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Research Article

CpG Site–Specific Hypermethylation of $p16^{INK4\alpha}$ in Peripheral Blood Lymphocytes of PAH-Exposed Workers

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Abstract

Background: Sufficient epidemiologic evidence shows an etiologic link between polycyclic aromatic hydrocarbons (PAH) exposure and lung cancer risk. While the genetic modifications have been found in PAH-exposed population, it is unclear whether gene-specific methylation involves in the process of PAH-associated biologic consequence.

Methods: Sixty-nine PAH-exposed workers and 59 control subjects were recruited. Using bisulfite sequencing, we examined the methylation status of $p16^{INK4\alpha}$ promoter in peripheral blood lymphocytes (PBL) from PAH-exposed workers and in benzo(*a*)pyrene (BaP)-transformed human bronchial epithelial (HBE) cells. The relationships between $p16^{INK4\alpha}$ methylation and the level of urinary 1-hydroxypyrene (1-OHP) or the frequency of cytokinesis block micronucleus (CBMN) were analyzed.

Results: Compared with the control group, PAH-exposed workers exhibited higher levels of urinary 1-OHP (10.62 vs. 2.52 μg/L), $p16^{INK4\alpha}$ methylation (7.95% vs. 1.14% for 22 "hot" CpG sites), and CBMN (7.28% vs. 2.92%) in PBLs. $p16^{INK4\alpha}$ hypermethylation in PAH-exposed workers exhibited CpG site specificity. Among the 35 CpG sites we analyzed, 22 were significantly hypermethylated. These 22 hypermethylated CpG sites were positively correlated to levels of urinary 1-OHP and CBMN in PBLs. Moreover, the hypermethylation and suppression of p16 expression was also found in BaP-transformed HBER cells.

Conclusion: PAH exposure induced CpG site–specific hypermethylation of $p16^{INK4\alpha}$ gene. The degree of $p16^{INK4\alpha}$ methylation was associated with the levels of DNA damage and internal exposure.

Impact: $p16^{INK4\alpha}$ hypermethylation might be an essential biomarker for the exposure to PAHs and for early diagnosis of cancer. *Cancer Epidemiol Biomarkers Prev; 21(1); 182–90. ©2011 AACR.*

Introduction

Cancer development involves the accumulation of multiple genetic mutations over time and epigenetic alterations (1). Genetic alternations have been the key mechanisms involved in chemical carcinogenesis. However, it is apparent that cancer development is fuelled by both DNA mutations and aberrant epigenetic patterns (2, 3). DNA methylation, as the most extensively investigated epigenetic modification of DNA, has been shown to be involved in chemical carcinogenesis (4, 5). The DNA methylation profiles of cancer cells are often characterized by global hypomethylation and gene-specific hypermethylation. Global DNA hypomethylation has been shown to induce genomic instability and promote carcinogenesis (6, 7). It is evident that aberrant hypermethylation of promoter CpG islands is an alternative to a mutation for inactivation of tumor suppressor genes (2, 8).

Silencing of the critical genes by promoter hypermethylation is a key event in human cancer development. It could be the potential biomarkers for early diagnosis, prognosis prediction, and the therapeutic targets (9, 10). Among numerous tumor suppressor genes found transcription silencing predominantly through promoter hypermethylation in human cancer (11–13), $p16^{INK4\alpha}$ was the first gene identified in primary lung cancers (14). The $p16^{INK4\alpha}$ gene, an inhibitor of cyclin D kinases (cdk) 2, 4,

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and 6, has critical functions in control of cell cycles. Suppression of its expression allows unregulated phosphorylation of the Rb protein and leads to uncontrolled cell-cycle progression and cell division (15). It has been reported that $p16^{INK4\alpha}$ is inactivated by methylation at prevalence up to 60% to 70% in primary lung cancers (10) with low frequency of mutations (16). $p16^{INK4\alpha}$ promoter hypermethylation appears to be an early alteration observed in the precursor lesions of squamous cell cancer and was more prevalent with each successive stage of the progression model, with 17%, 24%, and 50% at tissues of basal cell hyperplasia, squamous metaplasia, and carcinoma *in situ*, respectively (17). However, it is unclear whether $p16^{INK4\alpha}$ methylation change plays a causative role in chemical carcinogenesis or is merely a consequence of the malignant state of transformation. Furthermore, it is ambiguous how environmental factors affect the status of $p16^{INK4\alpha}$ methylation, which might contribute to the development of lung cancer.

Recently, the alterations of DNA methylation status by exposure to environmental chemicals have been reported. Investigations have identified several classes of environmental chemicals including metals, peroxisome proliferators, air pollutants, and endocrine-disrupting/reproductive toxicants were found to modify DNA methylation of specific genes in animal, human surrogates or *in vitro* studies (18). As for $p16^{INK4\alpha}$ gene, Kim and colleagues (19) studied the association between methylation of the $p16^{INK4\alpha}$ promoter region and the exposure to tobacco smoke in 185 primary non–small cell lung cancers. They found that levels of methylation of $p16^{INK4\alpha}$ were correlated with pack-years smoked, duration of smoking, and negatively with the time since quitting smoking. Higher frequencies of $p16^{INK4\alpha}$ methylation were also observed in smokers (20) and in human lung cancer associated with chromate exposure (21). Consistent with the results from patients with lung cancers, the frequency of $p16^{INK4\alpha}$ gene promoter hypermethylation in peripheral blood leukocyte was much higher in the patients with arseniasis (22) and in the workers exposure to radon (23). However, it is still unclear that whether $p16^{INK4\alpha}$ gene methylation can be used as an early biomarker for environmental and occupational exposure.

