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Association of *AS3MT* polymorphisms and the risk of premalignant arsenic skin lesions

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Abstract

Exposure to naturally occurring inorganic arsenic (iAs), primarily from contaminated drinking water, is considered one of the top environmental health threats worldwide. Arsenic (+3 oxidation state) methyltransferase (AS3MT) is the key enzyme in the biotransformation pathway of iAs. AS3MT catalyzes the transfer of a methyl group from S-adenosyl-L-methionine to trivalent arsenicals, resulting in the production of methylated (MAs) and dimethylated arsenicals (DMAs). MAs is a susceptibility factor for iAs-induced toxicity. In this study, we evaluated the association of the polymorphism in *AS3MT* gene with iAs metabolism and with the presence of arsenic (As) premalignant skin lesions. This is a case-control study of 71 cases with skin lesions and 51 controls without skin lesions recruited from a iAs endemic area in Mexico. We measured urinary As metabolites, differentiating the trivalent and pentavalent arsenical species, using the hydride generation atomic absorption spectrometry. In addition, the study subjects were genotyped to analyze three single nucleotide polymorphisms (SNPs), A-477G, T14458C (nonsynonymous SNP; Met287Thr), and T35587C, in the *AS3MT* gene. We compared the frequencies of the *AS3MT* alleles, genotypes, and haplotypes in individuals with and without skin lesions. Marginal differences in the frequencies of the Met287Thr genotype were identified between individuals with and without premalignant skin lesions ($p=0.055$): individuals carrying the C (TC+CC) allele (Thr) were at risk [odds ratio=4.28; 95% confidence interval (1.0–18.5)]. Also, individuals with C allele of Met287Thr displayed greater percentage of MAs in urine and decrease in the percentage of DMAs. These findings indicate that Met287Thr influences the susceptibility to premalignant As skin lesions and might be at increased risk for other adverse health effects of iAs exposure.

Keywords

Arsenic-skin lesion; *AS3MT* polymorphism; Arsenic metabolism

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Introduction

Human exposure to inorganic arsenic (iAs) occurs through its use in industry and agriculture; however, the natural occurrence of iAs in drinking water is a serious concern worldwide, particularly in low-income countries (IARC, 2004). In Mexico, several provinces have been reported to have drinking water containing arsenic (As) at concentrations higher than 10 µg/l, which is the maximum guideline value recommended by the World Health Organization (WHO, 2003).

The urine of people exposed to iAs contains trivalent and pentavalent iAs metabolites: monomethyl-As (MAs) and dimethyl-As (DMAs) (Valenzuela et al., 2005; Thomas et al., 2007). Before being excreted in the urine, iAs undergoes a series of reductions of the pentavalent species to the trivalent species followed by oxidative methylation to yield pentavalent methylated species. The methylation of iAs is catalyzed by arsenic (+3 oxidation state) methyltransferase (AS3MT), with *S*-adenosyl-L-methionine (SAM) serving as the donor of a methyl group (Waters et al., 2004; Thomas et al., 2007). In humans, biomethylation of iAs generally stops at the production of pentavalent and trivalent dimethyl arsenical (DMAs^V and DMAs^{III}). In addition, a sulfur-containing derivative of DMAs^V, dimethylthioarsinic acid (DMTA), has recently been found in urine of residents in arseniasis-endemic areas of Bangladesh (Raml et al. 2007). However, in some individuals with low exposures to iAs, the biomethylation process may proceed further to form trimethylarsenicals, mainly trimethylarsine oxide (TMAO) (Thomas, 2007). There is a large variation in the metabolism of iAs between species, human populations, and individuals. Most experimental animals methylate As efficiently to DMAs with essentially no MAs excretion and a faster overall excretion of As than humans (Vahter, 2002). Efforts have been made to characterize the toxic and carcinogenic effects of arsenicals and their metabolites. It has been demonstrated that individuals with greater proportions of MAs in urine, which are apparently due to a higher production of MAs in their tissues (Vahter, 1999), have a higher risk of adverse health effects, such as skin lesions and bladder cancer (Del Razo et al., 1997; Yu et al., 2000; Valenzuela et al., 2005; Steinmaus et al., 2006). Moreover, we previously reported that the urinary MAs in urine of these individuals are mainly in the trivalent oxidation state (Valenzuela et al., 2005). Human and rodent studies have indicated that chronic exposures to iAs are linked to several adverse health effects, including different forms of cancer (Chen et al., 2003; Pu et al., 2007; Liu and Waalkes, 2008). Based on these studies, iAs and its metabolites may act as complete carcinogens, co-carcinogens, or tumor promoters/progessors (Kitchin, 2001; Rossman, 2003). Also, As may trigger vascular disease, immunodepressed status, neurological effects, or diabetes mellitus (NRC, 2001; IARC, 2004).

