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Differential Gene Expression Profiling in Human Promyelocytic Leukemia Cells Treated with Benzene and Ethylbenzene

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Abstract

Benzene and ethylbenzene (BE), the volatile organic compounds (VOCs) are common constituents of cleaning and degreasing agents, paints, pesticides, personal care products, gasoline and solvents. VOCs are evaporated at room temperature and most of them exhibit acute and chronic toxicity to human. Chronic exposure of benzene is responsible for myeloid leukemia and also ethylbenzene is also recognized as a possible carcinogen. To evaluate the BE effect on human, whole human genome 35 K oligonucleotide microarray were screened for the identification of the differential expression profiling. We identified 280 up-regulated and 201 down-regulated genes changed by more than 1.5 fold by BE exposure. Functional analysis was carried out by using DAVID bioinformatics software. Clustering of these differentially expressed genes were associated with immune response, cytokine-cytokine receptor interaction, toll-like signaling pathway, small cell lung cancer, immune response, apoptosis, p53 signaling pathway and MAPKKK cascade possibly constituting alternative or subordinate pathways of hematotoxicity and immune toxicity. Gene ontology analysis methods including biological process, cellular components, molecular function and KEGG pathway thus provide a fundamental basis of the molecular pathways through BEs exposure in human lymphoma cells. This may provides a valuable information to do further analysis to explore the mechanism of BE induced hematotoxicity.

Keywords: Benzene, Ethylbenzene, Oligomicroarray, Gene ontology, KEGG pathway, Hematotoxicity

Benzene and ethylbenzene (BE) are volatile mono-aromatic hydrocarbons which are commonly found together in crude petroleum and petroleum products such as gasoline[1]. These compounds produced on the scale of megatons per year as bulk chemicals for industrial use as solvents and starting materials for the manufacture of pesticides, plastics, and synthetic fibers[2]. They are ubiquitous pollutants mainly due to engine emissions, tobacco smoke and industrial pollution. Benzene and ethylbenzene are in the top 50 chemicals produced and used worldwide. As a consequence of their usage, they are most common waste material from the industry[3]. Indoor exposure is thought to be of greater concern because indoor concentrations of many pollutants are often higher than those typically encountered outside[4]. The observed health effects in association with volatile organic compounds (VOC) exposure it seems to be true that high VOC concentrations mainly cause toxic effects[5,6] whereas moderate VOC exposure levels trigger inflammatory reactions in particular in the airways[7] and low exposure levels induce alterations in immune reactivity resulting in a subsequent higher risk for the development of allergic reactivity and asthma[8,9].

Children are a potentially at-risk population because they may be both more exposed to VOCs and more susceptible to diverse effects than adults. Children can be affected by different sources, pathways, and routes of exposure than adults; that children often have greater intake of air, food, beverages, soil, and dust per unit body weight and surface area; and that children differ from adults in terms of important pharmacokinetic and pharmacodynamic parameters[10,11]. Blood concentrations of 11 VOCs were measured up to four times over 2 years in a probability sample of more than 150 children from two poor, minority neighborhoods in Minneapolis, Minnesota. Blood levels of

benzene, carbon tetrachloride, trichloroethene, and *m*-/*p*-xylene were comparable with those measured in selected adults from the Third National Health and Nutrition Examination Survey (NHANES III), whereas concentrations of ethylbenzene, tetrachloroethylene, toluene, 1,1,1-trichloroethane, and *o*-xylene were two or more times lower in the children[12].

To predict the risk of environmental toxic substances or compounds, investigation of toxicological mechanisms or toxic biomarkers is noticed more and more. Toxicogenomic technologies are more helpful than traditional tools for investigation of these toxic substances. Recent advancements in high throughput gene profiling techniques such as DNA microarray allows simultaneous measurement of mRNA expression levels of tens of thousands of genes at once whereas previously only a handful of genes could be investigated[13]. Recently researchers are interested in analyzing the expression patterns of functionally related genes usually hope to improve the accuracy of their results beyond the boundaries of currently available experimental data. One of the most common problems encountered while deciphering results from expression profiling experiments is in relating differential expression of genes to molecular functions and cellular processes. A second important problem is that of comparing experiments performed by different labs using different microarray platforms, or even unrelated techniques. Gene Ontology (GO) data provides a novel way to measure the functional relationship between gene products[14]. Gene Ontology (GO) is now used to describe biological features, since GO terms are associated with genes, to overcome the apparent distance between expression profiles and biological comprehension[15].

Benzene and ethylbenzene were used as models of VOCs, this toxicogenomic study was carried out to compare BE-induced gene expression alterations in human lymphoma cells. We examined differentially expressed genes at multiple levels (biological process, pathway, gene) using Gene Ontology (GO) analysis, pathway mapping and cross-scatter plots. The Gene Ontology (GO) analysis of microarray data provides structured and controlled vocabularies and classifications for several domains of molecular and cellular biology[16]. GO is generally used to annotate the functions of genes and is composed of three domains, biological process, cellular components and molecular functions. However, it is not deal with the relations between pathways and sub-pathways or between pathways and their related biological phenomena[17]. So, the association of Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway containing information on biological pathways and GO is useful for analysis of function of genes. Combination of microarray and pathway data may highlight the process taking place in the cell providing information on the process-specific function of the genome. Biological process can be prospected according to combination of microarray results and biological information[18].

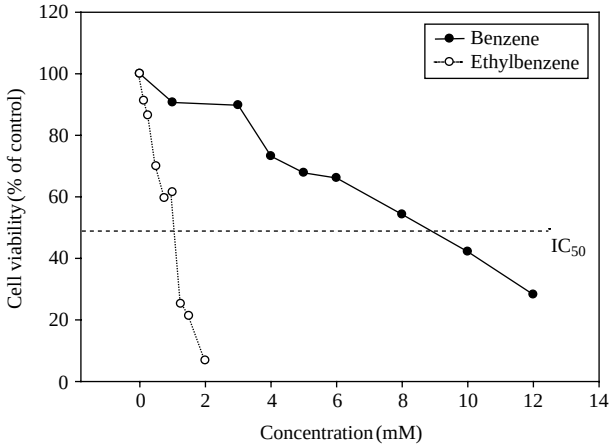

Figure 1. Cell viability after the exposure with BE were analyzed by the MTT assay. Three mL of HL-60 cells were seeded in 15 mL tube at 1×10^6 cells/mL in a shaking incubator. After 3 h treatment with BE, MTT solution (5 mg/mL) was added to each tube for 3 h. The reaction was stopped by adding DMSO and the absorbance (optical density) was determined at 540 nm. The change in absorbance which is the change in cell death is shown as % of control. Fifty percent inhibition concentration (IC_{50}) values of BE were calculated using SigmaPlot 8.0 statistical analysis software.

