Changes in DNA Methylation Patterns in Subjects Exposed to Low-Dose Benzene

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Abstract

Aberrant DNA methylation patterns, including global hypomethylation, gene-specific hypermethylation/hypomethylation, and loss of imprinting (LOI), are common in acute myelogenous leukemia (AML) and other cancer tissues. We investigated for the first time whether such epigenetic changes are induced in healthy subjects by low-level exposure to benzene, a widespread pollutant associated with AML risk. Blood DNA samples and exposure data were obtained from subjects with different levels of benzene exposure, including 78 gas station attendants, 77 traffic police officers, and 58 unexposed referents in Milan, Italy (personal airborne benzene range, <6–478 μg/m^3). Bisulfite-PCR pyrosequencing was used to quantitate DNA methylation in long interspersed nuclear element-1 (*LINE-1*) and *Alu*I repetitive elements as a surrogate of genome-wide methylation and examine gene-specific methylation of *MAGE-1* and *p15*. Allele-specific pyrosequencing of the *H19* gene was used to detect LOI in 96 subjects heterozygous for the H19 imprinting center G/A single-nucleotide polymorphism. Airborne benzene was associated with a significant reduction in *LINE-1* (−2.33% for a 10-fold increase in airborne benzene levels; $P = 0.009$) and *Alu*I (−1.00%; $P = 0.027$) methylation. Hypermethylation in *p15* (+0.35%; $P = 0.018$) and hypomethylation in *MAGE-1* (−0.49%; $P = 0.049$) were associated with increasing airborne benzene levels. LOI was found only in exposed subjects (4 of 73, 5.5%) and not in referents (0 of 23, 0.0%). However, LOI was not significantly associated with airborne benzene ($P > 0.20$). This is the first human study to link altered DNA methylation, reproducing the aberrant epigenetic patterns found in malignant cells, to low-level carcinogen exposure. [Cancer Res 2007;67(3):876–80]

Introduction

Exposure to benzene, a widespread airborne pollutant emitted from traffic exhaust fumes and cigarette smoking, has been consistently associated with acute myelogenous leukemia (AML), but the mechanisms relating benzene to AML risk are still unclear (1). Aberrant DNA methylation patterns, including global hypomethylation, gene-specific hypermethylation or hypomethylation, and loss of imprinting (LOI), are common in AML and other cancer tissues. Global genomic DNA methylation levels tend to decrease in each step of the progression from normal to AML cells as also shown for other cancers (2). Gene-specific hypomethylation has also been reported in cancer cells, including decreased methylation of *MAGE-1*, a gene hypomethylated in malignant cells (3, 4). In AML, gene-specific hypermethylation shows a specific pattern, including frequent methylation and inactivation of the *p15* tumor suppressor gene (5, 6). DNA methylation is responsible for imprinting in mammal cells or at least reflects the imprinted state of a locus produced during gametogenesis that causes specific genes to be expressed either by the paternally or maternally inherited chromosome. LOI represents an early epigenetic event in leukemogenesis that was found in blood from subjects with AML and myelodysplastic syndrome but not in blood or hematopoietic progenitor cells from normal individuals (7).

In the present study, we investigated the effects of low-dose benzene exposure on DNA methylation using peripheral blood DNA from subjects with well-characterized benzene exposure (8). The investigation was designed to evaluate (*a*) DNA methylation changes in *Alu*I and long interspersed nuclear element-1 (*LINE-1*) repetitive elements as a surrogate of genome-wide methylation, (*b*) promoter methylation of *p15* and *MAGE-1*, and (*c*) changes in allele-specific methylation of *H19* to detect LOI.

Materials and Methods

Subjects and Exposure Assessment

The study included 78 gasoline filling attendants and 77 urban traffic officers exposed to low-benzene levels in Milan, Italy (8). Fifty-seven office workers from the same area, frequency matched by gender, age, and smoking to the exposed groups, were used as referents (Table 1). Personal exposure to airborne benzene was higher in gas station attendants than in traffic officers, whereas levels were lowest in the reference group (Table 1). Benzene exposure was determined by a passive sampler (stainless steel tube, internal diameter of 9 mm, length of 90 mm) containing Chromosorb 106, worn by the study subjects near the breathing zone during the work shift. Benzene was determined by thermal desorption followed by gas chromatography/flame ionization detector analysis. Written informed consent was obtained from the study subjects, and approval was granted by the local Institutional Review Board.

