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Cytogenetic damage and genetic variants in the individuals susceptible to arsenic-induced cancer through drinking water

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In West Bengal, India, more than 300,000 arsenic-exposed people are showing symptoms of arsenic toxicity, which include cancers of skin and different internal organs. Since only 15–20% of the exposed population manifest arsenic-induced skin lesions, it is thought that genetic variation might play an important role in arsenic toxicity and carcinogenicity. A total of 422 unrelated arsenic-exposed subjects (244 skin-symptomatic and 178 asymptomatic) were recruited for this study. Cytogenetic damage, as measured by chromosomal aberrations in lymphocytes and micronuclei formation in oral mucosa cells, urothelial cells and binucleated lymphocytes, was studied in unexposed, skin-symptomatic and asymptomatic individuals with similar socioeconomic status. Identification of null mutations in *GSTT1* and *GSTM1* genes were carried out by PCR amplification. *GSTP1* SNPs, implicated in susceptibility to various cancers, were assessed by PCR-RFLP method. Symptomatic individuals had higher level of cytogenetic damage compared to asymptomatic individuals and asymptomatic individuals had significantly higher genotoxicity than unexposed individuals. No difference in allelic variants in *GSTT1* and *GSTP1* was observed between these 2 groups. Incidence of *GSTM1* null gene frequencies was significantly higher in the asymptomatic group. Individuals with GSTM1-positive (at least one allele) had significantly higher risk of arsenic-induced skin lesions (odds ratio, 1.73; 95% confidence interval, 1.24–2.22). These results show a protective role of *GSTM1* null in arsenic toxicity. This study also indicates that asymptomatic individuals are sub clinically affected and are also significantly susceptible to arsenic-induced genotoxicity.

Key words: arsenic; skin lesions; cytogenetic damage; *GSTT1*; *GSTM1*; *GSTP1*

Arsenic is one of the most toxic metals known in the environment. It is regarded as a paradoxical human carcinogen. Arsenic contamination in the ground water has been reported from different parts of the world, such as Taiwan, Mexico, Chile, Argentina, Thailand, USA, Canada, Hungary, Japan, Bangladesh and India. In West Bengal, India, the ground water of 9 out of 18 districts is contaminated with arsenic at concentrations far above the current maximum permissible limit laid down by both World Health Organization[1] and U.S. Environmental Protection Agency.[2] This is regarded as the greatest arsenic calamity in the world.

Epidemiological studies indicate that in West Bengal, India, more than 300,000 people show symptoms of arsenic toxicity.[3] A strong relationship between arsenic level in water with the prevalence of keratosis and hyperpigmentation in the exposed individuals has been reported earlier.[4] Long-term exposure to arsenic-contaminated water causes a wide range of adverse health effects, including skin pigmentations, keratosis, vascular diseases, conjunctivitis in the eyes, neuropathy, lung diseases and nonmelanocytic cancer of skin and different internal organs.[4] Since arsenic-induced skin lesions typically manifest after a latency period ranging from 6 months to 10 years of exposure, it is likely that over a period of decades, a larger number of people will show various diseases, including cancer. Thus, monitoring of cytogenetic damage is necessary for future preventive measures.

The most reliable biomarker of arsenic exposure is the measure of arsenic content in urine, hair and nails, which reflects a cumulative exposure to arsenic over a long period.[5] Arsenic content in hair and nails can estimate the arsenic exposure over the past 6–12 months, whereas urinary arsenic level is regarded as the best biological indicator for assessing current arsenic exposure, since more than 60% of the ingested arsenic is excreted through urine.[6,7] Genotoxic end-points have been utilized successfully as biomarkers, as these are considered to be markers of early biological effects of carcinogen exposure.[8] Genotoxic effects of arsenic have been implicated in the carcinogenic outcomes.[9] Higher incidences of micronuclei (MN), chromosomal aberrations (CA), sister chromatid exchanges (SCE) and aneuploidy have been reported from the human populations exposed to arsenic through drinking water in various countries, such as Mexico,[10] Finland,[11] Argentina[12] and Taiwan.[8] The exact mechanism of arsenic-induced carcinogenicity is not yet fully understood, since it does not by itself induce cancer in animal models. Short-term assays indicate that arsenic does not induce point mutations, but rather acts as a clastogen, inducing the CA and MN in animal and human systems.[13] Thus, arsenic is an ideal genotoxicant to be evaluated using both CA and MN assays. In 2002, we first reported an elevated frequency of MN in lymphocytes, oral mucosal cells and urothelial cells of individuals with arsenic-induced skin lesions inhabiting different arsenic-affected districts of West Bengal.[14] Subsequently, we also reported the elevated frequencies of CA, SCE and MN assays in 3 different cell types from the exposed individuals of North 24 Parganas district of West Bengal.[15,16]

Skin lesions are considered as the hallmark signs of chronic arsenicism, with hyperkeratosis being a precursor of nonmelanocytic skin cancer. A considerable variation in the manifestation of skin lesions in individuals exposed to arsenic-contaminated drinking water has been reported from West Bengal, India.[17] In these areas, although a large number of individuals are exposed to arsenic through drinking water, only 15–20% show arsenic-induced skin lesions. This observation suggests that genetic variation might play an important role for arsenic susceptibility. Studies on populations from different parts of the world exposed to com-

Abbreviations: CA, chromosomal aberrations; EDTA, ethylene di-amine tetra acetic acid; FI-HG-AAS, flow injection-hydride generation-atomic absorption spectrometry; GSH, glutathione; *GST*s, Glutathione S-Transferases; MI, mitotic index; MN, micronuclei; NBCS, new born calf serum heat-inactivated; PHA, phytohemagglutinin-M form; SCE, sister chromatid exchanges; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine.

