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Validation of Human HazChem Array Using VOC Exposure in HL-60 Cells

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Abstract

Volatile Organic Compounds (VOCs) have been shown to cause nervous system disorders through skin contact or respiration, and also cause foul odors even at low densities in most cases. Also, as a compound itself, VOCs are directly harmful to the environment and to the human body, and may participate in photochemical reactions in air to create secondary pollutants. In this study, HL-60 cells were treated with volatile organic compounds, including ethylbenzene and trichloroethylene, at a value of IC_{50}. Then, the in house-prepared Human HazChem arrayer was utilized in order to compare the gene expression between the two VOCs. After hybridization, 8 upregulated genes and 8 downregulated genes were discovered in the HazChem array. The upregulated genes were identified as SG15, TNFSF10, PRNP, ME1, NCOA4, SRXN1, TXNRD1, and XBP1. The downregulated genes were identified as MME, NRF1, PRARBP, CALCA, CRP, BAX, C7 or f40, and FGFR1. Such results were highly correlated with the quantitative RT-PCR results. The majority of the 16 genes were related with the characteristics of VOCs, including respiratory mechanism, apoptosis, and carcinogenesis-associated genes. Our data showed that our human HazChem array can be used to monitor hazardous materials via gene expression profiling.

Keywords: Toxicogenomics, VOCs (Volatile organic compounds), Ethylbenzene, Trichloroethylene, Microarray, Environmental hazards

Volatile Organic Compounds (VOCs) are important indoor air contaminants which evaporate into the atmosphere at room temperature. Paints, adhesives, building materials, cosmetics, furnishings, dry-cleaned clothes, cleaning agents, carpets, and tobacco smoke are all possible sources of indoor VOC exposure. It appears to be true that high concentrations of VOC principally cause toxic effects[1], and moderate levels of VOC exposure trigger inflammatory reactions in particular airways. In the case of low exposure levels, VOCs can still directly exert harmful effects on the environment or the human body, or can sometimes create secondary pollutants, such as photochemical oxidants, via photochemical reactions in air that induce alterations in immune reactivity, thus resulting in a higher risk for the development of allergic reactivity and asthma[2,3].

There have been a number of studies conducted regarding environmental hazard mechanisms, as a component of a more general attempt to evaluate the risks of such environmental hazards. In assessing risks with known and unknown materials, high-throughput screening technologies, such as microarray techniques, are considered to be very useful, and a great deal of effort has been exerted on the development of related technologies by the USA and other advanced countries. Currently, DNA microarray technology is sufficiently developed for the expression screening of almost all known genes of the species. Despite a great deal of effort, there remains no accurate analysis method for toxicity prediction using a DNA chip[4-6].

DNA microarrays are a powerful, high throughput tool for simultaneously monitoring the expression of thousands of genes. In environmental monitoring,

