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Methylation and expression analysis of tumor suppressor genes p15 and p16 in benzene poisoning

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1. Introduction

Benzene is an important industrial chemical and component of cigarette smoke, gasoline and automobile emissions. The production of pure benzene in China is increasing annually, and is expected to reach 10 million tons in 2010 [1]. The potential hazard from exposure to ambient benzene is a major public health concern. Benzene-induced hematotoxicity, referred to as benzene poisoning (BP), is the primary occupational harm of benzene. The hematopoietic toxicity of benzene includes aplastic anemia (AA), myelodysplastic syndromes (MDS), non-Hodgkin lymphoma and leukemia [2–4]. Epidemiological study has suggested that risk for acute nonlymphocytic leukemia (ANLL) and the related MDS was substantially increased among workers with a prior history of benzene poisoning [5]. However, the molecular mechanism in the development of benzene-induced leukemia remains unclear.

Among the leukemia subtypes, only AML incidence was significantly associated with benzene exposure [4,6]. In AML and MDS, inactivation of tumor suppressor genes p15 and p16 is one of the most common genetic events, and promoter DNA hypermethylation is the primary mechanism that leads to inactivation of these two genes [7,8]. p15 and p16 genes, both located in the 9p21 region, have a high degree of structural and functional homology and belong to the INK4 kinase family. They are cyclin dependent kinase inhibitors which negatively regulate the cell cycle. They can compete with cyclin D for CDK4 and 6, thus preventing retinoblastoma protein from phosphorylation and preventing progression into S phase, arresting the cell in the G1 phase [9]. A critical point in cell cycle control is the G1 to S transition. Therefore, functional loss of p15 and p16 may permit unlimited proliferation of the cell, and is thought to be associated with leukemogenesis [10]. Increased p15 promoter DNA methylation was reported in healthy individuals with low-dose benzene exposure [11]. In benzene poisoning, however, the link of promoter DNA methylation and the induction of inactivation of expression of p15 and p16 has not been established. In the present study, we have determined the gene expression level and promoter methylation status of p15 and p16 in patients with benzene poisoning and matched exposure controls. The results suggest that benzene could negatively affect the expression of p15 and p16 through DNA methylation.

2. Materials and methods

2.1. Subjects

Eleven BP workers were recruited from Shanghai, China, including 5 males and 6 females. BP was diagnosed from 1963 to 1994 by a

Abbreviations: AA, aplastic anemia; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ANLL, acute nonlymphocytic leukemia; BP, benzene poisoning; China CDC, Chinese Center for Disease Control and Prevention; CpG, dinucleotide cytosine-guanosine; DEAF-1, deformed epidermal autoregulatory factor 1; EBF, early B-cell factor; MDS, myelodysplastic syndromes; Olf-1, olfactory neuron-specific transcription factor; RT-PCR, real-time polymerase chain reaction; SD, standard deviation; WBC, white blood cell.

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Table 1
Sequence and PCR condition for each PCR analysis.

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Sequencing primer (5′ to 3′)	PCR condition
p15 (RT-PCR)	AAGCTGAGCCCAGGTCTCCTA	CCACCGTTGGCCGTAAACT	–	95 °C 3 min, 1 cycle;
p16 (RT-PCR)	GGGGGCACCAGAGGCAGT	GGTTGTGGCGGGGGCAGTT	–	95 °C 30 s, 66 °C 1 min,
β-Actin (RT-PCR)	GGTCATCACCATTGGCAATGA	GTAGTTTCGTGGATGCCACAGG	–	72 °C 30 s, 40 cycles
p15 (sequencing)	GTTTTTTTTTAGAAGTAATTTAGG	Biotin-TCCTTCTACRACTTAAAACC	TTTTTTTTAGAAGTAATTTA	95 °C 30 s, 52 °C 30 s, 72 °C 30 s, 45 cycles
p16 (sequencing)	GGGGGAGATTTAATTTGG	Biotin-CCCTCCTCTTTCTTCCTC	GGGGAGATTTAATTTGG	95 °C 1 min, 60 °C 1 min, 72 °C 1 min, 40 cycles

local Occupational Disease Diagnostic Team, and patients were registered in hospitals for prevention and treatment of occupational diseases. The diagnostic criteria for occupational BP, according to the Ministry of Health of the People's Republic of China, are as follows: (a) total WBC <4000/μl or granulocyte count <2000/μl with platelet count <60,000/μl, and persistence of such decreased cell counts in at least 3 consecutive months by a peripheral blood examination; (b) employment in a factory with documented benzene exposure for at least 6 months in the factory; and (c) exclusion of other causes of abnormal blood counts such as chloromycetin (chloramphenicol) use and ionizing radiation. None of the patients had any prior history of cancer, therapeutic radiation, or chemotherapy. Controls had the same factory, work unit and job title with the patients, and they were healthy without any clinical manifestation of benzene poisoning.