The objective of this study is the identification of function of the genes through the analysis of GO and to find potential gene-based markers induced by benzene and ethylbenzene. We have examined the global gene expression profiles in a small number of well matched exposed-control subject pairs. Differentially expressed genes were then ranked and selected for further examination using several forms of statistical analysis. Commonly expressed genes with both chemicals may assist in the identification of potential biomarker of BE and may understand molecular toxicological mechanism of benzene and ethylbenzene in human lymphoma cell line.

Cytotoxicity of Benzene and Ethylbenzene in HL-60 Cells

To determine the 50% inhibition concentration of cell proliferation on benzene, and ethylbenzene, various concentrations of BE were treated to the HL-60 cells. Benzene and ethylbenzene reduced cell viability gradually at increasing concentrations were

Table 1. Biological process of up-regulated genes in benzene and ethylbenzene treated HL-60 cell. Common expressed genes in two chemicals marked by bold.

GO	Benzene	Ethylbenzene
Immune response	BCL2, **BCL6**, CCL2, **CCL20**, **CCL3L1**, **CCL4**, **CCL4L1**, **CCL5**, **CD83**, CFHL5, CLECSF12, CLECSF5, CRTAM, **CXCL10**, CXCR4, DAF, DDIT3, **DDX58**, DEFB4, **EBI2**, ELF1, FASLG, FCAR, FCAR, FCGR1A, FLJ14466, FLJ45422, FTH1, **FYN**, G1P2, **GBP2**, HFE, HLA-DMA, HLA-E, HLA-G, **HMOX1**, IFI16, **IFIT1**, **IFIT2**, **IFIT3**, IGLC2, **IL18R1**, **IL18RAP**, IL1R1, IL1RAP, IL1RL1, IL23A, IL4R, INPPL1, KIR2DS2, LAX, LGP2, NFIL3, NFKB2, **OASL**, PGLYRP2, **PTGER4**, PTK9L, PTPN22, RELB, RNF125, SLC11A1, SQSTM1, TNF, **TNFSF10**, **TNFSF14**, **XBP1**	AIM2, BCL3, **BCL6**, **CCL20**, CCL23, **CCL3L1**, **CCL4**, **CCL4L1**, **CCL5**, **CD83**, CLEC4A, CLU, **CXCL10**, CXCL11, CXCR4, **DDX58**, **EBI2**, ETS1, F3, FASLG, FCGR1A, FTH1, **FYN**, **GBP2**, HLA-A, **HMOX1**, **IFIT1**, **IFIT2**, **IFIT3**, IFIT5, IFNA7, IFNB1, IL10, **IL18R1**, **IL18RAP**, IL1A, IL27RA, INPPL1, IRF1, IRF7, IRF8, ISG15, ISGF3G, LAX1, LTA, LTB, LY96, NCR3, NFKB2, OAS3, **OASL**, PRG3, **PTGER4**, PTX3, RBM24, RGS1, SFTPD, SLC11A1, SMAD3, SQSTM1, TAP1, TNF, TNFRSF9, **TNFSF10**, TNFSF13B, **TNFSF14**, TNFSF7, WAS, **XBP1**
Inflammatory response	AOC3, **BCL6**, BMP6, C3AR1, CCL2, **CCL20**, **CCL3L1**, **CCL4**, CCL4L1, **CCL5**, CFHL5, CLECSF12, **CXCL10**, **CXCR4**, DAF, **F11R**, FPR1, IL17F, **IL18RAP**, IL1R1, IL1RAP, IL23A, NOS2A, NR3C1, PLA2G2E, RAC1, **ZFP36**	APOL2, **BCL6**, C3AR1, **CCL20**, CCL23, **CCL3L1**, **CCL4**, **CCL5**, CLU, **CXCL10**, CXCL11, **CXCR4**, CYBB, **F11R**, FPR1, HRH1, IL10, **IL18RAP**, IL1A, IRF7, LY96, MIF, NCR3, NFKB1, PTX3, RBM24, SERPINA1, TLR8, TPST1, USP18, **ZFP36**
Cytokine production	IL17F, **NFAT5**, IGSF4, ELF1, CRTAM, **BCL6**, **HMOX1**, IL1RAP, ASB1	IRF1, **NFAT5**, SFTPD, ISGF3G, BCL3, **BCL6**, **HMOX1**, NFKB1, IL27RA, PRG3, TLR8
