Global and MGMT promoter hypomethylation independently associated with genomic instability of lymphocytes in subjects exposed to high-dose polycyclic aromatic hydrocarbon

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Abstract Global hypomethylation, gene-specific methylation, and genome instability are common events in tumorigenesis. To date, few studies have examined the aberrant DNA methylation patterns in coke oven workers, who are highly at risk of lung cancer by occupational exposure to polycyclic aromatic hydrocarbons (PAHs). We recruited 82 PAH-exposed workers and 62 unexposed controls, assessed exposure levels by urinary 1-hydroxypyrene, and measured genetic damages by comet assay, bleomycin sensitivity, and micronucleus assay. The PAHs in coke oven emissions (COE) were estimated based on toxic equivalency factors. We used bisulfite-PCR pyrosequencing to quantitate DNA methylation in long interspersed nuclear element-1 (LINE-1) and O^6-methylguanine-DNA methyltransferase (MGMT). Further, the methylation alteration was also investigated in COE-treated human bronchial epithelial (16HBE) cells. We found there are higher levels of PAHs in COE. Among PAH-exposed workers, LINE-1 and *MGMT* methylation levels (with CpG site specificity) were significantly lowered. LINE-1, *MGMT,* and its hot CpG site-specific methylation were negatively correlated with urinary 1-hydroxypyrene levels ($r = -0.329$, $p < 0.001$; $r = -0.164$, $p = 0.049$ and $r = -0.176$, $p = 0.034$, respectively). In addition, LINE-1 methylation was inversely associated with comet tail moment and micronucleus frequency, and a significant increase of micronucleus in low MGMT methylation group. In vitro study revealed that treatment of COE in 16HBE cells resulted in higher production of BPDE-DNA adducts, LINE-1 hypomethylation, hypomethylation, and suppression of *MGMT* expression. These findings suggest hypomethylation of LINE-1 and *MGMT* promoter could be used as markers for PAHs exposure and merit further investigation.

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental and occupational pollutants, which have

been classified as Group 1 carcinogens by IARC (IARC 1983). Coke oven workers are the representative PAH-exposed population who are regularly exposed to coke oven emissions (COE). Epidemiological studies have shown that subjects with a long-term exposure to COE are at significantly higher risk of developing lung cancer (Costantino et al. 1995). Studies in occupational populations provide great opportunities to understand the mechanisms through which exogenous agents cause cancer.

As a complex disease, cancer arises from both genetic and epigenetic errors. While the importance of genetic alterations in cancer, including chromosomal instability and genetic mutations, is evident, aberrant epigenetic regulation in malignant cell transformation is still poorly understood. Aberrant DNA methylation is one of the best known epigenetic changes in human cancers (Jones and Baylin 2002). Aberrant promoter methylation of a series of tumor suppressor genes has been detected in blood leukocyte DNA from lung cancer patients and healthy subjects exposed to carcinogens (Bowman et al. 2009; Chanda et al. 2006). The O^6-methylguanine-DNA methyltransferase (MGMT) is a DNA mismatch repair protein that removes a methyl group from the O^6-position in guanine and transfers it to cysteine residue at codon 145 within its own sequence without base excision (Pegg and Byers 1992). MGMT protein plays an important role in removing major premutagenic lesions induced by O^6-methylating agents, preventing cytotoxicity (Kaina et al. 2007). However, whether ambient PAHs can induce DNA methylation alterations in *MGMT* gene, which may be involved in air pollution-related lung carcinogenesis, has not been examined.

Genomic or global hypomethylation is believed to occur early in tumorigenesis, potentially facilitating the genomic instability currently thought to be necessary for cancer development (Suter et al. 2004). Long interspersed nuclear element 1 (LINE-1) represents a family of non-long terminal repeat retroposons that are interspersed all over the genomic DNA and account for approximately 20 % of the human genome (Lander et al. 2001). The level of LINE–1 methylation is regarded as a surrogate of global DNA methylation for its high frequency in the genome (Saito et al. 2010; Weisenberger et al. 2005). Quantitative methylation analysis on LINE-1 in lymphocytes is a useful marker for evaluating the effect of environmental and occupational pollutants on the genome (Nelson et al. 2011).

In the present study, we investigated the effects of chronic high-dose PAHs exposure on global and gene-specific methylation modification by quantitating LINE-1 and *MGMT* levels in peripheral lymphocyte DNA of subjects with well-characterized PAHs exposure. We also examine the relationship between PAH-induced DNA methylation and DNA damage, mutagen sensitivity, and chromosome instability occurrence by comprehensive

biomarkers in lymphocytes. In addition, we collected COE samples and examine whether aberrant methylation changes were early events on PAHs exposure by studying the mode of action in COE-treated cells. Our results will be reliable for population-based study combined with cellular model study.

Materials and methods

Study population

All the subjects came from a state-run steel company located in northeast China. Details of the study population and PAHs exposure have been described previously (Cheng et al. 2009). In brief, we enrolled 82 male PAH-exposed workers from a coking plant and 62 controls matched by age and gender to the exposed group. All participants were interviewed by an occupational physician using a detailed questionnaire that included demographic information, educational level, smoking history, alcohol consumption, occupational history of exposure, and personal medical history. Finally, 15 ml of 4-day shift-end urine and 4 ml of venous blood were collected from each subject. This study was approved by the Research Ethic Committee of the National Institute for Occupational Health and Poison Control, Chinese Center for Disease Control and Prevention, and informed consent was obtained from each participant.