Demographic characteristics among cases and controls were not statistically different ($p > 0.05$ for all). Cases were on average 58.6 years old (SD, 13.6), whereas controls were 60.9 years old (SD, 9.6). 45.4% of the cases and 25.0% of controls were male, 36.4% of the cases versus 18.2% of controls drank alcohol, and 18.2% of cases versus 12.5% of controls were cigarette smokers. The mean duration of working was 22.5 (SD, 7.4) years among cases and 21.0 (SD, 6.9) among controls.

The protocol was approved by the Ethical Review Committee at the National Institute of Occupational Health and Poison Control, Chinese Center for Disease Control and Prevention (China CDC) and informed consent was obtained using the approved procedures. A questionnaire was administered on age, sex, current and lifelong tobacco use, alcohol consumption, and medical and occupational history.

2.2. Bisulfite treatment

Genomic DNA was isolated using PureLink Genomic DNA Mini kit (Invitrogen). DNA samples were treated with sodium bisulfite with EpiTech Bisulfit kits (Qiagen) according to the manufacturer's instructions and then eluted in 50 μl elution buffer. Unmethylated human genomic DNA was used as the negative control and methylated human genomic DNA was used as the positive control to verify the bisulfite conversion. Bisulfite-treated DNA was stored at −20 °C until use.

2.3. PCR and pyrosequencing

Bisulfite-treated DNA was amplified by PCR. The PCR product was bound to Streptavidin Sepharose High Performance (GE Healthcare), and the Sepharose beads containing the biotin-labeled PCR product were purified, washed, denatured using 0.2 mol/L NaOH solution, and washed again using the Pyrosequencing Vacuum Prep Tool (Biotage) according to the manufacturer's instructions. Then the pyrosequencing primer was annealed and the pyrosequencing was performed using the Pyromark ID Pyrosequencing System (Biotage). PCR primers and conditions are given in Table 1.

2.4. Quantitative real-time PCR

Total RNA was isolated using the PureLink Micro-to-Midi Total RNA Purification System kit (Invitrogen) according to the manufacturer's instructions. Reverse transcription of total DNA-free RNA was performed with hexamer random primers using the SuperScriptIII First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. cDNAs were amplified and quantified by real-time PCR using specific primers for p15 mRNA, p16 mRNA and β-actin (Table 1). PCR reactions were performed using a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA). All samples were analyzed in duplicate. β-Actin was used as an internal control in order to normalize p15 and p16 expression levels. Non-template controls (NTCs) were included in each PCR. In detail, 2 μl cDNA was amplified by PCR containing 12.5 μl of 2× SYBR Green Supermix (Bio-Rad), 0.125 μl of each primer (50 nM β-actin, 50 nM p15, 100 nM p16, respectively) in a final volume of 25 μl. Melt curve analysis followed the final extension step, where the temperature was increased from 65 to 95 °C at a linear rate of 0.2°/s. In order to verify the results of the melt curve analysis, PCR products were analyzed by electrophoresis on 2.5% agarose gel. p15 and p16 transcript levels were calculated and normalized to each sample's housekeeping gene mRNA (β-actin). Standard curves were constructed from samples used in a series of consecutive dilutions for the p15, p16 genes and for the internal control (β-actin).

2.5. Statistical analysis

Normality was evaluated by the one-sample Kolmogorov–Smirnov normality test. Data not normally distributed were subjected to natural log-transformations to normalize the distributions before analyses. *t*-Tests were used to assess differences between the benzene poisoning group and control group for the gene expression level and methylation status of p15 and p16. Relationships between the gene expression and methylation status were calculated using Pearson correlations. All statistical analyses were performed with SPSS 11.5 for windows (SPSS Inc., Chicago, IL).

3. Results

3.1. Expression levels

Both p15 and p16 mRNA expression levels were down-regulated in the patients with benzene poisoning compared to the control group. The difference is statistically significant for p15 ($p < 0.05$), and borderline significant for p16 ($p = 0.064$) (Table 2). The mRNA expression levels of the 19 individuals in the cohort were distributed over a relatively wide range. We arbitrarily defined the high-expression group as having an adjusted expression value between 2 and 3, the low-expression group as between 0 and 1, and the equal-expression group as between 1 and 2. The low-expression group showed a high percentage of BP patients compared to the

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Table 2
Adjusted expression levels of p15 and p16 in BP patients and controls.