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TABLE I – DESCRIPTIVE CHARACTERISTICS OF STUDY PARTICIPANTS

Parameters	Unexposed group	Arsenic-exposed asymptomatic group	Arsenic-exposed skin-symptomatic group
Total Subjects (*n*)	102	178	244
Mean age (range; years)	39.16 (13–70)	38.65 (15–75)	41.19 (15–75)
Addiction [*n* (%)]			
Smoker	34 (33.3)	49 (27.53)	95 (38.93)
Nonsmoker	68 (66.7)	129 (72.47)	149 (61.07)
Gender [*n* (%)]			
Male	51	77	141
Smoker	20 (39.22)	33 (42.86)	76 (53.90)
Nonsmoker	31 (60.78)	44 (57.14)	65 (46.10)
Female	51	101	103
Smoker	14 (27.45)	16 (15.84)	19 (18.45)
Nonsmoker	37 (72.55)	85 (84.16)	84 (81.55)
Occupation [*n* (%)]			
Male	51	77	141
Farmer	26 (50.98)	42 (54.54)	75 (53.19)
Business	8 (15.68)	19 (24.68)	26 (18.44)
Daily wage earner	2 (3.92)	2 (2.6)	11 (7.80)
Teacher	4 (7.84)	1 (1.3)	5 (3.55)
Service	6 (11.76)	1 (1.3)	7 (4.96)
Student	3 (5.90)	5 (6.49)	4 (2.84)
Unemployed	2 (3.92)	7 (9.09)	13 (9.22)
Female	51	101	103
Housewife	43 (84.31)	84 (83.17)	86 (83.50)
Daily wage earner	2 (3.93)	13 (12.87)	15 (14.56)
Student	6 (11.76)	4 (3.96)	1 (0.97)
Service	–	–	1 (0.97)

parable levels of arsenic in drinking water also show varying degree of individual susceptibility to arsenic-induced genetic damage, metabolism, methylation capacity and other health effects.[13,18–20]

Glutathione S-transferases (GSTs) constitute a superfamily of ubiquitous, multifunctional enzymes, which play a key role in cellular detoxification.[21] The GSTs catalyze the conjugation of xenobiotics and endogenous substances with glutathione (GSH), a tripeptide consisting of glycine, glutamic acid and cystine, and thereby plays a significant role in the inactivation and occasional activation of many electrophilic substances.[22] GSTs also modulate the induction of other enzymes and proteins important for cellular functions, such as DNA repair.[23] This class of enzymes is, therefore, important for maintaining cellular genomic integrity, and as a result, may play important role in cancer susceptibility. About 60% of Asians, 40% of Africans and 20% of Caucasians do not express *GSTT1*.[24] Genetic variants of GST, alone or in combination with other genetic variations and environmental factors, were associated with a number of multifactorial diseases and conditions, such as smoking-related tumors, bladder cancer, colorectal cancer; esophageal cancer, myelodysplastic syndrome and leukemia, liver and renal diseases, and several other diseases like chronic obstructive pulmonary disease, bronchial asthma and solar keratosis.[25–30] *GSTP1* is widely expressed in normal epithelium and is also involved in the detoxification of carcinogens in different tissues.[31,32] *GSTP1* is overexpressed in tumors compared to normal tissues and has a role in cellular protection against oxidative stress.[33,34] Two cSNPs in *GSTP1*, resulting in missense changes of amino acids (*i.e.* Ile105Val and Ala114Val), have been implicated to susceptibility to various cancers.[35,36] *GSTM1, GSTT1* and *GSTP1* exhibit overlapping substrate specificity, suggesting that deficiencies of certain GST isoenzymes may be compensated by other isoforms.[23,33] To better understand the potential role of metabolic polymorphisms in arsenic toxicity, the combined effects of these 3 genes have been studied in this population. We have examined the cytogenetic damage and assessed the potential involvement of *GSTM1*, *GSTT1* and *GSTP1* genes in the skin-symptomatic and asymptomatic individuals exposed to arsenic. Attempt has also been made to correlate the genetic variation with cytogenetic damage studied in this population.

