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RESEARCH ARTICLE

Skin score correlates with global DNA methylation and GSTO1 A140D polymorphism in arsenic-affected population of Eastern India

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ABSTRACT
Arsenic is a potent environmental toxicant causing serious public health concerns in India, Bangladesh and other parts of the world. Gene- and promoter-specific hypermethylation has been reported in different arsenic-exposed cell lines, whereas whole genome DNA methylation study suggested genomic hypo- and hypermethylation after arsenic exposure in *in vitro* and *in vivo* studies. Along with other characteristic biomarkers, arsenic toxicity leads to typical skin lesions. The present study demonstrates significant correlation between severities of skin manifestations with their whole genome DNA methylation status as well as with a particular polymorphism (Ala 140 Asp) status in arsenic metabolizing enzyme Glutathione *S*-transferase Omega-1 (GSTO1) in arsenic-exposed population of the district of Nadia, West Bengal, India.

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Arsenic; whole genome DNA methylation; skin manifestation score; GSTO1*A140D; West Bengal; India

Introduction

More than 26 million residents of West Bengal, India are endemically exposed to high doses of inorganic arsenic (As) through drinking water, food and air at a level far above the acceptable limit of 10 µg/L (Chakraborti *et al.* 2009) leading to serious health concerns. According to West Bengal Pollution Control Board, ground water of 81 blocks of eight districts in West Bengal are contaminated with arsenic. Publication of Planning commission, Govt of India, reported arsenic contamination in ground water in West Bengal vary from 0.06 to 3.2 mg/L (Government of India Planning Commission 2007).

Pigmentation and keratosis are the characteristic skin lesions of chronic arsenic toxicity. It also produces various systemic manifestations over and above skin lesions; important ones being chronic lung disease, liver diseases like non-cirrhotic portal fibrosis, polyneuropathy, peripheral vascular disease, hypertension and heart disease, diabetes mellitus, non-pitting edema of feet/hands, weakness and anemia. Cancer of skin, lung and urinary bladder is typically associated with chronic arsenic toxicity (Guha Mazumder *et al.* 1988, Mazumder *et al.* 1998, NRC 1999, 2001, IARC 2004). However, it is observed that both skin and systemic manifestations are typically restricted to 10–15% of the exposed population. Remaining 85–90% (belonging to the same socio-economic and ethnic strata) remains asymptomatic: strongly indicating genetic predisposition. Eichstaedt *et al.* (2015) also hypothesized strong selection signatures in genes involved in arsenic metabolism in Colla population from the Puna region in Northwest Argentina who are exposed to arsenic in drinking water exceeding the recommended maximum by a factor of at least 20-fold since thousands of years.

Arsenic is readily absorbed after oral exposure and primarily metabolized through methylation and excreted through urine in most species (Hughes 2006). Methylation plays an important role in arsenic biotransformation. Genetic polymorphisms in enzymes responsible for arsenic metabolism, detoxification and urinary excretion are believed to account for the inter-individual variation in arsenic metabolism and thus susceptibility to arsenic toxicity (Wood *et al.* 2006, Schlawicke Engstrom *et al.* 2007, De Chaudhuri *et al.* 2008). Here, we have studied some of the genetic and epigenetic factors and their possible association with biomarkers of arsenic exposure and lifestyle habits.

Methylation occurs through repetitive reduction of AsV species to AsIII species in gastrointestinal tract and oxidative methylation of AsIII species in liver to yield methylated pentavalent metabolites [monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA)], respectively (Thomas *et al.* 2001). The methylated metabolites are readily excreted through urine (Hopenhayn-Rich *et al.* 1996). This pathway is incomplete in humans as some arsenic may remain either as iAs or MMA (Steinmaus *et al.* 2005).

In humans, the enzyme As(III) *S*-adenosyl-L-methionine (SAM)methyltransferase (hAS3MT gene on chromosome 10) plays an important role in the methylation process. As3MTcatalyzes the transfer of methyl group from *S*-adenosyl-L-methionine (SAM) to trivalent iAs (As^{III}) (Drobna *et al.* 2006, Dheeman *et al.* 2014). In a review, Agusa *et al.* summarized the association of AS3MT genetic polymorphisms with arsenic metabolism as well as human health effects.