Bisulfite Treatment

DNA (1 μg) was denatured in 50 μL of 2 mol/L NaOH for 20 min at 37°C. Then, 30 μL of freshly prepared 10 mmol/L hydroquinone and 520 μL of 3 mol/L sodium bisulfite (Sigma-Aldrich, St. Louis, MO) at pH 5.0 were

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Table 1. Characteristics and airborne benzene concentrations in the referent and benzene-exposed groups

	Referents	Benzene-exposed subjects		*P**
	Office workers (*n* = 57)	Urban traffic officers (*n* = 77)	Gas station attendants (*n* = 78)	
Mean age, y (min-max)	39 (25–66)	32 (24–48)	42 (19–74)	0.39
Gender, *n* (%)				
Male	38 (67)	47 (61)	69 (88)	
Female	19 (33)	30 (39)	9 (12)	0.30
Cigarette smoking, *n* (%)				
Nonsmoker	26 (46)	40 (52)	30 (38)	
Former smoker	8 (14)	9 (12)	16 (21)	
Current smoker	23 (40)	28 (36)	32 (41)	0.96
Cigarettes/day, *n* (SD)	14.3 (9.8)	14.7 (6.8)	16.9 (7.6)	0.27
Smoking, pack-years (SD)	7.4 (10.4)	5.0 (8.0)	14.0 (18.0)	0.72
Urinary cotinine, ng/mL (SD)	266 (434)	464 (784)	432 (642)	0.38
Median personal benzene exposure, $\mu g/m^3$ (IQR)	6.0 (<6–13.6)	22 (19–31)	61 (40–132)	<0.001

Abbreviations: IQR, interquartile range; SD, standard deviation.
*Test for differences between referents and benzene-exposed subjects.

added and mixed. The samples were incubated at 50°C for 16 h. The bisulfite-treated DNA was isolated using Wizard DNA Clean-Up System (Promega, Madison, WI). The DNA was eluted by 50 μL of warm water, and 5.5 μL of 3 mol/L NaOH were added for 5 min. The DNA was ethanol precipitated with glycogen as a carrier and resuspended in 20 μL water. Bisulfite-treated DNA was stored at −20°C until use.

PCR and Pyrosequencing

DNA methylation was quantitated using bisulfite-PCR and pyrosequencing (9). In brief, the samples were bisulfite treated and PCR amplified (see Table 2 for PCR primers and conditions). A biotin-labeled primer was used to purify the final PCR product using Sepharose beads. The PCR product was bound to Streptavidin Sepharose High Performance (Amersham Biosciences, Uppsala, Sweden), and the Sepharose beads containing the immobilized PCR product were purified, washed, denatured using a 0.2 mol/L of NaOH solution, and washed again using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Inc., Westborough, MA) as per the manufacturer's recommendations. Then, 0.3 μmol/L of pyrosequencing primer was annealed to the purified single-stranded PCR product and pyrosequencing was done using the PSQ HS96 Pyrosequencing System

Table 2. Primers and PCR conditions for DNA methylation analyses

Sequence ID	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Sequencing primer (5′ to 3′)	PCR conditions
Global DNA methylation markers				
*Alu*I	Biotin-TTTTTATTAAAAATATAAAAATT	CCCAAACTAAAATACAATAA	AATAACTAAAATTACAAAC	96°C for 90 s, 43°C for 60 s, 72°C for 120 s (40 cycles)
LINE-1	TTTTGAGTTAGGTGTGGGATATA	Biotin-AAAATCAAAAAATTCCCTTTC	AGTTAGGTGTGGGATATAGT	95°C for 30 s, 50°C for 30 s, 72°C for 30 s (35 cycles)
Gene-specific methylation				
MAGE-1	Biotin-TATTGTGGGGTAGAGAGAAG	AAATCCTCAATCCTCCCTCAA	GGTTTTTATTTTGAGGGA	95°C for 30 s, 50°C for 30 s, 72°C for 30 s (35 cycles)
p15	GTTTTTTTTTAGAAGTAATTTAGG	Biotin-CCTTCTACRACTTAAAACC	GTTAGGAAAAGTT	95°C for 30 s, 50°C for 30 s, 72°C for 30 s (35 cycles)
LOI				
H19 (1st PCR)	GGAGTTGTGTTTTGGGATAGATGT	AAACAATAAAATATCCCAATTCCA	—	94°C for 3 min (1 cycle), 94°C for 30 s, 53°C for 30 s, 72°C for 30 s (30 cycles), 72°C for 5 min (1 cycle)
H19 (nested PCR)	GTTTTTATGAGTGTTTTATTTTTAGATC	Biotin-CACATAAATATTTCTAAAAACTTCTCC	(C allele) GAATTTTAGTTG (A allele) GAATTTTAGTTT	94°C for 3 min (1 cycle), 94°C for 30 s, 62°C for 30 s, 72°C for 30 s (30 cycles), 72°C for 5 min (1 cycle)