Cellular defense response	**C3AR1**, **CCL5**, CLECSF5, HLA-G, KIR3DL2, **NCF2**, **TCIRG1**	**C3AR1**, **CCL5**, FOSL1, LY6H, LY96, **NCF2**, **TCIRG1**, TRA2, TYROBP
Apoptosis	AHR, AXUD1, **BBC3**, BCL2, **BCL2A1**, **BCL6**, BID, BIRC2, **BIRC3**, BIRC7, **BTG1**, CASP3, **CBX4**, CCL2, CDKN1A, CIDEC, CRTAM, **CXCR4**, DAPK2, DDIT3, **FASLG**, FLJ11259, **GADD45A**, **GADD45B**, **GCLM**, **HMOX1**, **HSPA1A**, IER3, IFI16, **IFIH1**, IGSF4, IKIP, JMY, **NFKBIA**, PHLDA1, PLEKHF1, **PMAIP1**, **PPP1R15A**, **PRNP**, **PTK2B**, RASSF5, RB1CC1, RTKN, RYBP, SART1, SGK, SHB, SIAH2, **SQSTM1**, **STAT1**, STK17B, **TNF**, **TNFAIP3**, **TNFSF10**, **TNFSF14**, **TRAF1**, **TRAF3**, **TRIB3**	ACVR1, AIFM2, ANGPTL4, ATF5, BAD, **BBC3**, **BCL2A1**, BCL3, **BCL6**, **BIRC3**, **BTG1**, BTG2, **CBX4**, CD38, CDKN1A, CLU, **CXCR4**, DAPK3, DEDD2, ERN1, **FASLG**, **GADD45A**, **GADD45B**, GCH1, **GCLM**, HIPK2, **HMOX1**, **HSPA1A**, **IFIH1**, IFNB1, IHPK2, IL10, IL1A, LGALS12, LTA, MIF, NFKB1, **NFKBIA**, NGFR, PHLDA1, PHLDA2, **PMAIP1**, **PPP1R15A**, **PRNP**, **PTK2B**, SART1, SGK, SMAD3, **SQSTM1**, **STAT1**, **TNF**, **TNFAIP3**, TNFRSF10B, TNFRSF9, **TNFSF10**, TNFSF13B, **TNFSF14**, **TRAF1**, **TRAF3**, **TRIB3**
Anti-apoptosis	BCL2, **BCL2A1**, BIRC2, **BIRC3**, BIRC7, **CBX4**, CCL2, **HMOX1**, **HSPA1A**, IER3, PRNP, RTKN, **TNF**, **TNFAIP3**	ATF5, **BCL2A1**, **BIRC3**, **CBX4**, CLU, **HMOX1**, **HSPA1A**, IL10, IL1A, NFKB1, PRNP, **TNF**, **TNFAIP3**, TNFSF13B
Regulation of I-kappaB kinase/ NF-kappaB cascade	BIRC2, **FASLG**, **HMOX1**, MAP3K7IP2, **SQSTM1**, **TNF**, **TNFAIP3**, **TNFSF10**	**FASLG**, **HMOX1**, LTA, **SQSTM1**, **TNF**, **TNFAIP3**, TNFRSF10B, **TNFSF10**, TRAF3IP2
Iron ion transport	**FTH1**, **FTHL17**, **FTHL3**, HFE, SFXN3, **SLC11A1**	**FTH1**, **FTHL17**, **FTHL3**, FTL, **SLC11A1**
MAPKKK cascade	DUSP16, **CXCR4**, **ITPKB**, **GADD45B**, BIRC7, TNF, DUSP2, **FPR1**, RB1CC1, **LAX**, MAP3K9, **DUSP4**, RAPGEF2	FGFR1, **CXCR4**, **ITPKB**, **GADD45B**, TNF, CARD9, MAPBPIP, **FPR1**, MAP3K11, **LAX**, HIPK2, **DUSP4**
Cell proliferation	ACHE, BCL2, **BCL6**, **BTG1**, CASP3, **CCL3L1**, **CDKN1A**, CDYL2, CLK1, CNTFR, COL8A1, CRTAM, **CXCL10**, **CXCR4**, **ELF5**, EMP1, EPHB4, **FTH1**, GLI1, HBEGF, HHIP, **HMOX1**, IFI16, **IFIT1**, IGSF4, **IRS2**, **JAG1**, KLF10, LAMP3, MAD, **NFKBIA**, PBEF1, PGF, PPAP2A, **PTK2B**, RARRES3, **SERTAD1**, **SH2D2A**, SHB, **TCIRG1**, **TNF**, **TNFSF14**, TRIB1, TSPAN1, TXNRD1	ARTN, **BCL6**, BIN1, **BTG1**, BTG2, CALCRL, CCL23, **CCL3L1**, **CDKN1A**, CDKN2B, CLU, **CXCL10**, **CXCR4**, **ELF5**, ETS1, FGFR1, FOSL1, FSCN1, **FTH1**, **HMOX1**, **IFIT1**, IFNB1, IGFBP6, IL10, IL1A, **IRS2**, ISG20, **JAG1**, KLF4, LTA, MAFG, MAP3K11, MIF, MXD1, **NFKBIA**, PLAUR, POLD4, **PTK2B**, RARRES3, RECQL4, **SERTAD1**, SFTPD, **SH2D2A**, SMAD3, TCF19, **TCIRG1**, **TNF**, TNFRSF9, TNFSF13B, **TNFSF14**, TP53I11, TXNRD1, WARS