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While most of the SNPs in AS3MT showed inconsistent results in terms of genotype-dependent differences in arsenic metabolism, two SNPs, AS3MT 12390 (rs3740393) and 14458 (rs11191439) were consistently related to arsenic methylation regardless of the study population (Agusa *et al.* 2015). As3MT variant 14458 (M287T, rs11191439, exon 9, T860C) was found to be associated with increased levels of enzyme activity and immune-reactive protein (Wood *et al.* 2006) as well as the percentage of monomethylated arsenic (MMA) in urine (Lindberg *et al.* 2007, Hernandez *et al.* 2008). Valenzuela *et al.* (2009) found its association with cancers, but the level of significance was marginally high ($p = .055$). Analyzing a genome-wide genotype data for 730,000 loci among Colla populations of Argentina, Eichstaedt *et al.* (2015), identified a strong signal of positive selection in the arsenic methyltransferase AS3MT gene.

GSTO1 (Glutathione *S*-transferase Omega-1) catalyzes the reduction of MMAV to MMAIII, the rate limiting step in arsenic biotransformation in humans (Zakharyan *et al.* 2001). Several known polymorphisms of GSTO1 have been studied and implicated in arsenic toxicity. GSTO1 variants, A140D and T217N were found to reduce enzyme activity inhibiting the enzyme's inorganic arsenic biotransformation capacity (Tanaka-Kagawa *et al.* 2003). Moreover, the GSTO1 A140D substitution (exon 4, C419A) was reported to have relationship with several cancer types, vascular dementia and stroke (Kolsch *et al.* 2004, Marahatta *et al.* 2006).

However, there is no report of whether any polymorphism has correlation with whole genome methylation level of arsenic-exposed individuals.

Arsenic does not directly interact with DNA (Rossman 2003); rather it fails to produce significant point mutation, either in bacterial test systems or in mammalian cells in culture (Chinese hamster V79 cells, *Escherichia coli*). Hei and coworkers suggested that arsenic can act as mutagen, depending on the presence of reactive oxygen species for its activity. It is a dose-dependent mutagen causing deletion mutation. Moreover, mutagenic activity of arsenic increases synergistically with UV light, observed in mammalian and human cells (Hei *et al.* 1998). Tezuka and coworkers (1993) demonstrated *in vitro* that exposure to 10 mM dimethylarsinic acid (DMAA) for 10 h caused significant single-strand breaks in DNA of human alveolar type II (L-132) cells. Using flow cytometry analysis Liu *et al.* (2016) showed that As_2O_3 at low concentrations led to enhanced accumulation of cell populations in G2/M phase with increasing exposure time, and increased levels of apoptosisin SK-N-SH cells. Arsenic potentiates adduct formation of benzo[*a*]pyrene with DNA synergistically enhancing the health effect of tobacco use strongly. Maier and coworkers (2002) reported that exposure of mouse hepatoma (Hepa-1) cells to low concentrations of arsenite increases benzo[*a*]pyrene-DNA adduct levels by as much as 18-fold. This was followed by many other works.

As arsenic has poor *in vitro* mutagenicity, epigenetic mechanisms of carcinogenesis like hyper/hypo-methylation of DNA have been invoked. Various researchers have tested the hypothesis on DNA extracted from cultured cell lines exposed to different doses of arsenic compounds. Mass and Wang (1997) demonstrated hypermethylation in the CpG island of p53 tumor suppressor gene in arsenic-exposed human lung cell adenocarcinoma A549 cell line. Later, they extended the work to human lung and kidney (UOK) cell lines, where using methyl sensitive PCR by degenerated primers, they could demonstrate both hyper- and hypomethylation (Zhong and Mass 2001). Such DNA hypermethylation occurs in arsenic-exposed humans too (Chanda *et al.* 2006). Reichard and coworkers (2007) suggested that long-term low dose arsenic exposure may result in DNA hypomethylation by measuring the effect of submicromolar and low-micromolar concentrations of arsenite on the methylation status of DNA and the biochemical reactions that regulate it. This study further demonstrated that arsenic causes the depletion of SAM, and represses the expression of the genes DNA methyltransferase 1 (DNMT1) and DNA methyltransferase 3 alpha (DNMT3A), the two possible complementary mechanisms which result in DNA hypomethylation.

