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# Gene Expression Pattern Analysis of PAHs and VOCs in Rat Blood Using HazChem Rat Array

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## Abstract

**The HazChem rat array includes 233 VOCs- and PAHs-specific genes in rat blood samples, 445 VOCs- or PAHs- or POPs-differentially-expressed genes in rat *in vivo* samples and 847 liver-toxicity-related genes in previous studies. In previous experiments, the expression levels of these genes were altered in whole genome microarray experiments using VOC-treated and PAH-treated rat blood cells. In this study, we employed the rat HazChem array to evaluate the gene expression patterns of the chemical groups. The chemical groups utilized in this study were PAHs and VOCs. We employed benzo[a]anthracene and benzo [a]pyrene as examples of PAHs, and dichloromethane, ethylbenzene, and trichloroethylene as examples of VOCs. Thus, a total of 5 chemicals were ultimately evaluated. We selected 239 genes that evidenced significant alterations in expression with 5 environmental toxicants. We also verified that the 5 chemicals utilized herein were divided into 2 chemical groups on the basis of the 62 genes. We also selected 117 commonly expressed genes associated with all types of chemicals. In the gene ontological data, these genes were associated with responses to stress, macromolecular biosynthesis, catabolism, cell adhesion, response to endogenous stimulus, etc. Thus, the results of this study show that the HazChem rat array can be used to predict the toxicity of chemical species, as well as the effects of environmental toxicants.**

**Keywords:** Toxicogenomics, PAHs (Polycyclic Aromatic Hydrocarbons), VOCs (Volatile Organic Compounds), Microarray, Environmental hazards

## Introduction

Toxicology is the study of the harmful effects of chemicals on living organisms. These effects include allostatic gene expression, which is defined as a drug- or environment-induced change in gene expression that is reflective of disease or pharmacological environmental exposure. The relationship between chemicals and their effects on the exposed organism are quite significant in toxicology.

Volatile organic compounds (VOCs) are emitted as gases from certain solids or liquids. VOCs include a variety of chemicals, some of which may exert short- and long-term adverse health effects. The concentrations of many VOCs are consistently higher indoors (up to ten times higher) than outdoors. Examples include: paints and lacquers, cleaning supplies, pesticides, and furnishings and office equipment.

Polycyclic aromatic hydrocarbons (PAHs) are chemical compounds that consist of fused aromatic rings and harbor no heteroatoms or substituents[1]. PAHs are one of the most widespread variants of organic pollutants. In addition to their presence in fossil fuels, they are also formed by the incomplete combustion of carbon-containing fuels including wood, coal, diesel, fat, tobacco, or incense[2]. As pollutants, PAHs are of great concern, as some of these compounds have been previously shown to exert carcinogenic, mutagenic, or teratogenic effects.

In this study, we evaluated the differential expression of genes in rat blood treated with high concentrations of benzo[a]anthracene, benzo[a]pyrene, dichloromethane, ethylbenzene and trichloroethylene, for exposure periods of 72 h using a HazChem rat array. The PAHS used were benzo[a]anthracene and benzo[a]

