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Functional Gene Analysis to Identify Potential Markers Induced by Benzene in Two Different Cell Lines, HepG2 and HL-60

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

[1]Cellular and Molecular Toxicology Laboratory, Korea Institute of Science & Technology P.O. 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)

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Abstract

Volatile organic compounds (VOCs) are common constituents of cleaning and degreasing agents, paints, pesticides, personal care products, gasoline and solvents. And VOCs are evaporated at room temperature and most of them exhibit acute and chronic toxicity to human. Benzene is the most widely used prototypical VOC and the toxic mechanisms of them are still unclear. The multi-step process of toxic mechanism can be more fully understood by characterizing gene expression changes induced in cells by toxicants. In this study, DNA microarray was used to monitor the expression levels of genes in HepG2 cells and HL-60 cells exposed to the benzene on IC20 and IC50 dose respectively. In the clustering analysis of gene expression profiles, although clusters of HepG2 and HL-60 cells by benzene were divided differently, expression pattern of many genes observed similarly. We identified 916 up-regulated genes and 1,144 down-regulated genes in HepG2 cells and also 1,002 up-regulated genes and 919 down-regulated genes in HL-60 cells. The gene ontology analysis on genes expressed by benzene in HepG2 and HL-60 cells, respectively, was performed. Thus, we found some principal pathways, such as, focal adhesion, gap junction and signaling pathway in HepG2 cells and toll-like receptor signaling pathway, MAPK signaling pathway, p53 signaling pathway and neuroactive ligand-receptor interaction in HL-60 cells. And we also found 16 up-regulated and 14 down-regulated commonly expressed total 30 genes that belong in the same biological process like inflammatory response, cell cycle arrest, cell migration, transmission of nerve impulse and cell motility in two cell lines. In conclusion, we suggest that this study is meaningful because these genes regarded as strong potential biomarkers of benzene independent of cell type.

Keywords: Volatile organic compounds (VOCs), Benzene, Microarray, Gene ontology

Volatile organic compounds (VOCs) such as benzene and toluene, and low molecular weight carbonyls like formaldehyde belong to the main air pollutants found in indoor environments. They are suspected to induce acute and chronic adverse health effects like asthma, allergic and cardiovascular diseases, and strongly affect well-being[1]. Among all the VOCs, benzene is the most widely used chemicals in our environment. Benzene is a ubiquitous industrial chemical (>2 billion gallons produced annually in the United States) and component of gasoline[2]. Benzene is considered as a human carcinogen by IARC (International Agency for Research on Cancer)[3,4]. Exposure to benzene induces many toxic effects in humans[5-8]. And its toxic effects on the blood and bone marrow include leukopenia, pancytopenia and aplastic anemia, and it is also an established cause of human leukemia[9-15]. In addition, benzene is also known to have toxic effects on the liver[16,17]. For comprehensive examination of cell specific effects of benzene on gene expression in human hepatocellular carcinoma cell line, HepG2 and human promyelocytic leukemia cell line, HL-60, microarray analysis using human oligonucleotide chip was carried out.

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. So, there is no doubt that the field of toxicogenomics will have a tremendous impact in the field of biotechnology and many other related studies. Investigations using toxicogenomic approaches such as DNA microarray have especially increased in number. Microarray analysis is a powerful tool to identify the biological effects of chemicals and this tool can provide a huge amount of data. So, to analyze these vast data effectively, it is necessary to use diverse analytical tools. The Gene Ontology (GO) analysis of microarray data provides structured and controlled vocabularies and classifications for several domains of molecular and cellular biology[18]. 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 subpathways or between pathways and their related biological phenomena[19]. 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[20].

The aim of this study is the identification of the potential gene-based markers on benzene independent of cell type. We examined the global gene expression profiles in a small number of well-matched exposed-control subject pairs. Genes with differentially expressed were then ranked and selected for further examination using several forms of statistical analysis. The identification of commonly expressed genes in two cell lines may assist in the identification of potential biomarker of benzene and may understand molecular toxicological mechanisms of benzene in human hepatocytes and human heamatological cells.

Cytotoxicity of Benzene in HepG2 & HL-60 Cells

Relative survival of HepG2 and HL-60 cells following exposure to a range of concentrations of benzene were determined by MTT assay. The survival percentage relative to solvent control (DMSO) was determined as a percentage of OD value measured after treatment. Benzene reduced cell viability gradually at increasing concentrations as shown in Figure 1. Especially, benzene is more sensitive in HL-60 cells than HepG2 cells. 20% inhibitory concentration (IC20) value for benzene in HepG2 cells was 65.62 mM and IC50 value in HL-60 cells was 8.75 mM.

