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Differential Gene Expression Analysis in K562 Human Leukemia Cell Line Treated with Benzene

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Even though exposure to benzene has been linked to a variety of cancers including leukemia, the detailed molecular mechanisms relevant to benzene-induced carcinogenesis remain to be clearly elucidated. In this study, we evaluated the effects of benzene on differential gene expression in a leukemia cell line. The K562 leukemia cell line used in this study was cultured for 3 h with 10 mM benzene and RNA was extracted. To analyze the gene expression profiles, a 41,000 human whole genome chip was employed for cDNA microarray analysis. We initially identified 6,562 genes whose expression was altered by benzene treatment. Among these, 3,395 genes were upregulated and 3,167 genes were downregulated by more than 2-fold, respectively. The results of functional classification showed that the identified genes were involved in biological pathways including transcription, cell proliferation, the cell cycle, and apoptosis. These gene expression profiles should provide us with further insights into the molecular mechanisms underlying benzene-induced carcinogenesis, including leukemia.

Key words: Microarray, Benzene, Cell proliferation, Gene expression, Leukemia

INTRODUCTION

Benzene (C_6H_6) is a broadly used chemical compound with a ring structure. It is a highly flammable molecule with a high melting temperature. It is broadly employed as an industrial solvent and a precursor of many products including drugs, plastics, dyes, and synthetic rubber.

The effects and toxicity of benzene in human health have been studied extensively. A well-known carcinogen, benzene induces leukemogenicity and myelotoxicity in both animals and humans (Huff, 1999). Benzene exerts extensive effects on genetic elements; it induces carcinogenesis though genetic changes resulting in chromosomal aberrations, translocations, long-arm deletions, and aneuploidy (Lan *et al.*, 2004). Previous studies have shown in detail that benzene affects tubulin, microtubules, and topoisomerase II, which might potentially induce DNA strand breakage, mitotic crossover, and chromosome translocations, and may interfere with chromosome segregation at anaphase to generate aneuploidy (Smith, 1996; Zhang *et al.*, 1999). Even relatively low-level exposure to benzene reduces the total numbers of leukocytes, granulocytes, and lymphocytes (Lan *et al.*, 2004). However, the detailed molecular mechanisms underlying benezene-induced carcinogenesis, including leukemia, remain to be elucidated.

As a cancer of the blood and bone marrow, leukemia is characterized by the uncontrolled proliferation of blood cells, usually leukocytes. Leukemia is divided into four types: acute myelogenous, acute lymphocytic, chronic myelogenous, and chronic myelogenous (Redaelli *et al.*, 2003). Among different leukemia cell lines, the K562 cell line was derived from chronic myelogenous leukemia (CML); K562 cells are generally characterized as highly undifferentiated leukemic cells. They also feature Philadelphia chromosomes, which evidence an unbalanced reciprocal translocation between the long arms of chromosome 15 and chromosome 17 (Lozzio and Lozzio, 1975).

cDNA microarray technologies enable the detection of global changes in gene expression. In this study, we employed this technology to detect altered gene expression patterns in

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a benzene-treated human leukemia cell line.

MATERIALS AND METHODS

Cell cultures. The K562 human leukemia cell line was grown in RPMI 1640 in 5% CO_2 at 37°C. The cells were grown to approximately 80% confluence and treated for 3 h with 10 mM benzene (Sigma, St. Louis, MO, USA).

MTT assay. K562 and HEK293 cells were seeded (48-well plates) 24 h prior to treatment with benzene. After treatment with 10 mM benzene, the cells were incubated for 3 h (Joo *et al.*, 2003). 20 μ*l* of MTT (3-(4,5-dimethyl-2-tiazolyl)-2.5-diphenyl-2H-tetrazoliumbromid) was subsequently added (final concentration 0.5 mg/m*l*) and incubated for 4 h, followed by the aspiration of the medium and the addition of 200 μ*l* of DMSO (Sigma). The OD was then determined using an ELISA reader at a wavelength of 570 nm (Biochrom, Cambridge, England).