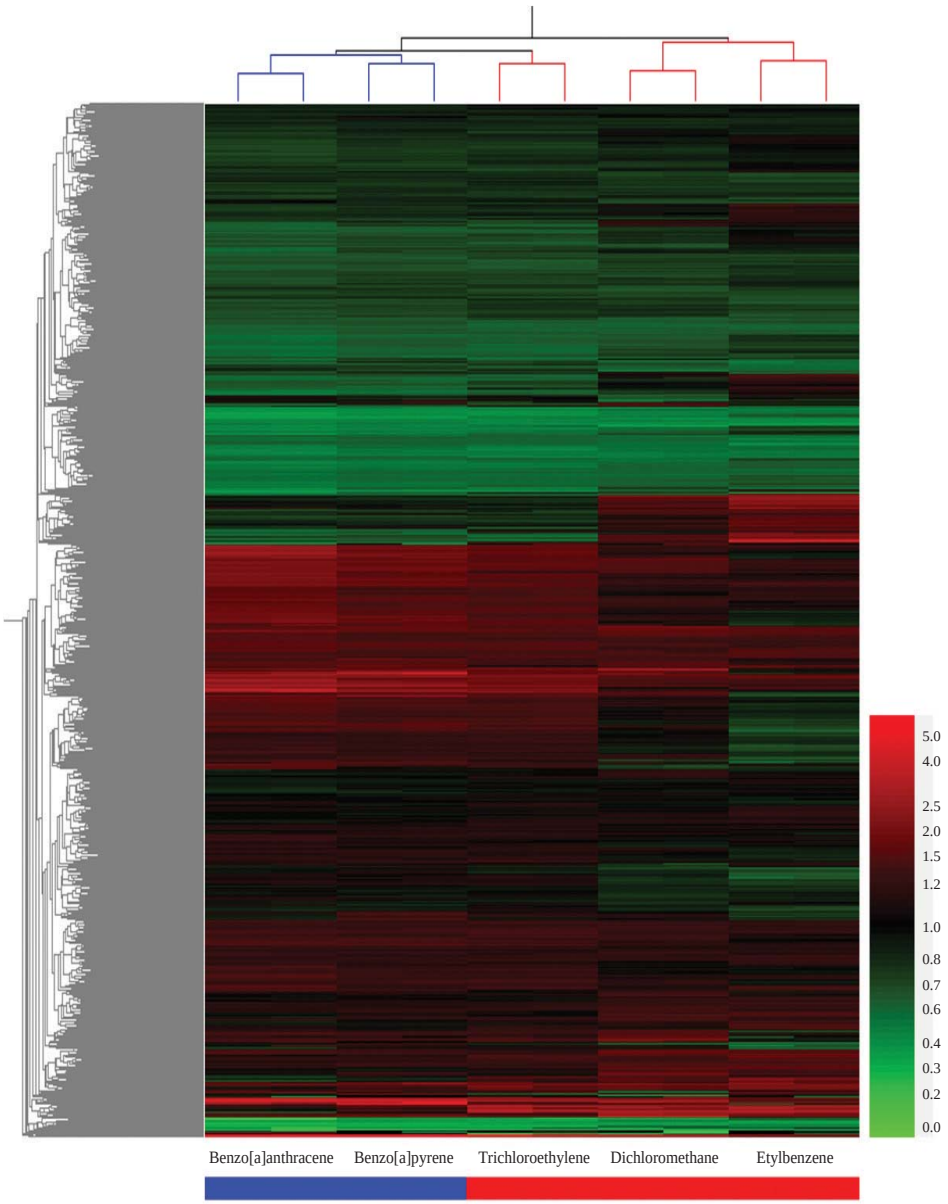


**Figure 1.** Hierarchical clustering analysis of total gene expression of chemical-treated samples. We applied the chemicals to rats. We used two chemical groups (blue bar: PAHs, red bar: VOCs).

pyrene, and the VOCs used were dichloromethane, ethylbenzene, and trichloroethylene.

The HazChem rat array includes 233 VOCs- and PAHs-specific genes in rat blood samples, 445 VOCs- or PAHs- or POPs-differentially-expressed genes in rat *in vivo* samples and 847 liver-toxicity-related genes in previous studies.

## Results

### HazChem Rat Array

Previously, in our study of expression patterns using an operon rat chip (not published) and a rat 4.8K cDNA toxarray chip (GenoCheck, Korea)[3], a HazChem rat array was developed that consisted of 233 VOCs- and PAHs-specific genes in rat blood samples, 445 VOCs- or PAHs- or POPs-differential expressed genes in rat *in vivo* samples from the microarray results using an operon rat chip and 847 genes from microarray results using a rat 4.8K cDNA chip. The HazChem rat array was manufactured using SurePrint technology with an Agilent custom array.

### Gene Expression Analysis

Rats were treated with PAHs and VOCs, after which the blood was isolated. The RNA was then subjected to HazChem rat array analysis. For each treatment,

