is the identification of potential gene-based markers. We examined global gene expression in a small number of well-matched exposed-control subject pairs. Genes with differential expression were then ranked and selected for further

examination using several forms of statistical analysis. The identification of differentially expressed genes (DEGs) may assist in the identification of potential biomarker and may understand molecular toxicological mechanisms of benzene, toluene and *o*-xylene in human lymphocytes.

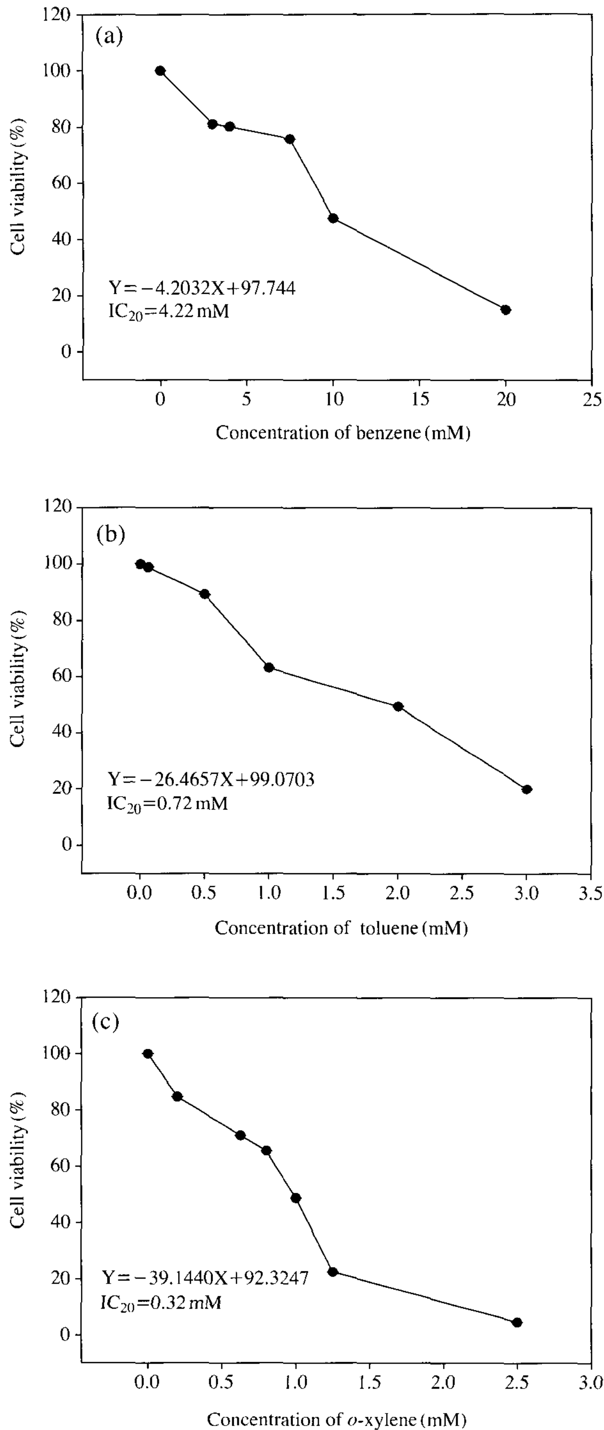

Fig. 1. Dose-response curves were assessed by the MTT assay after 3 h treatment. IC_{20} values of BTX were calculated as (a) 4.22 mM benzene, (b) 0.72 mM toluene and (c) 0.32 mM *o*-xylene.