RNA extraction. Total RNA was isolated from K562 and benzene-treated cells using RNAiso plus (Takara, Seoul, Korea) in accordance with the manufacturer's recommendations. For quality control, RNA purity and integrity were assessed via denaturing gel electrophoresis, OD 260/280 ratio measurements, and analysis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA, USA).

Amplification and labeling. Total RNA was amplified with Agilent's Low RNA Input Linear Amplification kit PLUS, in accordance with the manufacturer's instructions.

cDNA microarray. cDNA microarray experiments were conducted on a cDNA chip (Agilent Human Whole Genome 4X44K, Agilent Technologies) containing 41,000 cDNA clones selected from RefSeq, Goldenpath, Ensembl, Unigene, Human Genome Build 33.

Imaging and image analysis. Each hybridization array was scanned on an Agilent DNA microarray scanner (Agilent Technologies). The intensity of each hybridization signal was calculated using Feature Extraction Software. Agilent's Feature Extraction software (version 9.5) was used to extract data from raw microarray image files in preparation for analysis. Agilent Comparative Genomic Hybridization Analytics software (version 3.4) was employed for the visualization, detection, and analysis of aberrant patterns from array comparative genomic hybridization microarray profiles.

Data analysis. The selected genes were analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.nuaid.nih.gov/David/), which automatically classifies genes into known function/pathway groups. For biochemical pathway analysis, color objects in the KEGG pathway (http://www.genome.jp/kegg/tool/color_pathway.html) were used.

RT-PCR. After treating the K562 cells with 10 mM benzene, total RNA was extracted from the cells and cDNA was generated using 2.0 μg of RNA using random hexamers and reverse transcriptase (RTase) (Enzynomics, Daejeon, Korea). PCR was conducted for 10 min at 94°C, followed by 30 cycles at 94°C for 30 sec, primer Tm temperature for 30 sec, and 72°C for 30 sec with a 10 min final extension step at 72°C. The following primers were used for each of the genes: HDAC1, forward, 5'-GGCCTGTTTGAGTTCTGTCAGTTGT-3', reverse, 5'-TACTCTCCATACTTATGAAAGGACACAG-3'; FOS, forward, 5'-AACATGATGTTCTCGGGTTTCAA-3', reverse, 5'-GCTGGTGGAGATGGCTGTCAC-3'; LEF1, forward, 5'-GAGGTGGCCAGACAAGCACA-3', reverse, 5'-GGATGAGGGATGCCAGTTGT-3'; PKCα, forward, 5'-ATCCGCAGTGGAATGAGTC-3', reverse, 5'-GTTGTTTCCTGTCTTCAGAG-3'; HIFα, forward, 5'-TCACCACAGGACAGTACA-3', reverse, 5'-AAAGTTAAAGCATCAGGTTCC-3'; PDGFA, forward, 5'-ATGAGGACCTTGGCTTGCCT-3', reverse, 5'-TCACCTCACATCCGTGTCCTC-3'; Bcl-xL, forward, 5'-TTGGACAATGGACTGGTTGAGCCC-3', reverse, 5'- CGGCTCTCGGCTGCTGCATT-3'; β-actin, forward, 5'-GACCTTCAACACCCCAGCC-3', reverse, 5'-GATGACCTGGCCGTCAGGC-3'.

Western blot analysis and antibodies. Whole cell protein extracts were prepared in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8, 1 mM EDTA, 1% NP40, 0.1% SDS, 0.5% deoxycholic acid) and resolved via 12% SDS-PAGE (Bio-Rad, Hercules, CA, USA), transferred to nitrocellulose membranes (Pall, Port Washington, NY, USA), and probed with primary and horseradish peroxidase-conjugated secondary antibodies. The primary antibodies employed were Bcl-xL, β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and HDAC1 (Millipore, Billerica, MA, USA). Proteins were visualized with ChemiDoc (Bio-Rad).