In the present study, status of global DNA methylation has been studied in the Eastern Indian population and the data were analyzed against age, gender, tobacco usage, skin score and amount of arsenic in urine, hair and nail. The status of the particular polymorphisms in AS3MT and GSTO1 gene namely M287T and A140D, respectively and their association with skin score, amount of arsenic in urine, hair and nail, were also studied in the said population, anticipating that accumulation of such data may in future provide an explanation for the variability in inter-individual susceptibility to arsenic toxicity.

Methods

Subjects and selection criteria

The present study was conducted with 23 male and 15 female individuals exposed to arsenic through drinking water and food. All of them were residents of the district of Nadia, West Bengal, India and were drinking arsenic contaminated water (>100 μg/L) for at least 10 years. Individuals selected as control in this study ($n = 37$: 20 males and 17 female. None of them displayed any characteristic skin lesions or detectable concentration of arsenic in hair or nail) were all residents of Kolkata, the state capital and surrounding region drinking surface water purportedly free of arsenic contamination (about 10 μg/L). Due to limitation of resources whole genome DNA methylation was studied in 33 exposed and nine unexposed participants. All experiments were not done with all the samples.

Samples of peripheral blood, hair, nail and socio-economic and tobacco usage data were collected from these selected individuals. 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 concentrations

Arsenic levels in water, urine and hair and nail samples were measured using an Atomic absorption spectrophotometer with a flow-injection hydride generation system (Perkin Elmer AAnalyst 400, Waltham, MA) (Das *et al.* 1995).

Isolation and determination of DNA concentrations

Genomic 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.* 1988). DNA concentration was measured in a nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA).

Determination of skin score

The most common skin manifestations of arsenicosis are pigmentation and keratosis. Total skin score of the participants was determined following Mazumder *et al.* (2010) (No skin lesion: 0; mild: 1–2; moderate: 3–4; severe: 5–6).

Analysis of whole genome DNA methylation status

Whole genome DNA methylation status of the cases and controls were measured by ELISA-based Sigma Aldrich (St. Louis, MO) Imprint® Methylated DNA Quantification Kit (Cat No. MDQ1) with DNA extracted from peripheral blood leukocytes. The kit used antibody against the methylated base.

Genotyping of AS3MT M287T and GSTO-1 A140D polymorphisms

A PCR-restriction fragment length polymorphism (PCR-RFLP) analysis was carried out to detect both the substitutions. After amplification, the products were digested with restriction enzyme as per manufacturer's protocol and resolved on 2.5% agarose gel.

In the case of AS3MT, sequence of primers used was AS3MT-MF 5′-GTGCTGGAGATGAACCGTGAA-3′ (forward) and AS3MT-MR 5′-GCAAGGGCAAGAGCAGAAAGA-3′ (reverse). Amplification was performed with a protocol referred in Fujihara *et al.* (2007) with Primus 25 advanced® (PEQLAB, Germany). The (T/C) substitution creates a site for the restriction enzyme HpyCH4 IV (New England Biolabs, Ipswich, MA) (5′-A/CGT-3′). The HpyCH4 IV digestion of the amplified product yields 154 and 78 bp fragments in case of the mutant and an undigested 232 bp DNA fragment in case of the wild type.

Validity of the PCR products was confirmed by sequencing using ABI 3500 Genetic Analyzer (GCC biotech, Kolkata, India) (data not shown).

To detect the GSTO-1 A140D substitution, a 254 bp fragment was amplified using forward and reverse primers 5′-GAACTTGATGCACCCTTGGT-3′ (GSTO1-MF) and 5'-TGATAGC TAGGAGAAATAATTAC-3' (GSTO1-MR), respectively, as described earlier (Polimanti *et al.* 2010). The wild type fragment contained a site for the restriction endonuclease Cac8I (New England Biolab, Ipswich, MA), which is lost by the substitution. Thus, upon digestion of the PCR product, wild type alleles produce two fragments of 186 and 68 bp, whereas mutant alleles produce a single fragment of 254 bp. Sterile distilled water substituting the restriction enzyme served as the negative control.

Statistical analysis

Statistical analysis for this study was performed using non-parametric methods.