Table 2. Biological process of down-regulated genes in benzene and ethylbenzene treated HL-60 cell. Common expressed genes in two chemicals marked by bold.

GO	Benzene	Ethylbenzene
Regulation of progression through cell cycle	PLCB1, BUB1, BUB1B, CDKN2C, TADA2L, DLG1, IL1B, CCNE1, **NF1**, **CDC25A**, MKI67, CDKL1, RUNX3, **SMC1A**, HEXIM1, MTCP1, GTSE1, **ANLN**, CDC25C, CCNB1, PML, CCNE2, PKMYT1, **TRIM13**	TRRAP, CCNJ, CCNYL1, SLC12A6, **NF1**, CUL4A, **CDC25A**, E2F5, MYC, SHC1, TGFB2, VEGFA, LATS1, SLC12A6, EGFR, PRMT5, **SMC1A**, BRCA1, ABL1, PPP2R1A, ANKRD15, SUFU, CENPF, **ANLN**, MPKK6, UHMK1, SHC1, **TRIM13**
G1/S transition of mitotic cell cycle	GPR132, **GFI1**, CDKN2C, CDCA5, CCNE1	**GFI1**, ACVR1B, CUL4A, BCAT1, SKP2, POLE, GPR132
Cell motility	TOP2B, SLIT1, ABI3, NCAN, AKAP4, TUBB, IL1B, DOCK2, GAB1, CCL25, **NF1**, TPM1, GDNF, NPY, ATP1A1, PALM, TLX3, CENTD3, LPAL2, GDNF, ITGA6, MAP3K1	DNAH2, UNC5C, TAOK2, LRP5, ROBO3, ATP1A3, CDK5R1, **NF1**, ITGA4, MSN, MUC2, TGFB2, VEGFA, NTN1, CRKL, EGFR, NTN2L, TPBG, LTB4R2, MAPK8, ARX, F11R, CABP4, APBB2, NF2, LAMA3, MIA3
Mitosis	BUB1, BUB1B, **SPAG5**, DLG1, CIT, **ASPM**, CDCA5, NCAPH, **CDC25A**, NDC80, **SMC1A**, KIF2C, **TARDBP**, TPX2, GTSE1, ANLN, CDC25C, CNB1, PML, **CDCA2**, PKMYT1	DCTN1, TRRAP, **SPAG5**, PES1, PBRM1, **ASPM**, STAG1, STAG2, ATM, **CDC25A**, SHC1, LATS1, SMC4, PRMT5, **SMC1A**, **TARDBP**, PES1, CENPF, KIF15, **CDCA2**, SHC1, CDC27
Ras protein signal transduction	**WDR67**, TBC1D13, HRBL, **PLEKHG5**, ABRA, RASGRP4, **NF1**, ARHGEF17, **KIAA1415**, **ABR**, CENTD3, MRAS	**WDR67**, PLEKHG6, TBC1D14, **PLEKHG5**, HRBL, ARHGEF2, **NF1**, G3BP2, CDC42EP4, TBC1D3G, **KIAA1415**, SPATA13, RREB1, PSD, **ABR**, ARHGEF6, CRKL, TRIO, DDEFL1, ARHGEF7
Regulation of signal transduction	SNF1LK2, ACVR2B, FGF18, GEFT, MGC21654, CD8A, **ABR**, KIAA0720, FLJ40244, STARS, RASGRP4, IL1B, DKFZP434I092, **NF1**, **DTX1**, **HRBL**, RASA4, **TMOD2**, ARHGEF17, TBC1D13, CTNNBIP1, PREX1, CENTD3, APOL3, RFP2, KIAA1272, FLJ35390	ARHGEF2, RGS4, RGS11, RAPGEF4, TAOK2, SPRY4, MIER1, NOD1, WDR67, PLEKHG6, **ABR**, RASA3, RIC8B, RAMP3, PLEKHG5, **HRBL**, ARHGEF6, ACVR1B, DDEFL1, SPRY1, IL6ST, TBC1D3G, RGS22, SPATA13, KIAA1219, **NF1**, IL2, ABR, PSD, SHC1, VEGFA, **DTX1**, TRIO, PPP2R1A, **TMOD2**, PDCD11, OTUD7B, BC1D14, APOL3, ARHGEF7, SHC1, SOCS4, TRIM13
Cell migration	SLIT1, ABI3, FOXC1, IL1B, DOCK2, GAB1, CCL25, **NF1**, TOP2B, NTN2L, TLX3, CENTD3, LPAL2, GDNF, ITGA6	UNC5C, TAOK2, LRP5, ROBO3, CDK5R1, **NF1**, ITGA4, MUC2, TGFB2, VEGFA, NTN1, EGFR, LTB4R2, ARX, APBB2, LAMA3, MAP3K1, MIA3
Positive regulation of cell differentiation	**THRB**, **ACIN1**, ZNF488, **GFI1**, WNT7B	**THRB**, **GFI1**, ACVR1B, VHL, RUNX1, CD276, CALCA, TGFB2, NKX6-1, **ACIN1**, WNT7B
Cell cycle	PLCB1, BUB1, BUB1B, **SPAG5**, WWOX, FOXC1, TAF1L, TADA2L, MPHOSPH9, **GFI1**, DLG1, CIT, ASPM, IL1B, CDCA5, TCF19, CCNE1, BRRN1, **CDC25A**, MKI67, RAD54L, PRC1, CDKL1, RUNX3, TOP3A, KNTC2, SMC1L1, HIS1, KIF2C, **TARDBP**, TPX2, RACGAP1, GPR132, MTCP1, GTSE1, **ANLN**, CDC25C, CCNB1, PML, CCNE2, EXO1, CDCA2, PKMYT1, MCM7, **E2F7**, RFP2	ABL1, ACVR1B, ANKRD15, **ANLN**, APBB2, ASPM, ATM, BANP, BCAT1, BRCA1, CCNJ, CCNYL1, **CDC25A**, CDC27, CDCA2, CDK5R1, CENPF, CUL4A, DCTN1, DMTF1, DUSP13, E2F5, **E2F7**, EGFR, ERBB2IP, FLCN, FSD1L, **GFI1**, GPR132, KCNK17, KIF15, LATS1, MYC, PBRM1, PES1, PES1, POLE, PPP2R1A, PRMT5, RIF1, SHC1, SHC1, SKP2, SLC12A6, SLC12A6, SMC1A, SMC4, **SPAG5**, STAG1, STAG2, SUFU, SYCP2, **TARDBP**, TGFB2, TRIM13, TRRAP, UHMK1, VEGFA ,VHL
Localization of cell	ABI3, AKAP4, **ATP1A3**, CCL25, CENTD3, CSPG3, DOCK2, FOXC1, GAB1, GDNF, GDNF, IL1B, ITGA6, LPAL2, NPY, NTN2L, PALM, SLIT1, TLX3, TOP2B, TPM1, TUBB	APBB2, ARX, **ATP1A3**, CABP4, CDK5R1, CRKL, DNAH2, EGFR, F11R, ITGA4, LAMA3, LRP5, LTB4R2, MAP3K1, MAPK8, MIA3, MSN, MUC2, NTN1, ROBO3, TAOK2, TGFB2, TPBG, UNC5C, VEGFA