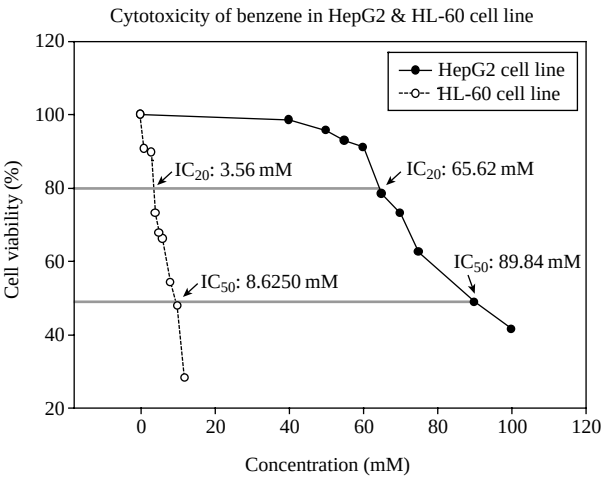

Figure 1. Viability of HepG2 and HL-60 cells after exposure to benzene.

Gene Expression Profiles Altered by Benzene in HepG2 & HL-60 cells

HepG2 cells were treated with IC20 dose of benzene for 48 hrs and HL-60 cells were treated with IC50 dose of benzene for 3 hrs, respectively, and their RNA were subjected to microarray analysis. For each treatment, genes with statistically significant expression changes were identified ($P < 0.05$). Three independent experimental samples for each treatment group were analyzed to determine RNA at transcription 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 two types of cell lines, using a combined list of genes identified to be altered statistically significant in at least one of the sample studied relative to control. In the clustering analysis of gene expression profiles, although clusters of HepG2 and HL-60 cells by benzene were divided differently, expression pattern of many genes observed similarly (Figure 2). Also, Venn diagram shows the gene expression profiles; Nine hundred sixteen genes were up-regulated and 1,144 genes down-regulated in HepG2 cells and also 1,002 genes were up-regulated and 919 genes down-regulated in HL-60 cells through benzene exposure, respectively. Sixty four up-regulated and 143 down-regulated genes were commonly expressed by both HepG2 and HL-60 cells (Figure 3).