Previously, we established malignant transformation models of human bronchial epithelial cells (16HBE) by various known carcinogens including benzo(*a*)pyrene (BaP; ref. 24). We also found that occupational exposure to polycyclic aromatic hydrocarbons (PAH) induced higher frequency of micronucleus in peripheral blood lymphocytes (PBL; ref. 25) and resulted in defect in DNA repair capacity (26). To explore the role of $p16^{INK4\alpha}$ methylation in occupational PAHs exposure and chemical carcinogenesis, we examined the $p16^{INK4\alpha}$ methylation in PBLs from PAH-exposed workers and in BaP-transformed human bronchial epithelial cells (HBERT) using a quantitative sequencing method. Here we showed that $p16^{INK4\alpha}$ methylation was associated with PAH exposure in a dose-dependent manner, indicating that epigenetic modifications could be used as biomarkers to monitor exposure to environmental or occupational carcinogenic agents and to identify individuals at high cancer risk.

Materials and Methods

Study population and sample collection

Information about the PAH-exposed workers have been described previously (26). In brief, all study participants were employed at Anshan Iron and Steel Group Cooperation. The 69 coke-oven workers with exposure to PAHs and a group of 59 workers without exposure to PAHs were recruited as nonexposed controls. We excluded workers who had suffered from acute infectious diseases, chronic diseases (such as autoimmune disease), or exposed to mutagenic agents (such as X-ray radiation) within 2 months. The demographic data, detailed information about alcohol consumption, and smoking history of all participants were collected using a structured questionnaire by an occupational physician. Informed consent was obtained from each participant. Individuals who had smoked >100 cigarettes in their lifetime were considered as smokers. Among these smokers, individuals who still smoked at the time of the interview were defined as current smokers; others were treated as former smokers. Field urine samples were collected from each subject at the end of a work shift after at least 4 consecutive days of work. Four milliliters of venous blood samples was collected from each subject for the cytokinesis block micronucleus (CBMN) assay and $p16^{INK4\alpha}$ gene methylation analysis. The detection of urinary 1-hydroxypyrene (1-OHP) concentrations and micronucleus in PBLs were carried out according to the methods described previously (26). The protocol was approved by the Research Ethics Committee of the National Institute for Occupational Health and Poison Control, Chinese Center for Disease Control and Prevention.

Cell lines and primary peripheral blood mononuclear cell culture

HBE cells, 16HBE14o, were kindly provided by Dr. D.C. Gruenert (University of California, San Francisco, CA) and authenticated by UCSF Office of Technology Management. The expression of SV40 oncoproteins by immunoblotting was tested prior to chemical treatment. HBER cells were generated by introducing an oncogenic allele of *H-Ras* to HBE cells (24). HBERT-BaP and HBERNT-BaP were generated as described previously (24). Peripheral blood mononuclear cells (PBMC) were isolated from blood by Ficoll-Hypaque (27) and treated with 10 μmol/L BaP for 12, 24, and 48 hours, respectively.

Bisulfite conversion, subcloning, and sequencing

Genomic DNA from cell lines and PBLs of the subjects were extracted with phenol/chloroform mixture. Two micrograms of genomic DNA was denatured by adding freshly prepared 3 mol/L NaOH and transfered to a tube with total volume of 200 μL mixture (pH 5.0) containing

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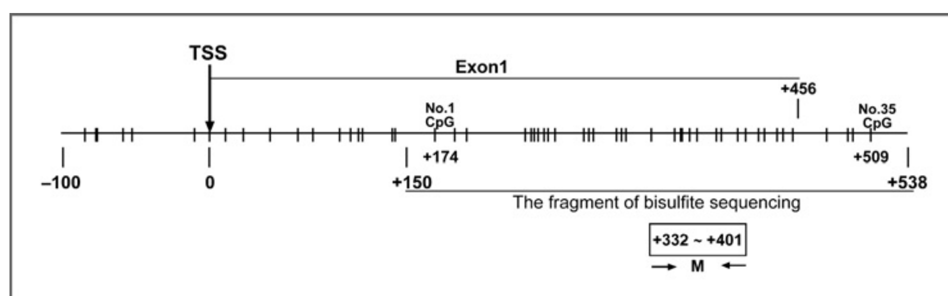

Figure 1. The distribution of CpG sites of the $p16^{INK4\alpha}$ across transcription starting site (NM_000077.4). The transcription starting site was indicated as TSS. Each vertical bar represents the presence of a CpG dinucleotide. No.1 CpG and No.35 CpG were named by their location in order of the fragment sequenced. M presents regions analyzed by MethyLight assay.

freshly prepared 3.1 mol/L sodium bisulfite and 0.5 mmol/L hydroquinone. After mixed and incubated for 12 to 16 hours under mineral at 50°C in the dark, the DNA was purified using the Wizard DNA Clean-Up System (Promega) and eluted in 100 µL of water. Desulfonation was carried out in 0.3 mol/L NaOH solution for 15 minutes at 37°C. The solution was then neutralized by adding 1/10 volume of 3 mol/L sodium acetate (pH 5.3). The DNA was ethanol precipitated, washing in 70% ethanol, and dissolved in 25 µL of sterile water. Sodium bisulfite–modified DNA was used as a template for PCR with the bisulfite-sequencing primers (sequences shown in Supplementary Table S1). The amplification (+150 to +538; transcription starting site, +1 or −1; Fig. 1) of the 389-bp fragment containing 150-bp MSP amplicon (+229 to +378) reported by several studies (28–30). The amplification annealing conditions were 60°C. The PCR products were purified by Gel Extraction Kit (Qiagen) and subcloned into a pMD19-T vector. Eight clones from each human sample and 10 clones for each cell line were selected for sequencing with M13 primers using an ABI BigDye Terminator Cycle Sequencing Kit (BigDye Terminator v3.1 K) on a 3130 ABI 96-capillary sequencer systems equipped with capillaries of 36 cm separation length.