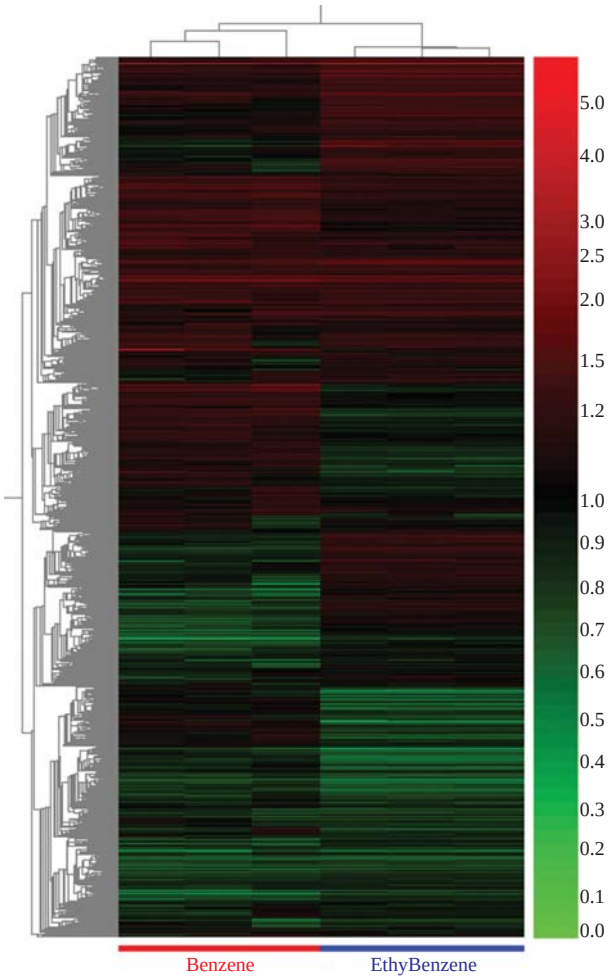

Figure 2. A cluster analysis was carried out using mean of differentially expressed genes of each chemical. HL-60 cells were individually exposure to IC_{50} concentration of BE, total RNA was extracted using Trizol reagent and purified with RNeasy mini kit according to manufacturer's instructions. Three independent experiments were performed for each chemical. Rows represent genes and columns represent samples. Red and green blocks respectively represent high and low expression relative to reference RNA, while black blocks indicate equal expression.

determined MTT assay. The survival percentage relative to solvent control (DMSO) was determined as a percentage of optical density value measured after treatment. Dose dependent cell viability curves were obtained after 3 hrs exposure to BTX and there inhibition concentration of were shown in Figure 1. The IC_{50} value for benzene was 8.75 mM and for ethylbenzene was 0.99 mM.

Gene Expression Analysis

HL-60 cells were treated with 8.75 mM benzene and also 0.99 mM ethylbenzene for 3 hrs, and the total RNA were subjected to microarray analysis. Gene expression changes were analyzed by comparing with treated and control group using a statistical criteria of ≥ 1.5 fold changes with $P < 0.01$. In this analysis, 1,007 genes were up-regulated and 919 genes were down-regulated by benzene exposure and also 929 genes were up and 1,455 genes were down regulated by ethylbenzene individually exposure. Two hundred eight genes were up and 201 genes were commonly down regulated through benzene and ethylbenzene exposure.

These genes were classified according to KEGG pathway to analyze molecular mechanism exposed to BE. The up and down regulated genes were listed in Table 1 and 2. Differentially changed genes involved in 1) MAPK signaling pathway, 2) Cytokine-cytokine receptor interaction, 3) Natural killer cell mediated cytotoxicity, 4) Toll-like receptor signaling pathway, 5) Apoptosis, 6) p53 signaling pathway, 7) Adipocytokine signaling pathway, 8) T cell receptor signaling pathway, 9) Type I diabetes mellitus, 10) Small cell lung cancer, 11) Chronic myeloid leukemia, 12) Cell cycle, 13) Hematopoietic cell lineage, 14) Antigen processing and presentation (Figure 4). In KEGG pathway analysis, Cytokine-cytokine receptor interaction, natural killer cell mediated cytotoxicity, toll-like receptor signaling pathway, apoptosis, small cell lung cancer, T cell receptor signaling pathway, p53 signaling pathway, Adipocytokine signaling pathway, Type I diabetes mellitus were commonly up regulated.

We have investigated an enrichment of GO annotations in the up-regulated and down-regulated genes related to the toxic effect of BE. The categories of the function in differentially expressed genes are presented in Figure 5. The biological process profiles of up-regulated genes were subdivided in to 208 groups and also 30 subdivisions for down regulated genes. The major up-regulated genes subdivisions were apoptosis, cellular defense response, anti-apoptosis, regulation of I-kappaB kinase/NF-kappaB cascade, inflammatory response, iron ion transport, cell proliferation, cell development, system development and immune response. As well the major subdivision of down-regulated genes were regulation of progression through cell cycle, G1/S transition of mitotic cell cycle, cell motility, mitosis, Ras protein signal transduction, Regulation of signal transduction, cell migration, positive regulation of cell differentiation, localization of cell, and cell cycle. Differentially expressed common genes were listed as bold in the Table 2. Analysis of cellular localization reveals the genes associated with nucleus, cytoplasm, cytosol, splicesome, chromosome, intracellular organelle part

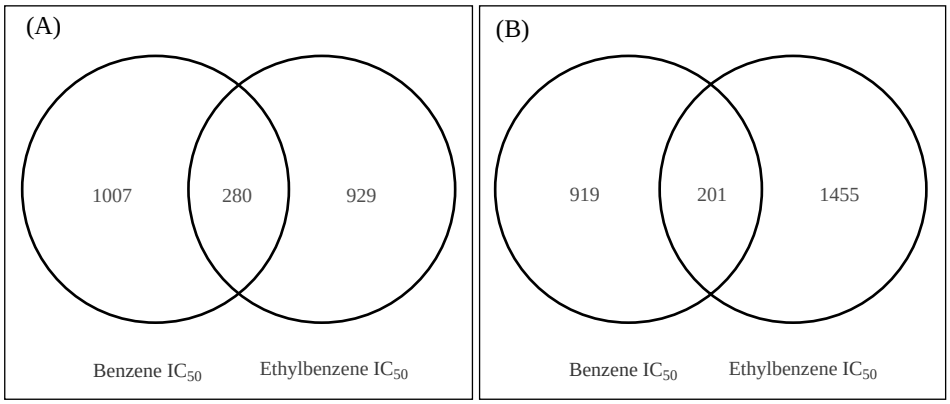

Figure 3. Venn diagram showed the differentially expressed genes in HL-60 cells exposed with BE for 3 h with IC_{50} doses. The diagrams were generated from the list of up (A) and down (B) regulated genes obtained from the microarray analysis. At least 1.5 fold increase or decrease genes were considered to generate these diagrams. The numbers in the diagram show the number of gene regulated by BE exposure.