(Pyrosequencing). The degree of methylation was expressed for each DNA locus as percentage methylated cytosines over the sum of methylated and unmethylated cytosines. We used non-CpG cytosine residues as built-in controls to verify bisulfite conversion. Each marker was tested in three replicates and their average was used in the statistical analysis.

Repetitive elements *Alu*I and *LINE-1*. *Alu*I and *LINE-1* element PCR was used for pyrosequencing-based methylation analysis using previously published methods (10) with the following modifications. A 25 μL PCR was carried out in 10 μL Eppendorf HotMaster Taq DNA kit (Eppendorf, Westbury, NY), 1 pmol biotinylated forward primer, 1 pmol reverse primer, 50 ng bisulfite-treated genomic DNA, and water.

***MAGE-1* and *p15*.** To measure methylation of the *MAGE-1* and *p15* promoters, a 25 μL PCR was carried out in 10 μL Eppendorf HotMaster Taq DNA kit, 10 pmol forward primer, 10 pmol reverse primer, 50 ng bisulfite-treated genomic DNA, and water.

H19 (LOI Assay)

H19 allele-specific methylation was done as a surrogate marker of LOI. An article describing this newly developed technique is under consideration (11). This technology relies on two individual primers that are directed against one allele but not the other of a single-nucleotide polymorphism (SNP) so that nucleotide extension analysis by pyrosequencing can be done on each allele individually. Briefly, the protocol involves bisulfite treatment of DNA, PCR amplification, and then pyrosequencing of the CTCF-binding domain using allele-specific primers.

H19 genotyping. First, heterozygosity for the G/A SNP within the *H19* imprinting center (rs2071094; dbSNP build 124; Genbank AF125183, nucleotide 8008) was determined by pyrosequencing. A 135-bp region was amplified on human genomic DNA with the PCR primers 5′-GGTCT-CACCGCCTGGATC-3′ and 5′-biotin-GACCCGGGACGTTTCCAC-3′ and genotyped with the sequencing primer 5′-ACAGCCCGAGCCCGC-3′ using standard pyrosequencing.

LOI assay. A nested PCR was done on DNA from heterozygote subjects. Bisulfite-modified DNA (2 μL) was amplified in a primary PCR followed by a second nested PCR using 1 μL of the primary PCR product. PCR was done in 25 μL volume using 10 pmol of each primer and the Eppendorf HotMaster Taq DNA kit at reagent concentrations as per the manufacturer's instructions.

Statistical Analysis

Student's *t* test and ANOVA were used to test for differences in methylation levels by categorical variables. We used linear regression analysis to assess the association of logarithmic airborne benzene, or other continuous variables, with DNA methylation. All statistical tests were two sided.

Results

DNA methylation by occupational groups. *LINE-1* methylation was lower in the benzene-exposed subjects compared with the referents (Table 3). The average percentage of methylated cytosines in *LINE-1* sequences was 62.2% (SD, 6.6) in gas station attendants, 62.3% (SD, 6.5) in traffic officers, and 65.7% (SD, 5.2) in referents ($P = 0.003$ for differences among the occupational groups). *Alu*I methylation showed a moderate nonsignificant decrease in gas station attendants (mean, 26.4%; SD, 2.5), with no change in traffic officers (mean, 27.5%; SD, 3.9), compared with referents (mean, 27.3%; SD, 3.4; $P = 0.12$).