Material and methods

Chemicals

RPMI medium-1640 powder (supplemented with L-glutamine and 25 mM HEPES buffer), new born calf serum heat-inactivated (NBCS), phytohemagglutinin powder (PHA M-form, lyophilized) and penicillin–streptomycin were purchased from Invitrogen (Carlsbad, CA). Cytochalasin B, trizma hydrochloride and ethylene di-amine tetra acetic acid (EDTA), colchicine powder (~95%, HPLC), sodium arsenite and Giemsa stain powder were purchased from Sigma Chemical Company (St. Louis, MO). Acrylamide, bis-acrylamide, ammonium persulfate and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were purchased from Amersham International, Buckinghamshire, UK. Sodium bicarbonate (GR), concentrated hydrochloric acid, nitric acid (minimum 69% GR), sulfuric acid, glacial acetic acid, methanol, potassium chloride (analytical reagent), sucrose, magnesium chloride, sodium perchlorate, isopropanol and chloroform were obtained from E. Merck (India). AciI and BsmA1 enzymes were purchased from New England BioLabs, Beverly, MA.

Study design and selection of subjects

The districts of North 24 Parganas and Nadia were selected for our study, which were located about 100–120 km from the main metropolis Kolkata. Five villages from the 4 administrative blocks (Gaighata, Habra, Deganga and Baduria) of North 24 Parganas and one village (*i.e.* Dasdia) from Haringhata block of the district of Nadia were selected, since these villages were highly affected with arsenic contamination in their ground water.[37] In these 2 districts, tube wells are the main source of drinking water in the rural villages and the contaminated tube wells are scattered irregularly through out the study site. Initially, 4 trained volunteers were sent to the different villages for a door-to-door survey to find out the presence of skin lesions among the villagers. Then, both the individuals with skin lesions and without skin lesions were requested to attend the medical camp. Medical camps were organized in this area to collect biological samples (blood, hair, nail and urine) from the donors residing in the same area and sometimes belonging to the same family. First, physicians and dermatologists examined the study participants, and then, each participant was asked to complete a questionnaire that

elicited information on demographic factors, life style, occupation, diet, addiction and medical and residential histories. In addition, water used by the donors was also collected for assessment of arsenic content. After the clinical diagnosis, the arsenic-exposed individuals in this study were classified into 2 groups, *i.e*. skin-symptomatic subjects who had cutaneous signs of arsenicism, like raindrop pigmentation, palmar and planter hyperkeratosis, hypo- and hyper-pigmentation, ulcerative lesions, gangrene and/or Bowen's disease and asymptomatic subjects who had no sign of arsenic-related skin lesions.

Blood samples were collected from a total of 422 unrelated donors comprising of 244 skin-symptomatic and 178 asymptomatic individuals, on the basis of their skin lesions (Table I). Among the skin-symptomatic individuals, 72.54% had raindrop pigmentation, 82.78% had keratosis and 47.54% had pigmentation. Out of the 422 individuals, analysis of cytogenetic damage was performed in a total of 204 subjects comprising of 105 skin-symptomatic subjects and 99 asymptomatic subjects (Tables IV and V). The cytogenetic data of exposed skin-symptomatic and asymptomatic individuals were compared with 102 unexposed subjects from east and west Midnapore districts. The unexposed group was matched to the exposed group by age, sex and socioeconomic status. This study was conducted in accord with the Helsinki II declaration and approved by the Institutional Ethics Committee.

In our study area, a large number of people smoke (mainly bidi) and chew betel quid and tobacco, which are the common habits among Indians. We excluded the heavy smokers (who smoke more than 7 bidi/day) and only included individuals with mild smoking (mainly bidi and/or with habit of chewing betel quid with tobacco) in our cytogenetic study. Thus, all the groups were further subdivided into 2 categories, smokers and non-smokers. The smokers represented the current smokers and non-smokers were those who never smoked. The smoker group included both betel quid chewers and few tobacco chewers. Occupational exposure to arsenic was ruled out, since majority of the study participants were farmers, businessmen and daily wage earners. Therefore, drinking water was the principal source of arsenic exposure in this region.

Collection of water and biological samples for the estimation of arsenic content

Study participants were provided with acid-washed [nitric acid–water (1:1)] polypropylene bottles for collection of drinking water (~100 ml) samples into which nitric acid (1.0 ml/l) was added later on as preservative.[38] First morning voids (~100 ml) were collected in precoded polypropylene bottles for arsenic estimation, as these give the best measure of the recent arsenic exposure.[6] Immediately after collection, the samples were stored in salt–ice mixture and brought to the laboratory where they were kept at −20°C until estimation of arsenic was carried out. Nail (~250–500 mg) and hair (~300–500 mg) samples were collected using blade/nail cutter. Hair samples were taken from more or less similar region of head, close to the scalp.[39] The arsenic content of the samples was analyzed at the School of Environmental Studies, Jadavpur University, Kolkata. Both the nail and hair samples were thoroughly cleaned for removal of exogenous arsenic, following the method of Das *et al*.[39] Before estimation, the nail and hair samples were dried in a hot oven after treating them with concentrated nitric acid.[39] Flow injection-hydride generation-atomic absorption spectrometry (FI-HG-AAS) was used for the estimation of arsenic in the collected samples. A Perkin–Elmer Model-3100 (Boston, MA) spectrometer equipped with a Hewlett–Packard (Houston, TX) Vectra computer with GEM software, Perkin–Elmer EDL System-2, arsenic lamp (lamp current 400 mA) was utilized for the purpose.

