Increased leukemia-associated gene expression in benzene-exposed workers

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Long-term exposure to benzene causes several adverse health effects, including an increased risk of acute myeloid leukemia. This study was to identify genetic alternations involved in pathogenesis of leukemia in benzene-exposed workers without clinical symptoms of leukemia. This study included 33 shoe-factory workers exposed to benzene at levels from 1 ppm to 10 ppm. These workers were divided into 3 groups based on the benzene exposure time, 1-<7, 7-<12, and 12-<24 years. 17 individuals without benzene exposure history were recruited as controls. Cytogenetic analysis using Affymetrix Cytogenetics Array found copy-number variations (CNVs) in several chromosomes of benzene-exposed workers. Expression of targeted genes in these altered chromosomes, NOTCH1 and BSG, which play roles in leukemia pathogenesis, was further examined using real-time PCR. The NOTCH1 mRNA level was significantly increased in all 3 groups of workers, and the NOTCH1 mRNA level in the 12-<24 years group was significantly higher than that in 1-<7 and 7-<12 years groups. Compared to the controls, the BSG mRNA level was significantly increased in 7-<12 and 12-<24 years groups, but not in the 1-<7 years group. These results suggest that CNVs and leukemia-related gene expression might play roles in leukemia development in benzene-exposed workers.
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### Methods

**Study population.** Methods used in this study were carried out in accordance with the approved guideline by the Committees for Ethical Review of Research involving Human Subjects at Tianjin Medical University. Informed consents were obtained from all subjects. This study included 33 workers from a shoe factory who were exposed to benzene levels from 1 ppm (the American occupational exposure level) to 10 ppm for various times. Assessment for the level of benzene exposure was performed as described previously. Air monitoring was conducted every month for 3–4 months prior to biological sample collection. These workers were divided into 3 groups based on the benzene exposure time, 1- < 7, 7 - < 12, and 12 - < 24 years. All benzene-exposed workers had normal levels of hemoglobin, RBCs, WBCs, platelets, and alanine aminotransferase (Table 1). These workers had no leukemia symptoms or other health problem at the time when blood samples were collected. 17 unexposed subjects were recruited from factories in the same region without benzene exposure as controls. In the control region, benzene and toluene were not detected in the air. The unexposed subjects were matched with the exposed workers for age and smoking history (Table 1).

Information was obtained from answering a questionnaire by all subjects including working history, past and current tobacco and alcohol use and medical history, including infections and ionizing radiation exposure, medication, and family disease history.

**Blood sample collection.** Fresh blood specimens were collected from the median cubital vein. EDTA-K2 was added to blood samples to prepare fresh anticoagulation blood for DNA extraction. Blood tests, including hemoglobin, RBCs, WBCs, platelets, and Alanine aminotransferase, were performed.

**Cytogenetic microarray.** Blood samples were collected from 6 benzene-exposed workers (3 female and 3 male, age: female: 39.7 ± 6.7, male: 44.7 ± 5.0, benzene exposure time: female: 6 ± 1 years, male: 6 ± 2) for microarray Affymetrix cytogenetic microarray analysis.

Genomic DNA was isolated from peripheral blood cells using a Gentra Puregene Blood kit (Santa Clara, California, USA) according to the manufacturer’s instructions. Genomic DNA (0.1 μg) was labeled using an Affymetrix Cytoarrays Reagent Kit, and the labeled DNA was applied to an Affymetrix Cytogenetics Whole-Genome Array, including 2.7 million probes for the detection of copy number variation (Affymetrix Inc., Santa Clara, California, USA) according to the manufacturer’s instructions. The array was scanned and the data were analyzed using the Affymetrix Chromosome Analysis Suite. The control group included 35 healthy Asian people (17 male and 18 female). The chromosomal structure of this control group was used as the standard, and array data from benzene-exposed workers were compared to this standard. Significant chromosomal changes were chosen by selecting the area with size >200 kbp, confidence level > 0.8, and mean marker distance <2.5 kbp.

**Real-time PCR assay.** Real-time PCR analyses were performed using 2X EvaGreen qPCR Master Mix in a 20 μl reaction mixture containing forward and reverse primers (10 μM each) and 25 ng of DNA. Reactions were run on ABI Stepone plus real-time PCR as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Double-stranded DNA fluorescence was repeatedly detected at the end of the elongation phase of each PCR cycle. The forward and reverse primers used were as follows: 5’-GGATGCAGGAGGAGGATGATG-3’ and 5’-CAATCTCA-TCTTGTGTTCCG-3’ (135 bp) for β-actin; 5’-CATGTGCTGCTGCAAGG-GCTCAG-3’ and 5’-CCGGTCATGAGGGCTTCTGTC-3’ (194 bp) for BSG; 5’-GACA-GGCCTACTCTCAGTC-3’ and 5’-ACAGTCATCGGATTCTGAC-3’ (154 bp) for NOTCH1.