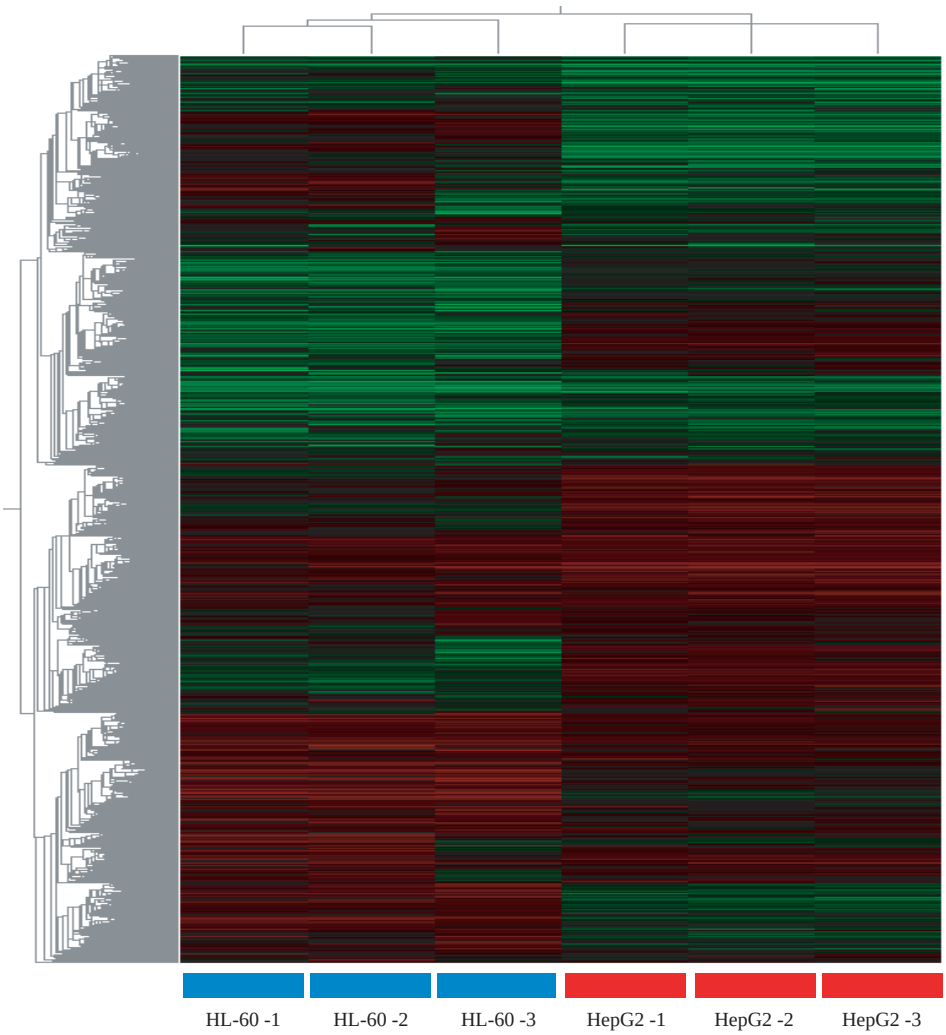

Figure 2. Hierachical cluster image showing the differential gene expression profiles of benzene exposed in HepG2 and HL-60 cells.

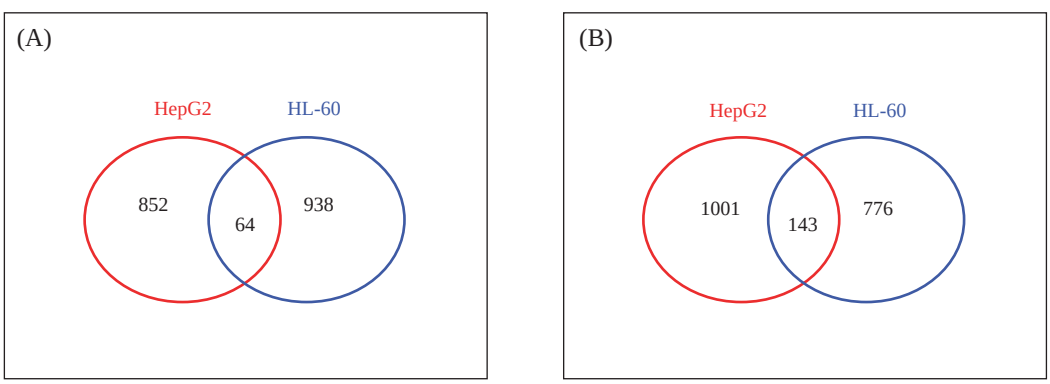

Figure 3. Venn diagram showed the differentially expressed genes by benzene in HepG2 and HL-60 cells, (A) showed the up-regulated genes and (B) showed the down-regulated genes.

Pathway Analysis & GO Analysis

Up-regulated 916 genes and down-regulated 1,144 genes in HepG2 cells exposed to benzene and also 1,002 up-regulated and 919 down-regulated genes were expressed in HL-60 cells were classified according to KEGG pathway to analyze molecular mecha-