Cytotoxicity of Benzene, Toluene and o-xylene in Jurkat Cells

Relative survival of Jurkat cells following exposure to a range of concentrations of benzene, toluene and *o*-xylene was determined by MTT assay. The survival percentage relative to solvent control (DMSO) was determined as a percentage of OD value measured after treatment. Based on the results of MTT assay, 20% inhibitory concentration (IC_{20}) of each compound was calculated. Dose dependent cell viability curves were obtained after 3 h of exposure to BTX in Jurkat cells as shown in Fig. 1. Benzene, toluene and

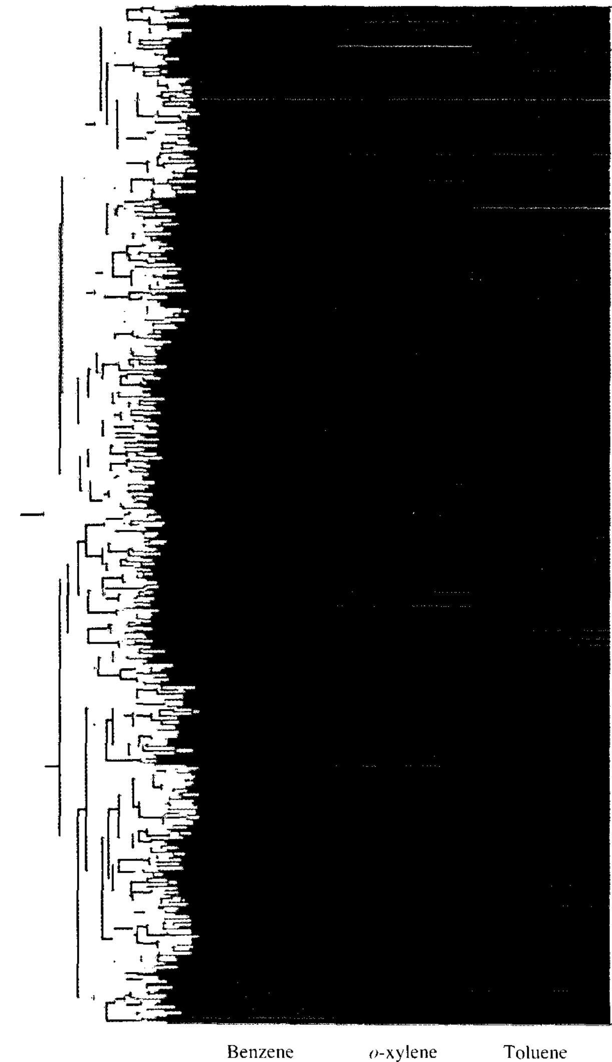

Fig. 2. Hierachical cluster image showing the differential gene expression profiles of BTX exposed in Jurkat cells.

o-xylene reduced cell viability gradually at increasing concentrations. The IC_{20} for benzene, toluene and *o*-xylene were 2.72 mM, 0.72 mM and 0.32 mM, respectively (Fig. 1).

Gene Expression Altered by Benzene, Toluene and o-xylene

Human lymphoblastoid T (Jurkat) cells were treated with 2.72 mM benzene, 0.72 mM toluene and 0.32 mM *o*-xylene for 3 h, and their RNA was subjected to microarray analysis. For each treatment, genes with statistically significant expression changes were identified by microarray. Three independent experimental samples for each treatment group were analyzed to determine RNA transcript levels. Only those genes, which displayed either greater than or equal to a 1.5 fold up- and down-regulation, have been considered for this study. Hierarchical clustering was used to aid in visualization and biological interpretation of this extensive data set, and in particular, to identify correlated expression patterns. Hierarchical clustering was applied across the three compounds, using a combined list of genes identified to be altered statistically significant in at least one of the sample studied relative to control (Fig. 2). From the clustering analysis, we can assumed that, the gene expression profiles of benzene and toluene are similar than *o*-xylene. Oligonucleotide microarray analysis was employed to characterize the cells response to the BTX. Comparison of the gene expression profiles in Jurkat cells exposed to these three VOCs at one dose and one time point. Venn diagram shows the gene expression