TABLE II – PRIMERS AND PCR CONDITIONS FOR THE TEST GENES (GST) AND CONTROL GENE (CYP)

Gene	Primer (5′–3′)	Location	PCR condition	Remarks
GSTT1	F1-ACTCCCTCTGGTTTCCGGTCAGG; R1-TGGGAGAAGTTCCCTAAGCTATG	Exon 1	94°C for 30 sec, 63°C for 30 sec and 72°C for 30 sec for 30 cycles, using 1.5 mM $MgCl_2$	These two exons of *GSTT1* were amplified to estimate null genotype
	F5-CCCTGCCCCTCACAACCATCCAT; R5-GTGGACTGCTGAGGACGGTGC	Exon 5	94°C for 30 sec, 65°C for 30 sec and 72°C for 30 sec for 30 cycles, using 1.5 mM $MgCl_2$	
GSTM1	F2-AGTGTAAACTGGGGGCTTCCCT; R2-CCACAGGTGGCCACCATGGGTT	Exon 2	94°C for 30 sec, 69°C for 30 sec and 72°C for 30 sec for 30 cycles, using 2.0 mM $MgCl_2$	These two exons of *GSTM1* were amplified to estimate null genotype
	F7-GCTTCACGTGTTATGGAGGTTC; R7-TACATGCGAGTTATTCTGTGTG	Exon 7	94°C for 30 sec, 58°C for 30sec and 72°C for 30 sec for 30 cycles, using 2.0 mM $MgCl_2$	
CYP2D6	F6-CATCTGGGCTGACAGGTGCAG; R6-TCCCAGCAAAGTTCATGGGCCC	Exon 6	94°C for 30 sec, 69°C for 30 sec and 72°C for 30 sec for 30 cycles, using 1.5 mM $MgCl_2$	Control gene
CYP2E1	F6-CCGGTCTGTCTCCGGTATCTCAG; R6-ATGCAGCCGCACGCACGGTGT	Exon 6	94°C for 30 sec, 65°C for 30 sec and 72°C for 30 sec for 30 cycles, using 1.5 mM $MgCl_2$	Control gene
GSTP1	F5-CAGTGACTGTGTGTTGATCAGGCG; R5-CCCTGACCCAAGAAGGGGTCAGC	Exon 5	94°C for 30 sec, 69°C for 30 sec and 72°C for 30 sec for 30 cycles, using 1.5 mM $MgCl_2$	To estimate G allele frequency by BsmA1 RFLP
	F6-GCAGAGGAGAATCTGGGACTCT; R6-GGCTCACACCTGTGTCCATCTG	Exon 6	94°C for 30 sec, 62°C for 30 sec and 72°C for 30 sec for 30 cycles, using 1.5 mM $MgCl_2$	To estimate C allele frequency by AciI RFLP

TABLE III – ARSENIC CONTENTS IN WATER AND BIOLOGICAL SAMPLES OF THE UNEXPOSED AND ARSENIC-EXPOSED INDIVIDUALS

Parameters	Unexposed group	Asymptomatic group	Comparison of unexposed with asymptomatic group		Skin-symptomatic group	Comparison of unexposed with skin-symptomatic group		Comparison of asymptomatic with skin-symptomatic group	
	Mean arsenic content (N = 102)	Mean arsenic content (N = 99)	Confidence interval (95%)	p-value	Mean arsenic content (N = 105)	Confidence interval (95%)	p-value	Confidence interval (95%)	p-value
Water (μg/l)	7.16	202.33	170.3–220.1	0.001*	242.06	201.2–268.6	0.001*	−81.4 to 1.90	0.061
Urine (μg/l)	26.16	181.13	135.9–178.0	0.001*	185.35	135.0–183.4	0.001*	−29.1 to 33.6	0.889
Nail (μg/g)	0.83	3.16	1.805–2.841	0.001*	3.58	2.222–3.262	0.001*	−0.291 to 1.129	0.246
Hair (μg/g)	0.53	2.04	1.169–1.859	0.001*	2.13	1.310–1.903	0.001*	−0.536 to 0.352	0.683

Two-sample t-test (large sample approximations).
*Significant at 1% level, p-value corrected up to three decimal.

