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Homeobox Gene Fragment Hypermethylation in Chronic Low Level of Arsenic Exposure in Residents of West Bengal

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Abstract—*Arsenic, though a poor mutagen, is an accepted environmental carcinogen. Perturbation of DNA methylation pattern leading to aberrant gene expression has been chosen as a mechanism for arsenic induced carcinogenesis. We had already demonstrated the presence of one hypermethyled fragment from human GMDS gene in arsenic induced skin cancer patients (Chanda et al.2013). In this study we are going to demonstrate another hypermethylated fragment from chronically highly arsenic exposed patients with Bowen's disease and arsenic induced bladder cancer patients. The fragment identified is from human homeobox gene and it is identified by methyl-sensitive arbitrarily primed polymerase chain reaction from urothelial cell DNA and the peripheral blood leukocyte DNA. The gene is responsible for developmental accuracy. Any mutation or transcriptional inactivation may lead to developmental defects. In lung and other solid tumors Homeobox gene hypermethylation have also been reported.*

Keywords: *Arsenic exposure, Arsenic induced cancer, homeobox gene, hypermethylation.*

1. INTRODUCTION

According to (International agency for research on cancer 1997) and National Research Council (NRC 1999) arsenic is an important environmental toxicant and carcinogen. However, the mechanism of arsenic mediated carcinogenesis is not due to any point mutation as arsenic is a poor mutagen (Rossman et al. 1980; Jacobson and Moltanbano 1985; Lee et al. 1985). Biotransformation of arsenic is the mechanism by which arsenic is transformed to less clastogenic methylated metabolites which are more electrophilic and excreted through urine (Vahter 1999). The interference of the DNA methylation pathway with arsenic detoxification pathway, as both the pathways require SAM, can lead to aberrant DNA methylation, resulting aberrant expression and/or silencing of genes (Georing et al. 1999). Therefore, epigenetic alterations, particularly aberrant DNA methylation has been mooted as a possible mechanism of arsenic induced carcinogenesis (Ren et al. 2010; Reichard and Puga 2010). Methylations of gene regulatory elements are known as epigenetic alteration which involves DNA methylation and histone methylation. Both of these causes altered chromosome compaction and transcriptional alteration (Fang et al 2004) which may cause cancer.

Cytosine-5 methylation at the CpG islands in the regulatory sequence of an active gene is one of the key mechanisms of gene inactivation. DNA methylation/demethylation seems to regulate a plethora of biological processes involving transcription, differentiation, development, DNA repair, recombination, and chromosome organization. Perturbation of DNA methylation has been correlated with many cases of cancer (Jones and Baylin 2002). The hypothesis that arsenic perturbs DNA methylation has been tested successfully on tissue culture system (Mass and Wang 1997), and later we demonstrated dose response pattern of hypermethylation of the promoter region of *p53* and *p16* genes in DNA extracted from human peripheral blood leukocytes of persons exposed to different doses of arsenic (Chanda et al. 2006). A few highly exposed persons also showed *p53* hypomethylation (Chanda et al. 2006). Further arsenic induced genome wide hypermethylation has been demonstrated by us in DNA extracted from same population (Majumder et al. 2010). We have also demonstrated hypermethylation human GMDS gene in persons chronically exposed to drinking water arsenic with arsenic induced cancer (Chanda et al 2013). The sequence isolated was hypermethylated in its first exon.

In this report we have further evaluated the hypothesis on a subsection of exposed population chosen from of West Bengal. It is studied by isolating hypermethylated gene fragment from genomic DNA of arsenic exposed persons by methyl sensitive arbitrarily primed polymerase chain reaction

(MS-AP-PCR). Differentially methylalated fragments have been identified and isolated from chronically arsenic exposed people. 4 persons of arsenic induced Bowen's disease (all the patients were recruited from NAIP study conducted in 2008-2009 in which field study was conducted in various blocks of Nadia district, West Bengal), have one common hypermethylated fragment of 580 bp. This hypermethylated fragment was also isolated from chronic arsenic exposed person with bladder cancer. The sequences were then analyzed by bioinformatic tools (NCBI BLAST) to indicate that the fragment is actually situated in the human homeobox gene. Though have been found here in small proportion this hypermethylated fragment may be act as a potential target (probe) for detecting aberrant methylation in chronic high level of arsenic exposure.

2. MATERIALS AND METHODS

Subject selection

Subjects of this study were chosen from one arsenic exposed district of West Bengal, Nadia. Two blocks were selected where the ground water arsenic concentration were high enough.

