CpG Site–Specific Hypermethylation of p16\(^{INK4a}\) 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\(^{INK4a}\) 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\(^{INK4a}\) 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 \(\mu\)g/L), p16\(^{INK4a}\) methylation (7.95% vs. 1.14% for 22 'hot' CpG sites), and CBMN (7.28% vs. 2.92%) in PBLs. p16\(^{INK4a}\) 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\(^{INK4a}\) gene. The degree of p16\(^{INK4a}\) methylation was associated with the levels of DNA damage and internal exposure.

**Impact:** p16\(^{INK4a}\) hypermethylation might be an essential biomarker for the exposure to PAHs and for early diagnosis of cancer. *Cancer Epidemiol Biomarkers Prev; 1–9. ©2011 AACR.*

Introduction

Cancer development involves the accumulation of multiple genetic mutations over time and epigenetic alterations (1). Genetic alterations 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\(^{INK4a}\) was the first gene identified in primary lung cancers (14). The p16\(^{INK4a}\) gene, an inhibitor of cyclin D kinases (cdk) 2, 4,
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 p16INK4a is inactivated by methylation at prevalence up to 60% to 70% in primary lung cancers (10) with low frequency of mutations (16). p16INK4a 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 p16INK4a 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 p16INK4a 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 animals, human surrogates or in vitro studies (18). As for p16INK4a gene, Kim and colleagues (19) studied the association between methylation of the p16INK4a promoter region and the exposure to tobacco smoke in 185 primary non-small cell lung cancers. They found that levels of methylation of p16INK4a were correlated with pack-years smoked, duration of smoking, and negatively with the time since quitting smoking. Higher frequencies of p16INK4a 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 p16INK4a 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 p16INK4a 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 p16INK4a methylation in occupational PAHs exposure and chemical carcinogenesis, we examined the p16INK4a 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 p16INK4a 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 p16INK4a 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 transferred to a tube with total volume of 200 μL mixture (pH 5.0) containing
fresly 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 ABI96-capsillary sequencer systems equipped with capillaries of 36 cm separation length.

MethyLight analysis

After sodium bisulfite 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\textsuperscript{INK4a} 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\textsuperscript{INK4a} (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 \(\chi^2\) 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\textsuperscript{INK4a} methylation between 2 groups. The Spearman correlation analysis was carried out to address the correlations between p16\textsuperscript{INK4a} 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\textsuperscript{INK4a} 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\textsuperscript{INK4a} 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\textsuperscript{INK4a} 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\textsuperscript{INK4a} 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\textsuperscript{INK4a} 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.
indicating that these 22 CpG sites might be the regulatory "hot CpG sites" participating in gene transcriptional suppression. We then recalculated the frequency of p16INK4a 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 p16INK4a gene hypermethylation occurs more frequently in workers exposed to PAHs in a CpG site–specific manner.

The frequency of p16INK4a 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 p16INK4a 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 p16INK4a methylation is correlated with the levels of PAHs exposure and DNA damage.

The effects of BaP treatment on p16INK4a 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 p16INK4a contributes to chemical carcinogenesis, we examined the expression and methylation status of p16INK4a gene during cell culture treatment with BaP.

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

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

a Two-sided 2-sample t test.

b Two-tailed χ² test.

c Two-sided 2-sample t test.

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**Figure 2.** Level of p16INK4a 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.
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 \( \text{mol} / \text{L} \) 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 p16INK4a 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 p16INK4a 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 p16INK4a hypermethylation and this action was reversible. These results revealed that p16INK4a hypermethylation could be a potential biomarker

<table>
<thead>
<tr>
<th>Smoking status</th>
<th>Controls</th>
<th>PAH-exposed workers</th>
</tr>
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<tbody>
<tr>
<td>Nonsmoking</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>Smoking</td>
<td>46</td>
<td>56</td>
</tr>
<tr>
<td>Drinking status</td>
<td>24</td>
<td>15</td>
</tr>
<tr>
<td>Nondrinking</td>
<td>1.60 (0–2.5)</td>
<td>5.71 (1.97–8.57)</td>
</tr>
<tr>
<td>Drinking</td>
<td>1.43 (0–2.5)</td>
<td>6.42 (2.85–11.7)</td>
</tr>
</tbody>
</table>

**Table 3.** The relationships between p16INK4a methylation and the levels of urinary 1-OHP and CBMN in PBLs

<table>
<thead>
<tr>
<th></th>
<th>1-OHP</th>
<th>CBMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total CpG sites</td>
<td>0.450</td>
<td>0.356</td>
</tr>
<tr>
<td>Hot CpG sites (n = 22)</td>
<td>0.474</td>
<td>0.369</td>
</tr>
</tbody>
</table>
for the exposure to environmental PAHs and prediction of high risk of tumorigenesis. Thus, we could place p16INK4a 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 p16INK4a hypermethylation was associated with CBMN frequency in PAH-exposed population. We speculate that BaP-induced DNA damage and p16INK4a 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 p16INK4a 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), p16INK4a 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, p16INK4a aberrant methylation, and lung cancer limits the applications of hypermethylated p16INK4a gene 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 p16INK4a 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 p16INK4a 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 p16INK4a gene was not affected.
In this study, we found that the levels of \( p16^{\text{INK4a}} \) hypermethylation were much higher in PAH-exposed workers. Consistent with the human population study, \( p16^{\text{INK4a}} \) methylation was also found in BaP-induced transformed HBER cells. These results provide evidence that epigenetic biomarkers, such as \( p16^{\text{INK4a}} \) 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 experiment 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 \( p16^{\text{INK4a}} \) 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). \( p16^{\text{INK4a}} \) 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 \( p16^{\text{INK4a}} \) 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, \( p16^{\text{INK4a}} \) 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 \( p16^{\text{INK4a}} \) hypermethylation in vitro and in vivo and \( p16^{\text{INK4a}} \) 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 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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