Methylation study:

ANOVA was applied for the comparison between means of different biomarkers listed below:

1. Skin manifestations (skin score).
2. Arsenic deposition in hair and nail (mg/kg).
3. Urinary excretion of arsenic (μg/L).
4. Smoking and tobacco chewing habit (yes/no).
5. Gender (male/female).
6. Age of the subject (years).

In all cases, the data of the 33 case samples were divided into two groups according to the median value of the methylation data and subsequently subjected to one way ANOVA analysis, using SPSS Statistics 17.0 (Spss Inc., Chicago, IL). Multiple linear regression analysis was also performed with the data to exclude the effects of confounding factors.

Analysis of correlation between genomic polymorphism and the following biomarkers by ANOVA are as follows:

1. Skin manifestation (skin score).
2. Arsenic deposition in hair and nail (mg/kg).
3. Urinary excretion of arsenic (μg/L).
4. Methylation status.

The level of significance was set at 0.01 instead of 0.05 applying Bonferroni correction when multiple hypotheses are tested simultaneously.

Result

Arsenic concentrations

Patient characteristics like age, gender, tobacco usage, skin score, the amount of arsenic in hair, nail and urine are described in Table 1. They were aged between 25 and 63 years (median value of 43.5 years): 14 of them being smokers or addicted to tobacco in other forms. The concentration of arsenic in hair and nail of these individuals ranged between 0.208 and 6.464 mg/kg and that in urine was 9–879 μg/L. Individuals selected as control were aged between 28 and 56 years (median value of 45.4 years): 13 of them were smokers.

Analysis of whole genome DNA methylation

One way ANOVA showed that whole genome DNA methylation value of the arsenic unexposed group differed significantly from that of the arsenic-exposed group ($p = .016$). The value for the exposed group was scattered over a large range. These were further divided in two groups, above and

Table 1. The sample data containing age, gender, skin score, tobacco usage, arsenic concentration in hair/nail and urine.

Sample no.	Age (yrs)	Gender	Skin score	Tobacco usage	Hair/nail arsenic (mg/kg)	Urine arsenic (µg/L)
Sample 1	25	M	3	N	0.655	23
Sample 2	39	M	2	N	0.371	103
Sample 3	58	M	3	Y	0.662	159
Sample 4	42	F	1	N	1.115	142
Sample 5	38	M	1	Y	0.849	76
Sample 6	39	M	1	N	0.533	165
Sample 7	53	M	1	N	0.995	84
Sample 8	39	F	3	Y	6.411	103
Sample 9	32	M	2	Y	0.711	71
Sample 10	46	M	4	Y	3.977	222
Sample 11	39	F	2	N	0.837	152
Sample 12	44	F	1	N	0.563	18
Sample 13	45	M	2	Y	0.208	40
Sample 14	57	F	2	N	0.513	121
Sample 15	46	M	4	N	0.669	82
Sample 16	28	M	2	Y	6.464	43
Sample 17	56	M	3	Y	1.118	400
Sample 18	46	M	4	N	0.669	82
Sample 19	48	F	3	N	0.918	55
Sample 20	39	M	2	N	2.781	64
Sample 21	24	M	4	N	0.439	14
Sample 22	57	M	1	N	2.195	441
Sample 23	56	M	4	N	1.036	11
Sample 24	39	F	2	Y	0.904	272
Sample 25	63	M	3	Y	1.925	879
Sample 26	56	M	1	Y	0.909	341
Sample 27	53	F	2	N	1.599	139
Sample 28	43	F	2	N	0.704	24
Sample 29	27	F	2	N	1.033	355
Sample 30	33	F	1	N	2.103	182
Sample 31	51	F	1	N	0.504	88
Sample 32	54	M	0	Y	1.23	135
Sample 33	51	M	3	Y	2.137	335
Sample 34	26	F	0	N	2.65	142
Sample 35	30	F	0	N	1.37	128
Sample 36	51	M	0	Y	1.257	71
Sample 37	40	M	0	N	0.83	47
Sample 38	36	F	2	N	0.682	9

Y: yes, N: no, M: male, F: female.

below the statistical median value of 0.753. Distribution of other parameters like skin score, urinary arsenic, arsenic in hair and nail, smoking habit, age and gender between the two groups is studied by ANOVA and the result is presented in Table 2. Only skin score showed a significant difference between the two groups (Figure 1) ($p = .004$). Multiple Linear Regression Analysis has also been performed to exclude the effects of confounding factors. In agreement with ANOVA, this result also indicated that whole genome DNA methylation is significantly correlated only to skin score at 5% level of significance (Table 3).