Exposure and effect biomarkers analysis

Urinary 1-hydroxypyrene (1-OHP), MN, and comet assay were done according to the methods as described previously (Leng et al. 2004). We used a modified version of the comet assay for bleomycin (BLM) sensitivity measurement (Schmezer et al. 2001). Lymphocytes were treated with 8 μg/ml BLM (Sigma-Aldrich) at 37 °C for 30 min, and the difference in Olive tail moment (TM) measured before and after BLM treatment reflects sensitivity to the mutagens.

Bisulfite treatment and pyrosequencing

Genomic DNA of subjects was extracted with phenol/chloroform mixture. We did bisulfite conversion on 1 μg genome DNA using the EpiTect bisulfite kit (Qiagen) according to the manufacturer's instructions. The PCR-pyrosequencing method to quantitate LINE-1 and *MGMT* promoter methylation was described previously (Bollati et al. 2007; Pavanello et al. 2009). In brief, 40 ng bisulfite-treated genomic DNA was amplified in 40 μl PCR system including 5 pmol forward and reverse primers, 0.2 μmol/l

of dNTPs, 1 × PCR buffer (Qiagen), and 2.5 U HotStar polymerase (Takara). After purification of PCR products using Sepharose beads on PyroMark Vacuum Prep Worktstation (Qiagen), pyrosequencing was performed using the PyroMark Q96 ID System (Qiagen) according to manufacturer's protocol. PCR and pyrosequencing primers of LINE-1 and MGMT are given in Supplementary Table S1, and nine individual CpG sites in *MGMT* promoter are shown in Supplementary Figure S1. The built-in controls were used to verify bisulfite conversion efficiency. We tested each marker in two replicates and used their average in the statistical analyses.

COE collection, PAHs exposure, cell culture, and BPDE-DNA adduct assay

Coke oven emissions were collected by glass fiber filter at the top oven area of the coking plant. Extraction of the organic matter of COE (OE-COE) and priority PAHs was performed according to a standard protocol described previously (Zhai et al. 2012). OE-COE was dissolved in DMSO to a final concentration of 1 mg/ml and stored at −20 °C. The benzo[a]pyrene (BaP) equivalent (BaPeq) concentration of the individual PAHs was calculated by multiplying their concentration by their toxic equivalency factors (TEF) (Nisbet and LaGoy 1992). The human bronchial epithelial cell line (16HBE14^{O-}, abbreviated as 16HBE) (Cozens et al. 1994) was a gift from Dr D.C. Gruenert (University of California, San Francisco, USA). Before chemical treatment, cells were plated in 100-mm culture dishes at a density of 5×10^4/dish. The 16HBE cells were treated by 1 μmol/l BaP (Sigma-Aldrich) for 48 h to induce CYP1A1 expression (Pang et al. 2008) and then exposed continuously to OE-COE for 5 days at the concentrations of 2.5, 5, 10, and 20 μg/ml, or vehicle (DMSO) at 37 °C in 5 % serum media. After 48 h treatment, cells were washed twice using 1 × PBS, and fresh OE-COE media was added. Cell DNA was extracted with DNA purification kit (Promega). DNA samples were diluted to 2 μg/ml by cold PBS, and benzo(a)pyrene diolepoxide (BPDE)-DNA adducts were measured using the BPDE-DNA Adduct Kit (Cell Biolabs). The assay was done according to the protocol, and the cells treated with DMSO were used as a control.

Reverse transcription-PCR

RNA was extracted with TRIzol Reagent (Invitrogen, USA), and cDNA was synthesized using the Reverse Transcription System (TaKaRa, Dalian,). PCR reactions were carried out with Taq polymerase (TaKaRa, Dalian). The primers were shown in Supplementary Table S1. The PCR products were analyzed on a 2 % agarose gel and visualized by staining with ethidium bromide.

Immunobloting analysis

For Western blot analysis of MGMT protein expression, 70 μg protein per sample was separated by 8–16 % SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes by electroblotting. The membranes were blocked in 5 % milk powder in PBS-T for 2 h and probed with antibodies against MGMT (1:2000 dilution; Proteintech) overnight at 4 °C or β-actin (1:10000 dilution; Sigma-Aldrich) for 4 h at 4 °C.

Statistical analysis

Medians and interquartile ranges (IQR) were calculated for LINE-1 and *MGMT* promoter methylation continuous variables. Ln-transformed 1-OHP, Olive TM, BLM sensitivity, and MN values were used in the analysis. Statistical comparisons for methylation status were made between various groups using the Mann–Whitney U test and Kruskal–Wallis test. Comparison of levels of Olive TM, BLM sensitivity, and MN frequency between different LINE-1 and *MGMT* promoter methylation status were by Student's t test. Correlation between methylation level and urinary 1-OHP concentration was analyzed using Spearman correlation. We also carried out linear regression analyses, modeling each methylation on ln-transformed Olive TM, BLM sensitivity, and MN frequency. The independent variables used in multivariable linear regression models were selected a priori and included general characteristics potentially associated with biomarkers modification. All statistics were two-sided and performed using SPSS software (SPSS 15.0, Chicago, USA).