TABLE IV – MICRONUCLEI FREQUENCIES IN THREE CELL TYPES IN THE UNEXPOSED AND ARSENIC-EXPOSED INDIVIDUALS

Cell type	Unexposed group (N =102)	Asymptomatic group (N = 99)			Skin-symptomatic group (N = 105)			Comparison of asymptomatic with skin-symptomatic group	
	Mean MN /1,000 cells	Mean MN /1,000 cells	Confidence interval (95%)	p-value	Mean MN /1,000 cells	Confidence interval (95%)	p-value	Confidence interval (95%)	p-value
Lymphocyte	2.03	6.30	−4.74 to −3.79[1]	0.001*	9.13	−7.55 to −6.65[2]	0.000*	2.26–3.40	0.000*
Oral mucosa cells	1.67	3.56	−2.25 to −1.53[1]	0.001*	5.62	−4.40 to −3.50[2]	0.000*	1.56–2.57	0.000*
Urothelial cells	1.70	4.18	−2.78 to −2.15[1]	0.001*	6.01	−4.33 to −3.85[2]	0.000*	1.37–2.28	0.000*

Two-sample t-test (large sample approximations).
[1]Comparison of unexposed with asymptomatic.–[2]Comparison of unexposed with skin-symptomatic.–*Significant at 1% level; p-value corrected up to three decimal.

Collection of oral mucosa and urothelial cells for micronucleus assays

From each subject, oral mucosa cells were collected and processed in a buffer solution (0.1 M EDTA, 0.01 M Tris HCl and 0.02 M NaCl; pH 7.0) as described earlier.[16] Urothelial cells were recovered by centrifuging (1,000 rpm for 10 min) urine samples collected from study subjects and washing the cell pellets with 0.9% NaCl.[40] Cell density was checked with a phase contrast microscope. The cell solution was either concentrated by centrifugation or diluted in the buffer solution (for oral mucosa cells) or 0.9% NaCl (for urothelial cells), as required. Once the desired cell density ($1.5–2.0 \times 10^6$) was attained, 50 μl of the cell suspension of both cell types was laid and spread well on clean, preheated (40°C) glass slides and allowed to air-dry for 5–10 min. The slides were coded and fixed in methanol:-acetic acid (3:1), stained with Giemsa[41,42] and observed under the microscope. MN in oral mucosa cells were scored in accordance with the criteria reported by Tolbert *et al*.,[43] while urothelial cells were analyzed following the method of Reali *et al*.[44] At least 3,000 oral mucosa cells and 1,000 urothelial cells were scored per individual.

Micronucleus assay from lymphocytes

Venous blood samples (5–7 ml) were obtained from each individual by venipuncture, and whole blood lymphocyte cultures were carried out for MN analysis as described earlier.[16] All the MN slides were coded and at least 2,000 binucleated cells from each subject were examined for MN under the microscope. Smeared, clumped, overlapped, necrotic cells or those without intact nuclei were not recorded. Only those MN were noted which were (*i*) rounded or oval shaped; (*ii*) less than 1/3 the diameter of the main nucleus; (*iii*) in the same focal plane as the nucleus; (*iv*) of the same color, texture and refraction as the main nucleus and (*v*) clearly separated from the main nucleus. Two trained research fellows crosschecked all MN scores to obviate the risk of bias, and the mean values were recorded.[45] Variability of repeated scoring of the slides by the same scorer was extremely low. The scorers were highly consistent on repeat counts and the concurrence between 2 scorers was good. All questionable MN were additionally assessed by a third scorer and a consensus result was accepted.

CA assay

From each subject, 5–7 ml venous blood was drawn and lymphocyte culture was carried for 72 hr, as described earlier.[15] All the slides of CA assay were coded, and from each individual subject, depending on the availability of good scoring metaphase plates, 50–100 metaphases were randomly scored for CA (chromatid and chromosome type). Gaps were also recorded but not included in the aberrations per cell. For mitotic index (MI) 1,000 cells per individual was scored from the same slides and expressed as a percentage.

Analysis of GSTT1, GSTM1 and GSTP1 allelic variants

Genomic DNA was prepared from fresh whole blood by salting out method as described earlier.[46] Three glutathione transferase genes (*i.e*. *GSTT1*, *GSTM1* and *GSTP1*) were amplified by polymerase chain reaction, as described in Table II. *GSTT1* and *GSTM1* genes were assessed for the difference in frequency of homozygous null mutation (if any) between the skin-symptomatic and asymptomatic groups of arsenic-exposed population. For this purpose, 2 terminal exons (*i.e*. 1 and 5) of *GSTT1* and 2 internal exons (*i.e*. 2 and 7) of *GSTM1* were amplified. To ensure that the absence of PCR products for any template was due to the presence of null mutation and did not result from the failure of the amplification reaction, 2 control genes (exon 6 for both *CYP2E1* and *CYP2D6*) were also amplified.