MethyLight analysis

After sodium bisulfate conversion, methylation analysis was carried out by a real-time PCR assay (MethyLight) as described previously (30). The classic primers (as shown Supplementary Table S1) of MethyLight were used to detect the 70-bp methylated fragment (+332 to +401) of $p16^{INK4\alpha}$ exon 1 using a SYBR Green Real-time PCR Master Mix kit (ABI).

Immunoblotting

Cells were suspended in a lysis buffer [50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 2 mmol/L EDTA, protease inhibitor cocktail (Roche) and 1.0% NP-40] and cleared insoluble material by centrifugation. Soluble proteins (70 µg) were subjected to SDS-PAGE. The antibodies used included: $p16^{INK4\alpha}$ (1:2,000 dilution; Epitomic).

Statistical analysis

Numerical variables were checked for normality before analysis. The Student t test was used to compare the age and the log-transformed urinary 1-OHP levels between exposed group and control group. The χ^2 test was used to compare the frequencies of current smokers and alcohol users between 2 groups and the Mann–Whitney U test was used to compare the levels of $p16^{INK4\alpha}$ methylation between 2 groups. The Spearman correlation analysis was carried out to address the correlations between $p16^{INK4\alpha}$ methylation status and level of urinary 1-OHP or DNA damage. All statistical tests were 2 sided ($\alpha = 0.05$) and carried out using SPSS for Windows (release 11.5, SPSS Inc.).

Results

Hypermethylation of $p16^{INK4\alpha}$ in PAH-exposed workers in a CpG site–specific manner

The demographic information of participants was described in Table 1. All subjects were male. There were no significant differences between 2 groups in terms of age and smoking habit.

The levels of $p16^{INK4\alpha}$ methylation in PBLs were examined quantitatively by bisulfite sequencing (primers sequences shown in Supplementary Table S1). The selected fragment which was previously showed to regulate $p16^{INK4\alpha}$ gene transcription activity (28, 29, 31) contained 389 bps (+150 to +538 bp around the transcription start site) and 35 CpG sites in total as illustrated in Fig. 1. Within this region, the first CpG located at +174 bp was defined as No.1 and the last CpG located at +509 bp was defined as No.35. Average percentage of methylation across 35 CpG sites was calculated by the number of methylated CpGs divided by the total number of CpGs as reported previously (32, 33). The average percentage of methylation across 35 CpG sites in all subjects is shown in Supplementary Fig. S1. As shown in Fig. 2A, we found that the median level (interquartile range) of $p16^{INK4\alpha}$ methylation was 5.71% (2.85%–10.54%, $n = 67$) in PAH-exposed workers, which was 4 folds higher than that in control workers (median, 1.43%; interquartile range, 0%–2.5%; $n = 59$; $P < 0.001$). The methylation levels of each CpG site were shown in Supplementary Table S2. Smoking status and drinking status had no influence on $p16^{INK4\alpha}$ methylation status (Table 2). No methylation modification was detected at the 4th, 5th, and 19th CpG site in 2 groups. When analyzing the difference at each CpG site between 2 groups using a Mann–Whitney U test, we found that 22 sites located at No. 1, 2, 8, 9, 11, 12, 17, 18, 20–24, and 26–34 were significantly higher in PAH-exposed workers

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Table 1. Distribution of select variables and biomarkers in PAH-exposed workers

Variables	Controls	PAH-exposed workers	*P*
Number	59	69	
Age (mean ± SD), y	41.95 ± 4.75	42.18 ± 6.51	0.82[a]
Current smokers (yes/no), %	46/16 (74.2)	56/11 (83.6)	0.19[b]
Alcohol user (yes/no), %	38/24 (61.3)	52/15 (77.6)	0.044[b]
Urinary 1-OHP levels [GM (95% CI)], μg/L	2.52 (2.28–2.77)	10.62 (8.13–13.87)	<0.001[c]
CBMN frequencies (mean ± SD), %	2.92 ± 3.04	7.28 ± 4.16	<0.001[c]
Coking history (mean ± SD), y	—	21.29 ± 7.55	—

Abbreviations: CI, confidence interval; GM, geometric mean.
[a]Two-sided 2-sample *t* test.
[b]Two-tailed χ^2 test.
[c]Two-sided 2-sample *t* test.

(Fig. 2B), indicating that these 22 CpG sites might be the regulatory "hot CpG sites" participating in gene transcriptional suppression. We then recalculated the frequency of $p16^{INK4\alpha}$ methylation using 22 CpGs as a denominator and found that it was 7.95% (3.98%–13.35%) in exposed group and 1.14% (0%–2.27%) in control group ($P < 0.001$). The frequencies of DNA molecules (clones) with different number of methylated CpG sites were summarized in Supplementary Table S3. Methylated CpG sites tend to distribute randomly. Only 18.7% and 2.5% DNA molecules have more than six methylated CpG sites in PAH-exposed group and in control group. Among methylated DNA molecules, the overwhelming majority had five or less methylated CpG sites in both groups. Taken together, these observations show that $p16^{INK4\alpha}$ gene hypermethylation occurs more frequently in workers exposed to PAHs in a CpG site–specific manner.