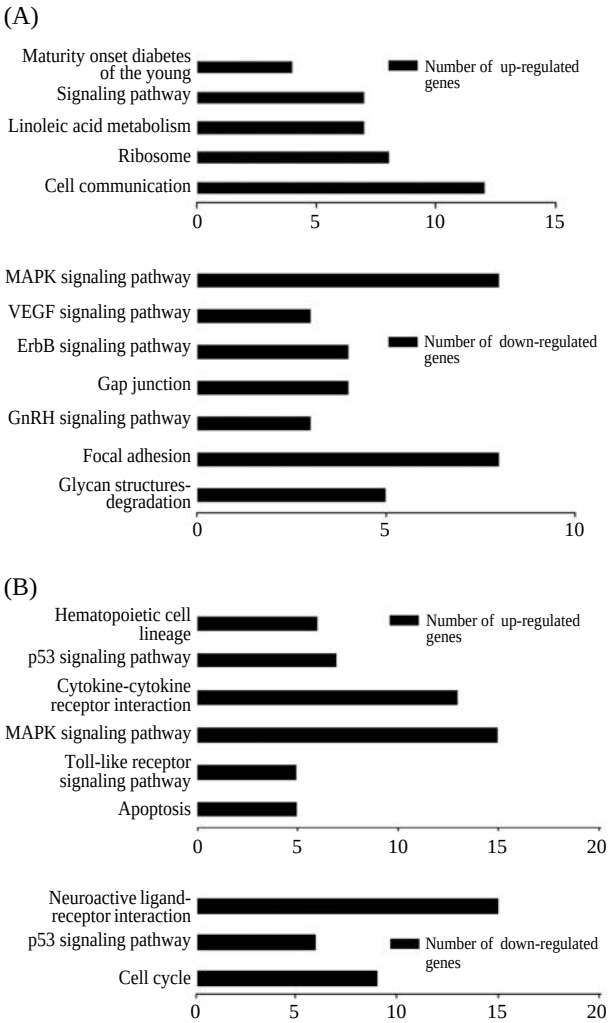

Figure 4. The pathway analysis of differentially expressed genes in HepG2 (A) and in HL-60 (B) cells using KEGG pathway data base.

nism. Figure 4 showed key KEGG pathway statistically significantly changed in HepG2 and HL-60 cells; cell communication, signaling pathway, glycan structures-degradation, GnRH signaling pathway, ErbB signaling pathway, MAPK signaling pathway are regulated in HepG2 cell and apoptosis, toll-like receptor signaling pathway, p53 signaling pathway, T cell receptor signaling pathway and cell cycle in HL-60 cells. Through the classification with these genes into KEGG pathways, as shown in Figure 4, we can infer that different pathways are involved in molecular mechanisms of different cell types.

We have investigated an enrichment of GO annotations in the up-regulated and down-regulated genes in HepG2 and HL-60 cells, respectively. The categories of the function in differentially expressed genes are presented in Figure 5. The biological process profiles of up-regulated genes in HepG2 cells were subdivided into ion transport, cellular ion homeostasis, inflammatory response, cell cycle arrest, cell adhesion, cell-cell signaling, cell migration, innate immune response, nervous system development and cellular biosynthetic process. And down-regulated genes in HepG2 cells were subdivided into cellular biosynthetic process, cell motility, synaptic transmission, vesicle-mediated transport, transmission of nerve impulse, Wnt receptor signaling pathway, skeletal development and cell adhesion. Also, the biological process profile of up-regulated genes in HL-60 cells were subdivided into iron ion transport, inflammatory response, cell cycle arrest, organic acid transport, innate immune response, leukocyte differentiation, angiogenesis and hemopoiesis. And down-regulated genes in HL-60 cells were subdivided into cell morphogenesis, intracellular signaling cascade, vesicle-mediated transport, localization of cell, blood circulation and cell cycle. From this analysis, we can infer that several different biological processes are involved in HepG2 and HL-60 cells. However, there are also same biological processes like ion transport, cellular ion homeostasis, inflammatory response, cell cycle arrest, cell adhesion, cell proliferation, cell migration, cell morphogenesis, cell motility, synaptic transmission and vesicle-mediated transport. Table 1 and Table 2 showed the list of up-regulated and down-regulated genes which belong in the same biological process in HepG2 and HL-60 cells.

From this, we can suggest that these biological processes are commonly involved in molecular toxicological mechanisms of benzene independent of cell types. And we also found 16 up-regulated and 14 down-regulated commonly expressed total 30 genes that belong in the same biological process like cellular ion homeostasis (BCL2), inflammatory response (BMP6, F11R, PLA2G2E), cell cycle arrest (SART1), cell surface receptor linked signal transduction (ELTD1, FST, OPN1MW, OR7G2, TAS2R38), cell-cell signaling (TH, WISP3), behavior (ACSL4), cell proliferation (ELF5), cell migration (VAV3), organ development (SH2D2A), cell morphogenesis (AR, NOX4, OSM), potassium ion transport (KCNAB2), cell motility (TLX3), intracellular signaling cascade (ABR, SPHK2), synaptic transmission (CSPG5), regulation of signal transduction (FGF18, RASA4), vesicle-mediated transport (AP1B1), extracellular matrix organization and biogenesis (FOXC1), transmission of nerve impulse (AKAP4, LPAL2). From that remark, we can infer that these genes regarded as strong potential biomarkers of benzene independent of cell type.