Results

PAHs exposure and effect biomarkers in subjects

Polycyclic aromatic hydrocarbons-exposed subjects were middle-aged (mean ± SD: 41.73 ± 6.63 years), similar to that of the unexposed controls, and heavy exposed to PAHs as their urinary 1-OHP were significantly higher than that of the controls (median = 9.31 μg/l, IQR = 2.77–22.77 versus 1.42 μg/l, IQR 0.7–2.34; $p < 0.001$) (Supplementary Table S2). PAH-exposed workers exhibited significantly higher levels of Olive TM ($p < 0.001$), BLM sensitivity ($p = 0.015$), and MN ($p < 0.001$) than controls.

Levels of global and *MGMT* methylation among PAH-exposed workers

Overall, the median 5 %-mC of LINE −1 was significantly lower in PAH-exposed subjects compared to controls

[59 % (Interquartile range (IQR) = 58.3–59.7 %) vs. 63 % (IQR = 58.9–64.1 %), respectively ($p < 0.001$)] (Fig. 1a).

DNA methylation levels at the individual *MGMT* promoter CpG sites showed inconsistent patterns (Table 1). Of the nine CpG sites, the methylation levels of site 3, 6, 7, 8, and 9 were significantly lower than that of controls. The median level decreased by 20.5, 16.9, 21.1, 19.5, and 7.6 %, respectively; all of them have $p < 0.05$. Only the median level of methylation at CpG site 2 increased 47.9 % in PAH-exposed subjects, significantly higher than controls ($p = 0.004$). The mean methylation level decreased 14.6 % in PAH-exposed workers compared with that of controls ($p = 0.018$). The remaining three CpG sites showed no significant change in PAH-exposed workers compared with controls, indicating that other six CpG sites might be the "hot CpG sites" participating in gene regulation. We then recalculated median level (IQR) of *MGMT* promoter methylation using 6 CpGs as a dominator and found that it was decreased by 17.6 % in exposed group, which is significantly lower than that in controls ($p = 0.005$). Univariate linear regression analysis determined no influence of covariates (age, smoking, and alcohol status) on LINE-1, *MGMT* promoter methylation status (data not shown).

DNA methylation and PAHs exposure

We divided controls, workers at oven bottom and side, and workers at oven top area into the low, middle, and high PAHs exposure groups, respectively. A significant dose–effect relationship was found between different PAHs exposure groups ($p < 0.001$). The median of LINE-1 methylation

Fig. 1 PAHs exposure and DNA methylation. Levels of LINE-1 methylation in lymphocytes of PAH-exposed workers and controls (**a**). The median methylation level was 59 % in PAH-exposed workers and 63 % in controls. The difference of methylation level of LINE-1 in the low, middle, and high PAHs exposure groups (**b**). The horizontal line in the box represents the median. The lower and higher edges of box are the 25 and 75 percentiles of the data, respectively. Subjects' PAHs internal exposure associated with LINE-1 methylation (**c**), MGMT promoter methylation (**d**), and hot CpG site-specific methylation of MGMT promoter (**e**). **$p < 0.001$ compared with control (Low-PAHs exposure) group
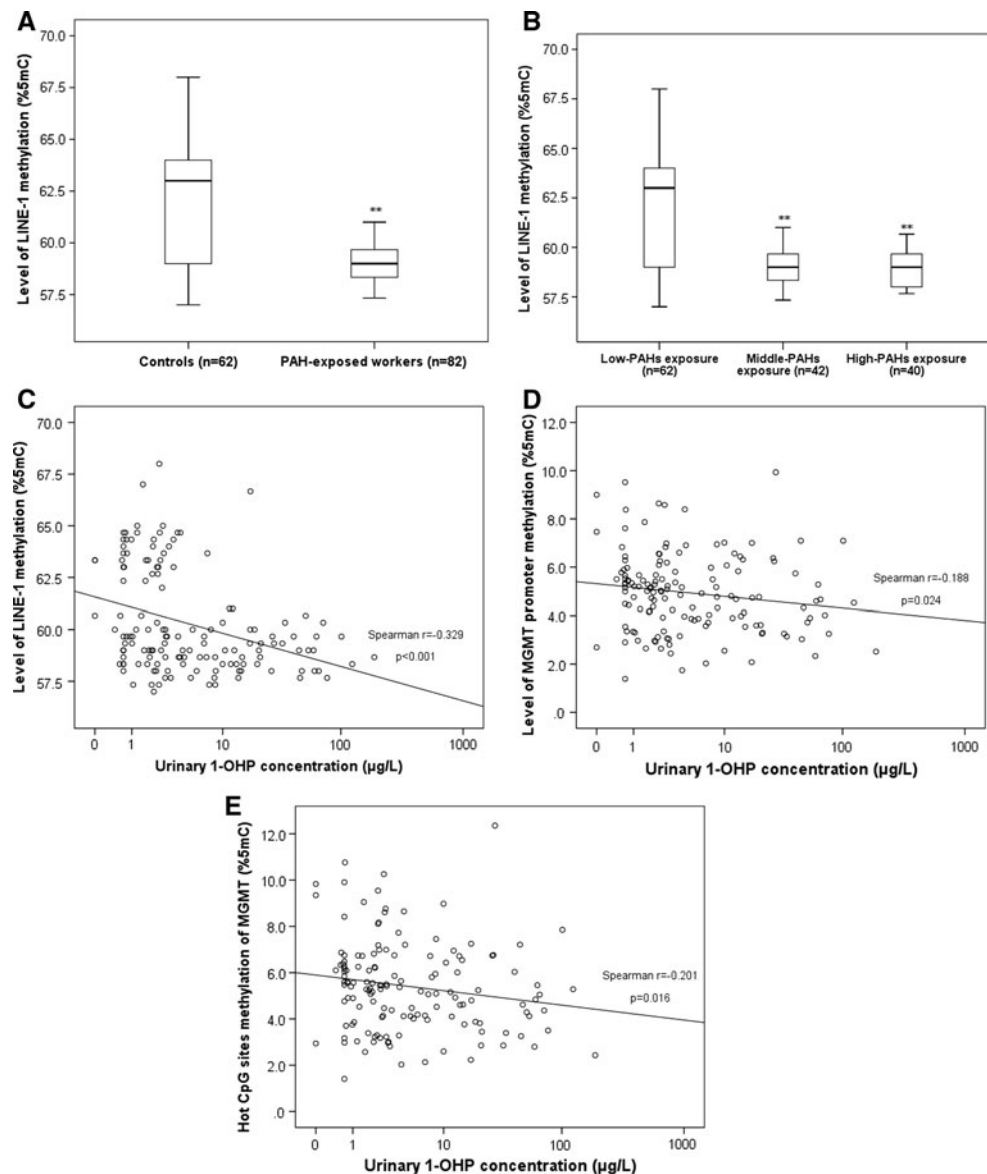
Table 1 Promoter CpG site DNA methylation of MGMT measured in PAH-exposed workers and controls