Criteria of diagnosis of arsenicosis and its severity are based on the parameters described earlier (GuhaMazumder,1998; 2001; Chanda et al. 2006). In this study morning void urine samples were collected in sterile vial with protease inhibitor. DNA was isolated from urothelial cells. Participants had been divided into the three groups A, B, C according to the concentration of arsenic in their drinking water, i.e. 0–50, 51–250, 251–500 µg/l respectively and a small fraction designated as group D have arsenic induced skin and bladder cancer having exposure level similar to group C. As the concentration of arsenic in group A is within the permissible limit according to WHO and Medical Council of India, it was considered as the unexposed control group (NRC 1999). Initially the number of participants in each group was 33, 52, 60 in group A, B, and C respectively and in group D there was 11 subjects. Among those 11 subjects 4 had arsenic induced Bowen's, disease and 7 subjects had arsenic induced bladder cancer. All of the subjects chosen for MS-AP PCR have hypermethylated *p16* promoter region compared to normal unexposed persons (Chanda et al. 2006). All of the subjects studied for MS-AP-PCR were compared to normal unexposed subjects treated in similar way.

The studies on isolation of hyper/hypo methylated fragments of genomic DNA was performed from urothelial cell DNA of highly arsenic exposed persons of group C and group D. Later, lower exposure group B was also evaluated for the presence of such hyper/hypomethylated gene fragments. Among 16 of the group C participants studied, 2 had a hypermethylated DNA fragment of 580 nucleotide long sequence. Among 11 subjects of group D, 3 had that hypermethylated fragment. The fragment identified was from the participants of group C and D and but not from any lower exposure groups. Although there is an overlap between group C and D in respect to the concentration of arsenic in water but the difference is one group have arsenic induced Bowen's disease and bladder cancer with higher degree of skin manifestations (group D) while the other group (group C) is only characterized by higher degree of skin manifestations without cancer.

Table 1: Demographic data of study subjects taken from different arsenic exposure groups with p16 methylation

Age group	Sex	Group A (0-50 µg/l)	Group B (51-250 µg/l)	Group C (251-500 µg/l)	Group D (251-500µg/l)
<20 years	Male	N = 2; 0.091, 0.19	N= 3; 0.87, 0.923, 1.01	N = 2; 1.34,1.62,	
	Female			N = 2; 1.50,1.45	
21-40 years	Male	N = 4; 0.21,0.33, 0.25, 0.23	N = 2; 1.30, 1.11	N = 5; 2.43, 1.38, 5.10	
	Female	N = 2; 0.15; 0.41		3.14, 2.34	
41-60 years	Male	N = 6;0.17,0.32,0.17 0.31,0.42, 0.18	N=5; 1.14, 1.74, 2.02	N = 4; 1.56, 2.4, 4.12,1.87	N = 6;2.21,3.38,1.09,3.69
	Female	N = 4; 0.018,0.029, 0.13,0.43	0.91, 0.887	N = 2; 1.95, 3.54	2.31, 1.67
>60 years	Male		N= 5; 0.74, 1.32, 0.97,	N= 1; 1.39	N = 4; 3.20,1.23,1.62,1.69
	Female		0.89, 1.31		N = 1; 2.73
Smoking status	Smoker	12		9	11
	Nonsmoker	6		2	1
	Exsmoker				
Average duration of Exposure (years)		7	10	9	8.5
Total number of samples	60	18	15	16	11

Note: numerical values in each cell indicate the degree of p16 methylation for individual study subjects recruited in the study.

Written informed consent was obtained from all participants before drawing their blood. The name of the institute where human clinical studies were carried out is DNGM Research Foundation.

Molecular Biological and *in silico* experiments were carried out in CSIR- Indian Institute of Chemical Biology ,Kolkata which is run by Govt. of India and Govt. of West Bengal. 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 declaration.

Determination of Arsenic concentration in urine and water

Level of arsenic in drinking water and urine was determined by atomic absorption spectrophotometer with hydride generation system (AAS) (Attalah and Kalman 1991).

DNA isolation from Blood & Urine

DNA was extracted from whole blood by conventional chloroform extraction method using 0.01% SDS and Proteinase K (0.1 mg/ml) (Miller et al. 1998).

***P16* methylation status analysis**

The p16 tumor suppressor gene methylation status was analyzed in each subject by the method described earlier (Chanda et al. 2006).

Determination of clinical symptom score

Each subject was assigned a clinical symptom score which reflects the severity of his/her skin manifestations. Control subjects have no pigmentation and keratosis and therefore have a clinical symptom score 0. The detail structure of the scoring system for pigmentation and keratosis is given in Table 2.