Epidemiologic studies have linked chronic exposure to iAs to increased risks of premalignant skin lesions, such as hyperkeratosis on the palms and soles or hyperpigmentation combined with small areas of hypopigmentation on the neck and back (NRC, 2001; Centeno et al., 2002). These skin lesions have been used as a bio-marker of chronic exposure to iAs (Chen et al., 2006). However, the occurrence of these skin lesions varies among exposed individuals, suggesting that the susceptibility is determined in part by genetic differences (Vahter, 2000).

The *AS3MT* gene, which is located in the chromosome 10q24.32 region and is comprised of 10 introns and 11 exons, has many polymorphic sites (Lin et al., 2002; Meza et al., 2005; Wood et al., 2006). The -477 (A→G) transition variant may be implicated in iAs metabolism because it is located 477 base pairs upstream from exon 1 in the promoter region (Wood et al., 2006). This polymorphism may be associated with the expression of the *AS3MT* gene, and in its turn, the amount of enzyme produced. A second relatively common

polymorphism is 35587 (T→C), which is located in intron 10 and results in a decreased relative proportion of MAs in the urine. This polymorphism is expected to be associated with a lower risk of adverse health effects following As exposure (Meza et al., 2007). The exonic Met287Thr (T→C) polymorphism is associated with high AS3MT activity and increased production of MAs in *in vitro* studies (Drobná et al., 2004; Wood et al., 2006). Furthermore, epidemiological studies of a European population (Lindberg et al., 2007) and a Chilean group (Hernandez et al., 2008) have shown that individuals carrying the C allele (Thr287) excrete a higher percentage of MAs in their urine.

To date, little is known about the association of the AS3MT polymorphism and the adverse health effects caused by chronic iAs exposure. Clearly, more data is needed to better understand the role of AS3MT polymorphism as a susceptibility marker in human populations. In the present study, we hypothesized that common AS3MT polymorphisms affect As biomethylation, mainly the production of trivalent As metabolites. Additionally, we investigated the associations between AS3MT polymorphism and As-induced premalignant skin lesions.

Materials and methods

Chemicals and reagents

Arsenic acid, disodium salt, ($\text{Na}_2\text{HAS}^{\text{V}}\text{O}_4$; > 99% pure), sodium *m*-arsenite ($\text{NaAs}^{\text{III}}\text{O}_2$; > 99% pure), and dimethylarsinic acid [DMAs^{V} ; $(\text{CH}_3)_2\text{As}^{\text{V}}\text{O}(\text{OH})$; 98% pure] were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Methylarsonic acid (MAS^{V}), disodium salt, [$\text{CH}_3\text{As}^{\text{V}}\text{O}(\text{ONa})_2$; 99% pure] was obtained from Ventron (Danvers, MA). The trivalent methylated arsenicals, methyloxoarsine ($\text{MAS}^{\text{III}}\text{O}$; $\text{CH}_3\text{As}^{\text{III}}\text{O}$) and iododimethylarsine of DMAs^{III} [DMAs^{III} ; $(\text{CH}_3)_2\text{As}^{\text{III}}$], were synthesized by W. R. Cullen (University of British Columbia, Vancouver, Canada) using previously described methods (Stýblo et al., 1997). Working standards of these arsenicals, which contained 1 μg of As/ml, were prepared daily from stock solutions. Sodium borohydride (NaBH_4) was obtained from EM Science (Gibbstown, NJ, USA). Tris hydrochloride was purchased from J. T. Baker (Phillipsburg, NJ, USA). Creatinine kits were purchased from Randox (San Diego, CA, USA). All other chemicals used were at least analytical grade. Standard reference material (SRM) water (SRM 1643e) and urine [SRM 2670; National Institute of Standards and Technology (NIST), Gaithersburg, MD] were used for quality control of total arsenic (TAs) measurements in water and for urine analyses, respectively.

Selection of study participants

In a cross sectional, case-control study was carried out in a total of 122 residents of Zimapan, Hidalgo (Mexico), all of Hispanic origin, were included. They were recruited for a baseline visit, where one hundred and forty people were invited, 15 refused to participate (10.7%) and 3 were excluded because they did not attend the appointment for urine collection. In Zimapan-endemic region, long-term iAs exposure has been recorded since 1992. The concentrations of iAs in wells and the potable supply was as high as to 1100 μg As/l. In 1999 the most contaminated well was closed by the local authorities, and the mean of iAs concentration in water decreased significantly; however, the iAs concentration in this region is still significantly higher than the recommended value 10 μg As/l for drinking water, with mean values of 110 μg iAs/l (Valenzuela et al., 2007). Subjects were recruited through door-to-door contact. They had to be at least 15 years old and had to be living in the town for the previous 2 years. Participants were recruited between January and March 2006. The ethnicities were determined through questionnaire and required both parents and all grandparents to be of the same ethnicity as the subject. Water, urine, and buccal cells were collected. Additionally, the As-induced skin lesion status was evaluated, quantified, and

validated by our study physicians and expert dermatologists. As-related skin lesions are known to be a hallmark of chronic iAs poisoning. These lesions include alterations in pigmentation and discoloration of skin, and in many cases, these symptoms are accompanied by keratosis, the thickening of the skin of the palms, soles and trunk (Yeh, 1973). The clinical examination protocols used to assess skin lesions were previously described (Valenzuela et al., 2005). The study physicians were not aware of the level of As in the participants' drinking water supplies. They identified 71 individuals with As skin lesions (case) and 51 without As skin lesions (control). The final decision for eligibility of participant in this study was based on to have one group of individuals (approximately 50%) presenting at least one skin sign of arseniasis, such as hypo/hyperpigmentation, palmoplantar hyperkeratosis and ulcerative lesions as described by Yeh (1973). The protocols used in this study were approved by the Institutional Committee at Cinvestav-IPN, and written informed consent was obtained from all participants in the study.

