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Association analysis of toluene exposure time with high-throughput mRNA expressions and methylation patterns using *in vivo* samples

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ABSTRACT

The emission of volatile organic compounds (VOCs) resulting from outdoor air pollution can contribute to major public health problems. However, there has been limited research on the health effects in humans from the inhalation of VOCs. Therefore, this study conducted an *in vivo* analysis of the effects of toluene, one of the most commonly used chemicals in many industries, on gene expression and methylation over time using the high-throughput technique of microarray analysis. We separated participants into three groups (control, short-term exposure, and long-term exposure) to investigate the influence of toluene exposure time on gene expression. We then comprehensively analyzed and investigated the correlation between variations in gene expression and the occurrence of methylation. Twenty-six genes were upregulated and hypomethylated, while 32 genes were downregulated and hypermethylated. The pathways of these genes were confirmed to be associated with cell survival and the immune system. Based on our findings, these genes can help predict the effects of time-dependent exposure to toluene on human health. Thus, observations from our data may have implications for the identification of biomarkers of toluene exposure.

1. Introduction

Among the diverse compounds used for industrial purposes, volatile organic compounds (VOCs) easily evaporate into the atmosphere, where they undergo changes through various physical and chemical processes (Mögel et al., 2011). VOCs in the air affect the indoor quality and, when inhaled, exert harmful effects on human health (Bernstein et al., 2008; Choi et al., 2013; Kim et al., 2015).

Toluene is a VOC commonly used in solvents and as a component in commercial and industrial materials. This chemical is typically emitted into the environment with industrial gases and automobile exhaust and is dangerous to both human health and the ecosystems. Toluene accumulates in tissues, including those of the liver, and parts of the brain with a high lipid content. It is distributed quickly through the body and can cause headaches, fatigue, nausea, and athymia (Tas et al., 2011). Exposure to toluene also affects gene expression. With an increase in the exposure time, there is a greater probability of the occurrence of related diseases because the accumulation of toluene in lipids, such as the blood–brain barrier, as well as of genetic variations caused by toluene (Seeber et al., 2005). Thus, it is important to identify how this substance genetically affects people in the industrial workplace and to understand the adverse effects associated with its periodic exposure.

The microarray is one of the many useful methods used to identify gene expression level. Expression profiles using high-throughput methods are performed along with bioinformatic studies, and the changes in molecular expression patterns from specific toxins are studied to determine how these toxins cause diseases. The microarray has been used to discover biomarkers in research on system toxicogenomics (Chai et al., 2013). DNA methylation profiles of various genomes have also been studied extensively. Methylation plays a significant role in repressing the transcription of downstream genes, leading to adverse effects in mRNA expression during development and differentiation (Gibbs

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et al., 2010). Recent studies have indicated that a change in the methylation pattern of an area with local genetic variations can have a significant effect on gene expression. However, most studies have concluded that there is no clear and convincing evidence of this mechanism (Boks et al., 2009; Schalkwyk et al., 2010; Suzuki and Bird, 2008; Zhang et al., 2010). This study examined the effects of toluene exposure on 42 employees who have worked in a confined place with chemicals added to toluene. We compared the variations in gene expressions and in methylation patterns of CpG islands over time to investigate the relationship between toluene exposure time and health effects. Toluene was selected because of its known role in respiratory diseases (Kotzias, 2005; Saijo et al., 2004). Our findings confirmed that the adverse effects of toluene exposure to human health can be predicted based on the exposure time.

2. Materials and methods

2.1. Sample selection

Healthy participants were selected from a factory and chemical production company in Ansan, Korea, where chemical concentrations in the work environment are maintained according to the regulatory standards. The 42 participants agreed to provide samples of their blood, and they were enrolled in an institutional review board (IRB)–approved human subject blood collection protocol (IRB #AS 14039). They were divided into three groups based on the exposure time: control (unexposed workers; $n=14$), short-term exposure (workers exposed to VOCs for <6.4 yr; $n=14$), and long-term exposure (workers exposed to VOCs for >6.4 yr; $n=14$).