Gene-specific analysis showed low *p15* methylation in the referent group (mean, 1.2; SD, 0.9) that increased significantly in traffic officers (mean, 2.0; SD, 1.0) and gas station attendants (mean, 2.0; SD, 1.2; $P < 0.001$). *MAGE-1* was highly methylated and showed no differences in the three groups (Table 3).

DNA methylation and airborne benzene exposure. *LINE-1* and *Alu*I methylation decreased with increasing airborne benzene exposure levels (Fig. 1*A* and *B*). This decrease, estimated for a 10-fold increase in airborne benzene, was equal to 2.33% [95% confidence interval (95% CI), −4.06 to −0.60; $P = 0.009$] for *LINE-1* and 1.00% (95% CI, −1.87 to −0.11; $P = 0.027$) for *Alu*I.

Airborne benzene was associated with increased methylation in p15 (+0.35%; 95% CI, 0.06–0.64; $P = 0.018$; Fig. 1*C*). *MAGE-1* exhibited decreased methylation (Fig. 1*D*), but the association with airborne benzene was borderline significant (−0.49%; 95% CI, −0.96 to −0.00; $P = 0.049$).

In multivariable regression analyses, the estimated changes in DNA methylation did not differ when several combinations of possible confounders, including age, sex, smoking (never, ex, and current), urine cotinine, cigarettes/day, pack-years, years since smoking cessation, alcohol, metabolic polymorphisms (NQO1 and CYP2E1), and percentage lymphocytes in the differential count (data not shown).

DNA methylation exhibited no consistent association with smoking status, number of cigarettes/day, urinary cotinine, pack-years, and years from smoking cessation ($P > 0.05$ for all DNA sequences considered). Notably, *Alu*I methylation exhibited a borderline negative correlation with pack-years of smoking ($r = -0.13\%$; $P = 0.06$).

Table 3. Methylation levels (Cm%) of global and gene-specific methylation markers in the exposed groups (urban traffic officers and gas station attendants) compared with referent subjects (office workers)

	Referents		Benzene-exposed subjects			
	Office workers		Urban traffic officers		Gas station attendants	
	n	Mean (SD)	*n*	Mean (SD)	*n*	Mean (SD)
LINE-1 (Cm%)*	51	65.7 † (5.2)	74	62.3 † (6.5)	61	62.2 † (6.6)
*Alu*I (Cm%)*	57	27.3 (3.4)	76	27.5 (3.9)	72	26.4 (2.5)
p15 (Cm%)	52	1.2 ‡ (0.9)	76	2.0 ‡ (1.0)	74	2.0 ‡ (1.2)
MAGE-1 (Cm%)	56	93.4 (1.8)	74	93.6 (2.1)	76	93.4 (1.8)

Abbreviations: SD, standard deviation; Cm%, methylated cytosine percentage.
*DNA methylation of *LINE-1* and *Alu*I repeated elements was measured as a surrogate for global DNA methylation (10).
† $P = 0.003$, ANOVA test for differences among exposure groups.
‡ $P < 0.001$, ANOVA test for differences among exposure groups.