To study selected RFLPs (Ile105Val by BsmA1 and Ala 114Val by AciI) in *GSTP1*, exon 5 and exon 6 were amplified

TABLE V – CHROMOSOMAL ABERRATIONS AND MITOTIC INDEX IN LYMPHOCYTES OF UNEXPOSED AND ARSENIC-EXPOSED INDIVIDUALS

Variables	Unexposed group ($N = 102$)	Asymptomatic group ($N = 99$)			Skin-symptomatic group ($N = 105$)			Comparison of asymptomatic with skin-symptomatic group	
	Mean CA/cell (no. of subjects)	Mean CA/cell (no. of subjects)	Confidence interval (95%)	*p*-value	Mean CA/cell (no. of subjects)	Confidence interval (95%)	*p*-value	Confidence interval (95%)	*p*-value
CA/cell	0.024	0.07	−0.050 to −0.041[1]	0.001*	0.094	−0.076 to −0.063[2]	0.001*	−0.031 to −0.017	0.000*
% Aberrant cells	2.12	6.35	−4.666 to −3.788[1]	0.001*	9.08	−7.504 to −6.400[2]	0.001*	−4.666 to −3.788	0.000*
Mitotic index	1.73	1.64	−0.038 to 0.212[1]	0.172	1.44	0.165 to 0.409[2]	0.001*	0.078 to 0.323	0.001*

Two-sample *t*-test (large sample approximations).
[1]Comparison of unexposed with asymptomatic.–[2]Comparison of unexposed with skin-symptomatic.–*Significant at 1% level; *p*-value corrected up to three decimal.

using primers designed from the intronic region flanking the exons (Table II). The PCR products were digested with the restriction enzymes, according to the manufacturer's instructions (New England BioLabs). The 279-bp PCR product containing GSTP1 exon 5 was digested with BsmA1, which cleaved the DNA fragment to 147 and 132 bp fragments, if the polymorphic site was present. On the other hand, 280-bp PCR product containing GSTP1 exon 6, when digested with Aci I, cleaved into 170 and 110 bp fragments but the site was lost when the polymorphic site was present. All PCR products and the restriction digests, as appropriate, were analyzed by polyacrylamide gel (6%) electrophoresis; the gels were stained with ethidium bromide (0.5 μg/ml), and the stained DNA bands were visualized under UV light as described by Sambrook *et al*.[46]

Statistical analysis

Mean and confidence intervals (CIs; 95%) were calculated for each parameter. Standard two-sample *t*-test (large sample approximations) was performed to test for significant differences in all parameters (arsenic contents in water and biological samples; MN frequencies in 3 cell types; CA; MIs) taken pairwise, between the unexposed and exposed groups. Unexposed group was designated as the reference category, when compared either with skin-symptomatic and asymptomatic individuals. Where difference between asymptomatic individuals *versus* skin-symptomatic individuals was studied, asymptomatic group was kept as the reference category. Statistical significance was evaluated within the asymptomatic and skin-symptomatic groups after stratification based on sex (between male and female) and smoking (between smoker and nonsmoker) (data not shown). Cochran–Armitage trend test was conducted to compare the increasing trend in all the aforementioned parameters with levels of arsenic in drinking water. The test of significance was carried out at both 5% and 1% levels based on $\chi^2_{1,0.05} = 3.848$ and $\chi^2_{1,0.01} = 6.035$, respectively. To study the difference in genotype prevalence and association between the asymptomatic and symptomatic group, two-sample proportion test (binomial) was performed. The data was analyzed using statistical package MINITAB version 13. The association between GSTT1, GSTM1, GSTP1 and risks of manifesting arsenic toxicity were estimated with odds ratio (OR) and their 95% CIs derived from univariate unconditional logistic regression analysis. The null genotypes were considered as the reference category for GSTT1 and GSTM1, while homozygous Ile105Ile and Ala114Ala were kept as reference category for analyzing GSTP1.

Results and discussion

Arsenic-induced skin lesions

Long-term exposure to arsenic causes changes in skin pigments and hyperkeratosis. This promotes ulceration of skin and accelerates the risk of cancer of skin, liver, bladder and kidney. Hallmark signs of chronic arsenicism observed in our present study are raindrop pigmentation (72.54%), palmar and plantar hyperkeratoses (82.78%) and hypo- and hyperpigmentation (47.54%). Among 244 skin-symptomatic individuals, 10 were identified as having Bowen's disease and one was affected with squamous cell carcinoma. These observations are consistent with reported studies conducted in Bangladesh and West Bengal,[47,48] with the exception of a high incidence of Bowen's disease observed by us. No significant differences in the distribution of different skin lesions were observed as far as age or sex is concerned. Despite our attempt, it was not possible to find correlation between arsenic content in drinking water and the incidence of different skin lesions among study subjects, since some of them, when alerted, appropriately switched to drinking water with acceptable range of arsenic. Descriptive characteristics of the study participants (unexposed, exposed skin-symptomatic and asymptomatic subjects) are summarized in Table I.