2.2. mRNA preparation and gene expression experiments

Whole blood samples were collected for RNA isolation in PAXgene blood RNA tubes (PAXgene miRNA Kit, Qiagen, Valencia, CA, USA). The isolations of RNA yields were assessed using NanoDrop (Thermo Fisher Scientific, USA), and the qualities of RNA were confirmed using a 2100 Bioanalyzer (Agilent Technologies, CA, USA). For expression array analysis, total RNA (100 ng) amplification and cRNA labeling were carried out using a Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies). RNA samples from all participants were labeled with Cy3 dye and hybridized to Human GE 8×60 K (v2) oligo arrays (Agilent Technologies) following the manufacturer's protocols. Gene expression analysis was performed using an Agilent C scanner (Agilent Technologies).

2.3. DNA preparation and methylation microarray experiments

We used EDTA tubes to collect whole blood samples for DNA isolation (DNeasy Mini Kit, Qiagen). We evaluated the yields and the qualities of DNA using NanoDrop (Thermo Fisher Scientific) and agarose gel electrophoresis (2% agarose), respectively. First, to immunoprecipitate the DNA, we fragmented the Genomic DNA samples into 200–300-bp fragments using a sonicator (Sonics & Materials, Inc., USA). Using a MethylMiner Methylated DNA Enrichment Kit (Invitrogen, Carlsbad, CA, USA), 1 μg of fragmented genomic DNA was then immunoprecipitated. We confirmed the fragmented DNA (input) and immunoprecipitated (IP) DNA using a polymerase chain reaction (PCR) with Xist-specific and GAPDH-specific (a housekeeping gene) primers. DNA samples were then amplified using a Whole Genome Amplification 2 Kit (Sigma, St. Louis, MO, USA). Prepared DNA samples (input and IP samples; 1 μg each) were labeled using a SureTag DNA Labeling Kit (Agilent Technologies). Input samples were labeled with Cy3, while IP samples were labeled with Cy5 dye. According to the manufacturer's instructions, Cy3- and Cy5-labeled DNA samples were mixed and hybridized together on ChIP/CH3 2×400 K custom arrays (Agilent Technologies). Methylation analysis was performed using an Agilent C scanner.

2.4. Gene expression variation analysis

We scanned microarray images using an Agilent C scanner and analyzed them with Feature Extraction software 10.7.3.1 (Agilent Technologies). The raw data were extracted and analyzed using GeneSpring GX v13.0 (Agilent Technologies). Spots were flagged based on judgments of data reliability. We then normalized the 22,583 of 50,599 probes in expression data using quantile normalization and used unpaired *t*-tests for commonly and differentially screening the expressed genes. Using analysis of variance (ANOVA), which is a set of statistical models used to study the differences among group means developed by statistician and evolutionary biologist Ronald Fisher, we accomplished a *p*-value of <0.05 and a fold change of ≤1.5 for comparing the expressions of genes between the control and exposure groups. We analyzed the selected genes for functionality using gene ontology (GO); the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database; the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.7; and Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com/products/ipa).

2.5. Methylation pattern analysis

The scanned methylation microarray images were analyzed with Agilent Feature Extraction v9.1 software using aCGH protocols, and the G3 microarrays setting protocol Agilent G3_CGH was obtained by extracting the Cy5 (methylated DNA) signals over Cy3 (input DNA) signals. We then normalized the data with the Predefined Peak Shape Detection 2.0 algorithm provided within the CH3 Analytics software package (Agilent Technologies). We identified the significant methylation patterns among these data using default algorithm parameters with a score threshold of 2.0. The associations of methylation patterns of genes were confirmed by comparing the data of transcription start sites and the promoters, defined by the genetic refFlat table, with RefSeq Hg19 provided by the University of California, Santa Cruz (UCSC) genome browser database.

3. Results

3.1. Gene expression variation analysis

To investigate the toxicities of mRNA expression in humans, we verified the exposure times for individuals in the control, short-term exposure, and long-term exposure groups, as shown in Table 1. The average exposure time in the short-term exposure group

Table 1
Information on the exposure time of the participants. Total number of participants was 42, and they were divided into three groups: control, short-term, and long-term exposure.

Groups	Control group	Short-term exposure group	Long-term exposure group	Total
Number of participants	14	14	14	42
Average exposure time (yr)	—	1.6	11.2	