*Genotyping of the AS3MT*M287T and GSTO1*A140D polymorphisms*

Table 4 lists the genotype and allele distribution of the two polymorphisms AS3MT M287T and GSTO1 A140D in individuals exposed and unexposed to inorganic Arsenic. Figure 2(A,B) is two representative gel pictures of the study.

Our result showed that in this population, the frequency of AS3MT M287T allele was low, the allele frequency being 0.059. The observation is consistent with reports of other researchers on Indian and Asian population (De Chaudhuri *et al.* 2008, Agusa *et al.* 2011). We obtained an overall allele frequency of 0.25 for the GSTO1 A140D allele.

For statistical analysis, the study population was stratified into two groups according to the median values of (a) skin score (2), (b) arsenic deposition in hair and nail (0.957 mg/kg), (c) urinary excretion of arsenic (103 µg/L) and (d) whole genome DNA methylation status (0.8865).

One way ANOVA coupled with Bonferroni correction revealed that the GSTO1 A140D polymorphism to have significant association with genomic methylation value and skin score ($p = .00009$ and .0004, respectively). In case of arsenic in hair and nail, the significance was missed marginally (.011). The results of ANOVA are shown in Table 5.

However, the low frequency of AS3MT M287T allele in our study population and the moderate number of test participants make the number of mutants too low to do any statistical analysis for this variant.

Discussion and conclusion

Arsenic in hair and nail rises to ~6.5 mg/kg in our samples which is similar (5.39) to the other local study (De Chaudhuri *et al.* 2008). Report from another As-affected country, Chile, showed similar values in hair (3.2–6.1) and a little elevated value in nail clippings (10–15) (Borgono *et al.* 1977). DNA methylation is involved in several important functions in mammals, including regulation of gene expression,

Table 2. The statistical analysis of variation of confounding parameters between two methylation groups above and below the median value.

Parameter	Sum of squares		Df	*F*	*p* Value
DNA methylation vs. Urinary arsenic	Between groups	2415.144	1	0.079	.780
	Within groups	942379.765	31		
DNA methylation vs. Hair As deposition	Between groups	4.514	1	2.065	.161
	Within groups	67.756	31		
DNA methylation vs. Skin score	Between groups	12.500	1	10.033	.004
	Within groups	37.375	30		
DNA methylation vs. Smoking and Tobacco chewing	Between groups	0.008	1	0.031	.861
	Within groups	7.734	31		
DNA methylation vs. Gender	Between groups	0.352	1	1.475	.234
	Within groups	7.390	31		
DNA methylation vs. Age	Between groups	0.127	1	0.518	.477
	Within groups	7.615	31		

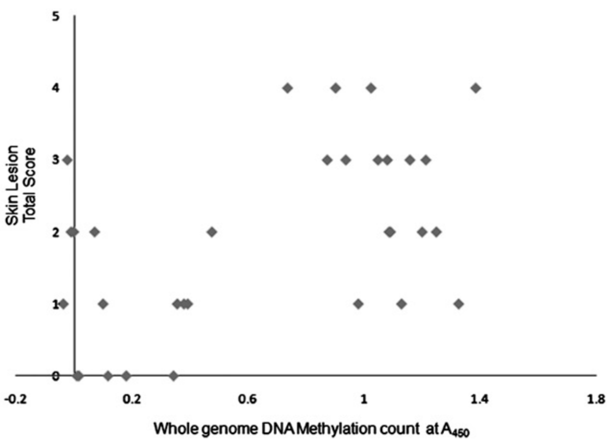

Figure 1. The variation of skin manifestation score in arsenic-exposed people with different levels of whole genome DNA methylation.

Table 3. Linear regression analysis using DNA methylation as the dependent variable and the confounding parameters as the independent variable DNA methylation $= \alpha + \beta_1$ skin score $+ \beta_2$ urine $+ \beta_3$ hair & nail $+ \beta_4$ age $+ \beta_5$ gender $+ \beta_6$ tobaco $+ e$.