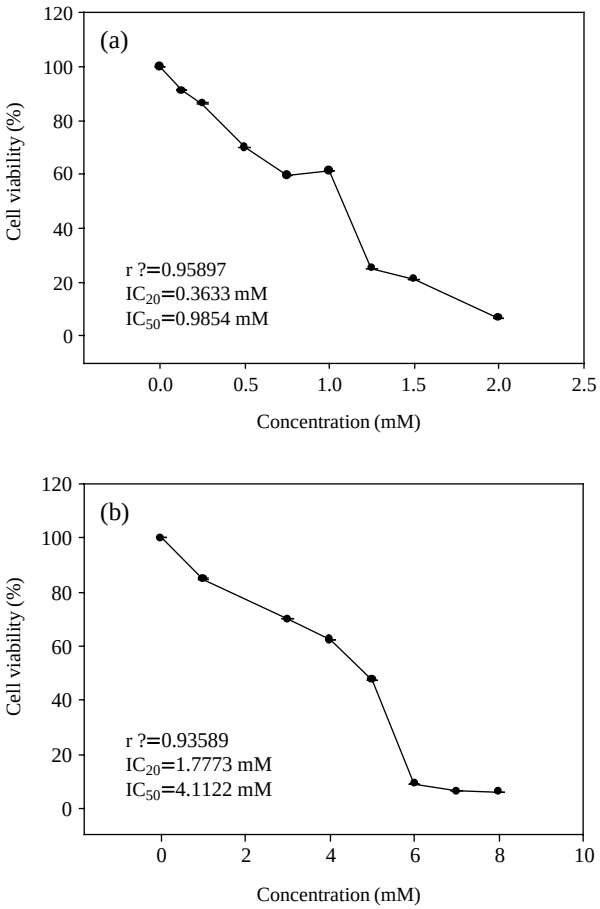


Figure 1. Cell viability after exposure to ethylbenzene and trichloroethylene was analyzed via MTT assays. Three mL of HL-60 cells were seeded in 20 mL tubes at a concentration of 1×10^6 cells/mL in a shaking incubator. After 3 hrs of treatment with ethylbenzene and trichloroethylene, the MTT solution (5 mg/mL) was added to each tube for 3 hrs. The reaction was halted via the addition of DMSO and the absorbance (optical density) was assessed at 540 nm. The change in absorbance, which is reflective of the change in cell death, is expressed as the % of control. 50% inhibition concentration (IC_{50}) values of two VOCs were calculated as (a) ethylbenzene IC_{50}: 0.9854 mM and (b) trichloroethylene IC_{50}: 4.1122 mM.

microarrays could provide not only a method by which chemicals can be quickly categorized and assigned a mode of toxic action, but would also allow for more sensitive end points to be addressed. Recently, a great deal of interest has become focused on the use of microarrays in toxicology for the rapid classification of toxicants based on characteristic expression profiles, and the use of these profiles as a means of identifying the putative mechanism of action[7-9].

In the case of DNA microarray technology, although it enables the screening of the expression of the entire set of known genes, it retains the problem of differing analysis results depending on the gene expression comparative analysis theories applied. Moreover, it has been reported that the degree of accuracy is higher when using an analysis method that utilizes a few genes selected from analysis on whole gene sets, rather than in the entire gene method itself. This study applies to an in-house generated human HazChem oligonucleotide array, which could be used to discover significant genes that evidence toxicity expression by processing VOCs including ethylbenzene and trichloroethylene in the HL-60 cell line. The results of this study are expected to contribute to the development of method by which VOCs, including ethylbenzene and trichloroethylene might be searched using the genes commonly expressed in both compounds.

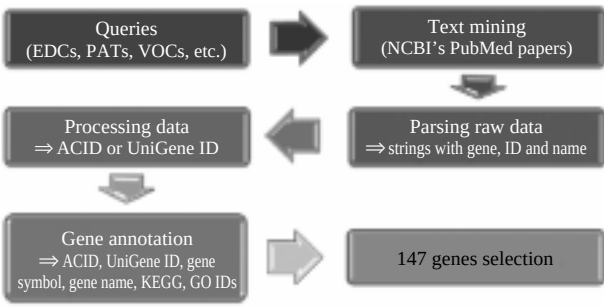


Figure 2. The database search diagram used to select the genes which are composed of the HazChem Human array.

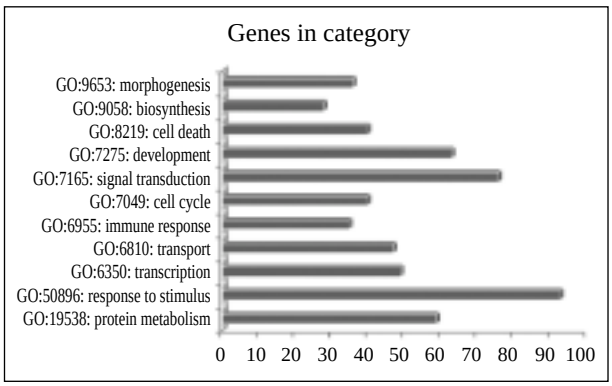
Cytotoxicity of VOCs Chemical in HL-60 Cell

The cell viability of HL-60 cells after exposure to a range of concentrations of VOCs compounds was determined via an MTT assay. Based on the results of the MTT assay, the 50% cell viability inhibitory concentration (IC_{50}) of each compound was calculated. Dose-dependent cell viability curves were obtained after 3 hrs of exposure to ethylbenzene and trichloroethylene in HL-60 cells, as shown in Figure 1. When the cell toxicity of ethylbenzene and trichloroethylene were assessed in the HL-60 cell line, the density (IC_{50}) results showing a 50% survival ratio were 0.9854 mM and 4.112 mM, respectively (Figure 1).