The frequency of $p16^{INK4\alpha}$ methylation is correlated with levels of urinary 1-OHP and CBMN

Urinary 1-OHP was used as an internal marker of PAH exposure. The degree of DNA damage was assessed using a CBMN assay. As we reported previously, PAH-exposed workers exhibited higher frequency of CBMN in PBLs than control subjects ($P < 0.001$; ref. 26). Next, we addressed the correlations between $p16^{INK4\alpha}$ methylation status and urinary 1-OHP and DNA damage by spearman correlation analysis. The correlations with each CpG site were shown in Supplementary Table S4. The methylation levels of 35 CpG sites or 22 hot CpG sites were both positively correlated with urinary 1-OHP or CBMN in PBLs (Table 3). Our results show that $p16^{INK4\alpha}$ methylation is correlated with the levels of PAHs exposure and DNA damage.

The effects of BaP treatment on $p16^{INK4\alpha}$ methylation and p16 expression

Previously, we had established several human cell models transformed by various chemical carcinogens. These transformed cells grow in an anchorage-independent manner and to form tumors in immunodeficient mice (24). To explore whether hypermethylation of $p16^{INK4\alpha}$ contributes to chemical carcinogenesis, we examined the expression and methylation status of $p16^{INK4\alpha}$ gene during

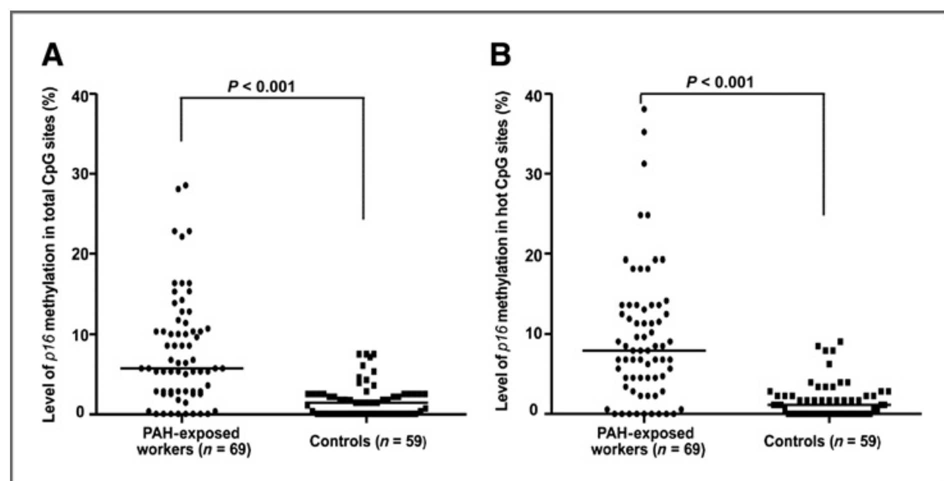

Figure 2. Level of $p16^{INK4\alpha}$ methylation in total CpG sites (A) and in hot CpG sites (B). The median (interquartile range) methylation levels of total 35 CpG sites were 5.71% (2.85%–10.54%) in PAH-exposed workers and 1.43% (0%–2.5%) in controls. The corresponding data of 22 hot CpG sites were 7.95% (3.98%–13.35%) in PAH-exposed workers and 1.14% (0%–2.27%) in controls. Mann–Whitney *U* test was used for statistical analysis.

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Table 2. The effects of smoking and drinking on $p16^{INK4\alpha}$ methylation (%) stratified by PAH exposure

	Controls			PAH-exposed workers		
	n	Median (25th–75th)	*P*	*n*	Median (25th–75th)	*P*
Smoking status						
Nonsmoking	16	1.60 (0–2.14)	0.726	11	5.36 (2.85–9.64)	0.115
Smoking	46	1.43 (0–2.5)		56	5.71 (2.5–10.44)	
Drinking status						
Nondrinking	24	1.60 (0–2.5)	0.85	15	5.71 (1.97–8.57)	0.28
Drinking	38	1.43 (0–2.5)		52	6.42 (2.85–11.7)	

NOTE: Mann–Whitney *U* test was used for statistical analysis.

the process of BaP-induced cell transformation. HBER cells at different stages of malignant transformation were named as nontransformed cells (HBER), pretransformed cells (HBERNT), and transformed cells (HBERT), respectively. Particularly, HBERNT cells were BaP-treated HBER cells without a malignant transformed phenotype (24). As shown in Fig. 3A, p16 mRNA level was downregulated by 51% in pretransformed cells (HBERNT-BaP) and hardly detected in transformed cells (HBERT-BaP). Consistent with the level of mRNA, the protein level of p16 was hardly observed in pretransformed HBERNT-BaP cells or transformed HBERT-BaP cells. Meanwhile, we observed that treatment with 5 µmol/L of 5-aza-2′-deoxycytidine (DAC), an inhibitor of DNA methyltransferase reversed methylation restored gene expression at both mRNA and protein levels in HBERNT-BaP and HBERT-BaP cells (Fig. 3A).

To address whether the suppression of p16 expression was because of gene methylation, we carried out bisulfite sequencing to analyze the status of $p16^{INK4\alpha}$ methylation in HBERNT-BaP, HBERT-BaP, and control HBER cells. Ten clones from each cell line were selected for sequencing with M13 primers. The methylation levels of 35 CpG sites or 22 hot CpG sites were calculated, respectively by the number of methylated CpGs divided by the total number of CpGs. The methylation levels of 35 CpG sites increased progressively during the different stage of cell-transformation with 0.57%, 8.57%, and 22.0% in HBER, HBERNT-BaP and HBERT-BaP cells, respectively. Treatment by DAC, the methylation status was reversed in almost all CpG sites in HBERNT-BaP and HBERT-BaP cells (Fig. 3C). The trend of methylation was similar if the analysis carried out on the basis of 22 CpG hot spots. Similar results were found when we treated primary PBMCs with BaP (Supplementary Fig. S2). For quality control, we also carried out a real-time PCR (MethyLight) analysis after sodium bisulfate conversion. As shown in Fig. 3B, MethyLight assay was well correlated with the results from bisulfite-converted method. Taken together, these *in vitro* studies reinforce the notion that downregulation of p16 expression resulting from gene hypermethylation is common in human cancers (34, 35).

