

# Modification of DNMTs Gene Expressions by GST O1 and GST O2 Polymorphism in Chronic Arsenic Exposed People with and without malignancy from West Bengal, India.

**Sarmishtha Chanda** (✉ [csarmishtha@yahoo.com](mailto:csarmishtha@yahoo.com))

Sister Nibedita Govt. General Degree College, for Girls, Hasting House, Alipur, Kolkata 700027.'s House,

**Jayashree Roy**

Indian Institute of Chemical Biology CSIR

**Aditi Mukhopadhyay**

Hooghly Mohsin College

**Tushar Chakraborty**

CSIR, Indian Institute of Chemical Biology

**Debendranath Guha Mazumder**

DNGM Research Foundation

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## Research Article

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## Abstract

**Background:** Chronic Arsenic exposure causes skin manifestations and even cancer. However, the response varies widely among persons despite receiving similar cumulative exposure through their food or drinking water or both. These differentiations in manifestations may be due to polymorphic distribution of arsenic metabolizing genes among exposed people.

**Method:** Polymorphism of GSTO1, GSTO2 and their frequency distribution may modify skin manifestations and development of arsenic induced cancer in exposed persons through food chain. Polymorphic variations of GSTO1 and GSTO2 have been studied on 112 subject including control. They were recruited from one of major arsenic affected district, Nadia of West Bengal, India, having high arsenic content in their food. Exposed subjects were categorized into three groups, i.e, with arsenical skin lesions and with out arsenical skin lesions and arsenic induced cancer. Control subjects were 33 in number. Concentration of arsenic in their urine, hair, drinking water, food, extent of clinical manifestations, GST O1 and O2 status was determined. DNMT1, 3A, and 3B were studied for their expression profile and analyzed with GSTO1 and O2 polymorphisms.

**Result:** Genetic polymorphism of GSTO1 gene polymorphism is significantly associated with arsenic induced skin scores in skin lesion positive cases and arsenic induced cancer cases and also significant increase is seen in DNMT expression and MDA level in exposed cases with homozygous wild type variants. Total urinary arsenic decreases significantly in wild type GSTO1 genotype, although, GSTO2 polymorphism showed no statistically significant differences in skin manifestations, and DNMTs expression. Frequency of GSTO1 and O2 polymorphic variety showed prevalence of wild type homozygous in arsenic induced cancer cases.

**Conclusion:** GSTO1 polymorphism shows significant association with DNMT expression profile in arsenic exposed people.

## 1. Introduction

Humans are exposed to environmental arsenic mostly through drinking water and food. The subsoil water of many countries of the world including India has been contaminated with arsenic. In India, the basin of river Ganga in West Bengal is highly contaminated with arsenic. The allowable limit for arsenic in drinking water set by the US

Protection Agency is 10 µg/l (WHO 2011, NRC 1999). At present, for most of the arsenic affected Asian countries; the permissible limit of arsenic in drinking water is 50 µg/l (BIS 1992). Though intensive programs has been taken by Government of West Bengal to block the arsenic affected tube wells and supplying arsenic free safe water to affected villages, no restriction and monitoring has been made to use this arsenic contaminated shallow wells for irrigation purpose. Due to irrigation with arsenic contaminated water vegetables are also acquiring inorganic arsenic in it and therefore transmitted to people consuming it which is reflected their urine arsenic values.

People, who have stopped the use of arsenic contaminated subsoil water still showed significant amount of arsenic in their urine. Presence of arsenic in urine is an indicator of current arsenic exposure. The subsoil water which contains inorganic arsenic is also used in irrigation purpose and therefore may be transmitted to plants and enter in to the food chain. Evidence coming from Bangladesh, India, Cambodia, Vietnam and other countries revealed that food grains can accumulate arsenic when harvested in arsenic contaminated soil and ground water (Zhao et al. 2010, Ma et al 2008, Abedin et al 2002). Outcomes of recent research indicate that rice is the more potent accumulator of arsenic (Abedin et al 2002, Ahmed et al.2011, Zhang et al. 2005, Su et al. 2010). Paddy field flooded with arsenic contaminated ground water produces paddy containing significant amount of inorganic arsenic in it (Dwivedi et al. 2010). Live stock, food grains and vegetables are exposed to environmental arsenic in this manner and enter into human body (Bera et al. 2010, Eisler et al. 2004, Biswas et al. 2014).

We observed in our previous studies that people receiving similar level of arsenic exposure with a similar duration reflecting differential degree clinical manifestations. This is due to different degree of arsenic metabolism in different people which is influenced by genetic polymorphism of arsenic metabolizing genes. Glutathione S Transferase omega (GSTO) and arsenic (III) methyltransferase (AS3MT) are involved in classic arsenic metabolism pathway in human and other animals. GSTO1 and GSTO2 are involved directly in the reduction of MMA(V) to MMA(III), which is the rate limiting step of arsenic methylation in human (Hsu et al 2011). The association of arsenic induced clinical manifestation and GSTO1 and GSTO2 polymorphism had been studied in different arsenic exposed population of the World (Esgtrom et al. 2007, Paiva et al. 2008, Lindberg et al. 2007). The polymorphism of GSTO1 and GSTO2 could be a significant modifier of arsenic methylation capacity of individuals exposed to arsenic and thereby may be involved in arsenic induced skin manifestation and arsenic induced urothelial carcinoma (Ahsan et al.2007, Wang et al.2009). Further, in our previous studies we have shown that arsenic induced cancer is associated with perturbation of DNA methylation status (Chanda et al. 2006, Majumder et al 2010, Chanda et al 2013). DNA methylation is carried out by a gene family called DNMT. Therefore any alteration in DNMT expression may perturb DNA methylation pathway. In this paper we are trying to find out the relationship between polymorphism of GSTO1 and GSTO2 genes and their frequency distribution in study population with expression pattern of DNMT gene and arsenic induced skin manifestations in chronically exposed people with arsenic through food chain contamination.