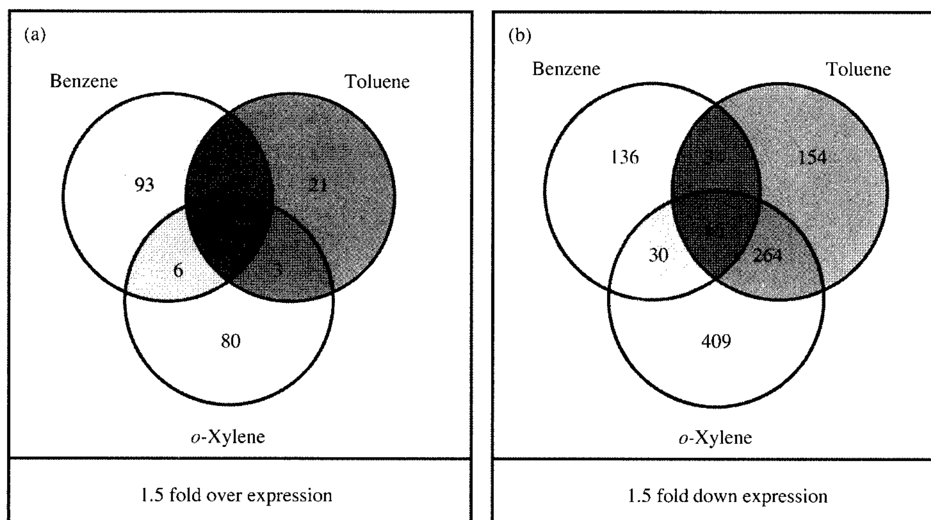

Fig. 3. Venn diagram showed the differentially expressed genes by BTX. The diagrams were generated from the list of up (a) and down (b) regulated genes that were >1.5 fold with microarray analysis.

Table 1. List of up-regulated genes by two BTX in Jurkat cell lines.

Accession No.	Gene symbol	Gene name	Mean intensity (Cy5/Cy3)			Function
			Benzene	Toluene	*o*-xylene	
Benzene and toluene common genes						
NM_207037	TCF12	transcription factor 12 (HTF4, helix-loop-helix transcription factors 4)	**1.69**	**1.52**	1.12	immune response
L20470	VLDLR	Very low density lipoprotein receptor	**2.17**	**1.57**	1.40	metabolism
AB007892	CDC5L	CDC5 cell division cycle 5-like (S. pombe)	**1.61**	**1.56**	1.26	transcription
AL832164	LOC283666	Hypothetical protein LOC283666	**1.55**	**1.52**	1.34	unknown
XM_496387	LOC440610	Similar to tripartite motif-containing 43	**1.53**	**1.53**	1.07	unknown
BC029537	MGC33951	Hypothetical protein MGC33951	**1.76**	**1.59**	1.42	unknown
Toluene and *o*-xylene common genes						
NM_006924	SFRS1	Splicing factor, arginine/serine-rich 1 (splicing factor 2, alternate splicing factor)	0.86	**1.57**	**1.55**	etc.
BC037570	FANCL	Fanconi anemia, complementation group L	1.27	**1.53**	**1.52**	etc.
AK095810	LOC283012	Hypothetical protein LOC283012	1.32	**1.61**	**1.52**	unknown

Table 2. List of down-regulated genes by benzene, toluene and *o*-xylene in Jurkat cell lines.

