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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.

Benzene, an aromatic hydrocarbon compound, is a primary industrial chemical produced at high levels in the manufacture of plastics, resins, and dyes. Occupational exposure to benzene occurs in the oil, shipping, automobile repair, shoe manufacture, and other industries[1]. Benzene is also a well-known environmental pollutant present in cigarette smoke and motor vehicle exhaust[2]. It has been widely studied that exposure to benzene leads to several adverse health effects. Benzene's toxic effects on the blood and bone marrow include increasing the risk of acute myeloid leukemia (AML), myelodysplastic syndrome, and other hematological malignancies, such as non-Hodgkin lymphoma[3–5]. Benzene also exerts hematotoxic effect at relatively low levels of exposure[6].

Studies regarding the mechanisms by which benzene increases the risk of AML reveal that benzene's metabolites induce chromosomal aberrations[7–9]. Benzene exposure has been associated with high levels of AML-related chromosomal changes, and these AML-related chromosomal changes have also been observed in human cell cultures treated with benzene metabolites[9–13]. Further studies have shown that target genes regulated by benzene's metabolites are critical for hematopoiesis in hematopoietic stem cells during leukemia development. It has been reported that 29 known genes in peripheral blood mononuclear cells are related to high benzene exposure[14]. Other studies including a larger number of samples have demonstrated that global gene expression changes involved in signaling pathways mediating AML, inflammatory and stress responses are associated with occupational benzene exposure[15,16].

The copy-number variations (CNVs), which may serve as a major cause for changes in RNA expression[17], is defined as a deletion, duplication, or size inversion of DNA fragments from one kilobase pair to several megabase pairs[18]. CNVs are common cytogenetic aberrations in healthy individuals, but they more frequently occur in cancers, including AML[19–22]. CNVs can influence gene expression by disrupting coding sequences, perturbing long-range gene regulation, or altering gene dosage. These effects contribute to phenotypic variations or increase disease risks[23–26]. Microarray technology is widely used in toxicological studies of CNVs[17,24–28]. However, information of CNVs in benzene-exposed workers is limited.

We show here that genetic alterations, such as long-segment copy-number variations in several chromosomes are found in blood cells of benzene-exposed workers without clinical symptoms of leukemia. Targeted genes of these altered chromosomes, which are related to development of leukemia, have been further evaluated by real-time PCR analysis. The *NOTCH1* and *BSG* mRNA levels are increased in workers with benzene exposure. These

results suggest that CNVs and leukemia-related gene expression might play roles in leukemia development in benzene-exposed workers.

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[29]. 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 include 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 Affymetric 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 mg) was labeled using an Affymetrix Cytogenetics 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 > 88%, and mean marker distance <2.5 kbp.

Real-time PCR assay. Real-time PCR analyses were performed using 2× 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 following: 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 following: 5′-GGATCAGCAAGCAGGAGTATG-3′ and 5′-CAATCTCA-TCTTGTTTTCTGCG-3′ (135 bp) for *β-actin*; 5′- CCATGCTGGTCTGCAAG-TCAG -3′ and 5′- CCGTTCATGAGGGCCTTGTC-3′ (194 bp) for *BSG*; 5′-GAC-AACGCCTACCTCTGCTTC-3′ and 5′-ACAGTCATCCAGGTTGATCTCG-3′ (154 bp) for *NOTCH1*.

Statistical analysis. Statistical significance was determined by one-way ANOVA followed by Newman-Keuls analysis using Prism 5.0 (GraphPad Software, Inc. San Diego, CA) for multiple comparisons. Data obtained from each group were expressed as the mean ± standard deviation. $P < 0.05$ was considered statistically significant.

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[30], 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[31,32].

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)	Control	1 - < 7	7 - < 12	12 - < 24
Male	9	3	9	2
Female	8	13	3	3
Age	34.7 ± 6.01	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, ×10⁶/ml)	5.3 ± 1.3	4.4 ± 0.4	4.9 ± 0.4	5.61 ± 1.1
White blood cells (WBCs, ×1000/ml)	4.9 ± 0.5	6.3 ± 1.8	6.4 ± 1.5	4.74 ± 0.6
Platelets (×1000/ml)	253.8 ± 48.3	250.3 ± 38.2	226.7 ± 31.8	244.2 ± 46.3
Alanine aminotransferase (ALT, U/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 (×10⁶/ml): 4.5–5.5 (male), 4.0–4.9 (female). WBCs (ml): 4,500–10,000. Platelets (ml): 100,000–450,000. ALT (U/L): 10–40 (male), 7–35 (female).