In the present report we are going to explain the association of polymorphic variation of GSTO gene (GSTO1 and GSTO2) with DNA methyltransferase (DNMT) expression, arsenic induced skin manifestations and arsenic induced urothelial cancer in persons with arsenic exposure through food. Subjects are selected from arsenic endemic area. Collection of samples and subject recruitment had been done during 2009-2010 and 2010-2011. Several reports came on GST polymorphism and arsenic induced skin manifestation indicated significant association between GSTO1 and O2 polymorphism and arsenic induced skin manifestations (Majumder et al 2017, Hsu et al 2011, De Chaudhuri et al 2008). Although, Majumder et al showed the correlation between arsenic induced skin manifestation, altered by individuals GSTO gene polymorphism status, and whole genome methylation status but not pointed out on the DNMT expression profile (Majumder et al 2017). We are therefore evaluated the, missing link, DNMT expression profile with GSTO1 and GSTO2 polymorphic status.

However, this is the first report of association of GSTO1 and GSTO2 gene polymorphism and its influence on DNMT gene expression with presence or absence of skin lesions and arsenic induced cancer in persons chronically exposed to arsenic through their food chain in West Bengal, India.

## 2. Materials And Methods

We have recruited a total 112 study subjects. The subjects were selected from Nadia district, a highly arsenic affected district of West Bengal. A total number of 79 arsenic exposed subjects have been chosen from this area. Primarily, they had been selected on the basis of their current arsenic exposure, urine arsenic concentration, and concentration of arsenic in hair. They were exposed to environmental arsenic through food. The total numbers of arsenic exposed subjects (79) are divided into three groups on the basis of their skin lesions and arsenic induced urothelial cancer and 33 subjects were arsenic unexposed people.

Selection of exposed and unexposed control is schematically presented in figure 1. During subject selection raw rice and cooked rice arsenic concentration were measured to detect the amount of arsenic consumed through rice. Arsenic concentration in cooked rice is lesser than raw rice in our study which is also at par with other studies (Ohno et al 2009, Mandal et al. 2019). The inclusion criteria for exposed subjects were arsenic in urine, food and hair above permissible limit with presence or absence of skin manifestations which are checked by group of specialized clinicians (GuhaMazumder et al. 2001, Chanda et al. 2006). The mean arsenic level in urine, hair and cooked rice of 79 exposed participants were 124 $\mu$ g/L, 1.59 mg/kg and 147 $\mu$ g/day. The exclusion criteria for exposed subject selection were less than 1 year of arsenic exposure through food. In group I there were 32 subjects having definitive arsenical skin manifestations and in group II there were 32 subjects without having any arsenical skin manifestations, although both the groups have received similar level of arsenic exposure through food (vide Figure 1). Group III is arsenic induced cancer (n= 15). Group IV is arsenic unexposed control (n=33). Group I is designated as arsenic induced skin manifestation positive, and group II is arsenic induced skin manifestation negative. Age, sex and socio economic status matched control subjects were selected from arsenic unexposed blocks of a district of West Bengal, North 24 Parganas, and designated as group IV. Participants of this group neither have any history of current or past arsenic exposure nor arsenical skin lesions. Exclusion criteria for unexposed control were any known chronic disease. The current and past drinking water arsenic concentration of this group was <10  $\mu$ g/L. Current drinking and cooking water samples, morning void urine samples and 24 hrs food samples, hair and nail samples had been collected in sterilized polypropylene container from all of the study subjects to detect the concentration of arsenic in their drinking water, urine, hair, nail and food. Controls were chosen from age, sex and socio economic status matched unexposed individuals with a food and urine arsenic level below detection limit. Their mean urinary arsenic level, hair arsenic and cooked rice arsenic were 9  $\mu$ g/L, 0.13 mg/Kg and 3.9  $\mu$ g/day respectively, at the time of inclusion in the study. Blood was collected by venipuncture from every participant in EDTA anticoagulant vial.

The mean concentration of arsenic in raw rice in arsenic exposed population was 315 $\mu$ g/kg and 298 $\mu$ g/kg for group I and group II respectively and the range of exposure of arsenic through food were 118-672  $\mu$ g/kg in group I and 212-478  $\mu$ g/kg in group II as measured in our laboratory from 24 hours food samples collected from the participants. Raw rice and cooked rice were collected from every participant's house and these were measured for arsenic concentration. In cooked rice the mean arsenic concentration were 217  $\mu$ g/kg and 138  $\mu$ g/kg in group I and II respectively. The method of collection of food samples are described in detail in our previous paper (Deb et al 2012). History of arsenic exposure from each participant was obtained in detail including duration and quantity of water intake, food sampling and food arsenic concentration. Demographic data, social characteristics and occupational data were collected for each subject (Deb et al. 2012, Biswas et al. 2013).

All male participants were involved in moderate works and smokers. Mostly they were small traders or farmers or involved in office jobs in small concerns. Women were mostly housewives or involved in sedentary works and non smokers. Written informed consent was obtained from all participants before drawing their blood. 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 and permission obtained from DNGMRF. The name of the institute where human studies were carried out is Indian Institute of Chemical Biology (IICB) which is run by Govt. of India, a CSIR research Unit.

### 2.1 Field Study:

A village level sampling frame was created with in two blocks, Chakdah and Haringhata of Nadia district, having at least one tube well contaminated with >10  $\mu$ g/L of arsenic.

### 2.2 Collection of Blood samples:

EDTA anticoagulated blood samples were collected from each participant and kept at  $-20^{\circ}\text{C}$  at DNGM research foundation, (DNGMRF), Kolkata. All the blood samples were transported in icebox from DNGMRF to the Department of Physiology, Gene Regulation laboratory, IICB, Kolkata for further storage at  $-70^{\circ}\text{C}$  and subsequent analysis after DNA extraction.