Accession No.	Gene symbol	Gene name	Mean intensity (Cy5/Cy3)		
			Benzene	Toluene	*o*-xylene
immune response					
AB209853	HMGA2	High mobility group AT-hook 2	0.63	0.62	0.61
M38056	HLA-DOA	Major histocompatibility complex, class II, DO alpha	0.53	0.51	0.52
AB208909	ITGB2	Integrin, beta 2 (antigen CD18 (p95), lymphocyte function-associated antigen 1; macrophage antigen 1 (mac-1) beta subunit)	0.61	0.54	0.60
NM_003151	STAT4	Signal transducer and activator of transcription 4	0.68	0.59	0.66
signal transduction					
NM_001004749	OR51A7	Olfactory receptor, family 51, subfamily A, member 7	0.60	0.56	0.61
AF045458	ULK1	Unc-51-like kinase 1 (C. elegans)	0.57	0.57	0.43
BC042688	RASD1	RAS, dexamethasone-induced 1	0.51	0.51	0.51
NM_002883	RANGAP1	Ran GTPase activating protein 1	0.54	0.50	0.66
NM_014891	PDAP1	PDGFA associated protein 1	0.61	0.47	0.45
transcription					
AK122813	POLR2E	Polymerase (RNA) II (DNA directed) polypeptide E, 25 kDa	0.57	0.46	0.43
NM_006532	ELL	Elongation factor RNA polymerase II	0.67	0.58	0.57
NM_002967	SAFB	Scaffold attachment factor B	0.55	0.56	0.62
XM_040592	ZNF469	Zinc finger protein 469	0.64	0.62	0.66
Protein buisynthesis					
NM_001961	EEF2	Eukaryotic translation elongation factor 2	0.48	0.54	0.60
XM_496245	WBSCR1	Myosin XVB pseudogene	0.38	0.43	0.39
apoptosis					
NM_005147	DNAJA3	DnaJ (Hsp40) homolog, subfamily A, member 3	0.58	0.45	0.42
cell cycle					
NM_006029	PNMA1	Paraneoplastic antigen MA1	0.59	0.56	0.63
BC051795	DRPLA	Atrophin 1	0.67	0.51	0.54
etc.					
NM_172206	CAMKK1	Calcium/calmodulin-dependent protein kinase kinase 1, alpha	0.55	0.60	0.62
AK124696	KCNAB2	Potassium voltage-gated channel, shaker-related subfamily, beta member 2	0.50	0.45	0.39
NM_198686	RAB15	RAB15, member RAS onocogene family	0.62	0.53	0.58
unknown					
BC053544	ZDHHC8	Zinc finger, DHHC-type containing 8	0.47	0.47	0.44
AB032966	TTC7A	Tetratricopeptide repeat domain 7A	0.60	0.52	0.61
AK123190	GCAT	Glycine C-acetyltransferase (2-amino-3-ketobutyrate coenzyme A ligase)	0.66	0.55	0.53
NM_004651	USP11	Ubiquitin specific peptidase 11	0.58	0.56	0.46
AB002308	KIAA0310	KIAA0310	0.52	0.49	0.41
AB002324	ZNF629	Zinc finger protein 629	0.46	0.44	0.55
NM_207119	LRRC20	Leucine rich repeat containing 20	0.56	0.58	0.60
AK056562	WDR6	WD repeat domain 6	0.49	0.58	0.55
AB017016	TPPP	Brain-specific protein p25 alpha	0.58	0.45	0.59
U10492	MEOX1	Mesenchyme homeo box 1	0.51	0.53	0.48
AK056318	LOC147093	Hypothetical protein LOC147093	0.43	0.44	0.45
XM_293937	LOC345711	Similar to RIKEN cDNA 0610012A05	0.47	0.47	0.43
AK122881	PRICKLE1	Prickle-like 1 (Drosophila)	0.65	0.50	0.54
BG184354		Similar to Cytosol aminopeptidase (Leucine aminopeptidase) (LAP) (Leucyl aminopeptidase) (Proline aminopeptidase) (Prolyl aminopeptidase)	0.67	0.46	0.56
AK128637		serpin peptidase inhibitor, clade B (ovalbumin), member 6	0.56	0.61	0.60

Table 2. Continued.

Accession No.	Gene symbol	Gene name	Mean intensity (Cy5/Cy3)		
			Benzene	Toluene	*o*-xylene
AB014551	ARHGEF2	Rho/rac guanine nucleotide exchange factor (GEF) 2	0.61	0.65	0.63
BC028205	CORO6	Coronin 6	0.55	0.56	0.63
NM_005066	SFPQ	Splicing factor proline/glutamine-rich (polypyrimidine tract binding protein associated)	0.57	0.63	0.54
BC035638	LSS	Lanosterol synthase (2, 3-oxidosqualene-lanosterol cyclase)	0.54	0.56	0.66
NM_014661	FAM53B	Family with sequence similarity 53, member B	0.55	0.66	0.64
NM_018996	TNRC6C	Trinucleotide repeat containing 6C	0.66	0.64	0.64
BC026343	MARLIN1	Multiple coiled-coil GABABR1-binding protein	0.63	0.52	0.51
NM_001325	CSTF2	Cleavage stimulation factor, 3′ pre-RNA, subunit 2, 64 kDa	0.65	0.52	0.60
AY672114	IGFL4	Hypothetical gene supported by AK096622	0.42	0.45	0.45
NM_014702	KIAA0408	Chromosome 6 open reading frame 174	0.63	0.49	0.56