Discussion

Epigenetic aberration is increasingly considered to play an important role in cellular response to environmental chemicals and induction of biologic consequences (36, 37). Alterations in gene expression, DNA repair, genome stability, and malignant cell transformation could be the results of epigenetic modifications induced by environmental chemicals. Thus, identification of specific epigenetic alterations, establishment of dose–response relationship, and showing a human relevance are essential for the application of epigenetic biomarkers in risk assessment. Here, we showed that $p16^{INK4\alpha}$ gene was hypermethylated in PAH-exposed workers and positively related to urinary 1-OHP and CBMN in PBLs. Consistent with the results from human study, we found that a major component of PAHs, BaP induced $p16^{INK4\alpha}$ hypermethylation and this action was reversible. These results revealed that $p16^{INK4\alpha}$ hypermethylation could be a potential biomarker

Table 3. The relationships between $p16^{INK4\alpha}$ methylation and the levels of urinary 1-OHP and CBMN in PBLs

	1-OHP		CBMN	
	r	*P*	*r*	*P*
Total CpG sites	0.450	<0.001	0.356	<0.001
Hot CpG sites ($n = 22$)	0.474	<0.001	0.369	<0.001

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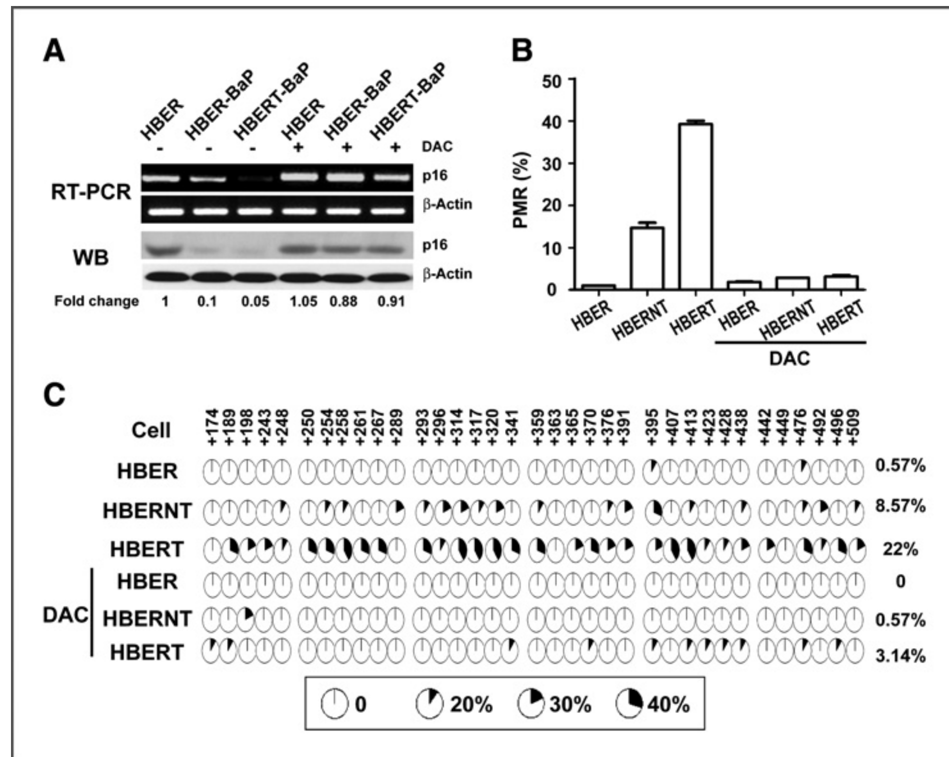

Figure 3. p16 expression and methylation status at the promoter of $p16^{INK4\alpha}$ gene in BaP-transformed HBER cells. A, mRNA levels (top) of and protein expression (bottom) of p16 in HBER, HBERNT-BaP, and HBERT-BaP cells with (+) or without (−) treatment of DAC. B, quantitative methylation levels of p16 by MethyLight assay. The results were expressed as the percentage of methylated reference (PMR, mean ± SEM). C, bisulfite sequencing on CpG island (+150 to +538 bp) of the $p16^{INK4\alpha}$ gene. For each cell line, 10 clones were selected for sequencing. The CpG position relative to transcription start site was shown at top of each CpG site. At each CpG site, the area filled with black represents the average percentage of methylation across all CpG sites tested in cells indicated. The value of average percentage is shown at the end of each row. RT-PCR, real-time PCR; WB, Western blotting.

for the exposure to environmental PAHs and prediction of high risk of tumorigenesis. Thus, we could place $p16^{INK4\alpha}$ hypermethylation at an early stage of carcinogenesis.