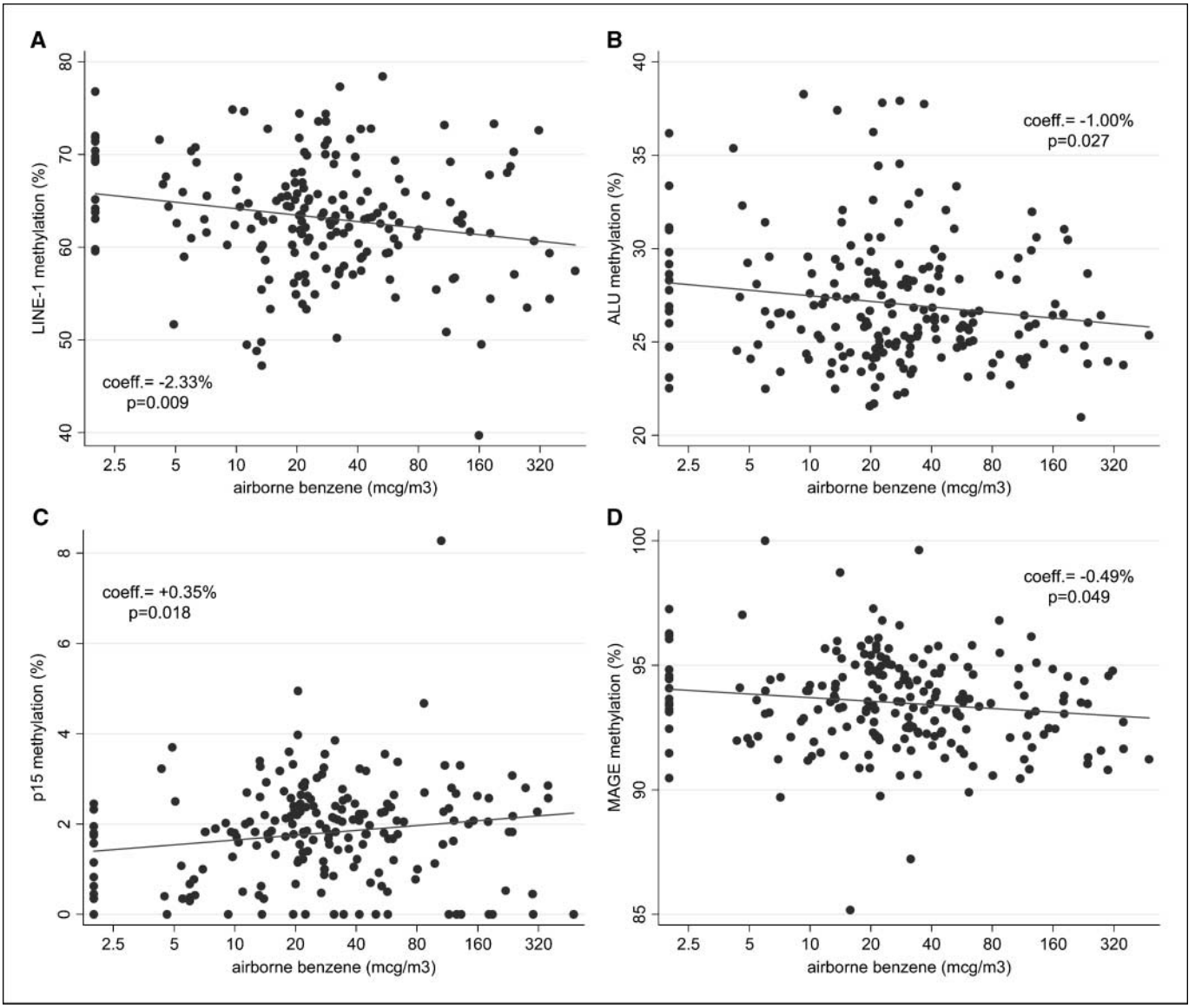

Figure 1. Subjects' airborne benzene exposure associated with *LINE-1* (*A*) or *Alu*I (*B*) sequences used as a surrogate for global methylation and gene-specific methylation of *p15* (*C*) and *MAGE-1* (*D*). Individual data points and univariate regression lines. Coefficients represent the change in percentage methylated cytosines estimated for a 10-fold increase in airborne benzene concentrations.

Loss of imprinting. We determined allele-specific DNA methylation of *H19* as a marker for LOI. Because this assay is applicable only to subjects who are heterozygotes in the *H19* G/A SNP (rs2071094; dbSNP build 124) used for allele-specific detection, only 96 subjects who had the G/A genotype were available for this test. We estimated the average methylation of the methylated allele (mean, 74.5%; SD, 20.5), of the unmethylated allele (mean, 2.85%; SD, 4.62), and of their difference (mean, 71.7%; SD, 21.9). None of these quantities was associated with airborne benzene ($P > 0.20$). We identified, however, four subjects that showed aberrant methylation patterns. Three of them showed both an increase of methylation in the unmethylated allele and a decrease in the methylated allele (subject 1: methylated, 22.9% and unmethylated, 9.0%; subject 2: methylated, 17.6% and unmethylated, 9.3%; subject 3: methylated, 25.2% and unmethylated, 12.1%). One more subject had one normally unmethylated allele with a large decrease in methylation of the methylated allele (subject 4: methylated, 16.5% and unmethylated, 0%). All of these four subjects were in the benzene-exposed groups, with airborne benzene levels equal to 34, 276, 47, and 28 $\mu g/m^3$, respectively.