In conclusion, although this data is not enough to

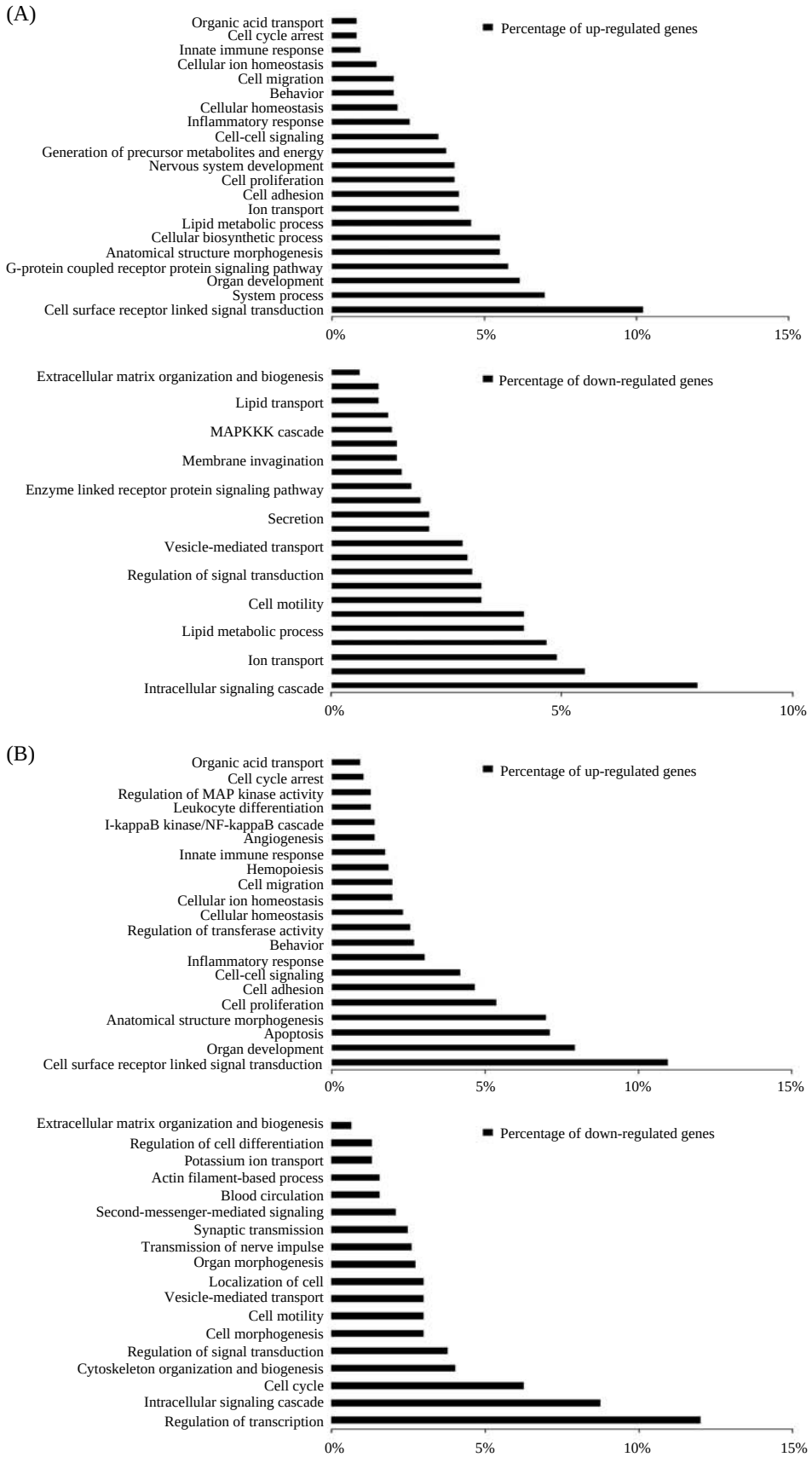

Figure 5. The distribution of up-regulated and downregulated genes according to gene ontology categories in benzene induced HepG2 (A) and HL-60 (B) cells.

Table 1. Biological process of up-regulated genes in benzene treated HepG2 cells and HL-60. Common expressed genes in two cell lines marked by bold.