Preparation of HazChem Array

For the selection of the HazChem array gene, the first selection was made on genes through the query search for each of the harmful materials, by means of the NCBI database and the previous literature and data, after which additional selection was conducted using the database ID, accession number, and unigene ID of the initially selected genes (Figure 2). By means

Table 1. Classification of the human HazChem arrays.



of analysis on the selected genes' functions and pathways, the genes were finally selected at the proportionate rate, which were associated with stimulus response, signal transduction, biosynthesis, morphogenesis, cell death, development, immune response, cell cycle, transport, transcription protein metabolism, and biosynthesis (Table 1).

Gene Expression Analysis

HL-60 cells were treated with 0.9854 mM ethylbenzene, and 4.112 mM trichlorobenzene for 3 hrs, after which the RNA was subjected to human HazChem array analysis. For each treatment, genes with statistically significant expression changes were identified via microarray. Only those genes displaying regulation (up or down) either greater than or equal to 1.5 fold, have been considered for this study. Hierarchical clustering was applied across the two compounds, using a combined list of genes (Figure 3). The results obtained using the human HazChem array and the comparison of the gene expression clusterings for ethylbenzene and trichloroethylene compounds showed a high correlation between the gene variances of the two compounds. Also, 8 upregulated genes and 8 downregulated genes were discovered based on the 1.5 fold threshold, and the upregulated genes were identified as ISG15, TNFSF10, PRNP, ME1, NCOA4, SRXN1, TXNRD1, and XBP1, whereas the downregulated genes were identified as MME, NRF1, PRARBP, CALCA, CRP, BAX, C7 or f40, and FGFR1 (Table 2, 3). These significant results were also highly correlated with the results of quantitative RT-PCR. Also, among the genes with significant changes were genes involved with immune reactions, carcinogenicity, and the respiratory system. With regard to the ethylbenzene and trichloroethylene-treated samples, the average linkage algorithm was applied in order to conduct the hierarchical clustering. The red color indicates overexpression, the green one indicates downexpression, and the black color shows the gene without variances in expression. Table 2 and 3 shows the genes whose expression patterns are commonly changed by ethylbenzene and trichloroethylene in HL-60 cells. These are lists of genes that were upregulated or downregulated by >1.5 fold as shown by microarray analysis.