### 2.3 Clinical Symptom Score:

Each study subject had been assigned a clinical symptom score according to their skin manifestations, i.e presence of pigmentation and keratosis in the exposed area of the body. Although in group II subjects absence of skin manifestation causes assignment of clinical score as 0. Both pigmentation and keratosis were graded 1,2 or 3, depending on the level of symptoms. Sum of the two was clinical symptom score, so that a person can have maximum score of 6. This scoring system is applicable in group I and group III subjects while group II and IV subjects had a score 0 (Guha Mazumder 2008) .

**2.4 Determination of Arsenic in drinking water, hair and urine:** The concentration of arsenic in drinking water, hair, nail, 24 hours food sample and urine was determined by atomic absorption spectrophotometry hydride generation (FI- AAS-HG) system according to manufacturer's instruction. Hair, nail and urine arsenic concentration was measured according to Das et al (Das et al. 1995) using FI-HG-AAS (PerkinElmer A Analyst 200). The limit of detection determined at the 90% confidence interval level was 3 $\mu$ g/L.

## 2.5 Genotyping of Glutathione-S-Transferase Omega

### PCR RFLP for GST O A 140 D:

In humans, five classes of GST genes exist:  $\alpha$  (*GSTA*),  $\mu$  (*GSTM*),  $\pi$  (*GSTP*),  $\theta$  (*GSTT*), and  $\zeta$  (*GSTZ*) with one or more genes in each class. The enzymes have different, but sometimes overlapping, substrate affinity (Buchard et al. 2007.). GST O1 A140 D genotype was determined by PCR RFLP. The primers for PCR were 5' AAA GTT GTT TCT TAA ACG TGC C-3' and 5' -AAG TGA CTT GGA AAG TGG GAA-3' (Hsu et al.2011). The reaction was incubated at 95° C for 10 min and subjected to 30 cycles of 94° C for 60 s, 55° C for 60 s, and 72° C for 60 s and a final extension step at 72° C for 10 min. The PCR product was digested for 12 hrs with one unit of Cac 8 I (New England, Biolabs) and then separated on 2.5% agarose gel for 2hrs. The genotypes were determined as follows: homozygous wild type C/C or AA: 243, 145 and 67 bp; heterozygous C/A or AD: 388, 243, 145, and 67 bp; and homozygous variant A/A or DD :388 and 67bp (Hsu et al 2011).

### PRC-RFLP for GST O 2 N 142 D polymorphism:

The PCR was performed using the primers 5' ACT GAG AAC CGG AAC CAC AG 3' and 5' GTA CCT CTT CCA GGT TG-3' (Hsu et al 2011). The reaction was incubated at 95° C for 10 min and subjected to 30 cycles of 95° C for 60 s, 60° C for 60s, and 72° C for 60 s and a final extension step at 72° C for 12 min. The PCR product was digested by Mbol (New England, Biolabs), and the products were resolved on 2.5% agarose gel. The genotypes were determined as followed: homozygous wild type A/A or NN: 280 bp, heterozygous A/G or ND: 280, 231 and 49 bp; homozygous variants G/G or DD: 231 and 49bp (Hsu et al. 2011).

## 2.6. RNA isolation and DNMT expression study:

Total RNA was isolated from anticoagulated whole blood by using a commercial kit Trizol according to the manufacturer's instructions (Gibco BRL). RT-PCR was done to study the transcript level of DNMT1, DNMT3A and DNMT3B.

### RT-PCR of DNMT1, DNMT3A and DNMT3B:

10  $\mu$ g of total RNA was used to perform RT-PCR in a 20  $\mu$ l of reaction buffer containing Superscript II reverse transcript (Life Technologies). At the end of 40 cycles of RT-PCR, the products were electrophoresed on a 2% agarose gel and viewed under transilluminator after stained with ethidium bromide. mRNA transcription level of DNMTs were normalized by expression of  $\beta$  actin gene which gives a 612bp fragment. The density of each bands after RT-PCR were divided by the density of  $\beta$  actin to get the normalized value for DNMTs. The RT-PCR conditions for DNMT1, DNMT 3A, DNMT3B and  $\beta$  actin are given bellow (Robertson 1999).

The quantitative value of expression thus obtained by  $(\text{Intensity of band in DNMT RT-PCR} - \text{background value}) / (\text{Intensity of band in } \beta \text{ actin RT-PCR} - \text{background value})$

**2.7. Statistical Analysis:** Along with Univariate analysis, we have done Multivariate ANOVA to find out the association between genetic polymorphism and arsenic induced skin manifestations, total urinary arsenic, and degree of DNMT expression. GSTO1 and GSTO2 gene polymorphism has been set as independent variables in two different study populations (GSTO1 and GSTO2 study population) and the dependent variables are skin score, total urinary arsenic and degree of DNMT expression in two different polymorphic groups with different genotypes. Therefore two separate MANOVA or Multivariate ANOVA has been performed for two study populations. The difference between AA with AD and DD group means for skin score, total urinary arsenic and DNMT expression have been studied in each of the exposure groups. Similarly, in GSTO2 study population the NN genotype has been set as independent variable. Multivariate analysis has been also done to interpret the intergroup means between different exposure groups (Group I, II, III and IV). Descriptive statistics has been done by SPSS and Roy's Largest Root has been performed to calculate the significance of difference. All the level of significance has been studied at 95% confidence interval. Intercept and Partial eta squared for multivariate analysis has been calculated to find out the strength of the test and reproducibility by which we can determine the limitations of the study and effect size of the sample respectively. Role of age and sex in GSTO1 and GSTO2 polymorphism and DNMT expression has been studied for group I, II and III by multivariate analysis. Raw data have been incorporated directly into the multivariate analysis.