TABLE VI – COMPARISON OF ARSENIC CONTENT AND FREQUENCIES OF MN AND CA WITH LEVELS OF ARSENIC CONTENT IN WATER AMONG ASYMPTOMATIC AND SKIN-SYMPTOMATIC INDIVIDUALS

Range of arsenic content (μg/l)	No. of subjects	Urine[1] (μg/l)	Nail[1] (μg/g)	Hair[1] (μg/g)	MN/1,000 lymphocytes	MN/1,000 oral mucosa cells	MN/1,000 urothelial cells	CA/cell[2]	% of aberrant cells	Mitotic index
Asymptomatic individuals										
51–150	46	124.19	2.02	1.41	6.35	3.44	4.17	0.064	5.89	1.76
151–250	27	182.93	3.10	1.93	6.16	3.91	4.16	0.071	6.31	1.56
>250	26	287.62	5.24	3.28	6.35	3.41	4.25	0.07	7.19	1.52
T^3_{CA}		2.94	16.40	19.40	1.45	0.41	0.45	6.52	9.46	−1.101
Significance		Insignificant	Significant at 1% level	Significant at 1% level	Insignificant	Insignificant	Insignificant	Insignificant	Significant at 1% level	Insignificant
Skin-symptomatic individuals										
51–150	43	117.67	2.69	1.73	8.54	4.95	5.25	0.096	9.23	1.39
151–250	25	154.23	3.31	1.92	8.84	5.45	5.84	0.092	8.77	1.41
>250	37	239.75	4.02	2.51	9.48	5.83	6.21	0.097	9.49	1.47
T^3_{CA}		6.34	7.02	4.11	9.03	5.38	7.58	−0.58	2.26	−0.177
Significance		Significant at 5% level	Significant at 1% level	Significant at 5% level	Significant at 1% level	Significant at 5% level	Significant at 1% level	Insignificant	Significant at 1% level	Insignificant

[1]All results are expressed as mean.–[2]Total number of aberrations (gaps not included)/total number of cells scored.–[3]Cochran–Armitage trend test.

TABLE VII – DISTRIBUTION OF GSTT1 AND GSTM1 NULL GENOTYPES IN THE ARSENIC-EXPOSED ASYMPTOMATIC AND SKIN-SYMPTOMATIC GROUPS

Genotypes	No. of subjects and prevalence (% total)		OR (95% CI)
	Asymptomatic (n = 178)	Skin-symptomatic (n = 244)	
GSTM1			
(−,−)	40 (53.33)	35 (46.67)	1.00[1]
(−,+) and (+,+)	138 (39.77)	209 (60.23)	1.73 (1.24–2.22)
GSTT1			
(−,−)	22 (40.00)	33 (60.00)	1.00[1]
(−,+) and (+,+)	156 (42.51)	211 (57.49)	0.91 (0.33–1.47)

Two-sample proportion test (binomial). OR, odds ratio; CI, confidence interval.
[1]Reference category.

Arsenic contents in water and biological samples

Arsenic contents in drinking water and other biological samples of exposed population (both skin-symptomatic and asymptomatic) were significantly higher ($p < 0.001$) when compared with the unexposed group (Table III). Within the exposed group (skin-symptomatic and asymptomatic), the difference in the arsenic contents of water, urine, nail and hair samples were not significant.

MN in lymphocytes, oral mucosa cells and urothelial cells

Cytogenetic damage in the arsenic-exposed and unexposed groups was assessed by MN assay in the aforementioned 3 cell types. A highly significant increase in MN frequency in all the 3 cell types was observed in the exposed group (Table IV). Exposed skin-symptomatic individuals, on an average, exhibited a 4.50-, 3.36- and 3.53-fold increase of MN in lymphocytes, oral mucosal and urothelial cells, respectively, over the unexposed group (Table IV). The relative increment for skin-asymptomatic individuals compared with unexposed group was lower (3.10-, 2.13- and 2.46-folds increase in lymphocytes, oral mucosa and urothelial cells, respectively). Within the unexposed group, no significant difference in MN was observed based on either sex or smoking habits (data not shown). However, between the 2 exposed groups, a significant increase in MN in all the 3 cell types was observed in the skin-symptomatic group over the asymptomatic group. MN analysis suggests higher cytogenetic damage in the lymphocytes ($p = 0.008$) and oral mucosa cells ($p = 0.026$) of skin-asymptomatic smokers compared to asymptomatic nonsmokers (data not shown). However, sex of the exposed study subjects (male *versus* females) did not influence the results. The results of the MN assays in 3 cell types show that the genotoxic effects vary in different tissues. These differences may be due to the differences in the exposure level of arsenic or its metabolite to cells and/or to different cellular kinetics of the different cells.[43] A slightly higher incidence of MN was observed in lymphocytes compared to the other 2 exfoliated epithelial cells. The difference could also be due to different concentrations at cellular level.[13]

CA in lymphocytes

As far as the MIs were concerned, a significant decrease in the mean MI values was observed in the exposed group compared to unexposed group (Table V). The decrease could suggest a slower progression of lymphocytes from S to M phase of the cell cycle.[49] No significant differences were observed in MI between asymptomatic and skin-symptomatic groups. CA was determined in 2 different ways, *i.e.*, CA per cell and the percentages of aberrant cells. A significant increase in CA per cell was observed in the 2 exposed groups (2.92- to 3.92-fold) compared to the unexposed group (Table V). No significant differences in CA were observed either within the unexposed or exposed groups based on their smoking habit and sex (data not shown). Between the 2 exposed groups, a significant increase (1.5-fold) in cytogenetic damage was noted in the lymphocytes of the skin-symptomatic individuals, when compared with asymptomatic individuals. Out of all types of aberrations, chromatid breaks and gaps were the predominant forms of CAs observed. Chromosome-type aberrations such as dicentrics were also observed. Some chromatid exchanges were also noted. Our data are consistent with the earlier reports that showed elevated incidence of CAs after chronic arsenic exposure.[10,11,50,51]