Table 2 | Genetic alterations in workers exposed to benzene

	Chromosome locus	Gene targets	Confidence
Gain			
1	p36.33	*PUSL1, MRPL20, CCNL2, TTLL10, AURKAIP1, SDF4, TNFRSF18, TAS1R3*	0.88
2	q21.1	*FAM128A, TUBA3D, CCDC74A*	0.90
8	q24.3	*C8ORFK29, LRRC14, NFKBIL2, GPT, FOXH1, MGC70857, DGAT1, FBXL6, CYHR1*	0.88
9	q34.3	*NOTCH1, EGFL7, RECGL4*	0.89
10	p15.3	*F108, DIP2C*	0.88
13	q34	*RASA3, FAM70b*	0.89
16	q22.3	*CLEC18B, PSMD7, GLG1*	0.90
16	q24.3	*C16, FAM38A, GALNS, CDT1, APRT, RNF166, CBFA2T3, TRAPPC2L*	0.88
19	p13.3	*BSG, SHC2*	0.89
22	q11.23	*LRP5L, IGLL3*	0.88
X	q26.3	*NCRNA00086, DDX26B, ZNF449*	0.92
Loss			
7	q11.21	*ZNF92, INTS4L*	0.89
19	q13.31	*PSG6, PSG8, PSG10, PSG11*	0.90

These results are in consistent with previous finding that cytogenetic alterations which can lead to altered gene expressions[33]. 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[30]. 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[34]. 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[35]. It has been reported that the significantly high expression level of *NOTCH1* is positively correlated with acute T-lymphocyte leukemia (ATLL)[36]. *NOTCH1* mutations occur in 10% of patients with chronic lymphocytic leukemia and are associated with poor prognosis[37–39]. 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[40]. This protein promotes tumor invasion and metastasis by inducing the expression of matrix metalloproteinases[41–44] and the degradation of the extracellular matrix[45]. 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[46,47]. In ATLL patients, most T-cells express significantly higher CD147 levels[48]. 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*.

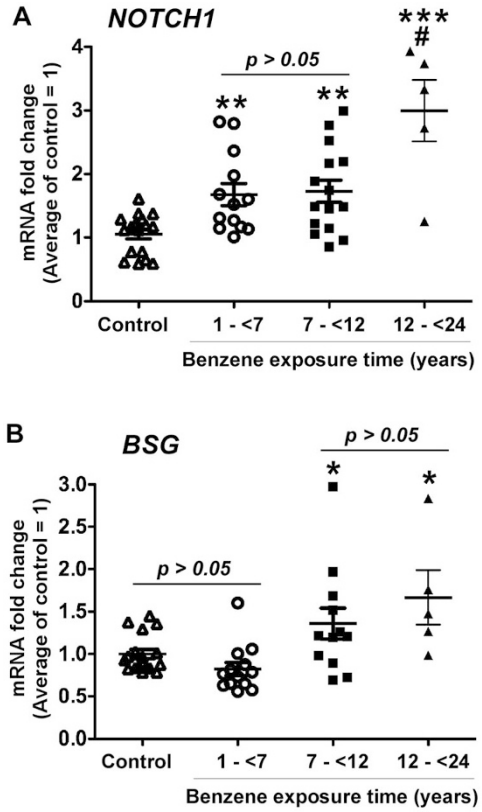

Figure 1 | Up-regulation of *NOTCH1* and *BSG* gene expression in benzene-exposed workers. Blood cell samples were collected from benzene-exposed workers and controls for DNA isolation and Real-time PCR analysis to detect the mRNA levels of *NOTCH1* (A) and *BSG* (B). The average of mRNA expression level in the control group was set as 1, and the mRNA expression level in each control subject and worker was compared to the average. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to the control group. # $p < 0.01$ compared to the 1 - < 7 years and 7 - < 12 years groups.

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 urolithiasis. *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 lysophosphatidic acid acyltransferase β. This protein is found within the endoplasmic reticulum membrane and converts lysophosphatidic 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 berzene exposure-induced leukiame and can be used as biomarkers for evaluation of the risk of leukemia development in benzene-exposed workers.

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Author contributions

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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