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# Arsenic Exposure Induces Genomic Hypermethylation

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**ABSTRACT**

Gene specific hypermethylation has previously been detected in Arsenic exposed persons. To monitor the level of whole genome methylation in persons exposed to different levels of Arsenic via drinking water, DNA was extracted from peripheral blood mononuclear cells of 64 persons. Uptake of methyl group from ^{3}H labeled S-Adenosyl Methionine after incubation of DNA with SssI methylase was measured. Results showed statistically significant (p = 0.0004) decrease in uptake of ^{3}H methyl group in the persons exposed to 250-500 μg/L arsenic, indicating genomic hypermethylation.

Key words: Genomic hypermethylation, Arsenic, hypomethylation, India

## Introduction

A large number of humans are chronically exposed to low levels of arsenic through ingestion of arsenic contaminated food and water and due to inhalation of arsenic from the ambient air. Arsenic, though accepted as a carcinogen by the US environmental protection agency, does not produce significant point mutation (Schwerdtle et al., 2003), and the underlying mechanisms of arsenic induced carcinogenesis are not clear.  As metabolism of arsenic occurs through repeated reduction and oxidative methylation of inorganic arsenic, perturbation of DNA methylation during detoxification of Arsenic has been hypothesized and tested in tissue culture system, using murine and human cell lines (Mass and Wang, 1997). We demonstrated hypermethylation of *p53* and *p16* genes in peripheral blood lymphocyte DNA of arsenic exposed population (Chanda et al., 2006). In an extension of that work, change in whole genome methylation status due to arsenic exposure has been studied in this work by monitoring the variation of methyl acceptance capacity of DNA extracted from peripheral blood of persons exposed to various doses of arsenic using SssI methylase assay.

## Materials and Methods

### Subjects and Selection criteria

All subjects of this study were selected from the arsenic clinic of Seth Sukhlal Karnani Memorial Hospital, Kolkata, India. They were residents of South & North 24 Parganas, West Bengal, India. Criteria of diagnosis of arsenicosis and its severity are based on the parameters described earlier (Chanda et al 2006, Guha Mazumder et al 1998, 2001). Briefly these are as follows.

a) History of exposure to arsenic contaminated water (>50μg/L) as a source of drinking water for more than 6 months. The average duration of exposure was about 10 years.

b) Presence of following characteristic skin manifestations of chronic arsenic toxicity:

Hypo pigmentation, Hyper pigmentation, Keratosis.

History of arsenic exposure of each participant was obtained in detail including duration of intake of water from the contaminated source. Samples of water from the source were collected for each participant. Level of arsenic in water was determined by atomic absorption spectrophotometer with hydride generation system (AAS) and expressed in μg/l. Participants have been divided into four groups according to concentration of arsenic in their drinking water.

All male participants were small traders or were involved in office jobs in small concerns. Women were housewives. Most of the men were smokers and women were all non-smokers.

For this study the total numbers of arsenic exposed samples of different groups were 64 (24 females, 40 males, median age 39.5 years, range19-84 years) [Gr. A- 15{6 females, 9 males, median age 47 years, range 23- 84 years}; Gr.B-15 {4 females, 11 males, median age 35 years, range 24-60 years}; Gr.C-19 {11 females, 8 males, median age 36 years, range 19-61 years}; and Gr.D-15 {3 females, 12 males, median age 42 years, range 22-65 years}].

All participants were Indians, from the state of West Bengal.

Written informed consent was obtained from all participants and institutional ethical committee approved the study. Ethical principles followed by the institute are guided by rules as formulated by Indian Council of Medical Research and these are in agreement with Helsinki rules.

**Determination of Arsenic concentration in water** –

Level of arsenic in water was determined by atomic absorption spectrophotometer with hydride generation system (AAS-HG) as described earlier (Atallah et al., 1991).

**Genome wide methylation assay-**

Genome-wide methylation was determined by an in vitro methyl acceptance capacity method described earlier (Woodson et al., 2001). Each sample was processed in duplicate.

**Statistical Analysis-**

Statistical analysis for this study was done using nonparametric methods. Fligner's test for the equality of variances showed that a significant inter group difference in variability exists between the groups, and standard ANOVA methods cannot be applied on the raw methylation data. However, if Fligner's test is applied on logarithmically transformed data, the difference in variability ceases to be significant (p>0.2). So the following ANOVA model was fitted to the logarithmically transformed data:

$\log (\text{Methylation}\_{ij}) = a + b\_i + e\_{ij}$

where $i$ = Group,   $j$ = observation number

The $e\_{ij}$'s are assumed to be independent normally distributed random variables with a common variance.