Table 2: Dermatological criteria and graduation of chronic arsenic toxicity for scoring sustem of skin manifestations

Pigmentation		
Mild 1	**Moderate score = 2**	**Severe score = 3**
Defuse Melanosis, Mild Spotty pigmentation, Leucomelanosis	Moderate Spotty pigmentation	Blotchy Pigmentation, Pigmentation of under surface of tongue, buccal mucosa
Keratosis		
Mild Score = 1	Moderate score = 2	Severe score = 3
Slight thickening, or minute papules (<2 cm) in palm and soles	Multiple raised keratosis papules (2 to 5 cm) in palm & soles with diffuse thickening	Diffuse severe thickening, large discreet or confluent keratotic elevations (>5 cm), palm and soles (also dorsum of extremely and trunk)

The underlined data represents the clinical symptom score

Restriction enzyme digestion for arbitrarily primed PCR

200 ng of total genomic DNA isolated from persons, unexposed or exposed to arsenic through drinking water, was digested with 3 units of RsaI and 3 units of HpaII restriction enzyme at 37°C overnight. The persons taken for MS-AP-PCR were from higher exposure groups of arsenic (251–500 µg/l, i.e. group C; and from arsenic induced cancer, i.e group D with similar exposure) and all have hypermethylated *p16* promoter. Out of 50 in group C, 16 subjects were taken for MS-AP-PCR.

Methyl sensitive arbitrarily primed PCR (MS-AP- PCR)

When RsaI and HpaII digested genomic DNA was used as template in MS-AP-PCR using random primers with 10 to 12 nucleotide that target CG-rich DNA sequences (Zhong and Mass 2001).

Isolation of candidate bands

The ethidium bromide stained PCR amplified DNA band of hypermethylated fragment was excised from the gel by a scalpel and recovered by the usual 'crush and soak' method (Sambrook et al. 1989). The candidate band isolated from subjects were from group C and D. Clinical symptom score, *p16* methylation status and degree of arsenic exposure for those subjects are given in Table 3.

Table 3: Demographic data and p16 methylation status of subjects having HUMAN HOMEOBOX gene hypermethylation

Sample	**Age (yr)/ sex**	**Smoking status**	**Conc. of arsenic in water µg/l**	**Duration of exposure yrs**	**Degree of pigmentation**	**Total urinary arsenic µg/l**	**P16 methylation value**
B/111/ DAS	43/ M	Non smoker	452	7	++ + 3	27	2.76
B/063/ GO	53/ M	smoker	342	5	++++ 4	63	2.35
B/126/ DAS	48/ M	smoker	425	5	++++ 4	116	3.16
B/026/ DPP	48/F	Non-smoker	414	7	++++ 4	182	2.05
B/043/ MIT	40/ M	Exsmoker	414	7	++++ 4	123	3.01

Note: The pigmentation and keratosis was assigned as a numerical score according to the degree of severity.

Purification and Sequencing:

The gel- recovered PCR product was re-amplified using the same PCR protocol with same primer for two times. The amplified product from each template was pooled and was then purified by ethanol precipitation and send for sequencing.

Result

Using the technique of MS-AP PCR, 1 common hypermethylated DNA fragment was identified from 5 different people with chronic high level of arsenic exposure with and with out arsenic induced cancer. Among 16 of the highly arsenic exposed persons from group C and among 11 of arsenic induced cancer patients of group D , we have identified a total 5 subjects having one common hypermethylated DNA fragment of 580 nucleotide long. Among 11 of group D subjects 3 and among 16 of group C subjects only 2 have been identified to harbour this hypermethylated DNA fragment. Demographic data for study subjects and *p16* methylation status for these 5 subjects (with hypermethylated DNA) has been listed in Table 1 & 3. Interestingly, people from lower arsenic exposure (group B) did not have this hypermethylated gene fragment. The identified and isolated hypermethylated fragment was then purified and sent for sequencing to Thermofisher Scientific for Sanger DNA sequencing. The sequence is from human *homeobox* gene. Once the fragment was identified and isolated from urothelial DNA of arsenic induced cancer patients, the procedure was cross-checked using DNA samples isolated from peripheral blood leukocyte of the same patients in case of group C and D subjects.