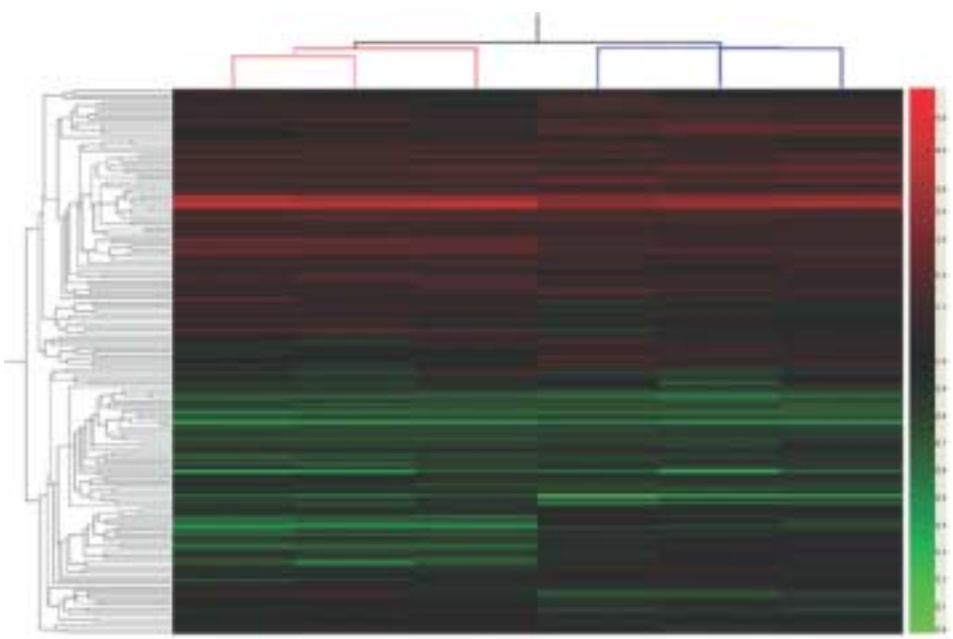


Figure 3. Hierachical cluster image showing the differential gene expression profiles between ethylbenzene and trichloroehtylene treatments in HL-60 cells.

Table 2. Up-regulated common genes by ethylbenzene and trichloroetylene in HL-60 cells.

GeneBank ID	GeneSymbol	GeneName	Fold change	
			Ethylbenzene	Trichloroethylene
NM_005101	ISG15	ISG15 ubiquitin-like modifier	4.303	2.852
NM_003810	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	3.915	4.053
NM_183079	PRNP	prion protein	3.103	2.572
NM_002395	ME1	malic enzyme 1, NADP (+)-dependent, cytosolic	2.978	3.259
NM_005437	NCOA4	nuclear receptor coactivator 4	2.728	2.302
NM_080725	SRXN1	sulfiredoxin 1 homolog	2.635	2.083
NM_003330	TXNRD1	thioredoxin reductase 1	1.588	1.567
NM_005080	XBP1	X-box binding protein 1	1.516	1.646

Table 3. Down-regulated common genes by ethylbenzene and trichloroetylene in HL-60 cells.

GeneBank ID	GeneSymbol	GeneName	Intensity	
			Ethylbenzene	Trichloroethylene
NM_007289	MME	membrane metallo-endopeptidase	−3.48432	−3.125
NM_005011	NRF1	nuclear respiratory factor 1	−3.23625	−2.73973
NM_004774	PPARBP	PPAR binding protein	−2.36967	−2.07039
NM_001741	CALCA	calcitonin/calcitonin-related polypeptide, alpha	−2.00401	−2.94985
NM_000567	CRP	C-reactive protein, pentraxin-related	−1.72712	−4.9505
NM_138765	BAX	BCL2-associated X protein	−1.71233	−1.65017
AK096179	C7orf40	chromosome 7 open reading frame 40	−1.58228	−2.1978
NM_023110	FGFR1	fibroblast growth factor receptor 1	−1.5083	−1.70068

Q-PCR was conducted in order to validate the results of the human HazChem array experiment. Q-PCR was conducted with the PRNP and TXNRD 1 genes, among the genes with commonly increased expression in ethylbenzene and trichloroethylene. The results were correlated significantly with those acquired from the Human HazChem array (Data not shown).

Discussion

Volatile organic compounds (VOCs) are familiar in routine life. They are volatile in air and produce foul odors or ozone, and are carcinogenic, causing nervous system disorders through skin contact or respiration. They have been shown to cause leukemia in cases of chronic addiction, and many studies are currently underway worldwide that endeavor to predict the risk of such environmentally harmful materials. In assessing the risk of the materials with known or unknown toxicity, massive screening technologies have proven fairly useful, and there are more efforts being made on the state-level in advanced countries, including the U.S.A. As a component of the development of such associated technologies, there have been great improvements in areas including DNA chip and protein massive screening. In particulay, DNA microarray technology is currently being developed to the point at which it will enable expression screening for almost all known genes[10-12].

Despite such development and the many attempts to develop toxicity prediction technology using DNA chip technology, no clear analysis method has yet been developed that allows for toxicity prediction. Thus, a variety of analytical approaches are being made which will allow for the prediction of risk. Although DNA chip technology may enable the screening of the expression of the entire set of known genes, the DNA chip has been reported to suffer from the problems of different results depending on the theories applied for the comparative analysis of gene expression. Moreover, it has been previously reported that the accuracy of the analysis method was increased when using a few selected genes, rather than utilizing the entire genome[13,14].