Model	ß
α	−0.092
Skin score	0.187[a]
Arsenic in urine	0.000
Arsenic in hair/nail	0.075
Age	0.007
Gender	0.170
Tobacco usage	−0.133

e: error term.
[a]Significant at 5% level of significance.

Table 4. The genotype and allele distributions of the AS3MT(M287T) and GSTO1(A140D) polymorphisms in individuals exposed and unexposed to inorganic arsenic.

Polymorphism		Genotype frequency (%)			Allele frequency (%)	
As3MT Met 287 Thr	*N*	TT	TC	CC	T	C
Case	34	30 (88)	4 (12)	–	64 (94.1)	4 (5.9)
Control	37	36 (97)	1 (3)	–	73 (99)	1 (1)
GSTO1 Ala 140 Asp		CC	CA	AA	C	A
Case	30	15 (50)	15 (50)	–	45 (75)	15 (25)
Control	31	17 (55)	14 (45)	–	48 (77)	14 (23)

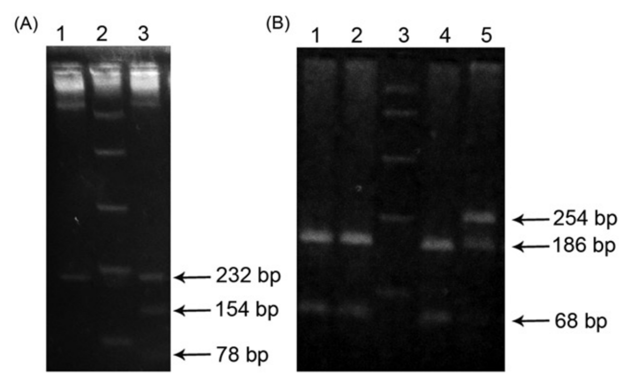

Figure 2. The representative electrophoresis gels of PCR-RFLP products. (A) Gel picture showing the HpyCH4IV digested PCR amplified product for As3MT M287T polymorphism; lane 1: sample homozygous for M287 (T/T); lane 2: molecular weight markers; lane 3: sample heterozygous for M287T (T/C). (B) Gel picture showing Cac 8I digested PCR amplified product for GSTO1 A140D polymorphism; lanes 1, 2 and 4: samples homozygous for A140 (C/C); lane 3: molecular weight markers; lane 5: samples heterozygous for A140D (C/A).

preservation of chromosomal integrity, parental imprinting and X-chromosome inactivation. In general, normal cells are characterized by gene-specific hypomethylation and global hypermethylation, while cancer cells portray a reverse profile to this norm. In cancer, the gene-specific hypermethylation of CpG-islands of promoter regions is associated with transcriptional repression and silencing of genes that function to prevent tumor growth and development. Also, a relative reduction in the overall level of methylation of non-CpG-island cytosines distributed throughout the genome is reported to be associated with reactivation of cellular proto-oncogenes leading to chromosomal instability (Robertson and Wolffe 2000).

In a recent publication, Li *et al.* claimed to have identified epigenetic lesions specific to carcinosarcoma. Hallmarks of DNA methylation abnormalities in uterine carcinosarcoma included global hypomethylation, especially in repetitive

Table 5. The statistical analysis of variation of mutation genotype (GSTO1 A140D) between two groups above and below the median value of each parameter.

Parameter	Sum of squares		Df	F	p Value
Skin score	Between groups	9.697	1	22.366	.000394
	Within groups	5.636	13		
Hair As deposition	Between groups	23.441	1	8.750	.011
	Within groups	34.828	13		
Urinary As	Between groups	217941.633	1	6.017	.029
	Within groups	470840.100	13		
DNA methylation	Between groups	2.479	1	31.328	.000087
	Within groups	1.029	13		

elements, and hypermethylation of tumor suppressor gene promoters (Li *et al.* 2017). The unique methylome displayed in cancer cells is induced after exposure to carcinogenic metals such as nickel, arsenic, cadmium and chromium. Exposure to arsenic has been found to be associated with alterations in DNA methylation and these and other epigenetic marks (Ramirez *et al.* 2008) have been proposed as mediators of arsenic-induced carcinogenesis (Mass and Wang 1997, Pilsner *et al.* 2007, Majumdar *et al.* 2010).