## 3. Results

Demographic distribution and frequency of occurrence of different polymorphic variations has been tabulated in table 1 and 2 respectively. In table 1 hair and nail arsenic data has not incorporated as these data are not directly involved into the study and statistics. Hair and nail arsenic had been measured here as a preliminary biomarker for chronic arsenic exposure and to cross check that whether a particular person assigned here as arsenic exposed is only currently exposed or exposed to arsenic through years (GuhaMazumder 2000). DNMT gene expression has been studied in such exposed participants whose hair and nail arsenic concentration is higher than control unexposed population. Urine arsenic is a biomarker for current arsenic exposure, therefore all the correlation has been studied with urinary arsenic values. We have only included those participants in our exposed study population whose hair and nail arsenic is greater than 1 mg/kg and 0.5 mg/kg respectively. The mean hair and nail arsenic concentration were 1.54 mg/kg and 1.61 mg/kg respectively in our arsenic exposed study population. Whereas, in unexposed subjects the mean arsenic concentration in hair and nail were of 0.13mg/kg and 0.11 mg/kg respectively.

In group I the occurrence of GSTO1 AA genotype is 63%, AD is 30 % and DD is 7 %. In group II, that is arsenic exposed but with out skin manifestation cases, the occurrence of AA is 56%, AD is 32% and DD is 12%. In group III category, i.e, similar level of arsenic exposure with arsenic induced cancer the occurrence of AA is 67%, AD is 23% and DD is 10%. Group IV is arsenic unexposed control, where the frequency of AA is 58%, AD is 30% and DD is 12%. Though the distribution pattern follows Hardy-Weinberg equation in each exposure groups , yet, it shows a significant deviation of AA genotype in arsenic induced cancer (group III) and non cancer patients with high skin manifestation (group I) when compared to normal unexposed person's AA genotype ( $p < 0.05$ ). In case of

GSTO2 N142D polymorphism, it is seen that the frequency distribution NN wild type is 45%, ND is 45% and DD is 10% in group I participants. In group II arsenic induced cases without skin manifestation, the frequency of NN genotype is 48%, ND is 50% and DD is 2%. In group III, the occurrence of NN genotype is 50%, ND is 42% and DD is 08 % only. In control population the frequency distribution of GSTO2 NN is 45%, ND is 45% and DD is 10%, which is similar to group I. In GSTO2 genotyping the frequency distribution and their differences are not statistically significant in between different groups and no significant deviation has been seen from Hardy-Weinberg Equation. The allelic frequency has been calculated for A allele for AA and AD genotype in all four groups. Similarly allele frequency has been calculated for NN and ND genotype in each group of the study population. It is seen that the allele frequency for A allele is high in group I and III in comparison to Group IV and almost in the range of Group IV in case of Group II. Allele frequency for N allele does not show comparable value in Group I and III. Allele frequency of N allele in Group II is 0.73 which is closure to the frequency found in Group IV (0.71) which hinders the interpretation of any association between GSTO2 and arsenic induced skin manifestation.

All the values expressed in table 3 are in mean  $\pm$  SD format. MDA level has been studied for each participant to assess oxidative damage induced by chronic arsenic exposure. It is seen from our study that MDA level is significantly higher in Group I, II and III participants in comparison to group IV participants. In AD and DD genotype the total urinary arsenic is significantly higher ( $p < 0.05$ ) in comparison to AA genotype of the same exposure level which was studied by paired two tail test. Moreover, the MDA concentration in urothelial cell lysate indicates a significantly ( $p < 0.05$ ) higher concentration in AA genotype in comparison to AD and DD genotype of GSTO1 polymorphism within the same exposure group. We have also analyzed the data obtained from our study population by multivariate ANOVA.

The mean values of total urinary arsenic and MDA level score is significantly higher Group I, III and II in comparison to arsenic unexposed group ( $p = 0.02$ , at 95% CI). The skin manifestation has been studied by multivariate ANOVA taking Group I, III and IV and shows significant difference ( $p = 0.04$ , 95% CI). Within GSTO1 study population in comparison to GSTO1 wild homozygous genotype, AA, AD or DD mutant heterozygous and homozygous genotype shows significantly decreased level ( $p = 0.043$ ) of total urinary arsenic within each exposure group. Univariate analysis was performed to study the total urinary arsenic in different genotypic groups within a particular exposure group which also showed significant decreased level in AA in comparison to AD genotype ( $p < 0.05$ ). Multivariate analysis shows increased level of MDA, Skin score and DNMT1 expression in AA genotype ( $p = 0.03$ , at 95% CI, table 3) in comparison to AD genotype in group I. Frequency of DD genotype in all four groups is very low, which may limit the study during statistical analysis. The groups where frequency of DD is less than 4, there statistical analysis has been performed without including DD group in both GSTO1 and GSTO2 polymorphic study population. Although a difference in total urinary arsenic and MDA has been notified in NN genotype in comparison to ND genotype of arsenic induced cancer patient and non cancer participant with and without skin manifestations (in Group I and II), it is not significant ( $p = 0.054$ , 95% CI). However, consistently low total urinary arsenic has been seen in wild type variations (AA and NN genotype) in both GSTO1 and GSTO2 study population in comparison to AD, DD and ND, DD genotype. The Skin manifestation has been studied by multivariate ANOVA taking Group I, III and IV and showed significant difference ( $p = 0.047$ , at 95% CI). In case of skin manifestation number of positive sign assigned in each genotypic group is multiplied by 1 to get the numerical of skin score in each genotype. In Group I and III the skin scores are significantly higher in comparison to Group I when studied by MANOVA ( $p = 0.025$ ,  $p = 0.036$ , at 95% CI respectively) in both GSTO1 and GSTO2 study population. .

