



Aberrant DNA Methylation of Two Tumor Suppressor Genes, $p14^{ARF}$ and $p15^{INK4b}$, after Chronic Occupational Exposure to Low Level of Benzene

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Abstract

Background: Exposure to benzene would be associated with many diseases including leukemia. Epigenetic alterations seem to be among the main mechanisms involved.

Objective: To determine if chronic occupational exposure to low level of benzene would be associated with DNA methylation.

Methods: Global DNA methylation and promoter-specific methylation of the two tumor suppressor genes, $p14^{ARF}$ and $p15^{INK4b}$, were assessed employing methylation-specific PCR using the DNA extracted from 40 petrochemical workers exposed to ambient benzene levels of <1 ppm, and 31 office workers not exposed to benzene or its derivatives.

Results: While an increase in global DNA methylation of 5% in $p14^{ARF}$ ($p=0.501$) and 28% in $p15^{INK4b}$ ($p=0.02$) genes was observed in the exposed group, no hypermethylation in either of the studied genes was observed in the unexposed group. No significant association was found between the frequency of aberrant methylation and either of age, work experience, and smoking habit in the exposed group.

Conclusion: Chronic occupational exposure to lower than the permissible exposure limit of benzene may still result in DNA methylation of tumor suppressor genes that may ultimately lead to development of cancer.

Keywords: Benzene; DNA methylation; Genes, tumor suppressor; Tumor suppressor protein p14ARF; Cyclin-dependent kinase inhibitor p15; Neoplasms

Introduction

Widely used in various industries, benzene (CAS 71-43-2) is one of the predominant air pollutants.¹ Chronic exposure to benzene can cause

aplastic anemia and increase the risk of some blood dyscrasia including acute myelogenous leukemia.^{2,3} Epigenetic changes in response to chronic exposures to benzene are among the main mechanisms of concern recently under extensive study.

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DNA methylation is an important epigenetic mechanism, which often occurs in response to environmental stimuli and is crucial in regulating gene expression. Global DNA hypomethylation and promoter hypermethylation of tumor suppressor genes are frequently observed in hematological malignancies.² Aberrant methylation changes of specific genes or regions associated with inefficient gene products can be induced by environmental pollutants.³⁻⁵ Hypermethylation of tumor suppressor genes has the potential to be a valuable biomarker for the extent of exposure to benzene.⁶

Located at 9p21 gene cluster region, *p14^{ARF}* and *p15^{INK4b}* anti-oncogenes have important roles in regulating cell proliferation.⁷ This makes this gene cluster a target for selective inactivation during carcinogenic process.⁸ High agglomeration of CpG islands on promoter regions of these genes predisposes them to often be inactivated by promoter methylation.^{9,10} Inactivation of *p14^{ARF}* and *p15^{INK4b}* genes by altered promoter methylation has been demonstrated in some types of malignancies.¹¹

We previously showed that exposure to low level of benzene does not increase the frequency of micronucleus, nucleoplasmic bridge, and nuclear budding in the peripheral blood lymphocytes, biomarkers for DNA damage, in a group of petrochemical workers.¹² We conducted this study to determine if chronic occupational exposure to low level of benzene is associated with the extent of methylation of the *p14^{ARF}* and *p15^{INK4b}* promoter CpG islands.

Materials and Methods

In a cross-sectional study, 40 workers exposed to low level of benzene and 31 unexposed workers were studied. The exposed people included 40 petrochemical male workers selected from the production unit of a petrochemical plant who had been exposed to benzene levels of <1 ppm (lower than the permissible exposure limit), confirmed based on periodic environmental monitoring, for at least five years. The 31 unexposed people were office workers selected from the same area who had not been exposed to benzene or other related derivatives.

Those with history of chemotherapy, radiotherapy, x-ray exposure, serious infections, and consumption of genotoxic drugs or supplemental vitamins during the previous six months were excluded from the study. Peripheral blood samples were collected in tubes containing 0.05 M EDTA. All samples were stored at 4 °C and transported to the laboratory within 8 hrs.

DNA Extraction

Genomic DNA was isolated from whole blood using a rapid genomic DNA isolation kit (MBST, Iran) according to the manufacturer's directions. The purity and concentration of the extracted genomic DNA were determined by nanodrop spectrophotometry.