Gene	Group	Median (range)	*p*-Value[a]
p15	BP	0.79 (0.36–1.64)	0.048
	Control	1.79 (0.24–2.67)	
p16	BP	0.53 (0.12–2.81)	0.064[b]
	Control	2.06 (0.09–9.15)	

[a] *t*-Test.
[b] *p*-Value obtained by *t*-test after log-transformations to normalize distributions.

Table 3
Distribution of case and control subjects in three different expression groups.

Gene	Group	↓ (%)	– (%)	↑ (%)
p15	BP	7/11 (63)	4/11 (36)	0/11
	Control	3/8 (38)	2/8 (25)	3/8 (38)
p16	BP	8/10 (80)	1/10 (10)	1/10 (10)
	Control	1/5 (20)	1/5 (20)	3/5 (60)

BP, benzene poisoning. ↓, low-expression; –, equal-expression; ↑, high-expression.

control group (63% versus 38% in p15, 80% versus 20% in p16, respectively), while the high-expression group had more people from the control group compared to the BP group (38% versus 0% in p15, 60% versus 10% in p16, respectively) (Table 3), suggesting that the BP patients have decreased expression of p15 and p16.

3.2. DNA methylation

Overall, the methylation status within the p15 promoter region was not significantly different between the BP and control groups. However, both the BP group and controls had higher methylation levels at the third CpG site than the genome DNA average methylation level which was used as a built-in control in the promoter region (12.6%, 10.8% and 9%, respectively) (Table 4). Although it is not statistically significant, we note that the BP group had higher methylation levels at the third site compared to the controls.

Table 5 presents the methylation status at the p16 promoter. The methylation level at the third CpG site is significantly higher in the BP group than in the control group (mean ± SD, 12.0 ± 3.6 and 8.7 ± 1.2, respectively; $p < 0.05$, *t*-test). The third CpG site is located within the consensus binding sequence for the deformed epidermal autoregulatory factor 1 (DEAF-1). Methylation level of the fourth CpG site was higher in the BP group than in controls (18.0%, 15.7%, respectively; $p > 0.05$).

3.3. Correlation between the level of mRNA expression and promoter methylation status

Significant negative correlation was observed between the levels of mRNA and methylation at the fourth CpG site in the promoter region of p16 (Pearson's $r = -0.88$, $p = 0.05$). The fourth CpG site is located within the consensus binding sequence for olfactory neuron-specific transcription factor (Olf-1). There was negative correlation between the levels of mRNA and methylation at the third CpG site in the promoter region of p16 (Pearson's $r = -0.63$, $p > 0.05$). Decreased p16 expression was correlated with increased average methylation level at the promoter region (Pearson's $r = -0.64$, $p > 0.05$). In addition, p15 mRNA expression was also negatively correlated with methylation level at the third CpG site in the promoter region of the gene (Pearson $r = -0.2$, $p > 0.05$).

4. Discussion

The most common type of leukemia caused by chronic benzene poisoning is AML, in which inactivation of tumor suppressor genes p15 and p16 is an important event [4,7,8]. We used Real-time PCR to evaluate mRNA expression of p15 and p16. p15 mRNA expression levels were significantly down-regulated in BP patients compared to the control group (median, 0.79 versus 1.79, $p = 0.048$) while down-regulation of p16 in BP patients was borderline significant (0.53 versus 2.06, $p = 0.068$). Further analysis showed that the low-expression group had a high percentage BP patients compared with controls (63% versus 38% in p15, 80% versus 20% in p16, respectively) (Table 3). Benzene exposure is known to be associated with dysregulated gene expression [12–14]. Down-regulated expression of JUN and PF4 was reported in benzene exposed workers [14]. Decreased levels of FOSB, DJ-1 [12] and p53 [13] mRNA were observed in BP patients. However, tumor suppressor genes involved in cell cycle regulation were rarely evaluated. p16 may function in intracellular growth regulatory pathways, and directly inhibits the activity of Cyclin D-CDK4/6 complex. This blocks retinoblastoma protein from phosphorylation and arrests cell growth. p15 may act as an effector of extracellular growth inhibitory signals [15]. Inactivation of p15 in leukemic cell lines correlated with loss of sensitivity to growth inhibition by TGF-β [16]. Thus, loss of function of both genes could simultaneously affect two major proliferation control pathways. It is possible that BP patients with p15 and p16 promoter DNA methylation are more likely to progress to AML, MDS and lymphoma.