In this study, we utilized a human HazChem array in order to identify the differentially expressed significant genes induced by ethylenebenzene and trichloroethylene in HL-60 cells. As a result, 8 upregulated genes and 8 downregulated genes were discovered on the basis of 1.5-fold microarray analysis. The upregulated genes were identified as ISG15, TNFSF10, PRNP, ME1, NCOA4, SRXN1, TXNRD1, and XBP1,

whereas the downregulated genes were identified as MME, NRF1, PRARBP, CALCA, CRP, BAX, C7 or f40, and FGFR1. However, according to the results of previously published works, it was determined that the majority of responding genes were associated with predominant changes in the expression of several proteins that regulate apoptosis & cellular growth, differentiation & stress response, all of which have been associated with VOCs toxicity[15-22].

In conclusion, although this data is not sufficient to demonstrate the mechanistic identification of gene expression, our results did suggest that the human HazChem array will facilitate the development of an efficient screening system for environmentally hazardous materials at the level of toxicogenomics in the future.

Methods

Chemicals and Reagents

Ethybenzene (CAS No, 100-41-4), trichloroethylene (CAS No. 79-01-6), Dimethylsulphoxide (DMSO) and 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chemical Company (USA). RPMI -1640 Culture Medium, Dulbecco's Phosphate Buffered Saline (PBS) and Fetal Bovine Serum (FBS) were obtained from GIBCO^{TM} (USA). All other chemicals used in this study were of analytical grade or the highest available grade.

Cell Line and Culture

The human promyelocytic leukemia HL-60 cell line was purchased from ATCC (CCL-240, USA) and maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum plus 100 U/mL of penicillin and 100 μg/mL of streptomycin, 1 mM sodium pyruvate, and 10 mM HEPES at 37˚C in a 5% CO_2 atmosphere. For cell growth, the medium was renewed every two or three days at a density of 5×10^5 cells/mL in T75 tissue culture flasks.

Determination of Cell Viability

An MTT assay[23] was performed using the HL-60 cell strain and the methods of Mossman *et al*. Ethylbenzene and trichloroethylene dissolved in DMSO was introduced to RPMI culture medium (Gibco-BRL, USA) in 15 mL tube with 1×10^6 cell/mL of cells. The cells were exposed to various concentrations of ethylbenzene and trichloroethylene in culture medium at 37˚C for an exposure time of 3 h at 200 rpm in a shaking incubator. 300 μL of MTT (5 mg/mL in PBS) solution was added to each tube and incubated for 3 hrs. DMSO was added to each tube and transferred into 96-well plates. The optimal density (OD) of the purple formazan product was measured at a wavelength of 540 nm. The 50% inhibitory concentration (IC_{50}) of cell proliferation in a particular chemical was defined as the concentration necessary to cause a 50% reduction in cell viability as compared to the solvent-treated controls. The IC_{50} values were determined directly from the linear dose-response curves.

RNA Extract from the Sample

Total RNA was extracted from the HL-60 cells treated with 0.9854 mM and 4.112 mM for ethylbenzene and trichloroethylene, respectively, for 3 hrs using Trizol reagent (Invitrogen Life Technologies) and purified using an RNeasy Mini Kit (Qiagen, USA) in accordance with the manifacturer's instructions. Genomic DNA was removed with an RNase-free DNase set (Qiagen, USA) during RNA purification. The quantity of each RNA concentration was quantified using Nanodrop, and the RNA quality was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies).

Preparation of HazChem Array

The human HazChem array was prepared to hold 316 genes in total after 16 control spots being added to 300 environmental hazard-related genes, and the probe to be spotted to the Human HazChem array was obtained from Qiagen. Normalization was conducted with probe DNA at a density of 50 pmol, then moved into the 384 well plates. They were then implanted onto the superamine-coated CMT-GAPS2 glass slides (Corning Ltd., UK), using a Genemachine pin-type arrayer. The reproducibility, reliability and accuracy of the HazChem array were assessed using the control materials in accordance with the internal guidelines, after which the microarray experiments were conducted using RNA extracted from the HL-60 cell line.