Comparison of arsenic content in biological samples, frequencies of MN and CA with arsenic content in water

The exposed group was divided into 3 subgroups based on the levels of arsenic exposure through drinking water—high (>250 μg/l), medium (151–250 μg/l), and low (51–150 μg/l) (Table VI). With the increment of arsenic content in drinking water, higher level of deposition of arsenic in nail and hair was observed in the skin asymptomatic group with similar trend in the percentage of aberrant cells in those study subjects. In the skin-symptomatic group, with the exception of CA/cell and MI, all other parameters showed significant increase with the increase in levels of arsenic in drinking water (Table VI).

Assessment of the role of selected GST genes towards susceptibility to arsenic toxicity

There are several studies trying to correlate the status of metabolizing enzymes or enzymes involved in DNA repair with a number of diseases.[25–30] However, the results are not unequivocal. We have examined the association, if any, between GST genotypes with arsenic-induced skin lesions. Frequencies of null genotype in *GSTT1* were 13.52% (33/244) and 12.92% (23/178) in skin-symptomatic and asymptomatic individuals and *GSTM1* null genotype were 13.90% (34/244) and 22.47% (40/178) in skin-symptomatic and asymptomatic individuals, respectively (Table VII). The distribution of *GSTM1* null genotypes was significantly higher in the asymptomatic group ($p = 0.026$). Compared to those with GSTM1 null genotype, individuals with GSTM1-positive (at least one allele) had significantly higher risk of arsenic-induced skin lesions (OR, 1.73; 95% CI, 1.24–2.22). The frequencies of the variations found in the study has been compared to the general distribution of these variations in the same ethnic population and observed to be similar to the overall exposed population, irrespective of their sensitivity towards arsenic (data not shown). To explore possible gene–gene interaction, we examined the joint effect of GSTT1 and GSTM1, which also suggests role of GSTM1 with arsenic toxicity (data not shown). We have carried out the analysis of genotoxicity data based on GSTM1 and GSTT1 status both for skin-symptomatic and asymptomatic individuals, but no significant difference was observed (data not shown). A total of 86 skin-symptomatic and 110 asymptomatic individuals were genotyped for GSTP1 Ile/Val variants at codon 105 and Ala/Val variants at codon 114 (Table VIII). There was no significant alteration in GSTP1 genotype frequencies between 2 arsenic exposed groups split by their skin phenotypes.

Contrary to the hypothesis of protective role of GSTs, we found increased risk of developing arsenic toxicity with *GSTM1*-positive phenotype. Similar findings suggesting protective role of *GSTM1* null genotype with several other diseases (*e.g.* Balkan endemic

TABLE VIII – DISTRIBUTION OF GSTP1 GENOTYPES IN ARSENIC-EXPOSED ASYMPTOMATIC AND SKIN-SYMPTOMATIC GROUPS

Genotypes	No. of subjects and prevalence (%)		OR (95% CI)
	Asymptomatic (n = 110)	Skin-symptomatic (n = 86)	
GSTP1 at codon 105			
Ile/Ile	82 (74.54)	65 (75.58)	1.00[1]
Ile/Val	25 (22.73)	18 (20.93)	0.91 (0.46–1.80)
Val/Val	3 (2.73)	3 (3.49)	1.26 (0.25–6.40)
GSTP1 at codon 114			
Ala/Ala	103 (93.63)	78 (90.69)	1.00[1]
Ala/Val	5 (4.54)	8 (9.3)	2.09 (0.67–6.53)
Val/Val	2 (1.82)	–	

Two-sample proportion test (binomial). OR, odds ratio; CI, confidence interval.
[1]Reference category.

neuropathy, chronic pancreatitis, POAG, lung cancer) have also been reported by several authors.[25,52–54] These findings are not surprising, since, although GSTs are generally recognized as detoxifying enzymes, they could also be involved in the generation and activation of toxic compounds.[55,56] Recent evidences support the role of bioactivation by GSTs.[57]

Conclusions

Thus, the overall results suggest that skin-symptomatic individuals are more susceptible to arsenic-induced toxicity and genotoxicity than the asymptomatic individuals. Although skin-symptomatic individuals are more susceptible to arsenic-induced genotoxicity, asymptomatic individuals are subclinically affected and are also significantly susceptible to arsenic-induced genotoxicity. This is the first report of the comparison of arsenic-induced genotoxicity between skin-symptomatic and asymptomatic individuals exposed to same arsenic-contaminated water and the possible protective role of *GSTM1* null in arsenic toxicity. These results also indicate that genetic variations may play an important role in arsenic-induced toxicity and genotoxicity through interplay of multiple gene products, which need to be explored further and are being currently investigated.

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