DNA sequence analysis revealed that the identified fragment has significant homology match (99%) to the sequence of human *homeobox gene (*Accession no.X56537), *Homo sapiens*) (taken from GENEBANK database) after BLAST search. The sequence is from the active transcriptional site of the gene and started from start codon. The sequence is from chromosome no. 2. Homeobox genes are conserved in evolutionary scale and served as transcription factors which bind to particular DNA segment for switching on or off of the transcribable genes. These factors thus regulate the developmental processes such as regional specification, patterning and differentiation. (Rodrigues & Nunes 2016). These groups of genes are characterized by a conserved 180 bp DNA sequence that code for a 60 amino acid DNA binding protein called homeodomain (Rauch et al. 2007). Mutation of homeobox gene is known to affect development of ectodermal appendages (Rodrigues et al. 2016).

Discussion

In the present work we have investigated that whether there is any probable common target for aberrant DNA methylation after arsenic exposure in exposed persons apart from *p53* or *p16* gene methylation. We have successfully identified one fragment of hypermethylated DNA , human GMDS gene fragment, from persons exposed chronically to arsenic and persons having arsenic cancer(Chanda et. al. 2013). The subjects have been chosen from our previous study population (Chanda et al. 2006) having hypermethylated p53 promoter region with chronic high level of arsenic exposure with and without arsenic induced cancer. Therefore persons having GMDS gene intron hypermethylation also have p53 promoter hypermethylation. Thus this study reflects an association between the p53 promoter hypermethylation with GMDS gene intron hypermethylation in chronic high level of arsenic exposed people. In the present study we have similarly identified and isolated one more hypermethylated fragment of gene from chronic low level of arsenic exposure with arsenic induced cancer and without cancer. The fragment isolated was from human homeobox gene. All the participants having human homeobox gene fragment hypermethylation also have p16 gene hypermethylation. Therefore, this study also is an association study of p16 gene hypermethylation and human homeobox gene hypermethylation in chronic low level of arsenic exposure with and with out cancer.The fragment was isolated from both peripheral blood leukocyte DNA and from urothelial cells of persons having chronic arsenic exposure with induced bladder cancer and Bowen's disease and with out arsenic induced cancer.

Homeobox gene function as a key regulator of genes responsible for morphogenesis and development and these genes are abnormally expressed in cancer. DNA methylation in homeobox gene alters its expression patterns and leads to development of cancer. More over, Homeobox gene hypermethylation has also been notified in numbers of human solid tumors (Rodrigues et al 2016).

Homeobox gene hypermethylation is associated with primary squamus cell carcinoma of lung (Rauch et al 2007). In breast carcinoma also homeobox gene hypermethylationhave been notified. Critical role of homeobox gene methylation in the insurgence and/or progression of breast cancer has been studied by Tommasi and co workers (Tommasi et al. 2008).

As it is reported that aberrant methylation of exons, introns or intergenic regions can regulate non coding RNA function to modify the degree of transcription of a gene and the exonal expression is dependent over the local methylation status rather than the promoter region (Cheung et al. 2011). Consequences of gene methylation beyond transcription start site have also been studied by Hoivik et al. (2011) and Jowaed et al. (2010) in two separate studies where it was reported that intron methylation is associated with altered expression. Moreover, dense methylation surrounding transcription start site or near the first exon is tightly linked with gene silencing (Brenet et al. 2011).

Till to date this is the first report of human homeobox gene hypermethylation in chronic arsenic exposure with and

without malignancy. Reports are also unavailable regarding association between *p16* gene hypermethylation and *homeobox* gene hypermethylation in human cancer as well as in arsenic induced cancer.

During the initial stage of the experiments we did observe some bands of hypomethylation, but we failed to clone them. It might be mentioned that in our previous investigations too, we observed far fewer hypomethylation cases. It is postulated that overexposure of arsenic and its biotransformation causes depletion of SAM, leading to hypomethylation of DNA. Hence extensive hypomethylation probably needs a very high exposure, which is achieved in artificial tissue culture systems, but rarely in real life situation. In the tissue culture experiments too, the study with cells exposed to arsenite for 2–4 weeks observed mostly hypermethylation and a few hypomethylation cases (Zhong et al. 2001). Chronic exposure of 18 weeks at low dose, on the other hand produced extensive hypomethylation and transformation in rat hepatocyte cell line (Zhao et al. 1997).

3. CONCLUSION

To sum up, this is the first report of *human homeobox* gene fragment hypermethylation in the peripheral blood leukocyte DNA and urothelial DNA of persons exposed to arsenic. To ascertain this fragment of hypermethylation as a biomarker for arsenic induced cancer and chronic arsenic exposure researchers require repetition of such work in large sample group.

4. COMPETING INTEREST

The authors declare that there is no competing interest exists.

Authors' contribution

CS and CT are contributing for the conception, design and planning of the work. The data analysis and interpretation has been done by CS. GDN is the contributor for clinical analysis of the subjects, All authors read and approved the final manuscript.

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