We have analyzed the level of expression of DNMT1, DNMT3A and DNMT3B through RT-PCR analysis. The overall expression of all the DNMTs increases in all exposure groups in comparison to control group. The rate of expression further varies within a particular exposure group according to their GSTO1 and GSTO2 polymorphic status. Expression of  $\beta$  actin gene is used here as an internal control. Degree of expression of DNMTs in each of the GSTO1 and GSTO2 polymorphic groups has been evaluated by Bio-Rad GelDoc densitometry scanning. No significant difference has been noted with respect to skin score in these three genotypes of Group I. The expression of different DNMT gene has been studied, it is seen that in comparison to unexposed group the DNMTs expression is 2 to 3 fold high in arsenic exposed group with skin lesions positive and in arsenic induced cancer group. Though the expression of DNMTs is also high in without skin manifestation group it is lies within a 2 fold increase. DNMT1 expression is considerably high in people with AA genotype of Group I in comparison to AD genotype. The expression of DNMT3A and 3B in Group I has increased upto 4-5 folds and 3 folds respectively in AA genotype in comparison to control group AA genotype. In arsenic induced cancer group (Group III) the DNMT3A expression is as high as 5-6 folds in comparison to control group (Group IV) and maximum expression is seen in AA genotype. DNMT3B expression is as high as 6 fold in arsenic induced cancer group (Group III) and also the maximum rate of expression is seen in AA genotype. Although with in an exposure group the expression of DNMTs are higher in AA genotype in comparison to AD or DD genotype, but the difference is not significant. Further, although, the people of Group I and II both are arsenic exposed at similar level, the DNMT1, DNMT3A and DNMT3B expressions are comparatively low in group II and further lower in AD and DD genotype, though not at a significant level. The comparative expression of DNMT1, 3A and 3B revealed that rate of expression is lower in DNMT1 in comparison to both of DNMT3A and 3B in all exposed groups. 5 to 7 fold increase in DNMT3A and 3B and 3 fold increase in DNMT1 in arsenic induced cancer group in as indicative of hypermethylation in chronic arsenic exposure and development of arsenic induced cancer. Also in this group the rate of expression are lower in AD and DD genotype in comparison to AA genotype of the same exposure group. Multivariate analysis by Roy's Largest Root revealed that expression of DNMTs are significantly ( $p = 0.038$ , at 95% CI) associated with GSTO1 genotypic variation in GSTO1 study population. The intercept is 0.007 and partial eta squared is 0.806.

DNMTs expressions also vary in different exposure groups according to their GSTO2 genotypic variations. The rate of expression in each exposed group (Group I, II and III) is highest in NN polymorphic genotype in comparison to ND and DD genotype. In comparison to control group NN genotype, group I, II and III showed 3-4, 3-5 and 2-3 folds increase respectively in DNMT1, 3A and 3B gene expression. Though, in GSTO2 study population the DNMT expression shows increased expression in NN genotype in a more stratified fashion the difference is not significant. Roy's Largest Root analysis in GSTO2 study population for DNMT gene expression fails to showed any significant association ( $p = 0.2$ , intercept 0.006, and partial eta squared is 0.932). DNMT expression is not significant ( $p = 0.14$ , at 95% CI; intercept 0.000 and partial eta squared 0.598) when only it is analyzed against GSTO2 polymorphism in GSTO2 study population by MANOVA. From the intercept and partial eta squared we can say that the size of the study population is the limitation for this study. However, we can say that study with a larger population may find significant positive association in relation to DNMT expression with GSTO2 gene polymorphism as we know that value of partial eta squared greater than 0.6 indicate a good effect size. The result of multivariate analysis for evaluation of the role of age and

sex in GSTO1 and GSTO2 polymorphism and DNMT expression in different exposure groups stated that no significant difference exist between in relation to age and sex.

## 4. Discussion

GST enzymes are highly polymorphic. Some of these polymorphisms affect enzyme expression and/or their activity. Functional alteration due to genetic polymorphisms of these enzymes thought to be linked with genotoxic effects as well as overall clinical manifestations of xenobiotics (Djukic et al. 2013, Chung et al.2011, Whitbread et al. 2005) The GST omega class belongs to the GST enzyme super family which has a cysteine amino acid in its active site. Two actively transcribed GST genes (GSTO1 and GSTO2) are located on the long arm of chromosome 10. Both of the GSTO1 and GSTO2 are polymorphically distributed among in our population. GSTO1, GSTO2 and Arsenic methyl transferase (AsMT or Cyt 19) are involved in arsenic methylation in variety of animals including human. The GSTOs can catalyze the reduction of MMA(V) to MMA(III), which is the rate limiting step for arsenic biotransformation in human (Hsu et al 2011). In addition to MMA(V) / DMA(V) reductase activity GSTOs also exhibit high thioltransferase activity as well as dehydroascorbate reductase activity (Kim et al 2017). The enzyme therefore can participate in intracellular thiolhomeostatic reactions and ascorbate recycling (Kim et al 2017).