profiles; benzene, toluene and *o*-xylene up regulated 99, 24 and 89 genes and also down regulated 265, 517 and 768 genes respectively (Fig. 3).

There were no common up-regulated gene expressed through BTX exposure, but 6 common genes were up-regulated by benzene and toluene, as well as 3 genes by toluene and *o*-xylene. On the other hand, 65 down-regulated common genes have changed their expression profiles through BTX exposure (Fig. 3). Table 1 showed the list of the up-regulated common genes and Table 2 showed some down-regulated genes, which statistically significantly changed their expression profiles, along with their function.

Discussion

Volatile organic compounds are the major concern to our environment because of their plenty use in industries as well as household works. Very few data is available about the alteration of gene expression with VOCs exposure. Microarray technique is one of the promising tools for the identification of expression profiles. In this study, we have used this approach to identify the gene expression profiles induced by BTX in Jurkat cells using 35 k whole human oligonucleotide microarray. Triplicate assays were performed for each chemical to avoid the error rates. From microarray study in conjunction with statistical analysis, benzene, toluene and *o*-xylene up-regulated 99, 24 and 89 genes and also down regulated 265, 517 and 768 genes respectively (Fig. 3).

Leukocyte adhesion deficiency (LAD) is characterized by the inability of leukocytes, in particular neutrophilic granulocytes, to emigrate from the bloodstream towards sites of inflammation. Infectious foci are nonpurulent and may eventually become necrotic because of abnormal wound healing. LAD-1 is characterized by the absence of the β_2 integrins (ITGB2) on leukocytes. When expression of ITGB2 is completely absent, patients often die within the first year. However, low levels of β_2 expression may result in a milder clinical picture of recurrent infection, which offers a better prognosis[21]. Signal transduction by IL-12 is mediated through the activation of STAT4, which belongs to a family of proteins termed signal transducers and activators of transcription (STATs)[22,23]. The signaling pathway involving STAT4 appears to be relatively specific to IL-12, although IFN-α has also been reported to activate STAT4[24]. Deletion of the STAT4 gene in knockout mice results in defective responses specific to IL-12[25]. Based on current knowledge, it is likely that the phosphorylation and activation of STAT4 are crucial steps in signal transduction by IL-12. IL-12 is involved in the differentiation of naive T cells in to Th1 cells, which is important in resistance against pathogens. Down-regulation of ITGB2 and STAT4 gene also reveals that BTX exposure to human may hampered our immune system, even than it may cause death.

Proteins encoded by the high mobility group AT-hook (HMGA) family are architectural transcription factors, which induce conformational changes in the DNA and thus influence gene expression. Despite the obvious association of the expression of high mobility group protein genes with human cancer, very little is known about the variation of the HMGA proteins within human populations[26]. HMGA2 plays a crucial role in pituitary tumorigenesis. HMGA2 mediated E2F1 activation is a crucial event in the onset of these tumors in transgenic mice and probably also in human prolactinomas[27]. The human leucocyte antigen (HLA) class II region of the human major histo-

compatibility complex (MHC) contains several genes which play a central role in the human immune response system. Among them, HLA-DO is a non-classical class II heterodimer consisted of α and β chains, which are encoded by the DOA and DOB genes located in the HLA class-II region of MHC[28]. Significant down regulation of these genes shows that the BTX exposures interrupt our immune system.