**Results**

**Study subjects and exposure assessment.** Since the incident rate of leukemia is significantly increased in individuals with long-term benzene exposure as compared to that in the un-exposed people, the goal of this study was to identify whether there are early effects of benzene exposure on genetic alterations in workers. This study included 33 benzene-exposed workers from a shoe manufacturer with benzene exposure time from 1 to 12 years. They did not have any clinical symptoms of leukemia. Their blood test results, including hemoglobin, RBCs, WBCs, platelets, and alanine aminotransferase, were among the normal ranges (Table 1). Thus, the health status of these workers was normal when this study was performed.

**Effects of benzene exposure on chromosome alterations.** The Affymetrix Cytogenetics microarray was applied to examine whether these benzene-exposed workers have chromosome alterations. Genomic DNA samples were prepared from blood cells of 6 benzene-exposed workers. Affymetrix Gene Chips (Human March 2006 (hg18) assembly) was used to screen the chromosomal structure. After filtering the area by using the criteria, size <200 kbp, confidence level < 88%, and mean marker distance >2.5 kbp, we found that chromosome gain with long-segment CNVs regions amplified from 200 kbp to 400 kbp in chromosomes 1, 2, 8, 9, 10, 13, 16, 19, 22 and chromosome X (Table 2). In addition, a 311–354 kbp deletion in chromosome 19 and a 458 kbp deletion in chromosome 7 were found (Table 2). These results suggest that chromosomal structural aberrations are related to occupational exposure to benzene in workers before symptoms of leukemia occurs.

**Effects of benzene exposure on expression of genes associated with leukemia.** We next used real-time PCR assay to detect expression of genes in these altered segments of chromosomes which are associated with leukemia. We detected expression of 2 genes, BSG (also known as CD147) and NOTCH1, which have been shown to be associated with occurrence and development of leukemia.

For this study, we categorized exposure groups based on the exposure time, 1- < 7 years, 7 - < 12 years, and 12 - < 24 years groups. Compared to the control group, the NOTCH1 mRNA level was significantly increased in workers in 1- < 7 years (p < 0.01), 7 - < 12 years (p < 0.01) and 12 - < 24 years groups (p < 0.001, Figure 1A). The NOTCH1 mRNA level in 12 - < 24 years group was significantly higher than that in 1- < 7 years (p < 0.001), and 7 - < 12 years groups (p < 0.001, Figure 1A). The BSG mRNA level was significantly increased in the 7- < 12 years and 12 - < 24 years groups (p < 0.05), but not in the 1- < 7 years group (p > 0.05, Figure 1B).

### Table 1 | Characteristics of the control subjects and workers exposed to benzene

| Benzene exposure time (year) | Male (n=3) | 1 - < 7 | 7 - < 12 | 12 - < 24 |
|-----------------------------|------------|--------|--------|--------|
| Male                        | 9          | 3      | 9      | 2      |
| Female                      | 8          | 13     | 3      | 3      |
| Age                         | 34.7 ± 6.0 | 39.4 ± 5.6 | 35.5 ± 5.6 | 40.2 ± 4.3 |
| Hemoglobin (g/dl)           | 14.6 ± 1.9 | 13.3 ± 1.6 | 15.1 ± 1.5 | 14.9 ± 2.5 |
| Red blood cells (RBCs, 10^12/m) | 5.3 ± 1.3 | 4.4 ± 0.4 | 4.9 ± 0.4 | 5.6 ± 1.1 |
| White blood cells (WBCs, 10^3/μL) | 4.9 ± 0.5 | 6.3 ± 1.8 | 6.4 ± 1.5 | 4.7 ± 0.6 |
| Platelets (10^3/μL)         | 253.8 ± 48.3 | 250.3 ± 38.2 | 226.7 ± 31.8 | 244.2 ± 46.3 |
| Alanine aminotransferase (ALT, IU/L) | 24.94 ± 9.4 | 19.0 ± 12.6 | 23.4 ± 10.7 | 21.6 ± 15.2 |

Values are mean ± SD.

Normal range of blood tests: Hemoglobin (g/dl): 13.5–16.5 (male), 12.0–15.0 (female); RBCs (10^12/m): 4.5–5.5 (male), 4.0–4.9 (female); WBCs (μL): 4,500–10,000. Platelets (μL): 100,000–450,000. ALT (IU/L): 10–40 (male), 7–35 (female).
These results are consistent with previous findings that cytogenetic alterations which can lead to altered gene expressions. Benzene exposure up-regulates leukemia-associated gene expression in workers and the longer benzene exposure time correlates with higher mRNA expression levels of leukemia-associated genes.

### Discussion

Long-term exposure to benzene has been shown to lead to an increased risk of acute myeloid leukemia and myelodysplastic syndrome. Benzene-induced decreases in blood cells could be observed within a few months after benzene exposure. However, there is a lag time of years between initial benzene exposure and the development of leukemia. In this study, we focused on investigating the potential effects of benzene exposure on leukemia-associated gene expression in workers who showed no clinical evidence of leukemia. We identified CNVs in chromosomes of benzene-exposed workers. By analyzing functions of genes in altered chromosomes, we further confirmed that the levels of BSG and NOTCH1, which are associated with the occurrence and development of leukemia, were increased in these workers. These genetic and gene expression changes might be used as biomarkers for evaluation of the risk of leukemia in benzene-exposed workers.