Discussion

In vitro and animal studies have shown that carcinogenic agents induce DNA methylation changes in normal tissues similar to those found in malignant cells (12–15). In our study, we observed DNA methylation alterations in subjects exposed to low-level airborne benzene that were qualitatively comparable with those observed in AML and other malignancies. To the best of our knowledge, this is the first study in humans to link altered DNA methylation patterns to exposure to any carcinogen at levels that are common in Western countries.

We used DNA methylation analyses of *LINE-1* and *Alu*I repeated sequences to evaluate global methylation. Due to the heavy

methylation of repetitive elements, these assays, which are easier to do than previous methods to quantitate total genomic 5-methylcytosine, can detect decreases in DNA methylation and serve as a surrogate for global methylation (10). Our results showed an exposure-related decrease in the methylation of *LINE-1* and *Alu*I repeated sequences. This observation is consistent with the global hypomethylation frequently observed in AML and other malignant cells, which has been linked to higher chromosomal instability and aberrant activation of cellular genes (2). The alterations we observed were small in size and may represent an early deviation induced by the exposure from normal methylation patterns.

In our study, *p15* promoter methylation in exposed individuals was nearly twice as high as that observed in the referent unexposed subjects. AML is one of the few neoplasms that show hypermethylation of *p15*, likely contributing to deregulated cell proliferation (5). The *p15* promoter shows low or no methylation in normal cells and is hypermethylated as the cell progresses through the multistep process leading to the development of the full malignant phenotype (5, 16). *MAGE-1*, which is usually heavily methylated in normal tissues, tended to exhibit lower methylation in association with airborne benzene levels in our study. This finding may indicate the initial activation of biological processes in exposed subjects, potentially preceding the profound *MAGE-1* hypomethylation observed in several tumors of various histologic types, including leukemias (3, 4).

LOI, also diffusely seen in malignant cells, was not significantly associated with benzene exposure in our study. Our LOI assay allows for the determination of LOI only in *H19* heterozygote subjects, thus limiting the statistical power for this analysis. However, the finding that changes in allele-specific methylation were present in four exposed subjects and no controls warrants further investigations.

Our study was based on quantitative analysis of DNA methylation using pyrosequencing methodology, which is highly reproducible and accurate at measuring small changes in DNA methylation. DNA methylation analysis was repeated thrice on each sample to minimize the assay variability.

The mechanism by which benzene interferes with DNA methylation remains unclear. Benzene enhances nitric oxide production in the bone marrow, possibly inducing a posttranscriptional increase in the activity of DNA methyltransferases (17). In addition, reactive oxygen species and oxidative DNA damage produced by benzene may reduce binding affinity of the methyl-CpG binding protein 2, thereby resulting in epigenetic alterations (18). At the same time, DNA strand breaks induced by benzene exposure may cause DNA methyltransferases to bind with higher affinity at specific sites (19).

Some of our findings open further questions warranting future investigation. *LINE-1* element methylation changes had a stronger correlation with benzene exposure than *Alu*I repetitive elements. Hypothetically, this could be explained as methylation of *LINE-1* and *Alu*I is controlled through different mechanisms (20). Alternatively, the lower concentration of CpG sites in *Alu*I elements may result in lower sensitivity for the use of *Alu*I methylation as a global DNA methylation marker. We found no consistent association of DNA methylation with smoking, which is the largest source of benzene in subjects without occupational exposure. However, tobacco smoke is a mixture of a high number of agents with complex synergic effects that could level off the effect due to tobacco-derived benzene. In addition, multivariable models indicated that smoking was not a confounder of the relation between benzene and DNA methylation.

In spite of the clear dose-response relationship between DNA methylation and airborne benzene, indicating that benzene exposure was the main factor responsible for DNA methylation changes in our study, we cannot exclude that other traffic pollutants, including particulate matter, polycyclic aromatic hydrocarbons, CO, SO_2, NO_2, toluene, or xylene, may have contributed to the observed changes.

In conclusion, our study showed for the first time that low-level benzene exposure is associated in normal subjects with DNA methylation changes that reproduce the aberrant epigenetic patterns found in malignant cells. Additional studies are required to better define the mechanisms by which benzene and other carcinogens produce such alterations.

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