TAKE-HOME MESSAGE

- Benzene, one of the most widely used industrial chemicals, is demonstrated to affect the health of a large number of workers.
- There is increasing experimental and epidemiological evidence that chronic occupational exposure to benzene is associated with an increased risk of hematological malignancies such as acute myelogenous leukemia.
- Several reports showed that epigenetic alterations in tumor suppressor genes induced by occupational and environmental exposure to genotoxic chemicals play a significant role in early stages of cancer development.
- Chronic occupational exposure to lower than the permissible exposure limit of benzene was associated with DNA methylation of *p15^{INK4b}* gene.

Table 1: Primers for methylation-specific PCR

Primer	Forward sequence	Reverse sequence	Product size (bp)
<i>p14^{ARF}</i> M	5' GTGTTAAAGGGCGGCGTAGC 3'	5' AAAACCCTCACTCGCGACGA 3'	122
<i>p14^{ARF}</i> UM	5' TTTTGGTGTAAAGGGTGGTGTAGT 3'	5' CACAAAACCCTCACTCACAACAA 3'	132
<i>p15^{INK4b}</i> M	5' GCGTTCGTATTTGCGGTT 3'	5' CGTACAATAACCGAACGACCGA 3'	148
<i>p15^{INK4b}</i> UM	5' TGTGATGTGTTTGTATTTGTGGTT 3'	5' CCATACAATAACCAACAACCAA 3'	154
<i>MyoD</i>	5' TGATTAATTTAGATTGGGTTTAGAGAAGGA 3'	5' CCAACTCCAAATCCCCTCTCTAT 3'	152

Bisulfite Treatment, Methylation-Specific PCR (MS-PCR)

The extracted DNA was treated with sodium bisulfite to convert all unmethylated cytosines to uracils. Bisulfite conversion was performed with the EpiTect Bisulfite kit (Qiagen, Germany) on 2 µg DNA.

MS-PCR included two separate PCR reactions using methylated/unmethylated-specific amplifiers flanking the CpG-rich *p14^{ARF}* and *p15^{INK4b}* promoter regions. MS-PCR was carried out according to the conventional procedure previously mentioned by Herman, *et al.*¹³

EpiTect control DNA (Qiagen, Germany) and *MyoD* gene amplification with primers selected in an unmethylated region, were served as positive methylated and bisulfite treatment controls, respectively. In the meantime, a blank control without DNA template was used for co-amplification. The primer sequences (Table 1) and PCR conditions for *p14^{ARF}*, *p15^{INK4b}* and *MyoD* genes are reported elsewhere.^{14,15} PCR products were electrophoresed on a 2% agarose gel.

Ethics

The study protocol was approved by the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran. Written informed consent was obtained from each study participant.

Statistical Analysis

IBM SPSS® for Windows® ver 22.0 was used for data analysis. The distribution of observed methylation frequencies was compared between exposed and unexposed groups using Fisher's exact test. A p value <0.05 was considered statistically significant.