Result of one Taiwanese study revealed that GSTO2 wild type (DD) is associated with excretion of increased inorganic arsenic, and, a study conducted on Mexican Population showed that GSTO1 homovariant (DD) is associated with increased inorganic arsenic either as AsIII or As V (Hsu et al 2011)in urine. Researchers showed that GSTO1 and GSTO2 are actively involved in disease activity of certain types of cancers. In human papilloma virus induced cervical cancer, it has been tested that GSTO1 wild type is responsible for poor prognosis and the frequency distribution of GSTO1 wild type (AA) is significantly greater in cervical cancer (Zamani et al. 2018).While in the same study carried out on Iranian population it has been reflected that GSTO2 wild type (NN) is not significantly involved in the disease activity or prognosis (Zamani et al.2018). In a separate Taiwanese study conducted over 764 subjects, role of GSTM1, GSTT1, GSTO1 and GSTO2 on the occurrence of arsenic induced urothelial carcinoma was analyzed (Hsu et al 2011). The result revealed that there was no correlation between GSTO2 or GSTO1 polymorphism and occurrence of non arsenic exposed urothelial carcinoma. However, when considered with cumulative arsenic exposure and arsenic exposed urothelial carcinoma, a significant correlation was observed between the polymorphism and urothelial cancer. The frequency distribution of GSTO1 A140D and GSTO2 N142D play pivotal role in clearance of arsenic expressed here as total urinary arsenic and subsequent disease progression in our study population. The distribution of A allele in GSTO1 polymorphism is different in our population from Chinese, Japanese, Thai or Mongolian population. Further, dissimilar distribution has been noted in German, Australian or American population in respect to GSTO1 A allele (Ada et al 2013, Shaban et al 2016). Similarly, differential distribution of GSTO2 N allele has also been noted in different ethnic groups through worldwide (Shaban et al 2016). Even, within a country interprovincial and interracial difference in frequency distribution had been reported (Fu et al. 2008) which can support differential distribution of A, N and D in our world. Gene environment interaction and GST polymorphism is associated with increased risk of several types of cancer and also determined as the prognostic marker in hepatocellular carcinoma (Qu et al. 2015, Tian et al 2020). DNMT gene polymorphism may also contribute the disease progression by epigenetic alteration (Saradalekshmi et al 2014). DNMT genes polymorphism may down regulate or up regulate the expression of different DNMT genes and may thereby affect the progression of disease by modifying the epigenetic events like DNA methylation. Further, incomplete arsenic metabolism and increased body retention of inorganic arsenic may contribute to perturb expression profile of genes involved in epigenetic regulation. Therefore, decreased total urinary arsenic may act as a contributing factor for altered DNMT expression, the key enzyme for DNA methylation. Till now, we have no reports about the expression profile of DNMTs which is shown to be modified by GST polymorphism status in arsenic induced cancer and arsenic exposed population.

Our study also shows significant association between arsenic exposure and urothelial cancer in homozygous wild variant of GSTO1 (AA) and also in homozygous wild variant of GSTO2 (NN) genotype with significant increased rate of DNMT expression, although one of the previous study showed that NN genotype of GSTO2 is not associated with an increased cancer risk in arsenic exposed persons in Taiwan and the risk is largely confined to high exposure (Hsu et al 2011). May be this variation in genotypic association with arsenic induced cancer is due to ethnicity and racial distribution of alleles in different geographical areas. We can see a low level of total urinary arsenic and arsenic induced skin manifestations. Both of these polymorphic varieties also show higher degree of MDA, and higher degree of DNMTs expressions in comparison to mutant varieties of same exposure groups. GSTO1 homozygous mutant variant (DD) and mutant heterozygous variant (AD) have significantly higher percentage of total urinary arsenic, reflecting, a good prognostic value of those genotype in chronic arsenic exposure. Lower incidence of arsenic induced urothelial cancer is also inversely correlated with higher total urinary arsenic and directly proportional with lower DNMTs expressions in those genotypes among our study subjects.

In two separate studies it was further demonstrated that GSTO2 N142D homozygous mutant genotype was associated with higher percentage of urinary inorganic arsenic (Chung et al. 2009). Studies displayed that GSTO1 E155 del (ND) was associated markedly altered degree of inorganic arsenic in urine when compared to wild homotype (NN) (Marnell et al. 2003, Agusa et al.2010). Surprisingly, the association of GSTO1 and GSTO2 in arsenic metabolism and different arsenic induced cancer shows inconsistent results due to small sample size, ethnicity, and differential nutritional status.

Despite its conclusive results regarding association of GSTOs polymorphism and arsenic induced urothelial carcinoma our present study has several limitations. Firstly, the small sample size with a limited number of urothelial cancer cases. Based on such a small number of participant we have got a very few number of cases in each of the genotypic variation under GSTO1 and GSTO2 polymorphism. A second limitation is that we do not study the urinary arsenic speciation by which we can specifically point out the role of GSTO1 and GSTO2 in arsenic metabolism. Lack of speciation of arsenic from urinary arsenic limits the power of the work to demonstrate the role of GSTOs in arsenic metabolism and potentiating the arsenic induced urothelial cancer. Finally, the estimate of exposure status from food source (24 hours recall process for food items and their amount) decreases the power of actual estimate of the type and amount of foods taken by each participant. Taking together these factors, the statistical analysis on which the data was estimated for study of significance and study of correlation, the interaction between genes, environment and disease become underestimated.

In conclusion, our present findings have shown an association of the wild type homozygous variant of the GSTO1 with arsenic induced skin manifestations, MDA value, DNMT expression in chronic arsenic exposed population with and without urothelial cancer. Such findings also had come from our GSTO2 study population though we do not get significant association in relation to DNMT expression or skin score and total urinary arsenic in it. Apparently, wild type GSTO1 and GSTO2 (AA and NN) constitutes a fairly high risk group of skin manifestations and future cancer. Occurrence of GSTO1 AA wild type homozygous variety is high in Group II and III which constitute a high risk group of arsenicosis and arsenic induced urothelial cancer in that genotypic group. However, the data does not show any correlation of polymorphism with the age, sex and occupational status of the exposed population. In our future studies we will look into the combined etiological roles of smoking, nutritional and dietary status vis-à-vis the methylation profile of arsenic within arsenic exposed population to identify other risk factors.

## Declarations

**Competing interest** Authors declare that they have no competing interests.

**Author's Contribution** SC standardized and performed wet lab experiments, analyzed the data and wrote the manuscript. TC designed the experiments, and furnished the manuscript, DNGM guided clinical assessments and patients selection. JR performed wet lab experiments and kept the records of data. AM has done the statistical analysis.