Finally, the group means were compared  pair wise using Tukey's method to correct for multiple comparisons.

### Results

Scatter diagram and box plots for the $^3$H methyl acceptance capacity of DNA isolated from peripheral blood of individuals pre exposed to different arsenic doses in drinking water is presented in Figure 1.

Side by side box plots of the methylation levels for the four groups indicate considerable variation in the within group variances.

As described in methods, Fligner's test was applied on logarithmically transformed data. On retransforming the data to the original scale, the following predicted group wise means together with 95% prediction interval was obtained.

| Group | Mean prediction | Lower 95% CI | Upper 95% CI |
|-------|-----------------|--------------|--------------|
| GroupA | 20551.591 | 14304.533 | 29526.86 |
| GroupB | 19628.572 | 13662.084 | 28200.74 |
| GroupC | 7324.018 | 5307.878 | 10105.97 |
| GroupD | 13852.905 | 9642.044 | 19902.73 |

The group means were compared pair wise to test for the significance of their difference by Tukey's method. The following are the p-values for the pair wise differences in (log)average group wise methylation levels:

| B-A | C-A | D-A | C-B | D-B | D-C |
|-----|-----|-----|-----|-----|-----|
| 0.9979279106 | 0.0004198711 | 0.4206910969 | 0.0007915062 | 0.5288856948 | 0.0515397600 |

Statistical analyses of the above data reveals that methyl acceptance capacity of group C is significantly low compared to that of group A and group B. However the persons exposed to >500 µg/L (Group- D) of arsenic dose show higher median value of the methyl acceptance capacity compared to that exposed to 251- 500 µg/L (Group-C). The data of group C only shows significant hyper methylation compared to group A, while the patients of group D show difference from group A on the basis of methyl acceptance capacity, but this fails to achieve significance.

## Discussion

Metabolism of arsenic occurs through repeated reduction and oxidative methylation of inorganic arsenic. Inorganic arsenic is reduced to arsenite in the gastrointestinal tract, which is then methylated mainly in the liver to monomethyl arsonic acid (MMA) and dimethyl arsinic acid (DMA). The methylated metabolites of arsenic are electrophilic, and are therefore readily excreted through urine (Vahter, 1999).

Reduced glutathione and methyl donor S-adenosyl-methionine (SAM) are requisites for metabolism of arsenic and glutathione-S-transferase omega class *hGSTO1* has been implicated in the reduction process and was found to catalyze the reduction of $MMA^V$ to $MMA^{III}$ in various tissues.

Poor in-vitro mutagenicity of arsenic and involvement of methylation machinery during metabolism of arsenic has led to the hypothesis of perturbation of genomic methylation in arsenic exposed people. Previous works with human adenocarcinoma cell line in tissue culture showed that arsenic induces significant changes in methylation status in tumor suppressor gene p53 (Mass and Wang et al.1997). Later, using arsenic exposed human kidney cell lines global hyper and hypomethylation has been demonstrated by the same group (Zhong et al., 2001). DNA sequencing and SssI methylase assay were used for estimation of genomic CpG methylation level. Arsenic exposure of A 549 cells in culture resulted in a dose dependent increase in cytosine methylation in *p53* gene. SssI methylase assay showed that arsenite could cause a small increase in global methylation (Mass. and Wang et al., 1997). Using methyl sensitive restriction enzyme and bisulfite treatment method, hyper-methylation of *p53* and *p16* genes respectively have been detected in DNA extracted from peripheral blood of Arsenic exposed persons (Chanda et al., 2006), and this is the first report of perturbation of methylation in the peripheral blood DNA of arsenic exposed persons.

This work is followed by other reports on methylation perturbation after arsenic exposure. From China, arseniasis patients were found to have increased level of *p16* methylation, and the effect increased with degree of arseniasis (Zhang et al., 2007).

The present work is the study on whole genome DNA methylation level of peripheral blood DNA of humans subjected to chronic arsenic exposure. There is a large dispersion of the data, and if the median values are considered, all arsenic exposed groups show some reduction of

methyl acceptance capacity, i.e. they are hypermethylated. There is, however, statistically significant increase in methylation level only in the group C persons, exposed to 250-500 μg/L of arsenic. This significance is lost in group D, exposed to >500 μg/L of arsenic in drinking water. This is in contrast to the result on gene specific methylation studies, where both group C and group D probands had statistically significant increase in methylation level over group A probands (Chanda et al., 2006). We discuss the possible reasons of this discrepancy below.

