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RESEARCH ARTICLE

BODY RETENTION OF ARSENIC IS ALTERED BY GST POLYMORPHISM

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ABSTRACT

Glutathione- S -Transferase (GST) might be involved in the initial reduction of arsenate to arsenite and subsequent oxidative methylation (Sampayo 2000, Zakharyan 2001). Humans with null genotype of GST M1 and T1 have been considered to be a high risk group of people who retain arsenic in their body due to incomplete metabolism of arsenic.In order to elucidate the relationship among clinical severity, urinary excretion of arsenic and genetic polymorphisms of GST M1 and T1, a total of 100 study subjects were recruited from the villages of southern region of WestBengal, India. Specimens of drinking water, blood and urine were collected from each study subjects. Concentration of arsenic in urine and water was determined by atomic absorption spectro photometry-hydride generation system. Multiplex polymerase chain reaction (PCR) was performed to determine the genetic polymorphism of GST M1 and T1. Genetic polymorphism of GSTM1 and T1 were significantly associated ($p<0.05$) with Clinical severity in higher exposure groups. Persons having null genotype have an increased clinical symptom score than persons with GSTM1 or GST T1 nonnull genotype. Persons with GSTM1 or GST T1 null genotype have decreased total urinary arsenic compare to persons having non null genotype.

INTRODUCTION

Arsenic is a ubiquitous element widely distributed in the environment. It is mainly occur in subsoil water in the form of oxides and oxi acids of arsenic. Humans are exposed to high level of inorganic and organic arsenic primarily through their drinking water though history of arsenic exposure also obtained from occupational and medicinal route. However, organic arsenic is much less toxic than inorganic arsenic. According to U.S Environmental protection agency the safe limit of arsenic in water is 0.05mg/L. In India, the basin of the river Ganga is highly contaminated with inorganic arsenic which contributes the contamination of subsoil water with inorganic arsenic in numerous districts of Bihar, West Bengal and Jharkhand. Shortage of bacteriologically safe surface water forces the people of these areas to drink arsenic contaminated water. Arsenic is a human carcinogen. Chronic environmental exposure to inorganic arsenic leads to sever skin manifestation like hyper/hypo pigmentation and hyper/hypokeratosis and skin, bladder and lung cancer. People who use arsenic contaminated water in different district of West Bengal also show severe skin manifestation, peripheral neuropathy, vascular abnormalities and skin, bladder and lung cancer.

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In subsoil water arsenic mainly occur in two forms, arsenite and arsenate. If inorganic arsenic entered into the body as arsenate then it is readily absorbed into the blood from GI tract where it is reduced to arsenite. Arsenite detoxified by methylation occurred mainly in the liver to produce monomethyl arsinic acid (MMA) and dimethyl arsenic (DMA) acid (Vahter *et al.,* 1999). MMA and DMA are less toxic and readily excreted in urine. Some of the unmethylated inorganic arsenic is also excreted in urine. So, the constituent of total urinary arsenic are inorganic arsenic, MMA and DMA. The ingested inorganic arsenic retained in the body is responsible for various genotoxic effects. It is well documented that glutathione and other thiols are required for arsenic methylation and subsequent detoxification. The sulfhydryl (SH) groups within the cell are potent receptors of trivalent arsenic. Arsenic binds to two thiol groups per molecule to form stable ring compounds, in preference to reacting with two thiol groups of two separate molecules (Goodman Gillman 1996). After prolonged feeding of arsenic contaminated water to mice, increased lipid peroxidation and plasma membrane damage have been noted which is associated with reduction of hepatic glutathione and oxidative enzymes (Santra *et al.,* 1998). The enzyme responsible for arsenic methylation is arsenic methyltransferase (Cyt 19). The methyl group donor is S-adenosyl methionine. Hayakawa and