GO term	Symbols of genes belong to each biological process	
	HepG2	HL-60
Ion transport	SCN2A, SLC11A1, SLC22A8, KCNE2, SLCO1B3, CTHRC1, KCNH5, TRPC4, CHRNB2, ATP5G3, FKBP4, SLC17A2, NMUR1, SLC5A3, FXYD7, FXYD5	SLC11A1, FTHL17, FTH1, SFXN3, HFE, FTHL3
Cellular ion homeostasis	TMPRSS3, CP, CCR2, **BCL2**, NR3C2, SLC34A3, LCK	CXCR4, CXCR3, PRNP, **BCL2**, CLN6, KCNMB3, C3AR1
Iinflammatory response	**PLA2G2E**, SERPINA3, RBM24, SAA3P, **F11R**, **BMP6**, NFATC3, SAA4, IL1F6, FN1	**PLA2G2E**, IL17F, **F11R**, CXCL10, AOC3, CCL3L1, BCL6, ZFP36, NOS2A, NR3C1, **BMP6**, FPR1, RAC1
Cell cycle arrest	CDKN1C, DST, **SART1**, CDKN2B, ERN1	PPP1R15A, SESN2, JMY, SESN3, GADD45A, **SART1**, HBP1, CDKN1A, DDIT3
Cell adhesion	CDH9, ITGA3, CELSR1, ZCCHC2, CLDN5, MYBPC1, THBS2, ITGA10, CLDN11, SRPX, XLKD1, PCDH21, VWA1, ITGB4	COL8A1, EMILIN2, ADAM12, FLJ23834, COL21A1, SPP1, HSPD1, CD58, PSCDBP, ARPC1B, LMO4, ITGA2, INPPL1
Cell surface receptor linked signal transduction	ZNF219, FLT4, OR7E5P, AGTRL1, OR10R3P, TAS2R14, **FST**, SIRPB1, GPR97, **ELTD1**, CDA, **OPN1MW**, GNAS, GABRA3, OR11L1, OR52M1, OR11H6, OR51D1, **OR7G2**, OR8K3, OR4A5, OR4A16, OR4B1, OR11H12, ADAM11, RGS1, RGS16, GPRC5A, RAMP2, DKK3, CIB3, **TAS2R38**	REPS2, ADAMTS7, CENTA1, ITPKB, TCF7L2, LPHN3, PYGO1, **ELTD1**, GPR148, P2RY11, ELF1, RGS13, IRAK3, NPY6R, GRB10, TGFBR3, GP1BB, ROS1, **OPN1MW**, HPGD, PTGER2, PTGER4, OR13C3, OR2T33, OR56A4, OR6Y1, **OR7G2**, OR51G2, OR2T6, BIRC3, CD69, PDC, PDPK1, RGS7, P2RY6, EBI2, FRAT1, IFNGR2, **FST**, PTPRE, FRS3, TLE4, OR1N1, BAIAP2, LAX, GPR85, GPR84, SUCNR1, TAS1R2, **TAS2R38**, ADCY5, KIAA1509, IL13RA1
Cell-cell signaling	GRM2, CPNE6, IL22, GABRG1, GDNF, AREG, FGF7, TNFSF4, TNFRSF11A, CBLN1, SLC6A8, WNT16, WNT7B, TSPAN32, GABRR3, **WISP3**, **TH**	AGRN, TNFSF10, PGF, SDFR1, NR4A2, SPAP1, STX1A, PBEF1, IFNA7, HSD11B2, FASLG, GRIK5, KCNK3, KCNN3, EGR3, G1P2, GLI1, NAB1, MERTK, **WISP3**, **TH**
Behavior	GALR3, LSP1, FZD9, PLAU, GHSR, NHLH2, **ACSL4**, CNR2	ENPP2, CCL20, EGR2, FOSB, CCL4L1, DEFB4, CIR, VLDLR, EGR1, CCL5, **ACSL4**, CKLFSF1
Cell proliferation	TM4SF4, TGFB1I1, AZGP1, VTI1B, TAL1, FABP6, FRAT2, NR6A1, **ELF5**	TNFSF14, IFI16, TSPAN1, SHB, SERTAD1, CRIP1, TCIRG1, LAMP3, PTK2B, MAD, CLK1, CASP3, RARRES3, ACHE, NFKBIA, TRIB1, CNTFR, **ELF5**, KLF10
Anatomical structure morphogenesis	AMBN, BRSK2, HOXD3, SERPINE1, DKK1, TWIST1, IGFBP1, TPD52, FLI1, SEMA3A, NTNG2, CDC42EP1	SOX15, EPHB4, CRIM1, FGD6, RASGRP2, IPMK, RGMA, MGC40405, SOX10, OKL38, BIRC2, EMP1, GCM1, IER3, DHRS9, CDC42EP3, EMCN, EYA3, NR4A3
Organic acid transport	AKR1C4, AKR1C1, SLC6A14, SLC16A8, SLC38A1	SLC25A10, TNF, OCA2, SLC3A2, SLC7A1, SLC22A6, SLC7A11, SLC1A5
Cell migration	NGFR, EPHB3, **VAV3**, GTPBP4, TNS1	JAG1, LENG5, HBEGF, CCK, NFASC, BTG1, MYH9, STC2, SEMA5A, **VAV3**, ABHD2, ZFHX1B, RELN, UNQ6077
Cellular homeostasis	ATP1A1, CNGB1, PPARGC1A, GLRX5	IFIT1, GCLM, CCL2, TXNRD1, ERP70, CLDN16
Innate immune response	C5, PGLYRP1, SIGIRR, C4BPA, MBL2	IL18RAP, CFHL5, LGP2, IFIH1, IGSF4, IL23A, CRTAM, CLECSF12, IL1R1, DAF, IL1RAP, IL18R1, DDX58, PGLYRP2, IL1RL1
Organ development	SRPK3, HEY1, SOX18, ODAM, **SH2D2A**, KRT17, ENG, KRT10, FSHB, ANXA2, COL4A2, CRYAB, FHL3, SOX3, TCF2, TAGLN3, TGM5, SHOX	FCMD, DLX2, LNK, MINPP1, HHIP, SMTN, HECA, MB, RELB, SGCA, CD44, IFRD1, HMOX1, LOX, IRS2, **SH2D2A**, CITED2, HLA-DMA, RB1CC1, PTPN22, ASB1, NFKB2