Tissue culture experiments demonstrated that exposure to inorganic arsenic in the form of arsenite or arsenate produces gene specific as well as genomic hypermethylation. A different group, on the other hand, subjected rat liver epithelial cells to chronic low level arsenic exposure and demonstrated hypomethylation of α-satellite DNA in the resulting aggressive tumor cells (Zhao et al., 1997). Exposure to high level (26mg/L) of  arsenite induced DNA hypomethylation in goldfish (Bagnyukova et al., 2007).

To explain the results, it is noted that inorganic arsenic induces cytosine methyltransferase in tissue culture system (Goering et al, 1999) and array analysis of tissue from mice exposed to different doses of arsenic also showed upregulation of DNA methyltransferase 3a gene (Ahlborn et at, 2008).  This can cause the initial hypermethylation of DNA after arsenic exposure. However, as arsenic load increases, its detoxification through enzymatic methylation causes depletion of the methyl donor SAM which has a vital role in the detoxification process, leading to inefficient arsenic excretion, and DNA hypomethylation. DNA methyltranferase (DNMT) uses the same methyl donor SAM and maintenance methylation is disrupted when the level of SAM in the body drops. It is expected that the extent of methionine intake would influence the rate of arsenic excretion and also the extent of DNA methylation.

Our results can thus be explained in terms of increase in hypermethylation with increasing arsenic dose from group A to C. In the last group with arsenic dose >500 μg/L, there is probably too much methionine requirement for excretion of the large amount of arsenic ingested, and the extent of hypermethylation is no longer statistically significant, due to the reduced  SAM level. This probably signifies the beginning of the process of hypomethylation. It might be mentioned that in our earlier work too, a small number of samples of group C and D showed hypomethylation, the level of significance increasing from .02  to .001 as the arsenic dose increased (Chanda et al., 2006).

Till date there is only one report on genome-wide DNA methylation level of humans chronically exposed to arsenic (Pilsner et al., 2007). Originally designed to detect hypomethylation, its findings corroborate with our conclusion that chronic arsenic exposure increases genome-wide methylation. In this work the concentration of arsenic in the drinking water varied between 100-200 µg/L. Methylation level varied with nutritional level, arsenic level in blood plasma, arsenic level in drinking water and age. In particular, methyl incorporation was found to be negatively correlated with arsenic level in water in persons with high plasma folate level, but this correlation is lost in persons with low plasma folate level. 5 methyl tetrafolate is the major source of methyl group for SAM, and its depletion is bound to reduce all methylation reactions, indicating that high methionine intake is essential for DNA hypermethylation.

It must be emphasized that in addition to exposure dose and nutritional level, there are other factors which influence the level of genomic methylation like, age (Pilsner et al., 2007), smoking habit, and possibly polymorphism status of *GSTO1* and *AS3MT* gene.  The wide dispersion of the data can thus be explained.

We have not detected significant variation of methylation acceptance with age, in contrast to the other study.

In essence, this work demonstrates that there is an increase in whole genome methylation following arsenic exposure of 200-500 µg/L, and >500 µg/L higher inorganic arsenic burden in the body causes reduction of genomic methylation.

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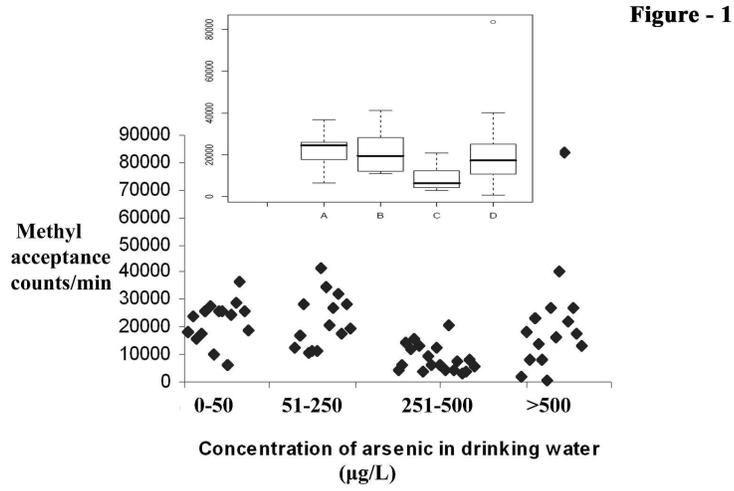
**Figure 1.**  Distribution of methyl acceptance in counts/minute in people exposed to different concentrations (μg/L) of arsenic in drinking water.

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**Figure - 1**



Distribution of methyl acceptance in counts/minute in people exposed to different concentrations (ìg/L) of arsenic in drinking water.

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