PAHs are a prominent class of carcinogens (especially of the lung) present in incomplete combustion such as coal-burning power plants, diesel- and gasoline-powered vehicles, home heating, and cooking, as well as released from tobacco smoke. It is estimated that an average of 6.5 per million people in China have lung cancer because of PAHs inhalation exposure (38). Although PAHs have been classified as class I carcinogens by the international agency for research on cancer (IARC; ref. 39), the exact underlying mechanism of PAH-derived carcinogenesis awaits further delineation. The accumulation of genetic and epigenetic changes permit cells to escape from the tight network of controls that regulate the homeostatic balance between cell proliferation and cell death, which might promote tumor development and progression (40, 41). The interaction of external factors with one or more internal factors drives the genetic and epigenetic changes, increasing the risk of tumor development (42, 43). In this study, we found that $p16^{INK4\alpha}$ hypermethylation was associated with CBMN frequency in PAH-exposed population. We speculate that BaP-induced DNA damage and $p16^{INK4\alpha}$ hypermethylation may confer susceptible cells a selective growth advantage. These cells can undergo clonal expansion, become genomically unstable, and transform into malignant cells. Several studies (33, 44, 45) showed that BaP diolepoxide (BPDE) DNA adduct preferentially bound to 5′-CpG sequences in the promoter region of *p53*, leading to an inhibition of DNA methylation by DNA methyltransferases. These observations suggest a direct role for BPDE–DNA adduct on the *p53* hypomethylation. Although it is hard to provide the direct evidence showing that $p16^{INK4\alpha}$ methylation is induced by a signal of DNA damage, we speculate that the interplay between genetic and epigenetic controls the biologic response of cells upon chemical exposure.

As the first gene identified in primary lung cancers (14), $p16^{INK4\alpha}$ is inactivated by methylation at prevalence up to 60% to 70% in primary lung cancers (10) with low frequency of mutations (16). But the missing link among environmental factors, $p16^{INK4\alpha}$ aberrant methylation, and lung cancer limits the applications of hypermethylated $p16^{INK4\alpha}$ as biomarker for early detection. In the last few years, several investigations have examined the relation between exposure to environmental chemicals and epigenetic biomarkers. They revealed that several toxicants such as metals, peroxisome proliferators, air pollutants, and endocrine-disrupting/reproductive toxicants could modify DNA methylation (18). For example, methylation of $p16^{INK4\alpha}$ in non–small cell lung cancer was significantly associated with pack-years smoked duration of smoking, and negatively with the time since quitting smoking (19). The reduced expression and aberrant hypermethylation of $p16^{INK4\alpha}$ are also induced by other chemicals such as nickel (46) and chromium (21). A recent study in Europe revealed that chronic exposure to PAHs led to a hypermethylation of global DNA and hypomethylation of *p53* and *HIC*, but $p16^{INK4\alpha}$ gene was not affected

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(33). In this study, we found that the levels of $p16^{INK4\alpha}$ methylation were much higher in PAH-exposed workers. Consistent with the human population study, $p16^{INK4\alpha}$ methylation was also found in BaP-induced transformed HBER cells. These results provide evidence that epigenetic biomarkers, such as $p16^{INK4\alpha}$ hypermethylation in PBLs, could be a potential biomarker for chemical carcinogen exposure.

As for the methods for detection of DNA methylation, there is a great demand for development of sensitive and reliable techniques to quantify methylation of CpG islands of specific genes. An ideal method should be quantitative, precise, fast and high throughput. It should be able to differentiate allele-specific changes in promoter methylation at a large variety of CpG sites efficiently. Bisulfite-sequencing PCR (BSP) established by Frommer and colleagues (47, 48) has been used for measuring DNA methylation for many years. The pyrosequencing method has emerged as an alternate technique to study DNA methylation (49). The great advantage of pyrosequencing is direct, precise, and high throughput, thus reducing biases and workloads (50, 51). However, Reed and colleagues compared BSP with pyrosequencing through head-to-head experimentation and found that there was a slight overestimation of methylation levels in mixtures containing very low percentages of methylated DNA (0%, 5%, and 10% mixtures) and a very clear underestimation of methylation levels in mixtures containing high percentages of methylated DNA (50%, 75%, and 100% mixtures; ref. 52). In the present study, the level of $p16^{INK4\alpha}$ methylation in majority of subjects was less than 10%, therefore BSP assay might be more reliable. However, it is not recommend using BSP in analyzing a great number of samples because of huge workloads. With the advantage of providing detail information of specific CpG sites methylation, BSP methods can be used to determine the region that is critical in control of transcriptional activity.

CpG islands were located in different regions of genes (53), and differential methylation can be observed in any region of these CpG islands (54). Methylation of each specific promoter seems to be more important in determining gene expression levels than overall methylation of a gene (55). $p16^{INK4\alpha}$ gene has three CpG islands. One is a large promoter CpG islands that spans the promoter region and exon 1-alpha, one is located over exon 2, and the other is located at intron 2 flanked exon 3. Differential methylation can be observed in any of these 2 CpG islands. However, transcription repression of $p16^{INK4\alpha}$ occurs consistently only when a 230-bp region that covers the transcription start site is methylated (56). These results suggest that there are "hot spots" of aberrant DNA methylation responsive to environmental toxicant. In our study, $p16^{INK4\alpha}$ hypermethylation in PAH-exposed workers exhibited CpG site specificity. Among the 35 CpG sites we analyzed, 22 were hypermethylated. These CpG sites almost locate in the core region spanning over 230-bp region. This phenomenon poses a challenge for methylation analysis to capture these "hot spots," although the underlying mechanism remains to be addressed.