Arsenic-induced DNA methylation is observed in both *in vivo* and *in vitro* systems including gene-specific promoter methylation as well as whole genome methylation. A study in West Bengal, India, reported significant promoter hypermethylation of p53 and p16 gene in peripheral blood leucocyte (PBL) DNA of humans exposed to arsenic (Chanda *et al.* 2006). This first study of DNA hypermethylation in arsenic-exposed human subjects has later been confirmed by others. Chronic arsenic exposure through drinking water has been reported to be positively associated with genomic methylation of PBL DNA among adults of Bangladesh (Pilsner *et al.* 2007). There are reports of increased whole genome DNA methylation upon chronic arsenic exposure from India too (Majumdar *et al.* 2010). Array analysis of tissue from mice exposed to different doses of arsenic showed up-regulation of DNA(cytosine-5)-methyltransferase 3A (DNMT3a), the gene that codes for the enzyme responsible for transfer of methyl groups to specific CpG structures in DNA (Ahlborn *et al.* 2008). Rat liver epithelial cells (TRL 1215) transformed by chronic arsenite exposure demonstrated a loss of methyltransferase activity by 40% though the DNMT1 mRNA activity in the same cells increased by up to 2-fold (Zhao *et al.* 1997). Depletion of SAM and SAM:SAH (*S*-adenosylhomocysteine) ratio may account for this loss of activity.

The early symptom of arsenic exposure is the characteristic skin lesions. Skin abnormalities including keratosis are the hallmark signs of chronic arsenic exposure and also potent biomarkers of arsenic toxicity. Arsenic-induced hyperpigmentation and keratosis are very unique. Hyperpigmentation is characterized by raindrop-shaped discolored spots, diffuse dark brown spots or diffused darkening of the skin on the limbs and trunk (Guha Mazumder *et al.* 1988). Simple keratosis can be usually marked as bilateral thickening of the palms and soles, while another one is nodular keratosis, where small protrusions appear on the palms and soles, with or without nodules on the dorsum of the hands, feet or legs. Advanced forms of keratosis are very painful. These skin lesions are generally detected within 5–10 years after exposure, unlike cancers which take decades to develop (Guha Mazumder *et al.* 1998). In a SILAC-based quantitative proteomic analysis, Mir *et al.* reported widespread molecular alterations in human skin keratinocytes upon chronic arsenic exposure. Human skin keratinocyte cell line, HaCaT, was chronically exposed to 100 nM sodium arsenite over a period of 6 months. They observed an increase in basal ROS levels in arsenic-exposed cells. SILAC-based quantitative proteomics approach resulted in the identification of 2111 proteins of which 42 proteins were found to be overexpressed and 54 down-regulated (twofold) upon chronic arsenic exposure (Mir *et al.* 2016).

A study in southwestern region of Taiwan reported that arsenic biotransformation, i.e. capacity of arsenic methylation, may have a role in the development of arsenic-induced skin disorders. They reported that the exposed individual with skin lesions have higher percentage of iAs, MMA and MMA:DMA but lower percentage of DMA than the matched controls (Yu *et al.* 2000). Another study demonstrated clear exposure–response relationships between the prevalence of skin lesions and both level of arsenic in water and dose per body weight (Guha Mazumder *et al.* 1998). Males were found to be more susceptible to both keratosis and melanosis (poorly demarcated, blotchy areas prevalent on the abdomen and back, portions of the body relatively unexposed to sunlight) than females (Watanabe *et al.* 2001).