coworkers showed that human recombinant methyltransferase, Cyt19, is capable of arsenic methylation in reducing environment. HPLC inductive plasma mass spectrometry analysis revealed that arsenic triglutathione (ATG) was generated from trivalent inorganic arsenic only when glutathione (GSH) was present at a concentration of at least 2mM. Methylation of arsenic was catalyzed only when ATG is present in the reaction mixture. According to Hayakawa arsenic glutathione complexes are the substrate for Cyt19 to produce finally MMA and DMA (Hayakawa *et al.,* 2004). Lin and co-workers purified SAM dependent arsenic methyltransferase from rat liver cytosol and reported that rat arsenic methyltransferase was a homolog to human Cyt19 (Lin *et al.,* 2002). It was also reported that rat recombinant Cyt19 catalysed the conversion of trivalent inorganic arsenicals to mono and dimethyarsenicals (Walton *et al.,* 2003). Kala *et al.,* reported that trivalent inorganic arsenic was metabolized in rat liver and excreted as arsenic glutathione complexes in bile (Kala *et al.,* 2000). Glutathione-S-transferase (GST) are a large family of detoxification enzyme that catalyze the conjugation of reduced glutathione to a wide spectrum of hydrophobic and electrophilic compounds. There are seven subclasses of GST in mammalian cells, namely α, μ, π, θ etc. The MI member of μ subclass and TI member of θ subclass are polymorphic and mainly distributed in the liver. As arsenic-glutathione complex is reduced by glutathione –S-transferase (GST) the status of this enzyme in arsenic exposed people is much relevant for arsenic metabolism. Glutathione –S-transferase omega (GST 01-1) has been shown to act as an enzyme in the reduction of pentavalent organic arsenicals (Sampayo *et al.,* 2000, Zakharyan *et al.,* 2001). The null genotype of GSTM1 has a decreases detoxification capability. It has been linked with an increased risk of cancer of the lung, bladder, breast, colon, skin and oral cavity. The null genotype of GSTT1 was reported to be associated with increased risk of brain and colorectal carcinoma (Chiou *et al.,* 2001). Humans with null genotype of GST have been considered to be at a high risk of cancer due to their GSH deficiency. The main site of arsenic detoxification is liver and GSTMI and GSTTI have a tissue specificity which shows that GST M1 and GSTT1 are mainly distributed in the liver. Therefore it is assumed that GSTMI and GSTTI null allele have some important role in arsenic metabolism and body retention of arsenic.

MATERIALS AND METHODS

Subjects

Subjects of this study were taken from the Arsenic clinic of Institute of Post Graduate Medical Education and Research, Kolkata, India. The Arsenic clinic is a tertiary referral center. All the cases recruited were referred cases from South 24 Parganas, one of the worst affected districts of West Bengal. Epidemiological studies were being carried out in this district since 1995 (GuhaMazumder *et al.,* 1998, Haque *et al.,* 2003). Arsenic exposure data of all these people were therefore known to the investigators. Criteria for diagnosis of arsenicosis were based on parameters described earlier (GuhaMazumder *et al.,* 2001). The skin lesions did not simulate with any other known skin disease.

Control selection

For this study population controls were living in the same arsenic area from where cases came to the arsenic clinic. They were drinking water from tube wells having arsenic level less than 50 μg/l and they did not have any skin lesion (Group A). History of arsenic exposure of each participant was obtained in detail including duration of intake of water from the source. Water samples were collected from tube wells used for drinking and cooking purpose by each participant. Level of arsenic in water and level of total arsenic (inorganic arsenic + MMA + DMA) in urine was determined by atomic absorption spectrophotometer with hydride generation system (AAS) and expressed as μg/l. Participants have been divided into following groups according to concentration of arsenic in their drinking water which are group A (control) <50 μg/l, group B 51–250μg/l, group C 251-500μg/l and group D 500-1000μg/l. Informed consent was taken from each participant before taking their blood and urine samples. The ethical guideline was maintained as framed by the medical council of India according to Helsinski rule. The degrees of skin manifestations have been classified into three grades according to their severity (Table 1). The degree of skin manifestation was given a score that is called clinical symptom score. The clinical symptom score thus reflects the severity of skin manifestation. According to this scoring system maximum total score = 6; Clinical score : No skin lesion = 0; Mild : ≤2; Moderate : ≤4; Severe : ≤6. The control subjects have no pigmentation and keratosis and therefore having a clinical symptom score of about 0.

DNA isolation from whole blood

Genomic DNA was isolated from whole blood using conventional chloroform extraction method, using 0.01% SDS and protienase K (0.1mg/ml).

Genotyping of Glutathione- S-Transferase (GST) M1 and T1:

The polymorphic deletion of M1 and T1 gene was genotyped using the multiplex PCR approach described by Mondal *et al.,* 2005.

Statistics

For finding the significance of the differences in total urinary arsenic between different arsenic exposure groups and different polymorphic groups of same arsenic exposure group we assumed no particular pattern of distribution of data. We therefore performed two tail Median test (Das and Das 1998) which is a non-parametric alternative of Student 't' test, for widely distributed data. For median test, the raw data of each of the group were compared with the data of control group and composite median were calculated in each case. Then the number of data in each group and that of the control group greater than or less than the respective composite median value is tabulated in 2 x 2 contingency table and the chi square value was calculated after Yate's correction wherever necessary. The calculated chi square value was then compared to critical chi square value at different level of significance.