Table 2. Biological process of down-regulated genes in benzene treated HepG2 cells and HL-60. Common expressed genes in two cell lines marked by bold.

GO term	Symbols of genes belong to each biological process	
	HepG2	HL-60
Cell morphogenesis	PTK7, OGFR, EFHD1, **AR**, LTBP4, MYO1A, PCNT, NTNG1, **NOX4**, **OSM**, FGFR3, CEP290, SEMA6C, GDNF	MARK2, CDKN2C, DLG1, RASGRP4, WISP3, **AR**, NPR1, TOP2B, PALM, **NOX4**, PC-LKC, **OSM**, PML, MGC27019, ITGA6, CEL
Ion transport	C16orf7, COL27A1, TRPM2, SLC4A2, KCNC3, TRPM1, SLC34A2, SLC26A9, CACNG3, **KCNAB2**, KCNS3, ATP11A, ACCN3, KCND1, SLC12A7, SLC22A17, SLC38A3, GLDN, ATP7A, SLC26A3, ABCC8, GRIA3, VDR, CLCN7, COL2A1, KCNAB3, SLC12A4, GIF, COL13A1, KCNA10, LASP1, NOX1, SLC12A5, UCP1, ATP6V0E2	**KCNAB2**, KCNG1, KCNMB4, FLJ40162, KCNE3, ATP1A1, KCNN1, KCNK12, KCNE4, KCNJ14
Cell motility	IL16, **TLX3**, SCNN1B, MUC2, SEMA4F, DNAH1, VNN2, F11R, CCL22	ABI3, CSPG3, TUBB, IL1B, GAB1, TPM1, **TLX3**, CENTD3
Intracellular signaling cascade	RHOBTB2, SH2B1, **ABR**, PDZD3, DVL1, RABL4, DEPDC6, SHANK2, ASB15, GNAI2, CDC42EP4, EVI5L, NLRP1, TNF, MAP2K7, PECAM1, PLA2G4C, DGKQ, SPATA13, RAB43, SSTR1, ADCY9, BIK, C5AR1, CALCA, PRKCB1, RREB1, DVL2, MAPKAPK2, GRB7, SRC, CAMKK2, TRIO, ABL1, KSR1, PLEKHG3, TBC1D16, **SPHK2**, TNS3, DOK3, MADD, TRAF5, RAB15, JAK3, SHANK3	PLCB1, ROCK2, ERAS, BUB1B, SPAG5, KIAA1536, DEPDC1, SHB, **ABR**, KIAA0720, STARS, CIT, PLCL3, MAPK11, CCNE1, MRAS, OPRL1, MT1X, PRKAR2B, MC5R, HIST1H4B, OXTR, CHRM2, RABIF, PIK3R3, BAD, TAF7, KNTC2, PDIP, RACGAP1, DEPDC5, ACIN1, CYCS, **SPHK2**, DUSP19, APBB3, TNFRSF19, FHL2, CNR2, GUCY1A2
Synaptic transmission	HAP1, SNPH, **CSPG5**, SCN1B, GABRB2, PLP1, HTR2C	BSN, **CSPG5**, CADPS, GJA7, DLG2, CPNE6, P2RX1, ADORA3, NPY, RIT2, VAMP2, LIN7B, GLRA3
Regulation of signal transduction	ARHGEF2, FARP2, CENTB5, TAOK2, **FGF18**, ARHGAP27, GRK4, **RASA4**, RGS22, SHC1, RAMP1, AKAP13, SYNGAP1, PPP2R1A, SIPA1L3, OTUD7B, DAB2IP	SNF1LK2, ACVR2B, **FGF18**, GEFT, MGC21654, CD8A, hSyn, FLJ40244, DKFZP434I092, C5orf13, DTX1, HRBL, **RASA4**, ARHGEF17, TBC1D13, CTNNBIP1, PREX1, APOL3, RFP2, KIAA1272, FLJ35390
Vesicle-mediated transport	KIF1C, AP1G2, EPN1, ABCA1, UNC13D, EHD1, DNM2, GSN, ZFYVE20, WAS, **AP1B1**, SH3GL3, TAPBP, MFGE8, CPLX2, RAB35, LRP10, RAB6B, LMBR1L	ARH, KIF20A, NAPB, RDC7, ELMO2, NKD2, AP3M2, RIMS3, AP2B1, **AP1B1**, SNX1, SORL1, COPA, RABEP1, CBL, CPNE1, PLA2R1
Extracellular matrix organization and biogenesis	COL5A2, **FOXC1**, NFKB2, COL12A1, COL11A1, COL11A2	**FOXC1**, COL6A2, MIA, NF1, MYH11