In summary, we showed that BaP-induced $p16^{INK4\alpha}$ hypermethylation *in vitro* and *in vivo* and $p16^{INK4\alpha}$ hypermethylation was correlated with DNA damage in PBLs of coke-oven workers. These results suggested that DNA hypermethylation and DNA damage interact in process of chemical carcinogenesis. It is increasingly recognized that altered DNA methylation in key regulatory genes can be an early and prominent event in human carcinogenesis. Accumulating data make it clear that epigenetic markers can be potentially applied in risk assessment. Further studies are required to determine the methylation signatures associated with carcinogen exposures and the biologic endpoints and to clarify dose–response and human relevancy to risk assessment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Mulero-Navarro S, Esteller M. Epigenetic biomarkers for human cancer: the time is now. Crit Rev Oncol Hematol 2008;68:1–11.
2. Baylin SB, Herman JG. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. Trends Genet 2000;16:168–74.
3. Stein GS, van Wijnen AJ, Imbalzano AN, Montecino M, Zaidi SK, Lian JB, et al. Architectural genetic and epigenetic control of regulatory networks: compartmentalizing machinery for transcription and chromatin remodeling in nuclear microenvironments. Crit Rev Eukaryot Gene Expr 2010;20:149–55.
4. Jones PA, Baylin SB. The epigenomics of cancer. Cell 2007;128: 683–92.
5. Esteller M. Epigenetics in cancer. N Engl J Med 2008;358:1148–59.
6. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R. DNA hypomethylation leads to elevated mutation rates. Nature 1998;395: 89–93.
7. Eden A, Gaudet F, Waghmare A, Jaenisch R. Chromosomal instability and tumors promoted by DNA hypomethylation. Science 2003;300: 455.

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8. Ushijima T, Asada K. Aberrant DNA methylation in contrast with mutations. Cancer Sci 2011;101:300–5.
9. Na YK, Lee SM, Hong HS, Kim JB, Park JY, Kim DS. Hypermethylation of growth arrest DNA-damage-inducible gene 45 in non–small cell lung cancer and its relationship with clinicopathologic features. Mol Cells 2011;30:89–92.
10. Belinsky SA. Silencing of genes by promoter hypermethylation: key event in rodent and human lung cancer. Carcinogenesis 2005;26: 1481–7.
11. Tsou JA, Hagen JA, Carpenter CL, Laird-Offringa IA. DNA methylation analysis: a powerful new tool for lung cancer diagnosis. Oncogene 2002;21:5450–61.
12. Belinsky SA. Gene-promoter hypermethylation as a biomarker in lung cancer. Nat Rev Cancer 2004;4:707–17.
13. Anglim PP, Alonzo TA, Laird-Offringa IA. DNA methylation-based biomarkers for early detection of non–small cell lung cancer: an update. Mol Cancer 2008;7:81.
14. Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, et al. 5′ CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nat Med 1995;1:686–92.
15. Lukas J, Parry D, Aagaard L, Mann DJ, Bartkova J, Strauss M, et al. Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. Nature 1995;375:503–6.
16. Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV, et al. A cell cycle regulator potentially involved in genesis of many tumor types. Science 1994;264:436–40.
17. Belinsky SA, Nikula KJ, Palmisano WA, Michels R, Saccomanno G, Gabrielson E, et al. Aberrant methylation of p16(INK4a) is an early event in lung cancer and a potential biomarker for early diagnosis. Proc Natl Acad Sci U S A 1998;95:11891–6.
18. Baccarelli A, Bollati V. Epigenetics and environmental chemicals. Curr Opin Pediatr 2009;21:243–51.
19. Kim DH, Nelson HH, Wiencke JK, Zheng S, Christiani DC, Wain JC, et al. p16(INK4a) and histology-specific methylation of CpG islands by exposure to tobacco smoke in non–small cell lung cancer. Cancer Res 2001;61:3419–24.
20. Divine KK, Pulling LC, Marron-Terada PG, Liechty KC, Kang T, Schwartz AG, et al. Multiplicity of abnormal promoter methylation in lung adenocarcinomas from smokers and never smokers. Int J Cancer 2005;114:400–5.
21. Kondo K, Takahashi Y, Hirose Y, Nagao T, Tsuyuguchi M, Hashimoto M, et al. The reduced expression and aberrant methylation of p16 (INK4a) in chromate workers with lung cancer. Lung Cancer 2006;53: 295–302.
22. Zhang AH, Bin HH, Pan XL, Xi XG. Analysis of p16 gene mutation, deletion and methylation in patients with arseniasis produced by indoor unventilated-stove coal usage in Guizhou, China. J Toxicol Environ Health A 2007;70:970–5.
23. Su S, Jin Y, Zhang W, Yang L, Shen Y, Cao Y, et al. Aberrant promoter methylation of p16(INK4a) and O(6)-methylguanine-DNA methyltransferase genes in workers at a Chinese uranium mine. J Occup Health 2006;48:261–6.
24. Pang Y, Li W, Ma R, Ji W, Wang Q, Li D, et al. Development of human cell models for assessing the carcinogenic potential of chemicals. Toxicol Appl Pharmacol 2008;232:478–86.
25. Duan H, Leng S, Pan Z, Dai Y, Niu Y, Wang C, et al. Biomarkers measured by cytokinesis-block micronucleus cytome assay for evaluating genetic damages induced by polycyclic aromatic hydrocarbons. Mutat Res 2009;677:93–9.
26. Cheng J, Leng S, Li H, Huang C, Niu Y, Zhang L, et al. Suboptimal DNA repair capacity predisposes coke-oven workers to accumulate more chromosomal damages in peripheral lymphocytes. Cancer Epidemiol Biomarkers Prev 2009;18:987–93.
27. Tchou-Wong KM, Kiok K, Tang Z, Kluz T, Arita A, Smith PR, et al. Effects of nickel treatment on H3K4 trimethylation and gene expression. PLoS One 2011;6:e17728.
28. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 1996;93: 9821–6.
29. Dong CX, Deng DJ, Pan KF, Zhang L, Zhang Y, Zhou J, et al. Promoter methylation of p16 associated with Helicobacter pylori infection in precancerous gastric lesions: a population-based study. Int J Cancer 2009;124:434–9.
30. Zhou J, Cao J, Lu Z, Liu H, Deng D. A 115-bp MethyLight assay for detection of p16 (CDKN2A) methylation as a diagnostic biomarker in human tissues. BMC Med Genet 2011;12:67.
31. Georgiou E, Valeri R, Tzimagiorgis G, Anzel J, Krikelis D, Tsilikas C, et al. Aberrant p16 promoter methylation among Greek lung cancer patients and smokers: correlation with smoking. Eur J Cancer Prev 2007;16:396–402.
32. Ji W, Yang L, Yu L, Yuan J, Hu D, Zhang W, et al. Epigenetic silencing of O6-methylguanine DNA methyltransferase gene in NiS-transformed cells. Carcinogenesis 2008;29:1267–75.
33. Pavanello S, Bollati V, Pesatori AC, Kapka L, Bolognesi C, Bertazzi PA, et al. Global and gene-specific promoter methylation changes are related to anti-B[a]PDE-DNA adduct levels and influence micronuclei levels in polycyclic aromatic hydrocarbon-exposed individuals. Int J Cancer 2009;125:1692–7.
34. Risch A, Plass C. Lung cancer epigenetics and genetics. Int J Cancer 2008;123:1–7.
35. Belinsky SA, Klinge DM, Dekker JD, Smith MW, Bocklage TJ, Gilliland FD, et al. Gene promoter methylation in plasma and sputum increases with lung cancer risk. Clin Cancer Res 2005;11: 6505–11.
36. Preston RJ. Epigenetic processes and cancer risk assessment. Mutat Res 2007;616:7–10.
37. LeBaron MJ, Rasoulpour RJ, Klapacz J, Ellis-Hutchings RG, Hollnagel HM, Gollapudi BB. Epigenetics and chemical safety assessment. Mutat Res 2010;705:83–95.
38. Zhang Y, Tao S, Shen H, Ma J. Inhalation exposure to ambient polycyclic aromatic hydrocarbons and lung cancer risk of Chinese population. Proc Natl Acad Sci U S A 2009;106:21063–7.
39. IARC. Monographs on the evaluation of the carcinogenic risk of chemicals to humans. In: Polycyclic aromatic hydrocarbons. Vol 34. Lyon, France: International Agency for Research on Cancer; 1984.
40. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100: 57–70.
41. Jones PA, Gonzalgo ML. Altered DNA methylation and genome instability: a new pathway to cancer? Proc Natl Acad Sci U S A 1997; 94:2103–5.
42. Herceg Z. Epigenetics and cancer: towards an evaluation of the impact of environmental and dietary factors. Mutagenesis 2007;22: 91–103.
43. Brait M, Ford JG, Papaiahgari S, Garza MA, Lee JI, Loyo M, et al. Association between lifestyle factors and CpG island methylation in a cancer-free population. Cancer Epidemiol Biomarkers Prev 2009;18: 2984–91.
44. Pogribny IP, James SJ. Reduction of p53 gene expression in human primary hepatocellular carcinoma is associated with promoter region methylation without coding region mutation. Cancer Lett 2002; 176:169–74.
45. Sadikovic B, Rodenhiser DI. Benzopyrene exposure disrupts DNA methylation and growth dynamics in breast cancer cells. Toxicol Appl Pharmacol 2006;216:458–68.
46. Govindarajan B, Klafter R, Miller MS, Mansur C, Mizesko M, Bai X, et al. Reactive oxygen-induced carcinogenesis causes hypermethylation of p16(Ink4a) and activation of MAP kinase. Mol Med 2002;8:1–8.
47. Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, et al. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci U S A 1992;89:1827–31.
48. Clark SJ, Harrison J, Paul CL, Frommer M. High sensitivity mapping of methylated cytosines. Nucleic Acids Res 1994;22: 2990–7.