RESULT AND DISCUSSION

A positive correlation exists between arsenic induced clinical symptom score and total urinary arsenic level ($r = 0.49$, $P<0.05$). Degree of arsenic exposure and clinical symptom

Table 1: Dermatological criteria and graduation of chronic arsenic toxicity for scoring system

Pigmentation (Score)		
Mild (1)	*Moderate (2)*	*Severe (3)*
Defuse Melanosis, Mild Spotty pigmentation, Leucomelanosis	Moderate Spotty pigmentation	Blotchy Pigmentation, Pigmentation of under surface of tongue, buccal mucosa
Keratosis (Score)		
Mild (1)	*Moderate (2)*	*Severe (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)

Table 2: Median value for clinical symptom score and Total Urinary Arsenic and corresponding chi square value for Total urinary arsenic in different Arsenic exposure group.

Group	Degree of arsenic exposure (µg/l)	Median value of Clinical symptom score	Median value of Total Urinary Arsenic (µg/l)	Chi-square value of Total urinary arsenic
A, n= 28	0-50	0	19.71	
B, n=24	51-250	2	119.00	25.07, p<0.001
C, n=22	251-500	4	129.32	29.72, p<0.001
D, n=15	501-1000	6	79.49	18.19, p<0.001

n =sample number, p<0.001 means probability of occurrence due to random sampling is 0.1 %.

Table 3: Total urinary arsenic in different polymorphic status of different arsenic exposure group.

Group	GSTM1T1 polymorphism	Individual group median of total urinary arsenic(µg/l)	Chi-Square value
A , n=28	M+T+, n=13	26.95	
	M-T+, n=8	21.01	0.27, p<0.65
	M+T-, n=5	19.85	0.53, p<0.55
	M-T-, n=2	-------	----------
B, n=24	M+T+, n=12	151.6	----------
	M-T+, n=6	115	4.50,p<0.05
	M+T-, n=4	66.85	5.92, p<0.05
	M-T- n=2	----------	---------
C, n=22	M+T+, n=8	200	
	M-T+, n=10	93.5	8.10, p<0.01
	M+T-, n=3	91	Not done
	M-T-, n=1	----------	----------
D, n=15	M+T+, n=8	101.00	
	M-T+, n=6	87.04	Not significant
	M+T-, n=1	92.55	Not done
	M-T-, n=0		

score is also shows positive correlation (r = 0.73, P<0.05). Total urinary arsenic and arsenic exposure are also correlated significantly (r= 0.46, P<0.05). Non-parametric median test indicate a significant increase in urinary arsenic concentration in group B and C in comparison to group A (table 2). In group D it is also significant but considering the degree of exposure the degree of total urinary arsenic is too small in this group. Probably this low degree of total urinary arsenic excretion may be the cause of clinical severity and highest clinical symptom score in this group. Total urinary arsenic concentration increases significantly in a dose dependent manner as the concentration of arsenic in drinking water increases up to 500µg/lof arsenic exposure. Level of total urinary arsenic shows significant variation with GST polymorphism status in different arsenic exposure groups (table 3). This difference is not significant in group A, but in group B and C the level of total urinary arsenic is significantly higher in GSTM1T1 non null allele (M+T+) population in comparison to GSTM1 null allele (M-T+) and GSTT1 null allele (M+T-) population respectively of the same arsenic exposure group. Ingested inorganic arsenate is first absorbed from the gastrointestinal tract and is rapidly reduced to arsenite in blood which is then readily taken up and methylated predominantly in the liver which is a suspected target organ for arsenic induced carcinogenesis (Donohue and Abernathy 2001, Goering *et al.,* 1999, Vahter 1999). After entering into the hepatocytes, inorganic arsenic is bound extensively to intracellular components and is metabolized to MMA and DMA, which are less toxic than unmethylated inorganic arsenic. The methylated forms are efficiently excreted in urine. Inorganic arsenic was the predominant arsenical in liver and kidney after 1 and 2 h of administration of arsenic with a dose of 10 and 100 µmol Arsenic/kg body weight, respectively (Kenyon *et al.,* 2005). At later times, DMA was the predominant metabolite in liver and kidney. The low capability of arsenic methylation might increase the body retention of arsenic and subsequently increase the risk of related health hazards. In the present study the total urinary arsenic was analyzed in four different arsenic exposure groups. A dose dependent increase in total urinary arsenic has been shown as the degree of arsenic exposure increases up to a certain limit of exposure (up to 500 µg/l). Hinwood *et al.,*have demonstrated that in an Australian population living in a gold mining area the soil arsenic concentration is positively correlated with urinary inorganic arsenic concentration. They have shown that soil arsenic concentration is a significant predictor of increased urinary