Localization of cell	PLXNB1, PAK4, LRP5, GDF7, DPYSL5, PKHD1, TBX5, MSN, VEGFA, ASTN1, ENPP2, ZEB2	SLIT1, DOCK2, CCL25, NTN2L
Transmission of nerve impulse	PARK2, GRIA1, **AKAP4**, PPARD, GNA11, AKAP5, **LPAL2**, GLRB, DLG4, ACHE, HTR7, DTNA, TH	**AKAP4, LPAL2**, SYT5, TMOD2, GRIK4

say the mechanistic inside of genotoxicity of benzene, we have identified commonly expressed genes in HepG2 and HL-60 cells treated to benzene. These genes could be a promising biomarker to detect benzene exposure in other type cells. Also, it can be suggested that oligonucleotide microarray is an efficient technology for evaluating the gene regulation and the possibility to identify the molecular biomarkers. In addition, GO analysis is good for a prediction of the mechanism associated with differentially expressed genes in cells or organisms influenced by chemical.

Materials and Methods

Chemicals and Reagents

Benzene, dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (USA). Dulbecco's Modified Eagle Medium (DMEM), RPMI-1640, Dulbecco's Phosphate Buffered Saline (PBS), 0.5% trypsin-EDTA and Fetal Bovine Serum (FBS) were the products of GIBCOTM (USA). Trizol reagent was produced by Invitrogen (USA) and RNeasy mini kit and RNase-free DNase set were purchased from Qiagen (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 (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[21]. 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 benzene in culture medium at 37˚C for 48 hrs and 3 hrs 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 hrs. 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 (IC20) and 50% inhibitory concentration (IC50) 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 IC20 and IC50 values were directly determined from the linear dose-response curves.

RNA Extraction

Total RNA was extracted from the HepG2 and HL-60 cells treated to 65.62 mM and 8.75 mM benzene, respectively, for 48 hrs and 3 hrs using the 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 was quantified using NanoDrop (Nanodrop, USA). Only samples with an A260/A280 ratio between 1.9 and 2.2 were considered for suitable use and 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, USA). Significance Analysis of Microarray (SAM) was performed for the selection of the genes with significant gene expression changes[22]. 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 0.667, 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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