RESULTS AND DISCUSSION

Abnormal cellular proliferation by benzene treatment. The results of previous studies demonstrate that benzene exposure induces the proliferation of myeloblasts and promyelocytes in CD-1 mice and an increased risk of leukemia in humans by inducing chromosomal aberrations in the blood leukocytes and bone marrow (Smith, 1996; Snyder *et al.*, 1982). In an effort to assess the cellular effects of benzene, we treated chronic myelogenous leukemia K562 cells and human embryonic kidney HEK293 cells for 3 h with 10 mM benzene. After treatment, we conducted an MTT assay to assess cellular viability and proliferation. As anticipated, benzene induced abnormal cellular

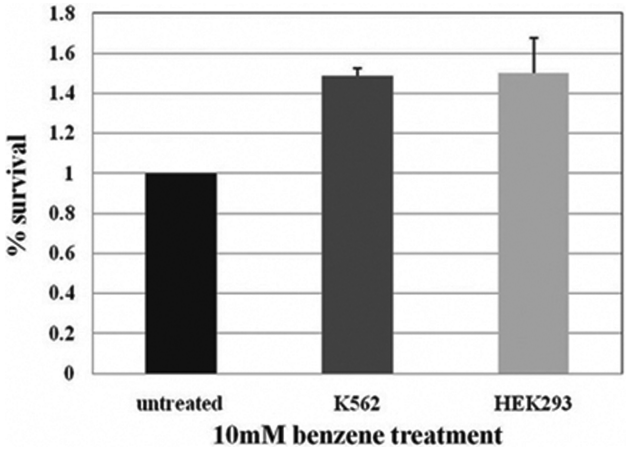

Fig. 1. Benzene induces the cellular proliferation of human cells. 10 mM benzene treated to K562 human leukemia cells and HEK293 human embryonic kidney cells for 3 h in the MTT assay.

proliferation in both the leukemia and embryonic kidney cells (Fig. 1). Relative to the untreated cells, the benzene-treated cells evidenced increased cell survival rates (up to 150%) in both cell lines (Fig. 1). These results demonstrate that benzene may influence the expression of a broad variety of genes associated with cell survival and proliferation.

Gene expression profiling of benzene treatment of K562 cells. In an effort to identify genes expressed differentially as the result of benzene treatment, we conducted gene expression profiling using Agilent human whole genome 44 K microarrays. K562 cells were treated for 3 h with 10 mM benzene and examined for altered gene expression patterns relative to those of untreated (0 h) cells.

Initially, we identified 6,562 genes evidencing expressional changes of more than 2-fold after benzene treatment. Among them, 3,395 upregulated genes were classified according to different biological processes using KEGG color pathways (Fig. 2A, B). Upregulated genes were classified into genes involved in signal transduction (21%), transcription (20%), apoptosis (9%) and proliferation (7%). Similarly, 3,167 downregulated genes were classified into biological pathways, namely signal transduction (21%), transcription (19%), apoptosis (9%), cell cycle (9%), and proliferation (8%).

The upregulated genes were associated with the carcinogenesis-related genes Krueppel-like factor 10 (KLF10), CD40, and jun (Table 1). Recent reports have implicated KLF10 in cell differentiation, and shown that it is a potential marker for human breast cancer, cardiac hypertrophy, and osteoporosis (Subramaniam *et al.*, 2010). CD40 is expressed in both normal lymphoid and hemopoietic cells, and has been previously implicated in oncogenic events (Loskog and Eliopoulos, 2009). The Jun oncogene is a transcription factor that performs important functions in promoting myeloid differentiation (Lord *et al.*, 1993).