**Table 1.** GO ontology classification of 117 significantly altered genes.

| Category | Genes in List in Category | p-Value |
|---|---|---|
| GO:9719: response to endogenous stimulus | 9 | 0.000999 |
| GO:9059: macromolecule biosynthesis | 14 | 0.00129 |
| GO:48015: phosphoinositide-mediated signaling | 5 | 0.00215 |
| GO:74: regulation of progression through cell cycle | 9 | 0.00269 |
| GO:7626: locomotory behavior | 10 | 0.00322 |
| GO:6092: main pathways of carbohydrate metabolism | 5 | 0.00436 |
| GO:51480: cytosolic calcium ion homeostasis | 4 | 0.00442 |
| GO:6950: response to stress | 17 | 0.00594 |
| GO:30182: neuron differentiation | 8 | 0.0074 |
| GO:16477: cell migration | 7 | 0.00757 |
| GO:9056: catabolism | 11 | 0.0106 |
| GO:6259: DNA metabolism | 9 | 0.0133 |
| GO:6816: calcium ion transport | 4 | 0.0151 |
| GO:7155: cell adhesion | 10 | 0.0159 |
| GO:30005: di-, tri-valent inorganic cation homeostasis | 5 | 0.0162 |
| GO:6725: aromatic compound metabolism | 4 | 0.0218 |

genes with statistically significant expressional changes were identified via microarray. Hierarchical clustering was applied across the five agents, using a combined list of genes. The results obtained using the human HazChem array and the comparison of the gene expression clusterings for dichloromethane (72 h high dose), ethylbenzene (72 h high dose), trichloroethylene (72 h high dose), benzo[a]anthracene (72 h high dose), and benzo[a]pyrene (72 h high dose) compounds evidenced differentially expressed gene patterns according to the chemical groups to which the compounds belonged (Figure 1).

### Significantly Expressed Gene Analysis

Rats were treated with PAHs and VOCs, after which the blood RNA was subjected to HazChem rat array analysis. Changes in gene expression were analyzed via comparison with the treated and control RNA. In all chemical groups, a total of 117 were selected. 24 genes were upregulated and 93 genes were downregulated. In the gene ontological data, these genes were associated with stress responses, macromolecule biosynthesis, catabolism, cell adhesion, response to endogenous stimulus, etc. (Table 1).

### Classification

We utilized a statistical method for the classification of chemicals using gene expression profiles[4,5]. We employed ANOVA as a statistical method, using Welch's T-test as an algorithm. We compared the expression profiles of the PAHs and VOCs via data analysis. We selected 239 genes, which could be classified into two chemical types. Variances were calculated using on-chip replicates. P-value cutoff 0.05, multiple testing correction: Benjamini and Hochberg False Discovery Rate. The following hierarchical clustering data evidenced different patterns (Figure 2).

Also, using the PCA software[6], we tested 63 genes with regard to their chemical groupings of PAHs and VOCs (Figure 3).

## Discussion

Recently, a great deal of interest has been focused on the use of microarrays in toxicology for the rapid classification of toxicants based on characteristic expression profiles[7-9].

In this study, we utilized a HazChem rat array to identify differentially expressed genes that were significantly induced by PAHs and VOCs in the blood of rat. The HazChem rat array was manufactured for the purpose of assessing toxicant action in the rat (*in vivo*). The HazChem rat array design was based on PAHs and VOCs-related genes and toxarray chip genes. In this study, we conducted HazChem rat array tests using 5 different chemicals. The result of microarray data analysis demonstrated a differentially expressed gene pattern as the result of exposure to PAHs (benzo[a]anthracene, benzo[a]pyrene) and VOCs (dichloromethane, ethylbenzene, trichloroethylene) in the blood of rats. We found 239 distinct genes associated with different patterns between PAHs and VOCs, on the basis of the gene expression profiles and genetic distances (Figure 2). We also detected 117 commonly expressed genes associated with both types of chemicals, on the basis of the results of our 1.5 fold microarray analysis. Furthermore, different analysis methods, including 1-way ANOVA and PCR, were utilized for the validation and classification of the