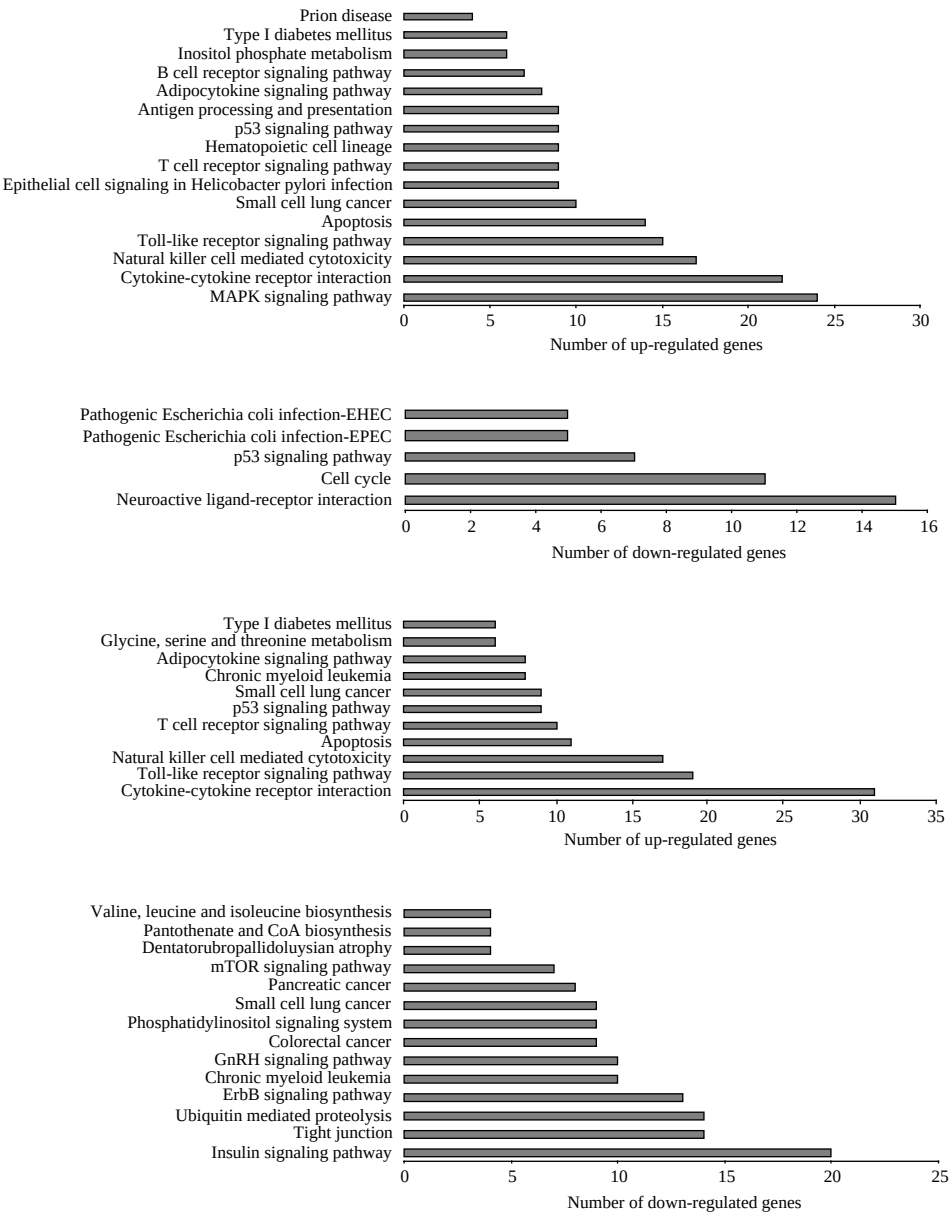

Figure 4. The pathway analysis of differentially expressed genes by benzene (A) and ethylbenzene (B) in HL-60 cells using KEGG pathway database.