arsenic concentration (Hinwood *et al.,* 2004). Mexican population exposed to environmental arsenic that a positive correlation exists between total arsenic intake by drinking water/day and the total arsenic concentration in urine ($r = 0.50$, $P < 0.001$) (Meza *et al.,* 2004). Our population also shows significant positive correlation between degree of arsenic exposure and level of total urinary arsenic in different arsenic exposure groups. In freshwater fish, in response to inorganic arsenic the activities of glutathione-S-transferases, glutathione peroxidase, glutathione reductase, and catalase initially decreases in the liver and kidney but prolonged exposure results in increase of such enzyme activity in liver and kidney with subsequent decline in arsenic concentration (Allen and Rana, 2004). Detoxification of endogenous and exogenous electrophilic compounds by glutathione conjugation generally requires GST activity, as the conjugation of thiol groups with electrophilic compounds requires an initial reduction of glutathione, which is a GST dependent step. Human recombinant arsenic methyl transferase (Cyt 19) is capable of arsenic methylation in presence of endogenous reductase and GSH is required for the initial reduction of arsenate to arsenite (Hayakawa *et al.,* 2004). It was hypothesized that arsenic-glutathione complex and trivalent inorganic arsenic compounds are in equilibrium depending on the GSH concentration (Hayakawa *et al.,* 2004). This metabolic process occurs in the liver. ATG (arsenic triglutathione) and MADG (methyl arsenic diglutathione) were found as major metabolites in the bile and the concentration of GSH exceeded to 10mM in inorganic arsenic injected rat (Kala *et al.,* 2000). Both of these metabolites are substrate of Cyt 19.

It has been showed that glutathione- S- transferase omega (hGSTO1) can catalyse the conversion of MMAV to MMAIII in various tissues (Sampayo *et al.,* 2000, Zakharyan *et al.,* 2001). Polymorphism in hGSTO1 (MMAV reductase) gene is responsible for interindividual variability in arsenic metabolism (Marnell *et al.,* 2003) and several new polymorphisms in hGSTO1 gene has been identified. According to Mernell persons having such polymorphism have an increased level of inorganic arsenic and reduced level of MMA in urine. Polymorphic deletion of GST O1 has been associated with abnormal monomethyl arsenic reductase and dimethyl arsenic reductase activity.GSTO1 with coding region deletion (deletion in E155) has been associated with abnormal arsenic excretion pattern (Schmuck *et al.,* 2005). Liu and Xie showed that arsenic increases the expression of GSTM1, GST T1 and glutathione reductase in tumorous and non tumorous liver tissue when administered orally in adult mice (Liu *et al.,* 2004, Xie *et al.,* 2004). RT-PCR analysis showed that rate of transcription of GSTT1 allele is increased in nontumorous and tumorous liver of pregnant mice exposed to 42.5 and 85 ppm of arsenic through their drinking water comparison to unaltered water control (Liu *et al.,* 2004).

A reduction of hepatic GSH level has been found to greatly decrease the urinary level of MMA and DMA in experimental animals. As low level of GST activity might decrease the level of reduced glutathione, it is hypothesized that persons having null genotype of GSTM1 or GSTT1 may have altered arsenic methylation capacity and therefore different body retention capacity from persons having non-null genotype. This hypothesis has been tested on arsenic exposed residents of Taiwan (Chiou *et al.,*1997). The null genotype of GSTM1 was associated with increased percentage of inorganic arsenic in urine in arsenic exposed population and the null genotype of GSTT1 was associated with increased percentage of DMA in urine. These findings indicated that GSTM1 might facilitate the methylation of inorganic arsenic (Chiou *et al.,*1997). The speciation of methylated metabolites has not been done in the present study. The polymorphic status of GSTM1 and GSTT1 allele has been studied in individual study subjects. The total urinary arsenic concentrations in different polymorphic status of same arsenic exposure group have been analyzed to find the correlation between total urinary arsenic and GSTM1 and GSTT1 polymorphism. In our population people having GST M1 and GSTT1 null genotype is associated with a decrease in total urinary arsenic in comparison to GSTM1 and GSTT1 non-null counterpart of the population. Persons having GSTM1 null allele (M-T+) have a reduced percentage of total urinary arsenic than persons having GSTM1 non-null allele. This finding is indicative of involvement of GSTM1 allele in arsenic metabolism. Persons with GSTT1 null allele (M+T-) have also significantly lower total urinary arsenic than GSTT1 non null allele (M+T+). In our study population the number of double null allele (M-T-) allele is very small. Therefore it has not been possible to study the level of total urinary arsenic in persons having GSTM1 and T1 null allele at statistically significant level. The mechanisms by which GSTM1 and GSTT1 modify the individual's arsenic metabolism status and body retention of arsenic deserve further investigation. The arsenic induced clinical symptoms in different arsenic exposure group were studied and the correlation coefficient was analyzed between degree of arsenic exposure and clinical symptom score. Level of urinary arsenic and arsenic induced clinical symptom score was analyzed to find out the correlation between these two variables. Both of these two variables have a significant positive correlation with clinical symptom score.

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