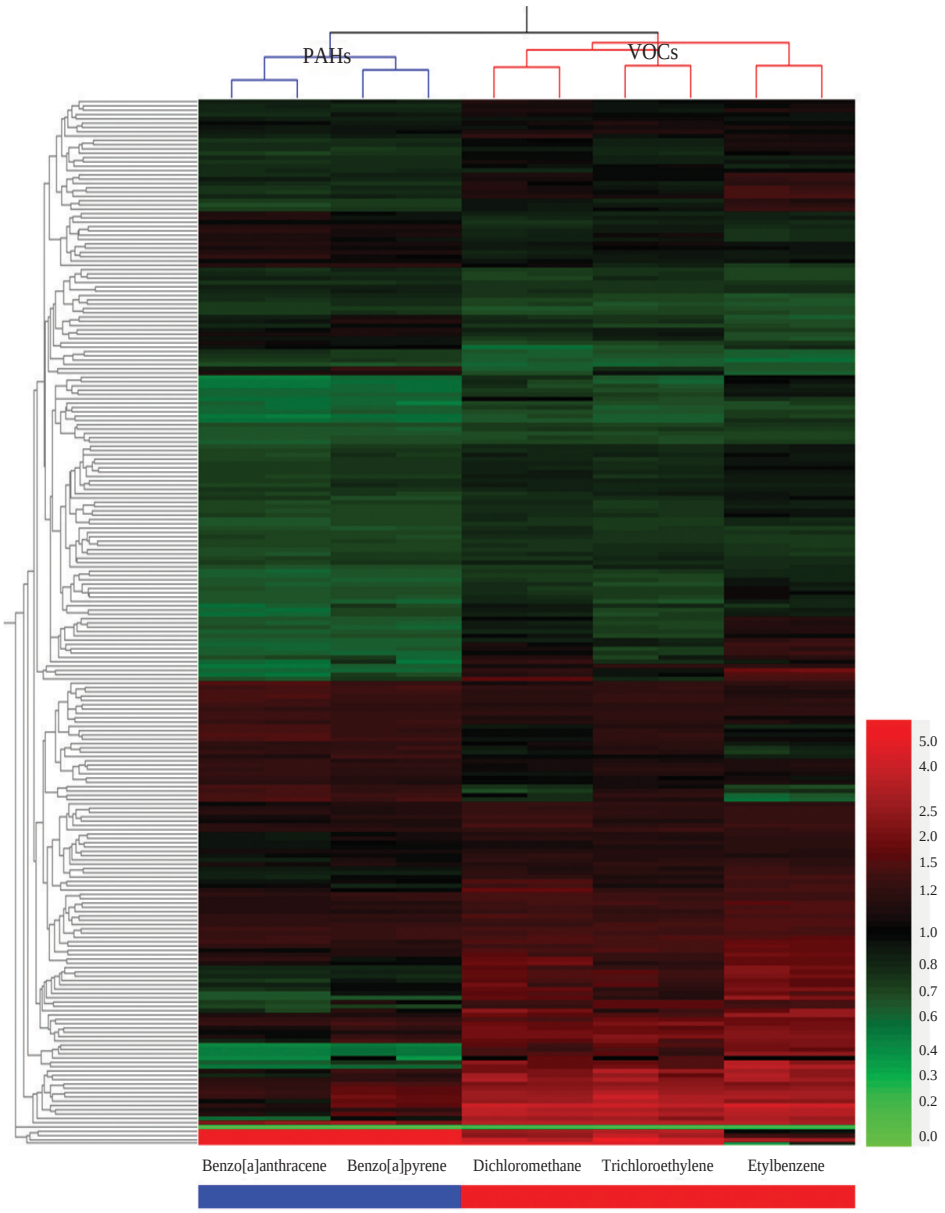


**Figure 2.** Classification of two chemical groups using genes selected via 1-way ANOVA. 252 genes selected by statistical method, 1-way ANOVA (blue bar: PAHs, red bar: VOCs).

HazChem rat array.

## Materials and Methods

### Animals and Treatments

Male Sprague-Dawley rats (11-week old) were purchased from ORIENT BIO INC (Korea). The animals were housed in a controlled environment in the institutional animal facility with free access to food and water throughout the experiment. The animal facility was operated at 23±3°C, at a relative humidity of 55 ±10% with a 12 h light/12 h dark cycle. Dichloromethane (DM), Ethylbenzene (EB), Trichloroethylene (TE) Benzo[a]anthracene (BA), and Benzo[a]pyrene (BP) (TCI-EP, Tokyo Kasei, Japan; CAS Number: 56-55-3) were prepared in accordance with the manufacturer's protocol. High toxic doses were designated as 50% of the published $LD_{50}$s and low toxic doses were designated as 10% of the published $LD_{50}$s for each Polycyclic aromatic hydrocarbons. The rats were sacrificed and the rat blood cells were extracted 72 h after the oral administration of DM, EB, TE, BA (25, 5 mg/kg), and BP (100, 20 mg/kg) with vehicle (corn oil), respectively. We decided the high (50% of $LD_{50}$) and low doses (10% of $LD_{50}$) for each of the Polycyclic