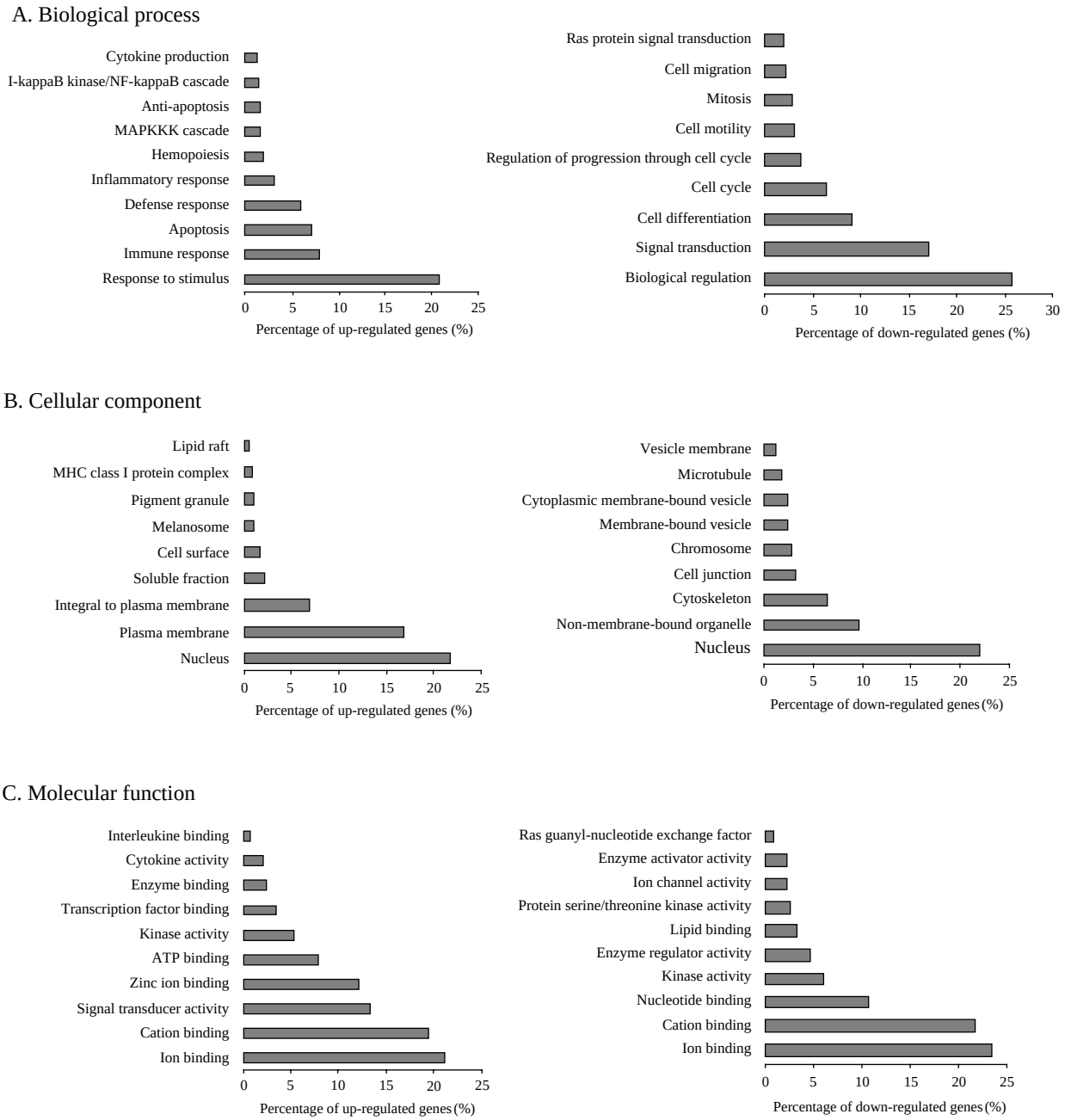

Figure 5. The distribution of up-and down-regulated genes according to gene ontology categories in HL-60 cells through benzene exposure.

and extracellular matrix. And the majority of profiles in molecular function are signal transduction activity, molecular transduction activity, transcription regulator activity, ATP binding activity, enzyme activator activity, cytokine binding activity and mostly transcription activities.

Discussion

Benzene is a well known carcinogen can cause acute myeloid leukemia (AML) and other hematotoxicity and leukemogenicity in human[21] and also ethylbenzene is known as possible carcinogen to

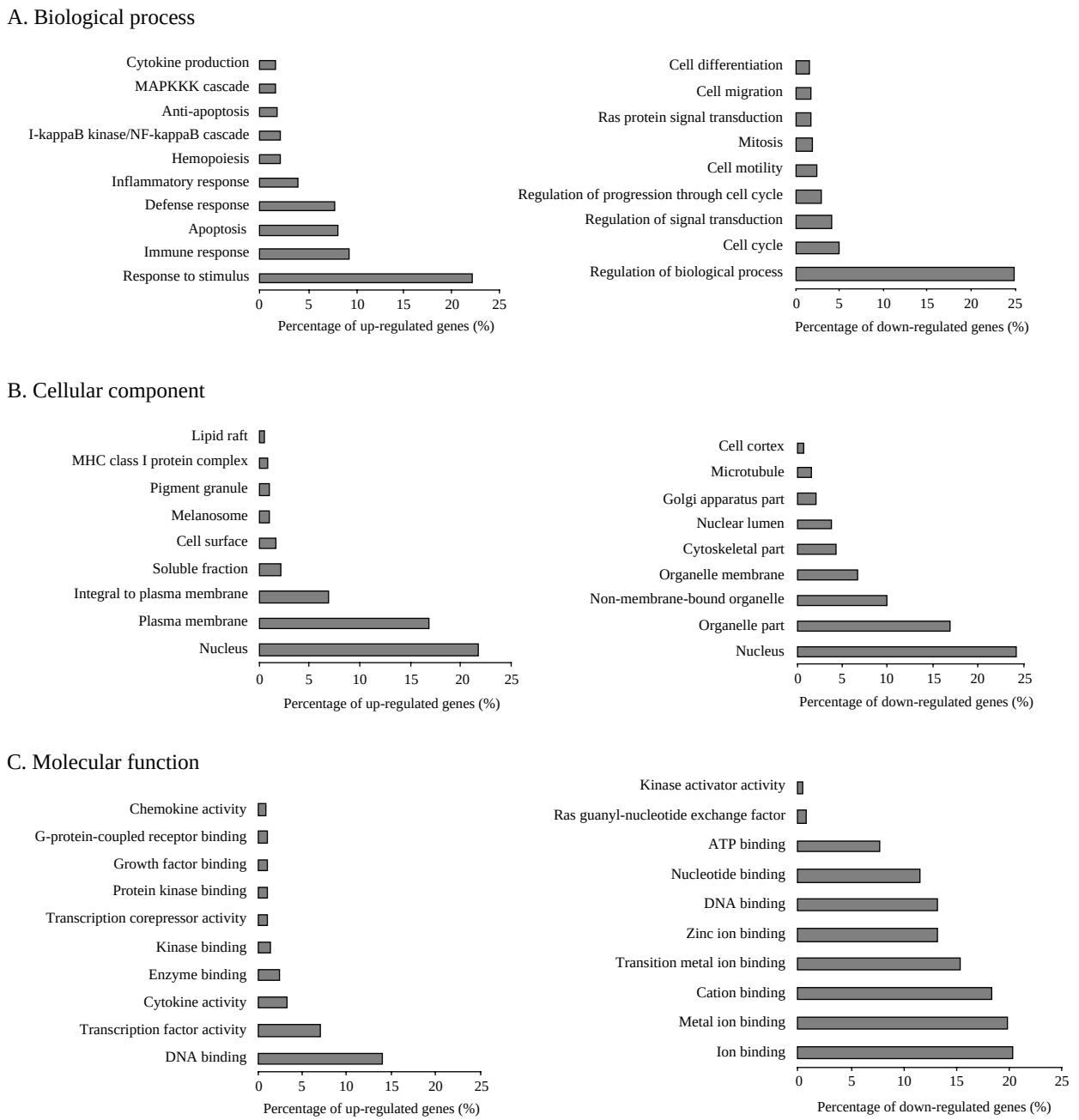

Figure 6. The distribution of up-and down-regulated genes according to gene ontology categories in HL-60 cells through ethylbenzene exposure.

human. Here in this study, we have demonstrated the gene expression profiles together with benzene and ethylbenzene using microarray in conjunction with statistical analysis. Earlier, very few studies were carried out only with benzene, but this study evaluated the gene expression profiles with BE in human cells lines.