Although homozygous deletions of p15 and p16 genes are common in leukemia and lymphoma, aberrant DNA methylation is the most frequent form of inactivation of these two genes [17]. Hypermethylation of p15 was an almost universal finding in AML

Table 4
Methylation levels of p15 in BP patients compared with controls.

	CpG site, mean ± SD (%)								
	1	2	3	4	5	6	7	8	Average
BP ($n = 11$)	2.7 ± 1.1	4.5 ± 1.2	12.6 ± 3.7	4.7 ± 1.7	6.3 ± 1.4	6.7 ± 1.5	7.7 ± 2.1	4.8 ± 2.0	6.3 ± 1.2
Control ($n = 8$)	3.6 ± 1.3	4.4 ± 1.5	10.8 ± 2.0	5.5 ± 1.7	7.1 ± 1.4	7.3 ± 1.4	8.4 ± 1.3	6.4 ± 2.2	6.5 ± 1.3

Table 5
Methylation levels of p16 in BP compared with controls.

	CpG site mean ± SD (%)										
	1	2	3	4	5	6	7	8	9	10	Average
BP ($n = 10$)	9.7 ± 4.2	21.7 ± 3.5	12.0 ± 3.6[a]	18.0 ± 3.5	13.7 ± 4.2	9.9 ± 3.2	12.0 ± 2.2	10.0 ± 3.2	9.7 ± 2.7	7.4 ± 2.5	12.4 ± 4.4
Control ($n = 4$)	7.0 ± 3.0	19.0 ± 2.0	8.7 ± 1.2	15.7 ± 2.5	11.3 ± 2.9	9.5 ± 1.3	12.8 ± 2.1	11.0 ± 0.8	10.5 ± 1.0	7.3 ± 0.5	11.3 ± 3.7

[a] $p < 0.05$ (*t*-test).

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and also occurred frequently in MDS and ALL [17]. Hypermethylation of p16 was found in AML [7], non-Hodgkin lymphoma [17] and B-lymphoproliferative disorders [18]. In our study, the average methylation level of p16 is higher in the BP group compared with the controls (Table 5). Interestingly, there was significant negative correlation between the levels of p16 mRNA and promoter methylation at the fourth CpG site (Pearson's $r = -0.88$, $p < 0.05$). The fourth CpG site had higher methylation levels in BP patients, and is located within the consensus binding sequence for olfactory neuron-specific transcription factor (Olf-1). Olf-1 is involved in the cell-specific expression of olfactory marker protein [19]. An isoform of Olf-1, early B-cell factor (EBF), plays a critical role in B-cell lymphopoiesis via interacting with the Olf-1 half-site TCCYYR [20]. We note that the methylation level of the fourth CpG site was higher in the BP group than in controls (18.0%, 15.7%, respectively), but the difference is not significant. It suggests that the observation in this study is preliminary and need to be confirmed in a larger number of samples. It is worth noting that both BP patients and control subjects examined in this study had more than 20-years exposure to benzene. Average p16 promoter methylation levels in these two groups (12.4% and 11.3%) are significantly higher than that reported in non-benzene exposure individuals (1–5%) [21,22]. Since we cannot completely rule out the possibility that confounding factors and/or different assay platforms resulted in the observed differences, additional study is needed to demonstrate the association between long-term benzene exposure and high levels of DNA methylation.

Until now, the mechanism by which benzene effects DNA methylation remains unclear. Benzene can induce DNA oxidative damage [23]. DNA damage occurring at a gene promoter region may be one key factor in inducing epigenetic gene silencing in association with abnormal CpG island DNA methylation [24]. Upon introduction of double strand breaks at gene promoter region, several factors, such as stress-related protein SIRT1 and DNA methyltransferases that play a role in gene silencing, are recruited to the break [24], binding to unrepaired lesions or mispairs [25], and retention of some of these factors can lead to sustained silencing. Although the basic mechanisms underlying promoter hypermethylation and gene silencing are still to be elucidated, the important translational implications of DNA modification are already receiving increasing attention. Monitoring DNA methylation status can be used in early tumor detection and prognosis prediction, and can be instrumental in the development of novel prevention and treatment strategies that rely on the potentially reversible nature of epigenetically mediated altered gene function [26]. Further in-depth studies, utilizing large number of samples, are needed to fully understand the molecular mechanism involved in tumor-suppressor gene inactivation in benzene-related diseases. These studies will have important implication in the prevention of benzene poisoning and BP-associated leukemia.

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