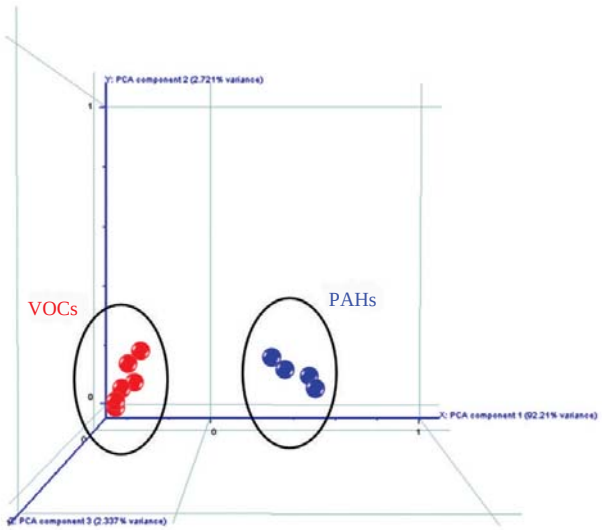


**Figure 3.** Classification of 2 chemical groups using genes selected by 1-way ANOVA. 63 genes selected by statistical method, 1-way ANOVA. 5 chemicals accurately classified as 2 chemical types using 63 selected genes.

aromatic hydrocarbons based on the reports in the literature. Animals were observed at least once per day for signs of overt toxicity and readily apparent clinical symptoms. In all instances, animals were humanely handled in accordance with IACUC (Institutional Animal Care and Use Committee) guidelines. After treatment, the rats were sacrificed, and the serum samples were preserved for the assessment of hematological parameters.

### RNA Extraction

Total RNA was extracted from rat whole blood cells using TRIzol (Invitrogen, Carlsbad, CA, USA) through RBC lysis buffer (150 mM ammonium chloride, 10 mM potassium bicarbonate, and 1 mM EDTA disodium salt), and quality control was conducted using RNA 6000 Nano chips on an Agilent 2001 Bioanalyzer (Agilent Technologies, Germany), as reported previously.

### Hybridization

Each extracted total RNA sample (1 μg) was amplified via a reverse transcription and an *in vitro* transcription reaction using RNA ampULSe: Amplification and Labeling Kit (KREATECH). The amplified RNA (2 μg) was labeled with Cy3-ULS or Cy5-ULS via a co-ordinative bond formation reaction (ULS labels DNA and RNA by binding to the N7 position of guanine) (KREATECH)[10]. The mixed two labeled aRNAs were suspended with 2Xhybridization solution (Agilent Biotechnologies), placed on a HazChem rat array (Agilent, Korea) and covered with a Hybridization Gasket Slide Kit-8 microarray per slide format (Agilent Biotechnologies). The slides were hybridized for 16 hr at 45°C in an oven. The hybridized slides were washed in 2XSSC, 0.1% SDS for 2 min, 1XSSC for 3 min, and 0.2XSSC for 2 min at room temperature. The slides were dried via 20 seconds of centrifugation at 3,000 rpm.

### Microarray Data Analysis

The hybridized slides were scanned with an Agilent scanner and the scanned images were analyzed with the software program Feature Extraction v10.7 (Agilent Technologies, CA) and GeneSpring GX 10 (Agilent Technologies, CA). Spots that were adjudged as substandard via the visual examination of each slide were flagged and excluded from further analysis. Spots harboring 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) ratios below 10 were excluded from the data. Data were normalized by intensity LOWESS normalization for data reliability. Expression profile data included clustered groups of genes that behaved similarly across the drug treatment experiments using GeneSpring GX 10 (Agilent Technologies, CA). We utilized an algorithm based on Euclidean distance and average linkage to separate genes evidencing similar patterns. To select genes with commonly altered expressions, we utilized significant analysis of microarray (SAM) methods. Thus, the Benjamini-Hochberg correction for false discovery rate (FDR) was used to evaluate type-dependent differences. We selected expressed genes with FDR-adjusted P values of less than 0.05 according to the results of ANOVA (test type is Welch's t-test) using GeneSpring GX 10. ArrayToKegg software (GenoCheck, Ansan, Korea) was used for the functional analysis of genes within the differentially expressed genes[11,12].

## Acknowledgements

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