CpG sites	N	Controls			PAH-exposed workers			p^{a}
		Median (%)	IQR		N	Median (%)	IQR	
site 1	62	2.86	1.97–3.51		82	2.64	1.84–3.29	0.29
Site 2	62	1.67	1.03–2.64		82	2.47	1.78–3.13	**0.004**
Site 3	62	10.64	6.49–11.88		82	8.46	6.38-10.34	**0.009**
Site 4	62	3.95	2.65–5.17		82	4.4	3.37–5.65	0.094
Site 5	62	5.38	2.86–6.09		82	4.51	3.49–5.62	0.455
Site 6	62	3.66	2.36–4.63		82	3.04	1.91–3.73	**0.008**
Site 7	62	8.53	6.63–12.38		82	6.73	5.17–8.83	**<0.001**
Site 8	62	7.28	4.37–8.17		82	5.86	4.47–7.07	**0.014**
Site 9	62	2.63	2.31–3.44		82	2.43	1.82–2.99	**0.034**
Average	62	5.34	4.12–6.12		82	4.56	3.59–5.55	**0.018**
Average of hot CpG sites[b]	62	5.85	4.61–6.77		82	4.82	3.86–6.01	**0.005**

Bold values indicate statistical significance

[a] Mann-Whitney U test was used for statistical analysis

[b] Hot CpG sites were site 2, 3, 6, 7, 8, and 9

were 63, 59, and 59 % in low, middle, and high PAHs groups, respectively (Fig. 1b). We then investigated the *MGMT* promoter methylation and hot CpG site-specific methylaion between PAHs external exposure groups. There was a significant dose–effect relationship between the hot CpG sites of *MGMT* promoter methylation and PAHs exposure ($p = 0.017$), but borderline significant relationship for *MGMT* promoter methylation ($p = 0.054$) (Table 2).

We used urinary 1-OHP as a marker of PAHs internal exposure. The inverse correlation was found between urinary 1-OHP levels and LINE-1 methylation in a statistically significant manner ($r = -0.329$; $p < 0.001$) for all subjects (Fig. 1c). *MGMT* promoter exhibited decreased methylation in PAH-exposed workers, and the association with urinary 1-OHP concentration was significant in all subjects ($r = -0.188, p = 0.024$; Fig. 1d). But, for the hot CpG sites assay, the association was more significant ($r = -0.201, p = 0.016$; Fig. 1e), indicating that LINE-1 and hot CpG sites hypomethylation of *MGMT* gene may be more sensitive and applicable in the surveillance of epigenetic damage for PAHs.

We did not find that the *MGMT* and hot CpG site-specific methylation were directly proportional to LINE-1

methylation ($p = 0.131$ and $p = 0.093$, respectively) for all subjects (Supplemental Figure S2A and B). The median of LINE-1 and *MGMT* promoter methylation did not differ substantially between younger and older age groups, between yes and no-smoking groups, or between yes and no-alcohol using groups in PAH-exposed workers and controls (Supplemental Table S3).