A study showed that the benzene metabolites induce the binding of chlorine to DNA. Halogenated DNA can induce both genetic and epigenetic changes that contribute to carcinogenesis. Halogenative stress may account for benzene-induced bone marrow disorders and myeloid leukemia. Thus, CNVs found in this study may represent a form of the halogenated DNA resulting from genetic changes.

**NOTCH1** is crucial in T-cell differentiation and proliferation. **NOTCH1** mutations appear in approximately 50% of acute T-lymphoblastic leukemia cases. It has been reported that the significantly high expression level of **NOTCH1** is positively correlated with acute T-lymphocyte leukemia (ATLL). **NOTCH1** mutations occur in 10% of patients with chronic lymphocytic leukemia and are associated with poor prognosis. Our results show that the copy number of **NOTCH1** in the benzene exposed workers exhibits amplified changes. Thus, the **NOTCH1** gene may be a target of benzene-affecting gene.

**BSG** encodes a protein called Basigin. This protein is a single transmembrane glycoprotein with a high degree of glycosylation and is a member of the immunoglobulin superfamily of adhesion molecules. Basigin has diverse biological functions and is involved in tissue repair, reproductive development, as well as energy metabolism. Basigin is up-regulated in tumors. This protein promotes tumor invasion and metastasis by inducing the expression of matrix metalloproteinases and the degradation of the extracellular matrix. Basigin is an ideal target for cancer therapy as a new tumor marker. BSG was recently identified as a part of a gene-expression signal associated with the recurrence of childhood acute lymphoblastic leukemia. In ATLL patients, most T-cells express significantly higher CD147 levels. In this study, increased copy number of BSG was found in benzene-exposed workers. Thus, benzene may affect the lymphocytes of exposed individuals, which eventually leads to the occurrence of leukemia by the amplification of BSG.

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**Table 2 | Genetic alterations in workers exposed to benzene**

| Chromosome locus | Gene targets | Confidence |
|------------------|--------------|------------|
| Gain 1 p36.33    | PUSL1, MRPL20, CCNL2, TTL10, AURKAIP1, SDF4, TNFRSF18, TAS1R3 | 0.88 |
| Gain 2 q21.1     | FAM128A, TUBA3D, CCDC74A | 0.90 |
| Gain 8 q24.3     | CBFORF2K9, LRRC14, NFKB1L2, GPT, FOXH1, MGCM70857, DGAT1, FBXL6, CYHR1 | 0.88 |
| Gain 9 q34.3     | NOTCH1, EGFL7, REGL4 | 0.89 |
| Gain 10 p15.3    | F108, DIP2C | 0.88 |
| Gain 13 q34      | RASA3, FAM70b | 0.89 |
| Gain 16 q22.3    | CLEC18B, PSMD7, GLG1 | 0.90 |
| Gain 19 q13.3    | BSG, SHC2 | 0.89 |
| Gain 22 q11.23   | LRPSL, IGL3 | 0.88 |
| Loss X q26.3     | NCRNA000086, DDX26B, ZNF449 | 0.92 |
| Loss 7 q11.21    | ZNF92, INTS4L | 0.89 |
| Loss 19 q13.31   | PSG6, PSG8, PSG10, PSG11 | 0.90 |
Other genes found in altered chromosomes in benzene-exposed workers are involved in normal human functions. **APRT** encodes adenine phosphoribosyltransferase. This enzyme catalyzes the formation of AMP and inorganic pyrophosphate from adenine and 5-phosphoribosyl-1-pyrophosphate. It also produces adenine as a byproduct of the polyamine biosynthetic pathway. A deficiency in this enzyme is associated with uricoteliasis. GALNS, encodes N-acetylgalactosamine-6-sulfatase. Mutations of this gene, including point, missense, and nonsense mutations, may lead to a lysosomal storage disorder. **AGPAT2** encodes lysophosphatic acid acyltransferase β. This protein is found within the endoplasmic reticulum membrane and converts lysophosphatic acid to phosphatidic acid, which is involved in phospholipid biosynthesis. **RECQL4** encodes a DNA helicase that belongs to the RecQ helicase family. Mutations in this gene may lead to chromosomal instability.

In summary, occupational exposure to benzene can cause CNVs in several chromosomes and increased **NOTCH1** and **BSG** expression in workers without symptoms of leukemia. Further investigations, including recruitment of additional workers and prolonged follow-up time are required to determine whether these changes can serve as the mechanisms underlying benzene exposure-induced leukemia and can be used as biomarkers for evaluation of the risk of leukemia development in benzene-exposed workers.

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L.K., J.Y., Y.C., L.S., Z.Y., H.X., L.F. and H.J. designed and carried out the experiments. L.K., J.Y., Y.C. and L.G. analyzed the data and wrote the manuscript. All authors reviewed the manuscript.

Additional information
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