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49. Dupont JM, Tost J, Jammes H, Gut IG. De novo quantitative bisulfite sequencing using the pyrosequencing technology. Anal Biochem 2004;333:119–27.

50. Colella S, Shen L, Baggerly KA, Issa JP, Krahe R. Sensitive and quantitative universal Pyrosequencing methylation analysis of CpG sites. Biotechniques 2003;35:146–50.

51. Tost J, Dunker J, Gut IG. Analysis and quantification of multiple methylation variable positions in CpG islands by Pyrosequencing. Biotechniques 2003;35:152–6.

52. Reed K, Poulin ML, Yan L, Parissenti AM. Comparison of bisulfite sequencing PCR with pyrosequencing for measuring differences in DNA methylation. Anal Biochem 2010;397:96–106.

53. Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci U S A 2002;99: 3740–5.

54. Ushijima T. Detection and interpretation of altered methylation patterns in cancer cells. Nat Rev Cancer 2005;5:223–31.

55. Yan PS, Shi H, Rahmatpanah F, Hsiau TH, Hsiau AH, Leu YW, et al. Differential distribution of DNA methylation within the RASSF1A CpG island in breast cancer. Cancer Res 2003;63:6178–86.

56. Gonzalgo ML, Hayashida T, Bender CM, Pao MM, Tsai YC, Gonzales FA, et al. The role of DNA methylation in expression of the p19/p16 locus in human bladder cancer cell lines. Cancer Res 1998;58: 1245–52.

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