DNA methylation correlated with DNA and chromosome instability

To determine the association between DNA methylation on DNA damage or chromosome instability, we conducted linear regression and multivariable regression analyses between LINE-1 methylation groups, comet TM, and BLM sensitivity or MN frequency (Fig. 2A). We chose to dichotomize the LINE-1 methylation levels by the median of LINE-1 methylation (59.33 %) in all subjects. For the DNA damage, the level of Olive TM was significantly higher in low LINE-1 methylation group (2.18 ± 1.54) than in the high group (1.69 ± 1.48, $p = 0.028$). The frequency of MN was significantly higher in low LINE-1

Table 2 MGMT and hot CpG sites methylation status analyzed by PAHs external exposure levels in all subjects

	N	MGMT		p	Hot CpG sites of MGMT		p
		Median	IQR		Median	IQR	
PAHs exposure levels[a]							
Low	62	5.34	4.12–6.13		5.85	4.61–6.77	
Medium	42	4.73	3.29–5.86		5.07	3.41–6.18	
High	40	4.4	3.74–5.25	0.054	4.69	4.03–6.01	**0.017**

Bold value indicates statistical significance

[a] Kruskal-Wallis test was used for statistical analysis

methylation group (6.34 ± 4.89 ‰) than in high group (4.67 ± 3.89 ‰, $p = 0.04$). The level of BLM sensitivity was higher in low LINE-1 methylation group (18.5 ± 8.37) than in the high group (16.7 ± 6.74), but the difference was not statistically significant ($p = 0.21$).

We then categorized each individual *MGMT* promoter methylation by median (4.93 %). Then, low MGMT methylation group had significantly higher MN frequency (6.64 ± 4.95) compared with that of the high methylation group (4.42 ± 3.71) ($p = 0.005$, Fig. 2b). The level of Olive TM and BLM sensitivity was not significantly different between the low and high MGMT methylation groups (2.11 ± 1.59 vs. 1.78 ± 1.45, $p = 0.1529$; 18.2 ± 8.29 vs. 17.1 ± 6.96, $p = 0.521$, respectively). When we adjusted age and smoking status in the multivariable linear regression, the results did not differ substantially (data not shown).

PAHs exposure and BPDE-DNA adduct in COE-treated cells

There are higher levels of PAHs in COE; PAHs components and calculated BaP equivalents are shown in Table 3. Based on the TEF values, we computed that the BaPeq of 1 ug/ml OE-COE is equivalent to 1 μmol/l BaP for the in vitro assay. The biological effect marker of COE was investigated by BPDE-DNA adduct assay. By the OE-COE 5-day long-term treatment, the BPDE–DNA adduct concentration was 39.8 and 102 ng/ml in 10 and 20 μg/ml OE-COE-treated group, respectively. But in the 5 μg/ml OE-COE or less treatment groups, the amount of BPDE-DNA adduct measured below the limit of detection (Fig. 3a).

MGMT methylation and expression induced by COE

To explore whether modification LINE-1 and *MGMT* promoter methylation contributed to PAHs carcinogenesis, we examined methylation status of LINE-1 and *MGMT* gene, and changes of MGMT mRNA and protein expression in different dose of OE-COE-treated cells. As shown in Fig. 3b, compared with control cells, the mean level of LINE-1 methylation was slightly decreased by 4.24 % in 2.5 μg/ml OE-COE-treated group and significantly decreased by 11.6, 11.6, and 10.1 % in 5, 10, 20 μg/ml treated groups, respectively. We observed strong methylated signals in vehicle-treated HBE cells. As a result, the level of *MGMT* methylation was significantly increased by 22.4 % in 2.5 μg/ml group. In contrast, there was a decrease of 37.4, 9.5, and 55.4 % in 5, 10, 20 μg/ml OE-COE-treated groups, respectively (Fig. 3c). For gene-specific methylation, low-dose COE exposure cause MGMT hypermethylation in cells and higher dose of COE cause hypomethylation.
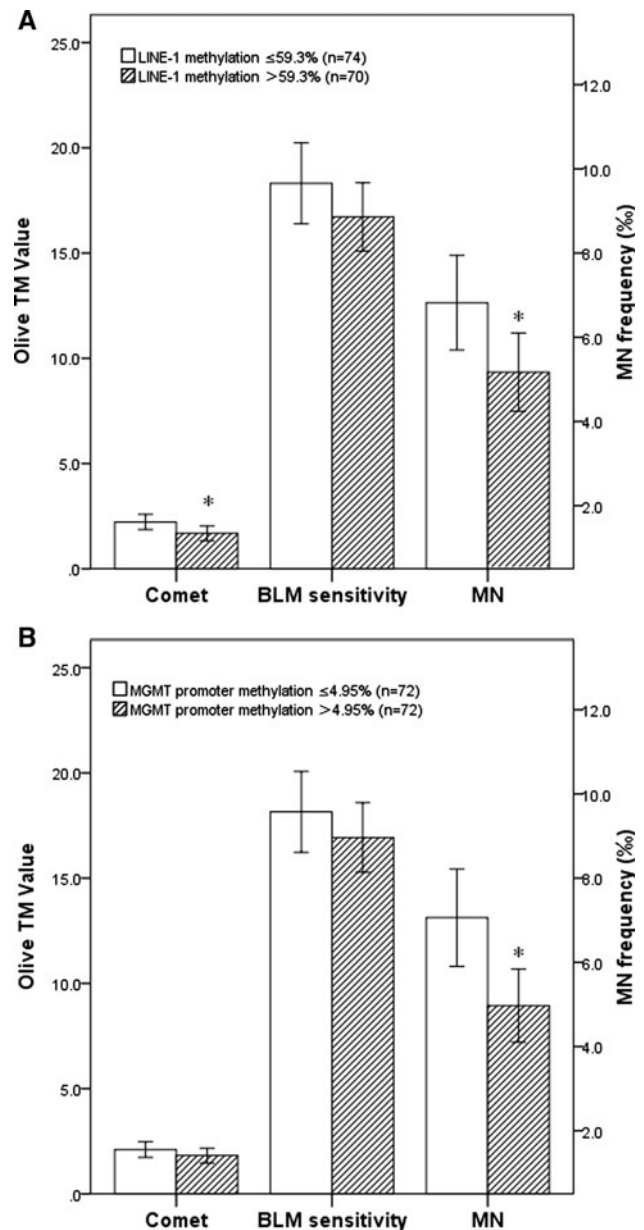
Fig. 2 Level of DNA damage, BLM sensitivity, and MN frequency in different groups of global methylation (**a**) and MGMT promoter methylation (**b**). Differences in comet TM, BLM sensitivity, and MN frequency were tested by linear regression, adjusting for relevant covariates as indicated in Materials and Methods. The data were shown as mean ± SE. *$p < 0.05$ compared with lower methylation group