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## Tables

**Table 1: Demographic data of the study population. All the data expressed as mean±SD.**

Group	Age (yrs)	Sex	Avg Ht/Wt	Smoking habit	Occupation	Avg duration of exposure	Avg food arsenic $\mu\text{g/Kg}$	Avg urine arsenic $\mu\text{g/L}$	Avg water arsenic $\mu\text{g/L}$	Hair & nail arsenic mg/Kg
Gr I N= 32 With Skin lesion	i).20-35 N= 6 36-50, N=18 51-65, N= 8	i).M=4, F=2 ii). M=14, F=4 iii).M= 6 F=2	M= Ht =167 cm, Wt=67 Kg F= 152 cm, Wt= 54 kg	M= all smokers F= all non smokers	M=20 farmers, 5 small traders, 1 service holder F=house wives	10 $\pm$ 2.3 years	237 $\pm$ 22.34	134 $\pm$ 21.23	117.4 $\pm$ 17.42	1.22 $\pm$ .34(hair) 1.27 $\pm$ .58 (nail)
Gr II N=32 With out skin lesion	i). 20-35 N=20 ii). 36-50 N= 15 iii).51-65 N= 10	i).M=7, F=5 ii).M=6 F=4 iii).M=80, F=2	M=Ht= 64cm Wt= 65 kg F=Ht= 152 cm, Wt=52 kg	M= all smoker F= non smoker	M= 20 farmers, 5 small traders, 1 service holder F=house wives (N=6)	10 $\pm$ 3.4 years	188 $\pm$ 19.77	119 $\pm$ 11.14	97.8 $\pm$ 10.32	1.34 $\pm$ .41(hair) 1.44 $\pm$ .26(nail)
Gr III As Cancer N=15	i).20-35 N=01 ii). 36-50 N= 11 iii).51-65 N=04	i).M=1, F=0 ii).M=8 F=3 iii).M=3, F=1	M=Ht= 159cm Wt= 63 kg F=Ht= 150 cm, Wt=51 kg	M= all smoker F= non smoker	M=all Farmer F= house wives	6 $\pm$ 1.5 years	329 $\pm$ 17.88	87 $\pm$ 11.44	129 $\pm$ 19.56	2.06 $\pm$ .53 (hair) 2.14 $\pm$ .62 (nail)
Gr IV N=33 control	i). 20-35 N=11 ii). 36-50 N= 15 iii). 51-65 N= 07	i).M= 7, F= 4 ii).M= 10, F=5 iii).M=6, F=1	M= Ht=159cmWt= 63kg F= Ht= 150 cm, Wt=51 kg	M= Smokers F=non smoker	16 farmers, rest M are small traders F=housewives	7 $\pm$ 1.5 yrs	8.9 $\pm$ 0.88	9 $\pm$ 0.33	6.6 $\pm$ 1.2	0.13 $\pm$ .05(hair) 0.11 $\pm$ .03(nail)

Table 2: Frequency distribution of different genotypic subgroups in GSTO1 and GSTO2 polymorphism study population.

Group Name	GST O1 A140D (frequency of occurrence)	GST O2 N142D (% of occurrence)	P value	Allele frequency for A&N allele
<b>Group I N=32</b> With skin manifestation	AA 63% (n= 20) AD 30% (n=10) DD 07% (n= 02)	NN 45% (n= 14) ND 45% (n= 14) DD 10% (n= 05)	AA Gr IV vs AA Gr I. P<0.05	A=0.78 N=0.67
<b>Group II N=32</b> Without skin manifestation	AA 56% (n= 18) AD 32% (n= 10) DD 12% (n=04)	NN 48% (n= 15) ND 50% (n=16) DD 02% (n=02)	P<0.05	A=0.72 N=0.73
<b>Group III N=15</b> Arsenic induced cancer.	AA 67% (n= 11) AD 23% (n= 03) DD 10% (n= 01)	NN 50% (n=08) ND 42% (n= 06) DD 08% (n= 01)	AA Gr IV vs AA GrIII. P<0.05	A=0.78 N=0.71
<b>Group IV N=33</b> Arsenic unexposed control.	AA 58% (n= 19) AD 30% (n= 10) DD 12% (n= 4)	NN 45% (n= 15) ND 45% (n= 15) DD 10% (n=03)		A=0.73 N=0.675

Table 3: Comparative chart for Mean value of T.U.As, Skinscore and MDA Level, DNMTs level (DNMT expression score is obtained by dividing the score obtained from  $\beta$  actin expression) in each GSTO1 and GSTO2 polymorphic groups