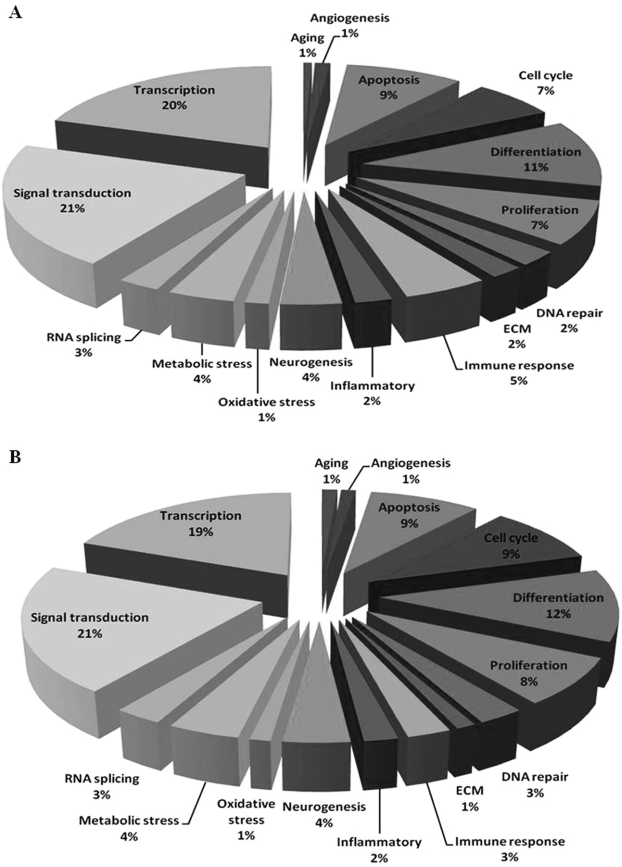

Fig. 2. Classification of genes differentially regulated by benzene. Differentially expressed genes (6,562 genes) up- or down-regulated by at least 2-fold after 3 h of treatment of the cells with 10 mM benzene. (A) The upregulated genes (3,395 genes) and (B) downregulated genes (3,167 genes) were classified by the Database for Annotation, Visualization, and Integrated Discovery (DAVID, http://david.nuaid.nih.gov/David/) and color objects in the KEGG pathway (http://www.genome.jp/kegg/tool/color_pathway.html).

Certain of the downregulated genes were associated with apoptosis, including the death inducer-obliterator 1 (DIDO1), fibroblast growth factor receptor 3 (FGFR3), and CD24 (Table 2). DIDO1 is upregulated at an early stage in apoptotic cell death (Garcia-Domingo *et al.*, 1999). FGFR3 knockdown in human bladder carcinoma cells induces cell-cycle progression arrest and influences tumorigenesis (Qing *et al.*, 2009). CD24 induces apoptosis in human B cells via the glycolipid-enriched membrane domain-mediated signaling system (Suzuki *et al.*, 2001).

Analysis of upregulated genes. Cellular proliferation and programmed cell death are among the key features of cell fate (Raff, 1996). The deregulation of cellular proliferation and apoptosis has been shown to lead to a variety of disease states, including inflammatory conditions, meta-

Table 1. Biological processes classification of genes with up-regulated in benzene treated cells

Classification	Accession ID	Symbol	Gene name	Fold change
Signal transduction	NM_002607	PDGFA	platelet-derived growth factor alpha	11.97
	NM_005655	KLF10	Kruppel-like factor 10	8.36
	NM_002010	FGF9	fibroblast growth factor 9 precursor	8.06
Transcription	NM_006195	PBX3	pre-B-cell leukemia homeobox 3	8.05
	NM_005934	MLLT1	myeloid/lymphoid or mixed-lineage leukemia	6.00
	NM_004040	RHOB	ras homolog gene family, member B	4.68
Apoptosis	NM_001191	BCL-XL	BCL2-like 1	3.41
	NM_001166	IAP	baculoviral IAP repeat-containing 2	3.14
	M13995	BCL2	B-cell CLL/lymphoma 2	2.57
Proliferation	NM_001250	CD40	TNF receptor superfamily member 5	9.02
	NM_002228	JUN	JUN oncogene	2.25
Immune response	NM_022168	OPRK1	opioid receptor, kappa 1	6.28
	NM_000912	CCR8	chemokine (C-C motif) receptor 8	5.4
	NM_005201	IFIH1	interferon induced with helicase C	5.13
Cell cycle	NM_033031	CCNB3	cyclin B3	8.06
	NM_006718	PLAGL1	pleiomorphic adenoma gene-like 1	5.43
	NM_003184	TAF2	TBP-associated factor 2	4.05
Angiogenesis	NM_002607	PDGFA	platelet-derived growth factor alpha	11.97
	NM_002010	FGF9	fibroblast growth factor 9 precursor	8.36
	NM_005560	LAMA5	laminin, alpha 5	2.4
Oxidative stress	NM_005252	FOS	oncogene	4.11
	NM_006793	PRDX3	peroxiredoxin 3	2.96
	NM_001430	HIF1A	Hypoxia inducible factor 1 alpha	2.35
Metabolic stress	NM_024767	DLC1	deleted in liver cancer 1	7.37
	NM_001978	EPB49	erythrocyte membrane protein band	6.93
	NM_001663	ARF6	ADP-ribosylation factor 6	4.59