We examined mRNA and protein levels of MGMT in long-term COE-treated cells. As shown in Fig. 3d, mRNA levels of MGMT were not significantly changed in 2.5, 5 μg/ml OE-COE-treated groups, while there was a reduction in both 10 and 20 μg/ml groups. The protein levels of MGMT in the cells examined were consistent with the mRNA expression. These results demonstrated that high doses of COE-treated cells down-regulates MGMT.

Table 3 Levels of PAHs and calculated BaP equivalent (BaPeq) exposure values in COE at the top site of coke oven

PAHs	Concentration in COE (μg/ml)	Ambient concentration(μg/m^3)	Toxic equivalence factor (TEF)[a]	BaPeq (μg/m^3)
Anthracene	2.98	1.1	0.01	0.011
Benzo[a]anthracene	14.1	5.22	0.1	0.522
Benzo[b]fluoranthene	12.25	4.54	0.1	0.454
Benzo[g,h,i]perylene	15.94	5.9	0.01	0.059
Benzo[k]fluoranthene	10.37	3.84	0.1	0.384
Chrysene	14.99	5.55	0.01	0.056
Dibenz[a,h]anthracene	25.16	9.32	5	46.6
Fluoranthene	19.67	7.28	0.001	0.007
Indeno[1,2,3-cd]pyrene	19.38	7.18	0.1	0.718
Phenanthrene	3.37	1.25	0.001	0.001
Pyrene	17.52	6.49	0.001	0.006
Benzo[a]pyrene	12.41	4.59	1	4.59
Tatal BaPeq				**53.41**

Bold value indicates the BaP equivalent in risk assessment

[a] TEF for PAHs are from Nisbet and Lagoy (1992)

Fig. 3 Changes of BPDE-DNA adduct, LINE-1 methylation, MGMT promoter methylation, and expression status in COE-treated 16HBE cells. After the metabolic enzymes induction, the cells were treated with OE-COE at final concentrations ranging from 2.5 to 20 μg/ml for 5 days, DMSO as vehicle control. BPDE-DNA adducts levels was tested by ELISA method (**a**). DNA methylation levels were expressed as the mean percentage of LINE-1 methylation (**b**) and MGMT promoter methylation (**c**) by pyrosequencing in bar chart. mRNA levels of and protein expression of MGMT in COE-treated 16HBE cells (**d**) RT-PCR, real-time PCR; *WB* Western blotting
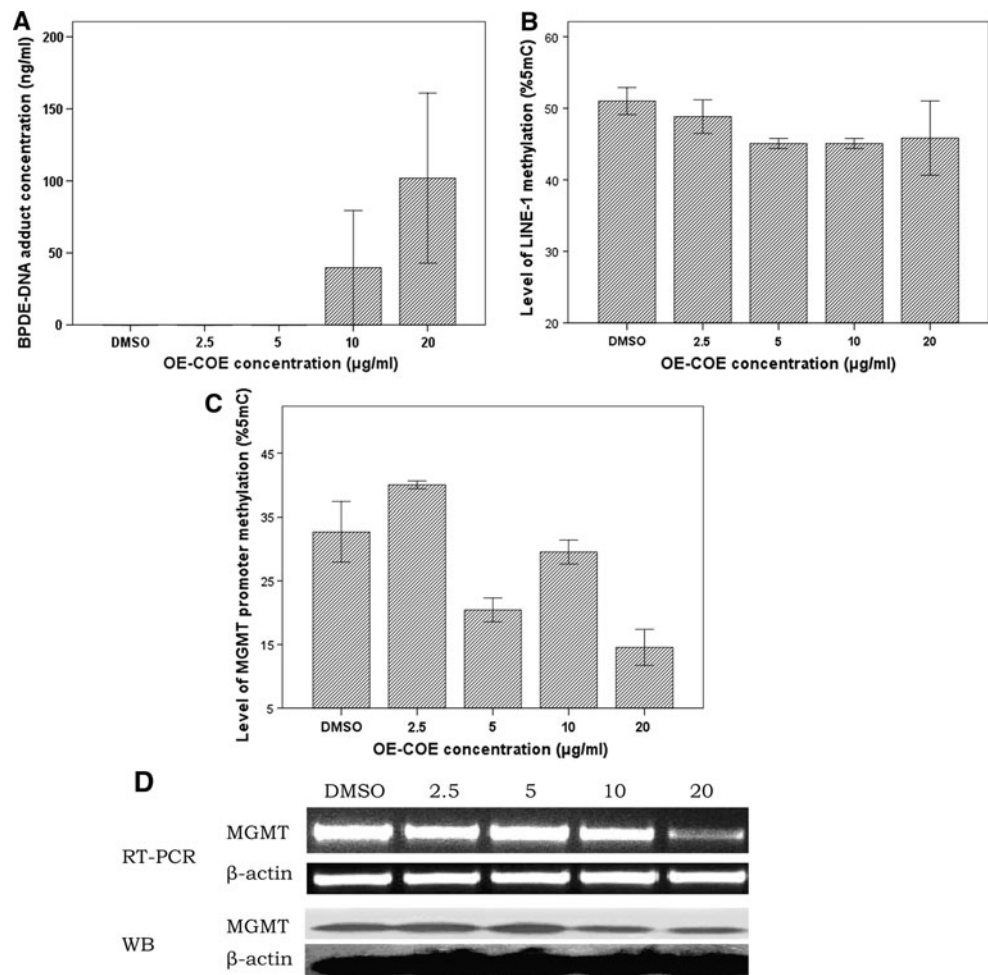
Discussion