GSTO1 Polymorphism						
Group	T.U.As $\mu\text{g/L}$	Skin score	MDA $\mu\text{mol/L}$	DNMT 1	DNMT 3A	DNMT3B
Group I						
AA	82.41 $\pm$ 6.76	++++	23.76 $\pm$ 2.68	0.194 $\pm$ 0.013	0.19 $\pm$ 0.014	0.16 $\pm$ 0.0318
AD	144.88 $\pm$ 9.21	+++	17.43 $\pm$ 3.0	0.134 $\pm$ 0.008	0.157 $\pm$ 0.007	0.14 $\pm$ 0.0056
DD	Not done	Not done		Not done	Not done	
Group II AA						
AD	83.72 $\pm$ 8.42	----	19.56 $\pm$ 2.97	0.132 $\pm$ 0.011	0.112 $\pm$ 0.004	0.121 $\pm$ 0.003
DD	128.43 $\pm$ 9.88	-----	15.23 $\pm$ 3.86	0.113 $\pm$ 0.026	0.077 $\pm$ 0.006	0.079 $\pm$ 0.002
	149.74 $\pm$ 10.3	-----	10.97 $\pm$ 2.32	0.110 $\pm$ 0.023	0.072 $\pm$ 0.003	0.073 $\pm$ 0.004
Group III						
AA	84.74 $\pm$ 7.34	+++++++	28.08 $\pm$ 4.35	0.212 $\pm$ 0.031	0.211 $\pm$ 0.003	0.314 $\pm$ 0.004
AD	113.23 $\pm$ 10.7	++++	18.77 $\pm$ 2.42	0.107 $\pm$ 0.024	0.162 $\pm$ 0.004	0.218 $\pm$ 0.001
DD	137.29 $\pm$ 11.5	++++	17.71	0.183	0.133	0.197
Group IV						
AA	3.8 $\pm$ 1.01	-----	11.41 $\pm$ 4.67	0.081 $\pm$ 0.0071	0.044 $\pm$ 0.005	0.042 $\pm$ 0.0036
AD	3.1 $\pm$ 0.94	-----	10.53 $\pm$ 3.23	0.055 $\pm$ 0.0067	0.032 $\pm$ 0.002	0.061 $\pm$ 0.0041
DD	1.4 $\pm$ 0.32	-----	9.37 $\pm$ 1.22	0.053 $\pm$ 0.0023	0.042 $\pm$ 0.006	0.028 $\pm$ 0.0049
GSTO2 Polymorphism						
Group	T.U.As $\mu\text{g/L}$	Skin score	MDA $\mu\text{mol/L}$	DNMT 1	DNMT 3A	DNMT3B
Group I						
NN	93 $\pm$ 6.47	+++	21.67 $\pm$ 4.19	0.182 $\pm$ 0.002	0.174 $\pm$ .032	0.143 $\pm$ 0.024
ND	127 $\pm$ 8.43	+++	18.47 $\pm$ 2.79	0.123 $\pm$ 0.004	0.153 $\pm$ 0.03	0.147 $\pm$ 0.049
DD	139 $\pm$ 8.96	+++	11.92 $\pm$ 2.44	0.102 $\pm$ 0.003	0.121 $\pm$ 0.02	0.091 $\pm$ 0.008
Group II						
NN	91 $\pm$ 9.61	-----	17.39 $\pm$ 3.56	0.156 $\pm$ 0.003	0.16 $\pm$ 0.004	0.106 $\pm$ 0.04
ND	115 $\pm$ 8.74	--	14.56 $\pm$ 2.77	0.138 $\pm$ 0.003	0.099 $\pm$ 0.00	0.121 $\pm$ 0.05
DD	114	--	11.74	0.155	0.104	0.164
			--			
Group III						
NN	81.8 $\pm$	+++++++	22.01 $\pm$ 3.54	0.182 $\pm$ 0.041	0.176 $\pm$ 0.00	0.182 $\pm$ 0.027
ND	127.1 $\pm$	+++++	17.78 $\pm$ 3.81	0.186 $\pm$ 0.055	0.107 $\pm$ 0.00	0.177 $\pm$ 0.031
DD	143.4	+++++++	13.11	0.21	0.124	0.113
Group IV						
NN	2.41 $\pm$ 0.089	-----	8.87 $\pm$ 1.47	0.041 $\pm$ 0.005	0.059 $\pm$ 0.006	0.054 $\pm$ 0.0073
ND	3.01 $\pm$ 0.074	-----	7.91 $\pm$ 2.12	0.042 $\pm$ 0.003	0.068 $\pm$ 0.005	0.043 $\pm$ 0.0071
DD	1.07 $\pm$ 0.085	-----	9.24	0.063	0.066	0.039

Footnote: T.U.As= total urinary arsenic, MDA=Malondialdehyde, SD= Standard Deviation, CI= confidence interval,

## Figures

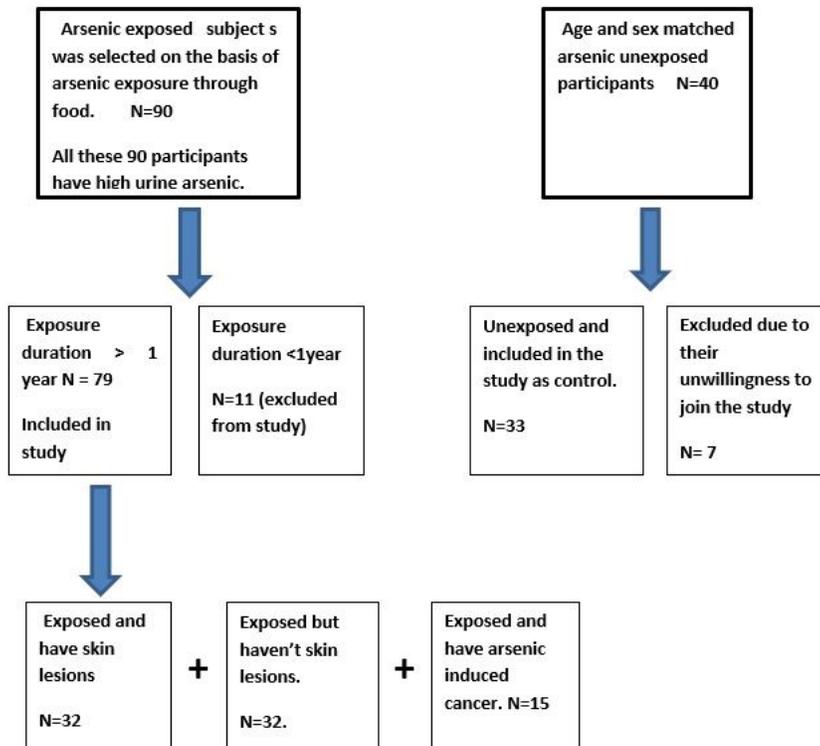


Figure 1

Flow Chart showing selection of exposed and unexposed subjects.