Table 2. Biological processes classification of genes with down-regulated in benzene treated cells

Classification	Accession ID	Symbol	Gene name	Fold change
Signal transduction	NM_016269	LEF1	lymphoid enhancer-binding factor 1	0.09
	NM_005292	GPR18	G protein-coupled receptor 18	0.11
	NM_032271	TRAF7	TNF receptor-associated factor 7	0.15
Transcription	NM_133334	WHSC1	Wolf-Hirschhorn syndrome	0.08
	NM_004689	MTA1	metastasis associated 1	0.14
	NM_080797	DIDO1	death inducer-obliterator 1	0.15
Apoptosis	L33930	CD24	CD24 molecule	0.23
	NM_001673	ASNS	asparagine synthetase	0.24
	NM_002737	PRCKA	protein kinase C, alpha	0.42
	NM_004964	HDAC1	Histone Deacetylase 1	0.48
Proliferation	NM_000142	FGFR3	fibroblast growth factor receptor	0.02
	NM_001554	CYR61	cysteine-rich, angiogenic inducer	0.09
Immune response	NM_017413	APLN	apelin, AGTRL1 ligand	0.17
	NM_145285	NKX2-3	NK2 transcription factor related	0.18
	NM_000022	ADA	adenosine deaminase	0.18
Cell cycle	NM_006031	PCNT	pericentrin (kendrin)	0.20
	NM_001238	CCNE1	cyclin E1	0.23
	NM_018365	MNS1	meiosis-specific nuclear structural 1	0.25
Angiogenesis	NM_005938	FOXO4	forkhead box O4	0.37
	NM_000314	PTEN	phosphatase and tensin homolog	0.4
Oxidative stress	NM_005809	PRDX2	peroxiredoxin 2	0.27
	NM_004972	JAK2	Janus kinase 2	0.35
	NM_012094	PRDX5	peroxiredoxin 5	0.46
Metabolic stress	NM_002735	PRKAR1B	protein kinase, cAMP-dependent	0.13
	NM_033407	DOCK7	dedicator of cytokinesis 7	0.2
	BC042998	ADD1	adducin 1 (alpha)	0.23

bolic diseases, and cancer (Kozma and Thomas, 2002).

Our microarray data showed that anti-apoptotic genes such as Bcl-xL, B-cell lymphoma 2 (Bcl-2), inhibitor of apoptotic protein (IAP), and Hypoxia inducible factor (HIF-1α) are upregulated by 2.57-fold in benzene-treated K562 cells (Table 1). Bcl-xL is one of several anti-apoptotic protein members of the Bcl-2 family. Bcl-2 family members control the coordination of apoptosis via signal transduction or cell cycle progression (Hsu *et al.*, 1997). IAP is an anti-apoptotic protein that binds to and inhibits caspases 3 and 7 (Schimmer and Dalili, 2005). Via the inhibition of caspases 3 and 7, IAP blocks the activation of caspase pathways and ultimately inhibits apoptosis. A previous report has demonstrated that Bcl-2 suppresses cellular apoptosis in benzene-activated metabolism and attenuates the activity of the machinery relevant to the repair of oxidative DNA damage (Kuo *et al.*, 1999). HIF-1α appears to be involved in a variety of responses to hypoxic conditions, and is also frequently upregulated in common human cancers (Tomita *et al.*, 2007).