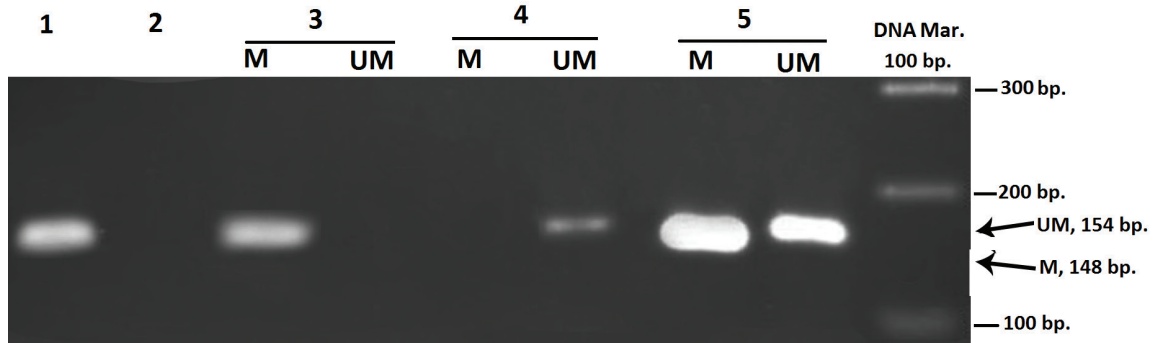
Results

The mean age of exposed group, 33.1 (SD 3.2) years, was significantly (p=0.011) lesser than that for unexposed group (35.8 [SD 5.4] years). The mean work experience in the exposed group, 8.7 (SD 2.7) years, was also significantly (p=0.014) lesser than that for the unexposed group (11.2 [SD 5.5] years). Five (13%) of 40 exposed and two (6%) of 31 unexposed workers were current smokers (p=0.457). None of the participants reported alcohol consumption.

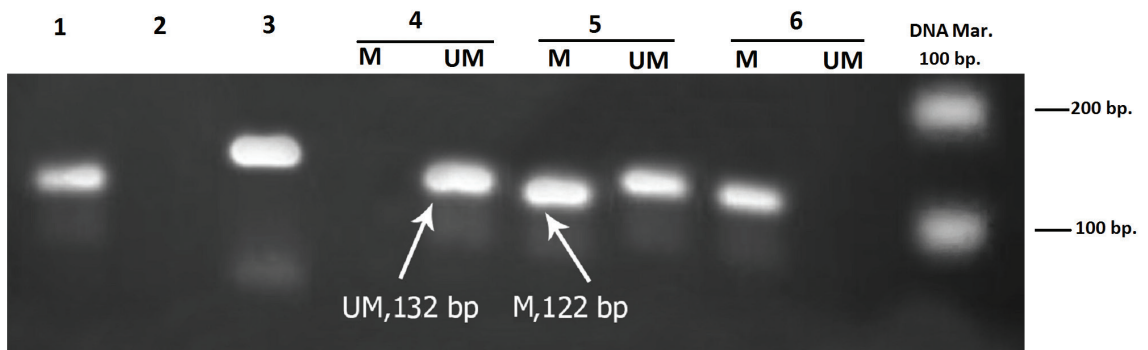
***p14^{ARF}* and *p15^{INK4b}* Methylation**

No one in the unexposed group had promoter hypermethylation in *p14^{ARF}* and *p15^{INK4b}* genes. Aberrant methylation of *p14^{ARF}* gene was detected in two (5%) exposed workers. *p15^{INK4b}* methylation was found in 11 (28%) exposed workers. Although the methylation rate was significantly (p=0.02) higher in *p15^{INK4b}* gene compared to that in the unexposed group, it was not significantly (p=0.501) different for *p14^{ARF}* gene between exposed and

Figure 1: A representative agarose gel demonstrating the methylation-specific PCR products of (A) *p14^{ARF}* and (B) *p15^{INK4b}*. The PCR products were run on a 2% agarose gel. EpiTect control DNA and *MyoD* amplification were used respectively, as positive methylated and bisulfite treatment controls. Blank control without DNA template was used as negative control. M: methylated primer; UM: unmethylated primer



a: Gel documentation for *p14^{ARF}* promoter region by methylation-specific PCR. Lane 1: positive *p14^{ARF}* methylated control (122 bp); Lane 2: negative control; Lane 3: positive bisulfite treatment controls (154 bp); Lanes 4–6: samples from exposed workers



b: Gel documentation for *p15^{INK4b}* promoter region by methylation-specific PCR. Lane 1: positive *p15^{INK4b}* methylated control (148 bp); Lane 2: negative control; Lanes 3–5: samples from exposed workers

unexposed groups. None of the exposed workers had methylation of both studied genes.