Coke oven emissions, a complex mixture of PAHs, are a known human carcinogen. We used comprehensive biomarkers including comet TM, BLM sensitivity, and MN assay, to assess the genetic damages, and observed a PAH-related decrease in the methylation of LINE-1 repeated elements and *MGMT* promoter which associated with

genetic damages in PAH-exposed population. Compared to average methylation, hot CpG site-specific hypomethylation in the MGMT promoter region was more significant in the PAH-exposed population. We did not find a direct correlation between levels of MGMT and LINE-1 methylation in all subjects and PAH-exposed workers, indicating the alteration of global methylation and gene-specific methylation occurred independently. Consistent with the results from the human study, we found that high levels of COE induced LINE-1 and MGMT hypomethylation with high levels of BPDE-DNA adducts in long-term treated cells. Thus, alterations of LINE-1 and MGMT promoter methylation can be biomarkers for assessing risk of PAHs.

Global DNA hypomethylation may have an important role in human tumorigenesis by increasing genomic instability and furthering the likelihood of developing cancer (Daskalos et al. 2009; Moore et al. 2008). To the best of our knowledge, this is the first report that LINE-1 hypomethylation in peripheral lymphocytes is strongly associated with the MN, a validated biomarker for chromosomal instability and risk of several cancers (Fenech 2006), among PAH-exposed workers. We demonstrated that hypomethylation of LINE-1 correlates with high MN frequencies indicating a strong link between genome stability and global methylation ($p = 0.038$). A similar observation has been reported in lung cancer in which LINE-1 hypomethylation correlated with microsatellite instability (Daskalos et al. 2009). We also found LINE-1 hypomethylation is associated with DNA damage by comet assay in all subjects. The formation of MN is the result of chromosome breakage and loss due to unrepaired or misrepaired DNA lesions or chromosome malsegregation due to mitotic malfunction. Recent studies found that DNA rearrangements and mutations acquired in MN could be incorporated into the genome of a developing cancer cell (Crasta et al. 2012). It may deduce that hypomethylation of LINE-1 promoter region causes transcriptional activation and overexpression of LINE-1, resulting in retroelement transposition and chromosomal alteration (Daskalos et al. 2009; Saito et al. 2010). In addition, hypomethylation of DNA can induce chromatin decondensation, centromere and telomere abnormalities, and chromosome segregation defects and cause the formation of MN (Rodriguez et al. 2006). Notably, our data indicate that LINE-1 hypomethylation may represent an effect biomarker that significantly correlates with urinary 1-OHP, DNA damage, and chromosome instability. This finding suggests that global DNA hypomethylation may contribute to tumor progression, likely promoting genomic instability.

Several studies have investigated the role of MGMT methylation in the etiology of lung cancer (Furonaka et al. 2005; Pulling et al. 2003). In the present study, we first conducted a cross-sectional study to evaluate altered MGMT promoter methylation to PAHs exposure and investigate the associations between MGMT methylation and DNA or chromosome damages. We found that MGMT promoter demethylation in PAH-exposed workers inversely correlated with urinary 1-OHP levels. Compared to average methylation, hot CpG sites-specific hypomethylation in MGMT promoter region was more significant for both PAHs external and internal exposure analysis. We also found MGMT promoter methylation could influence chromosome instability by MN assay, which suggests that this might have an effect on chromosome instability among the population exposed to the high level of PAHs. The mechanism may be that change in methylation of MGMT promoter induced the aberrant MGMT expression and lowered the repair capacity for repairing genetic damage. These results, although in need of confirmation, suggest that MGMT CpG site-specific methylation may play a role in PAHs induced carcinogenesis.