In conclusion, although this data is not enough to say the mechanistic inside of BTX toxicity, we have identified immune responsive genes including ITGB2, STAT4, HMGA2 and HLA-DOA which expressed in Jurkat cells treated to benzene, toluene and *o*-xylene. These genes could be a promising biomarker to detect other VOCs induced immune toxicity. Also, it can be suggested that oligomicroarray is an efficient technology for evaluating the gene regulation by BTX exposure and this approach also offers the possibility to identify the molecular markers.

Methods

Chemicals and Reagents

Benzene, toluene, *o*-xylene and dimethylsulphoxide (DMSO) were obtained from Sigma-Aldrich Chemical Company (USA). RPMI-1640, Dulbecco's phosphate buffered saline (PBS) and fetal bovine serum (FBS) was the products of GIBCO™ (USA). All other chemicals used were of analytical grade or the highest grade available.

Cell Lines and Culture

Jurkat, clone E6-1 cell line (human lymphoblastoid T-cells) was used throughout the study, purchased from Korean Cell Line Bank. The cells were grown in RPMI-1640 medium supplemented with 10% inactivated fetal bovine serum (FBS), 1 mM sodium pyruvate, 10 mM HEPES and antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin) at 37°C in 5% CO_2 atmosphere. For cell growth, the medium was renewed every two or three days at a density of 1×10^6 cells/mL in T75 tissue culture flask (TPP, Trasadingen, Switzerland).

Determination of Cell Viability

MTT assay was performed for the detection of cell viability[29]. Due to volatile compounds, 15 mL tube was used for cytotoxicity assay. 5 mL of Jurkat cells were seeded at a seeding density of 1×10^6 cells/mL. Cells were exposed to various concentrations of benzene, toluene and *o*-xylene in culture medium at 37°C for 3 h exposure time at 200 rpm in a shaking incubator. 500 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 transfer in 96 well plates. The optimal density (OD) of the purple formazan product was measured at a wavelength of 540 nm. The 20% inhibitory concentration (IC_{20}) of cell proliferation in a particular chemical was defined as the concentration that causes a 20% reduction in the cell viability versus the solvent treated control. The IC_{20} values were directly determined from the linear dose-response curves.

RNA Extraction

Total RNA was extracted from the Jurkat cells treated to 4.22 mM, 0.72 mM and 0.32 mM for benzene, toluene and *o*-xylene, respectively, for 3 h using the Trizol reagent (Invitrogen, USA) and purified using RNeasy mini kit (Qiagen, USA) according to the manufacturer's instructions. Genomic DNA was removed using RNase-free DNase set (Qiagen, USA) during RNA purification. The amount of each total RNA concentration was quantified using SmartSpec 3000 (Bio-Rad, Hercules, CA). Only samples with an A260/A280 ratio between 1.9 and 2.2 were considered for suitable use and its quality was checked by agarose-gel electrophoresis.

Oligonucleotide Microarray Hybridization

Gene expression analysis was conducted on the RNA samples using 35 k whole human genome microarray (Operon Biotechnologies, Inc. Germany). Triplicate analysis was performed for each chemical simultaneously. Labeling and hybridization were performed by instruction of Platinum Biochip Reagent Kit (GenoCheck Co. Ltd, Korea). This was followed by the coupling of the Cy3 dye for the controls (DMSO) or Cy5 dye for the treated samples. Hybridization was performed in a hybridization oven at 62°C for 12 h. After washing ($2 \times$ SSC/0.1% SDS for 2 min at 58°C, $1 \times$ SSC for 2 min at RT and $0.2 \times$ SSC for 3 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 ScanArray Lite (PerkinElmer Life Sciences, USA). Scanned images were analyzed with GenePix 3.0 software (Axon Instruments, 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 was used to perform intensity dependent normalization for the gene expression. 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[30]. Computing a q-value for each gene assessed the statistical significance of the differential expression of genes. 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 considered differentially expressed when logarithmic gene expression ratios in three independent hybridizations were more than 1.5 or less than 0.50, *i.e.*, 1.5-fold difference in expression level, and when the q-values were <5.

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