Chronic exposure to benzene and ethylbenzene characteristically causes hematotoxicity and myelogenous leukemia in humans. The relationships between levels of VOCs in blood and air have not been well characterized in the general population. Figure 4 showed the KEGG pathway of the genes, Figure 5 and Figure 6 showed the GO analysis of the genes

which were differentially expressed genes through BE exposure in HL-60 cells. Through the KEGG pathway genes were related to functions such as cytokine-cytokine receptor interaction, toll-like signaling pathway, small cell lung cancer, immune response, apoptosis, p53 signaling pathway and MAPKKK cascade. Cytokines is a chemical messenger that mediates intercellular communication. The regulation of cellular and nuclear functions by cytokines, growth factors, and peptide or protein hormones is initiated through the activation of cell surface receptors (Rc). Cytokines, chemokines, and cellular adhesion molecules are soluble proteins that play an important regulatory role in benzene induced hematopoiesis[22]. The activated receptor may then interact with other cellular components to complete the signal transduction process[23]. The first steps in the generation of the immune response is the recognition by T lymphocytes of peptide fragments (antigens) derived from foreign pathogens that are presented on the surface of antigen presenting cells (APC). T cell receptor (TCR) mediated this event by transduces these extracellular signals by initiating a wide array of intracellular signaling pathways[24]. Toll-like receptors lead to the synthesis and secretion of pro-inflammatory cytokines and lipid mediators, thereby initiating the inflammatory response that recruits both soluble immune components and immune cells from the blood[25]. GO analysis showed that genes belongs to the biological process of cytokine production, immune responsive, inflammatory responsive, hemopoiesis, cellular defense response, apoptosis and also MAPKKK cascade were significantly up-regulated through BE exposure. The p53-dependent pathways help to maintain genomic stability by eliminating damaged cells either by arresting them permanently or through apoptosis[26]. Apoptosis can be triggered by a wide range of stimuli, including cell surface receptors like Fas and FasL. It constitutes a system for the removal of unnecessary, aged, or damaged cells that is regulated by the interplay of proapoptotic and antiapoptotic proteins of the Bcl-2 family[27]. MAPK signaling is activated by Ras-family Guanosine triphosphatases (GTPases) and other protein kinases, and connects extracellular signals to transcription factors and other effectors that regulate many cellular programs such as cell proliferation, cell death and cytoskeleton remodeling[28].

In conclusion, KEGG and GO analysis showed that the changes of gene expression pattern were associated with hematotoxicity induced by benzene and ethylbenzene exposure. Toxicogenomic approaches improved the prediction of chemical carcinogenicity by considering multiple techniques like microarray. GO analysis gives a new way of analysis for the prediction of the mechanism associated with biological, cellular and molecular functions of genes using whole genome through chemical exposure.

Materials & Methods

Chemicals and Culture Media

All reagents used were of analytical grade; benzene, toluene, *o*-xylene, dimethylsulfoxide (DMSO) and 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co. (USA). Culture media and buffer solutions were purchased from Gibco™: RPMI-1640, fetal bovine serum and antibiotics (penicillin and streptomycin).

Cell Culture

The human promyelocytic leukemia HL-60 cell line was purchased from ATCC (CCL-240, USA). The cell lines were maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum plus 100 U/mL penicillin and 100 µg/mL streptomycin, 1 mM sodium pyruvate and 10 mM HEPES at 37°C in 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 flask.

Determination of Cell Viability

MTT assay was performed for the detection of cell viability[19]. 3 mL of HL-60 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. 200 µL of MTT (5 mg/mL in PBS) solution was added to each tube and incubated for 3 h. DMSO was added to each tube and 100 µL/well transferred in 96 well plate. The optimal density (OD) of the purple formazan product was measured at a wavelength of 540 nm. The 50% inhibitory concentration (IC_{20} & IC_{50}) of cell proliferation in a particular chemical was defined as the concentration that causes 50% reduction in the cell viability versus the solvent treated control. The IC_{20} & IC_{50} values were directly determined from the linear dose response curves.

RNA Extraction

Total RNA was extracted from the HL-60 cells treated to IC_{20} & IC_{50} doses of 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. RNA quality were assessed with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) (260/280 nm ratios) and only samples with an A260/A280 ratio between 1.9 and 2.2 were considered for suitable use. The RNA quality was checked with the Experion automated electrophoresis station (Bio-Rad Laboratories, USA) (28S/18S ratio). K-562 and U-937 cells were also treated with only IC_{50} doses of BTX and extracted the RNA for real time RT-PCR.

Oligonucleotide Microarray Hybridization

Gene expression analysis was conducted on the RNA samples using 35 K whole human genome microarray (Operon Biotechnologies, Inc. Germany). Inhibition concentration 50% (IC_{50}) dose treated BTX samples were performed for oligonucleotide microarray hybridization. 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 × SSC/0.1% SDS for 2 min at 58˚C, 1 × SSC for 2 min at RT and 0.2 × 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.

Microarray 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. Significance Analysis of Microarray (SAM) was performed for the selection of the genes with significant gene expression changes[20]. 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 0.66, i.e., 1.5-fold difference in expression level, and when the q-values were < 5.

Functional Grouping and Clustering 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

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