Preparation of Fluorescent DNA Probe and Hybridization

Each extracted total RNA sample (30 μg) was labeled with Cyanine (CY3) or Cyanine (Cy5) conjugated dCTP (Amersharm, Piscataway, NJ) via a reverse transcription reaction using reverse transcriptase, SuperScrip ll (Invitrogen, Carlsbad, California). The labeled cDNA mixture was then concentrated via ethanol precipitation. The concentrated Cy3 and Cy5 labeled cDNAs were resuspended in 10 μL of hybridization solution (GenoCheck, Korea). The two labeled cDNAs were then mixed, placed on a HazChem array Human 300 (GenoCheck, Korea) and covered

with a MAUI M4 chamber (Biomicro Systems, Inc. UT). The slides were hybridized for 12 hr at 62˚C with a MAUI system (Biomicro systems, Inc. UT). The hybridized slides were washed in 2XSSC, 0.1% SDS for 2 min, 1XSSC for 3 min, and then 0.2XSSC for 2 min at room temperature. The slides were then centrifuged for 20 seconds at 3,000 rpm to dry.

Microarray Data Analysis

The hybridized slides were scanned with an Axon Instruments GenePix 4000B scanner and the scanned images were analyzed using the software program GenePix Pro 5.1 (Axon, CA) and GeneSpring GX 7.3.1 (Sillicongenetics, CA). Spots that were adjudged as substandard via the visual examination of each slide were flagged and excluded from further analysis. Spots that harbored dust artifacts or spatial defects were manually flagged and excluded. In an effort to filter out the unreliable data, spots with signal-to-noise (signal-background-background SD) below 10 were not included in the data. Data were normalized by Global, lowess, print-tip and scaled normalization for data reliability. Fold change filters included the requirement that the genes be present in at least 200% of the controls for upregulated genes and lower than 50% of controls for the downregulated genes. The data were clustered groups of genes that behaved similarly across the drug treatment experiments using GeneSpring GX 7.3.1 (Silicongenetics, CA). We utilized an algorithm, based on the Pearson's correlation, to separate genes evidencing similar patterns[24].

Real-time Quantitative PCR

Real-time quantitative PCR was conducted in triplicate in 384-well plates. A 384-well high-throughput analysis was performed using the ABI Prism 7900 Sequence Detection System (PE Applied Biosystems), and white-colored 384-well plates (ABgene, Hamburg, Germany) for the intensification of the fluorescent signals by a factor of three. This system operates using a thermal cycler and a laser which is directed via fiber optics to each of 384 sample wells. The fluorescence emission from each sample was collected by a charge-coupled device-camera and the quantitative data were analyzed with Sequence Detection System software (SDS version 2.0, PE Applied Biosystems). The reaction mixtures contained 10 pmol/μL of each primer and 2X SYBR Green PCR Master Mix (PE Applied Biosystems, www.appliedbiosciences.com), which includes the HotStarTaqt DNA-Polymerase in an optimized buffer, the dNTP mix (with dUTP additive), the SYBRs Green I fluorescent dye, and ROX dye as a passive reference. 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 employed to generate relative standard curves for the genes. All primers were amplified under the same conditions. Thermal cycling conditions were as follows: 95˚C for 10 min followed by 40 cycles of 95 ˚C for 30 s and 60˚C for 30s, 72˚C for 30s. In order to exclude the presence of unspecific products, a melting curve analysis of the products was routinely conducted after finishing amplification via high-resolution data collection during an incremental temperature increase from 60˚C to 95˚C, with a 0.21˚C/sec ramp rate. We then converted the real-time PCR cycle numbers to gene amounts (ng) on the basis of the equation. Real-time PCR analysis was conducted using an Applied Biosystems Prism 7900 Sequence Detection System (PE Applied Biosystems).

Acknowledgements

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