One worker with methylated *p14^{ARF}* and five of those with *p15^{INK4b}* methylation had a PCR product corresponding to the unmethylated DNA sequence (Fig 1). No significant association was observed between the status of the studied genes promoter methylation and either of age, work experience, and smoking status. No association was also observed between the studied genes methylation and the frequency of

micronucleus, nucleoplasmic bridge, and nuclear budding in the peripheral blood lymphocytes (data not shown).¹²

Discussion

We found that methylation of *p15^{INK4b}* gene (but not *p14^{ARF}*) was significantly associated with chronic exposure to low level of benzene. This was not dose-dependent. Previous studies on environmental chemical exposures and human epigenetics show that exposure to low-level airborne ben-

zene is associated with alterations in DNA methylation in blood DNA,¹⁶ and hypermethylation of *p15^{INK4b}* gene.⁴ An increase in *p15^{INK4b}* methylation is reported after exposure to arsenic too.¹⁷ Bollati, *et al*, also show significant association between *p15^{INK4b}* hypermethylation with increasing airborne benzene levels in a group of gas station attendants and police officers exposed to low level of benzene.¹⁶

The significant role of epigenetic alterations in benzene carcinogenicity has recently been reported.⁶ Many of the epigenetic changes observed in response to environmental exposures may be mechanistically associated with susceptibility to several diseases.¹ Gene-specific DNA methylation has also been reported in individuals exposed to benzene.¹⁸

Contradictory results on methylation of tumor suppressor genes have been reported in various studies. For example, in a Bulgarian population, gene-specific hypomethylation of *p15^{INK4b}* is reported after exposure to benzene.¹⁹ This observation is in direct contradiction to the results reported by Bollati, *et al*.¹⁶ This reflects that epigenetic response to environmental stimuli is variable—the same environmental pollutant may produce different epigenetic changes in different ethnic groups, tissues, and even within the same tissue on different cell types.⁴ However, the majority of results support the increase in *p15^{INK4b}* promoter methylation in response to exposure to benzene in accordance to our results.

Studies on molecular changes of *p14^{ARF}* and occupational exposure are very limited and more focused on genetic modifications such as gene polymorphism.²⁰ Studies on the epigenetic alterations of *p14^{ARF}* are almost limited to cancer, not focused on airborne pollutants, especially benzene.

Higher rates of *p14^{ARF}* and *p15^{INK4b}* methylation are reported in coke oven workers exposed to airborne benzo[a]pyrene (10.7% and 22.3%, respectively) where the

frequency of *p14^{ARF}* hypermethylation depends on work experience; that of *p15^{INK4b}* does not.²¹ Our findings did not reveal any associations between the frequency of promoter methylation of *p14^{ARF}* and *p15^{INK4b}* and either of age, smoking habit, and work experience. That would be attributed to the low sample size of our study. Nonetheless, our observation of no consistent association between the aberrant methylation and smoking, as a source of benzene,¹⁶ was in line with previous reports.^{16,17} This might be a result of a mixture of a high number of agents with complex synergic effects that could level off the effect of the tobacco-derived benzene.¹⁶ DNA methylation is an age-dependent process.¹¹ However, all of our participants were almost young and no association was observed between the frequency of methylation and the age of participants.

Inactivation of *p14^{ARF}* would result in degradation of the p53 protein,²² a potential target for methylation in cancer. Hypermethylation of *p15^{INK4b}* would also result in a five-fold increase in the risk of *p53* methylation.²³ Both genes are members of the INK4 family of cyclin-dependent kinase (CDK) inhibitors located at the same chromosomal region. Accumulation of methylation aberrancies at their promoter region, which is common mechanism for loss of tumor suppressor genes function,²⁴ represent potential step towards development of malignancies.

Our previous work show no effect of chronic exposure to low level of benzene on peripheral blood elements.¹² However, findings of our current study of aberrant epigenetic changes in those chronically exposed to lower than the permissible exposure limit of benzene would raise the concern about the potential toxicity of benzene.

Our study had several limitations. Because of the cross-sectional nature of the study, we could not make any cause and

For more information on changes in peripheral blood cells after exposure to low level of benzene see <http://www.theijoem.com/ijoem/index.php/ijoem/article/view/785>



effect relationship between the observed DNA methylation and the exposure. Furthermore, we could not be sure how accurate was the periodic measurements of the ambient benzene in the studied unit. Another covariate was that the workers in the unit had not been solely exposed to benzene; they had been exposed to various chemicals the effects of which on DNA methylation were not considered in our analyses.

In conclusion, chronic exposure to low level of benzene might cause hypermethylation of tumor suppressing genes. This would result in many occupational-related malignancies. Further well-controlled studies should be conducted to shed light on this important issue.

Conflicts of Interest: None declared.

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