In order to confirm the array data, we conducted RT-PCR and Western blot analysis. As expected from the expression levels of these genes, the levels of Bcl-xL mRNA and protein were also increased in the benzene-treated cells (Fig. 3A, B). Similarly, cellular proliferation-associated genes including Jun, Fos oncogenes, platelet-derived growth factor subunit A (PDGFA), and HIF-1α were also confirmed to be upregulated after benzene treatment via RT-PCR analysis (Fig. 3A). These results show that many of the genes involved in cellular proliferation, carcinogenesis, and anti-apoptosis are upregulated by benzene treatment.

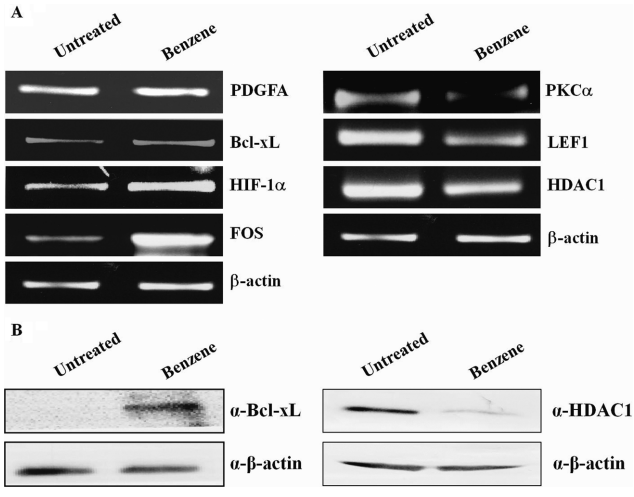

Fig. 3. Validation of the expression of regulated genes by benzene. (A) K562 cells were treated with 10 mM benzene. Total RNA was isolated from each sample, and genes regulated by benzene were confirmed via RT-PCR. β-actin was employed as a loading control. (B) Western blot analysis was conducted using Bcl-xL and HDAC1 antibodies. β-actin was used as a loading control.

Analysis of downregulated genes. Among downregulated genes, we detected many genes involved in signal transduction and apoptosis. The expression levels of lymphoid enhancer-binding factor-1 (LEF1), histone deacetylase 1 (HDAC1) and protein kinase C alpha (PKCα) genes were reduced by more than 12.5-, 2.08-, and 2.38-fold relative to the untreated cells, respectively (Table 2).

The LEF1 gene has been shown to function as both an oncogene and a tumor suppressor, and the results of recent studies demonstrate that the inactivation of LEF1 is associated with T-cell acute lymphoblastic leukemia (T-ALL) (Gutierrez *et al.*, 2010). HDAC1 performs important functions in the regulation of eukaryotic gene expression. The downregulation of HDAC1 affects ubiquitin ligase Chfr, which induces tumor metastasis via cell cycle progression (Oh *et al.*, 2009). PKCα phosphorylates a variety of protein targets in the signaling pathway, and the deregulation of PKCα has been previously implicated in both breast cancer and colon cancer (Martiny-Baron and Fabbro, 2007).

The downregulation of PKCα, LEF1, and HDAC1 expression in benzene-treated cells was confirmed via RT-PCR (Fig. 3A). Western blot analysis results also demonstrated the downregulation of HDAC1 expression (Fig. 3B).

Collectively, we analyzed differential gene regulation in human leukemia K562 cells treated with 10 mM benzene, via microarray analysis. We identified 6,562 genes whose expression was altered by benzene treatment. The array results show that a broad variety of genes involved in cellular proliferation and anti-apoptosis pathways were expressed differentially. The profiling of genes expressed differentially after benzene treatment should provide basic information for further research into carcinogenesis, including leukemia, in response to benzene exposure.

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