Relatively few epidemiologic studies have examined the effects of exposure to PAHs on DNA methylation. Gene-specific hypomethylation found in our study is partially consistent with Pavanello's study on coke oven worker from Poland (Pavanello et al. 2009). They found p53 promoter was hypomethylated in PAH-exposed workers, who exhibited significantly higher levels of BPDE-DNA adducts and MN. In addition, Bollati (Bollati et al. 2007) found MAGE-1 hypomethylation among individuals exposed to low-dose airborne PAH and benzene (urban traffic police officers). In contrast, our observations on global methylation are not consistent with that of Pavanello's, which reported that LINE-1 sequences are higher methylated in PAH-exposed workers. There can be several explanations for this finding. First, the construction of coke oven and technological process can be different, and the PAHs categories and concentrations generated from the workplace are different. Based on the urinary 1-OHP, subjects in our study had higher PAHs exposure than Polish workers; the median (interquartile range) of 1-OHP was 9.31 (2.77–22.77) µg/l. Second, the difference in country and life style: these two populations are likely to be very different in terms of diet, which can affect DNA methylation. In addition, our subjects were all recruited from one cokery with well-characterized PAHs exposure, which allowed for contrasting subjects over a wide range of different exposure levels.

One particular strength of this study is that we studied the epigenetic effect of PAHs exposure both in vivo and in vitro based on the mode of action of PAHs. LINE-1 was found demethylated in OE-COE long-term treated cells, which is consistent with the population study. MGMT was methylated in low-dose (2.5 µg/ml) of OE-COE exposure and demethylated in high-dose groups. MGMT gene was also suppressed in higher OE-COE-treated cells

predominantly due to higher BPDE-DNA adducts and aberrant hypomethylation of its promoter region. We also found higher levels of DNA damage, MN, nucleoplasmic bridges, markers for indicating chromosomal, and genomic damage in OE-COE-treated cells in our former study (Zhai et al. 2012). *MGMT* gene can be suppressed by DNA adducts, aberrant promoter methylation, and ubiquitin-mediated proteolytic systems induced by carcinogens (Hwang et al. 2009). Studies suggested that the loss of MGMT expression is also due to silencing of the gene by hypermethylation of the CpG islands; authors found aberrant methylation of MGMT in 25 % of non-small-cell lung carcinomas (Esteller et al. 1999). These observations confirm that long-term exposure to PAHs can induce DNA methylation alterations at an early stage of malignant transformation by epigene–environment interaction.

The mechanism by which PAHs and its metabolites interfere with global and gene-specific DNA hypomethylation remains unclear. Several toxicologic studies have suggested PAHs inhibit DNA methylation. In in vitro studies, PAHs treatment did inhibit gene-specific methylation in human breast cancer cells and global methylation in BALB/3T3 mouse cells (Sadikovic and Rodenhiser 2006; Wilson and Jones 1983). The inhibition could be due to a variety of mechanisms: (1) Binding of PAH to DNA adduct preferentially binds to guanines at 5′-CpG sequences or CG-rich regions in the promoter region where methylation sites are more frequent (Weisenberger and Romano 1999), and the BPDE-DNA complex could inhibit methylation by altering substrate conformation (Wilson and Jones 1983). (2) DNA damage and DNA adduct influence the expression and/or activity of DNA methyltransferase (DNMT) (Pogribny et al. 2008); or PAH-DNA adducts may influence patterns of DNMTs binding as intercalating agents making potential methylation sites inaccessible (Hogan et al. 1979). Teneng reported that BaP decreased levels of DNMT1 and DNMT3A and elicited concomitant decreases in methylation at several CpG loci within the gene promoter by in vitro study (Teneng et al. 2011).

In the current study, we quantitated the methylation levels of LINE-1 repetitive sequences and *MGMT* promoter in PAH-exposed workers and matched controls using the pyrosequencing technology. This approach is widely thought to have a great precision, accuracy, sensitivity, and reproducibility, and easier to carry out in epidemiologic studies (Irahara et al. 2010). However, there are some limitations in the present study. We did not have information on folate and nutrient intake among the subjects. Folate is required for supplying methyl groups during its recycling, and deficiency of methyl donors may be a major cause for loss of DNA methylation. Nevertheless, association between global DNA methylation in blood and dietary folate has not been generally detected in previous studies. In studies of cancer-free population and cancer patients, individuals with lower dietary folate showed no difference in LINE-1 methylation compared with individuals with higher dietary folate intake (Zhang et al. 2011). In our study, all the studied subjects lived in the same geographic region had comparable demographic and socioeconomic characteristics and were engaged primarily in physical work. The different dietary, genetic, and environmental factors, other than PAH exposure, are unlikely to explain the results.

In summary, our results support the hypothesis that exposure to PAHs may influence DNA methylation. The results of this study suggest the possible use of global levels of DNA methylation as biomarkers of effect for subjects exposed to PAHs. Further studies should aim to replicate these findings and examine additional gene-specific methylation modifications in larger cohorts.

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