Whole genome DNA methylation level may differ with nutritional level, arsenic level in blood plasma, arsenic level in drinking water and age. Arsenic-induced DNA methylation cannot occur under low folate availability (Pilsner *et al.* 2007). In addition to exposure dose and nutritional level, factors like age, smoking habit and polymorphism status of different genes involved in arsenic metabolism like GSTO1 and AS3MT may influence the level of genomic methylation. With adequate nutritional status, arsenic exposure is assumed to cause hypermethylation of DNA initially. However, with time and increase of exposure dose, there is depletion of SAM and gradually hypomethylation sets in. Thus, the correlation of DNA hypermethylation status with increase of arsenic dose is lost with nutritional deficiency in the probands (Pilsner *et al.* 2007)

Seow *et al.* (2014) conducted a prospective study among 10 incident skin lesion cases and 10 controls among adults in Bangladesh. Skin lesion cases were defined as the presence of at least one type of arsenical skin lesion. DNA methylation was measured at both baseline (2001–2003) and follow-up (2009–2011) in each study participant to identify DNA methylation changes associated with incident skin lesions based on percentage methylation difference between the baseline and follow-up assessments. Although no significant associations were observed, this study employed a repeated assessment of DNA methylation to evaluate changes in methylation in relation to skin lesion incidence.

The present study is the first of its kind, aimed to investigate the correlation between the biomarkers of arsenic exposure like skin manifestation severity, amount of arsenic deposition in hair and nail, urinary excretion of arsenic or factors like smoking and tobacco chewing habits (very common in this area), gender or age with global DNA methylation status of the arsenic-exposed population in this region of West Bengal, India. The grouped individuals (grouped by the median value of the methylation data) showed a statistically significant increase in skin manifestation score with the increase in global DNA methylation ($p = .004$). The methylation of cellular DNA after arsenic exposure is a kinetic process. It is proposed that, initially arsenic up-regulates DNMT3a gene causing genomic hypermethylation globally. However, as the process goes on the system gets depleted of SAM and removal of iAs becomes less efficient, which accumulates in the body and increases the skin lesions. We have not observed significant association of whole genome DNA methylation with urinary arsenic, age, gender or smoking and tobacco chewing habits.

When probands with genome hypomethylation are considered, hypomethylation is a risk factor for skin lesions (Pilsner *et al.* 2009). Thus, our probands are probably at an earlier stage of genome modulation where considerable hypomethylation has not set in.

To date two pathways of arsenic biotransformation have been proposed. In both the pathways, As3MT plays an important role. *In vitro*, human cases and epidemiological studies strongly suggested an association between As3MT polymorphism (M287T) and cancer (Agusa *et al.* 2011). Study with Mexican populations suggested a marginally significant association between skin cancer and this polymorphism (Valenzuela *et al.* 2009). The relatively low allele frequency of this variant in our population made us unable to test the association. De Chaudhuri and coworkers (2008) had similar experience. Genetically lesser susceptibility of Asiansto arsenic toxicity among the various global population (Agusa *et al.* 2011) may stem from this.

The enzyme GSTO1 has a cysteine residue in its active site (other GSTs have ser/tyr) (Girardini *et al.* 2002) and human GSTO1 is MMA^V reductase (Zakharyan *et al.* 2001). It has been suggested that the Ala140Asp (A140D) variant reduces thioltransferase activity of GSTO1 (Tanaka-Kagawa *et al.* 2003). A study in Taiwanese population also detected higher cancer incidence among the variants (Marahatta *et al.* 2006). However, a study by Whitbread *et al.* (2003) could not find alteration in enzyme activity in the variant. De Chaudhuri *et al.* (2008) reported no significant association between this polymorphism and presence or absence of skin manifestation among the arsenic-exposed population of West Bengal. Our study design is different and all probands have some manifestation. However, its severity is increased in the variants in a significant manner. Xu and co-workers (2009) found no association between urinary profile or oxidative stress status and the GSTO1 A140D, GSTO2 N142D polymorphism. A study conducted by Ada *et al.* (2013) suggested association of GSTO1 A140D gene polymorphism with susceptibility to non-small cell lung cancer in the Turkish population. Djukic *et al.* (2013) suggested that GSTO1 D140D may play a pharmacogenomic role in patients with muscle invasive bladder cancer.

To sum up, this is the first report till date where significant association between global DNA methylation status and skin score ($p = .004$) was found. The GSTO1 A140D polymorphism shows significant association with skin score ($p = .000394$) in the arsenic-affected population of West Bengal. In disagreement with others results, our data suggest significant association ($p = .000087$) between GSTO1 A140D polymorphism and global DNA methylation in the arsenic-affected population of West Bengal. Regression analysis showed that this association is not due to effect of confounding factors.

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Disclosure statement

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