Sample collection, storage, and processing

Water sample collection and As assay—Water samples were collected from the wells used by the study participants in 15-ml acid-washed tubes and stored at -20°C until they were assayed. A detailed description of the water-collection procedure has been reported previously (Valenzuela et al., 2007). Total As (TAs) concentrations were measured as previously described (Del Razo et al., 1990) by hydride generation-atomic absorption spectrometry (HG-AAS) using a Perkin Elmer 3100 spectrophotometer (Perkin Elmer, Norwalk, CT, USA) equipped with a FIAS-200 flow injection atomic spectroscopy system. SRM 1643e was used for quality control during the analyses of TAs in water. The certified TAs concentration in SRM 1643e is $60.45 \pm 0.72 \mu\text{g/l}$. The value determined in our laboratory, $61.3 \pm 0.68 \mu\text{g/l}$ ($n=3$) is in good agreement with the certified value.

Urine sample collection and As speciation analysis—Spot urine samples were collected in 100 ml acid-washed bottles. A detailed description of the urine collection procedure has been reported elsewhere (Valenzuela et al., 2007). The collected urines were aliquoted to 15 ml plastic tubes and shipped on dry ice to our analytical laboratory at Cinvestav-IPN, Mexico City. The trivalent As species were analyzed within 6 h of collection.

All urinary metabolites of As (iAs^{V} , iAs^{III} , MAs^{V} , MAs^{III} , DMAs^{V} , and DMAs^{III}) were measured by a pH-specific HG-AAS, using cryotrapping (CT) for preconcentration and separation of arsines (Del Razo et al. 2001; Devesa et al., 2004). The TAs value was calculated as the sum of the iAs^{V} , iAs^{III} , MAs^{V} , MAs^{III} , DMAs^{V} , and DMAs^{III} . We used the SMR 2670 with the certified As content of $480 \pm 100 \mu\text{g/l}$ to validate the calculated TAs in urine samples with high As concentrations. The low-As urine SRM 2670 with a reference value of $60 \mu\text{g/l}$ was used to validate analyses of urines with low levels of As. Triplicate measurements of the high and low SMR standards using pH-specific HG-AAS provided the values of $507 \pm 17 \mu\text{g/l}$ and $64 \pm 5 \mu\text{g/l}$, respectively.

Because trivalent methylated arsenicals rapidly oxidize, only 10 to 12 urine samples were analyzed each time. Quality assurance included the measurement recovery of fresh prepared methylated trivalent standard (10 ng for MAs^{III} and 20 ng of DMAs^{III}), analyzed in duplicate for each analytical batch. Recoveries ranged from 88 to 101% with a variation coefficient between 0.5 and 13%. The As species were measured from spot urine samples and were thus dependent on urine dilution. To correct for differences in urine dilution, the urinary As species were adjusted for urinary creatinine. This parameter was measured by the Jaffe reaction using a Randox commercial kit.

Buccal cell collection and DNA extraction—Buccal cells were collected with a cytobrush. All participants were instructed to brush their teeth and refrain from eating and drinking for at least 1 h before sample collection. Subjects had each cheek brushed for at least 30 s. Each brush was then placed in a 15 ml sterile plastic tube. Genomic DNA was isolated from the buccal cell samples using a commercial DNA purification kit according to the manufacturer's protocol (Amersham GFX Genomic blood, Amersham Pharmacia Biotech Inc., Uppsala, Sweden). The DNA concentration and purity were evaluated by the ratio of absorbances at 260 and 280 nm (range obtained was 1.6 to 1.8). DNA quality was assessed after separation by the ethidium bromide agarose-gel electrophoresis.

TaqMan SNP assays—We have genotyped three single nucleotide polymorphic sites (SNPs; A-477G, Met287Thr and T35587C) in the *AS3MT* gene. Quantitative real-time PCR assays (rtPCR) were performed with gene-specific fluorescent labeled probes in a PCR ABI Prism 7000 Sequence Detector using TaqMan Universal PCR Master Mix (Applied Biosystems; Foster City, CA, USA). The probes were labeled with 6-carboxyfluorescein and VIC as the 5'-fluorescent reporter. Nonfluorescent quenchers were designed at the 3'-end of the probes using Primer Express software (Applied Biosystems) and are listed in Table 1. The TaqMan genotyping assay was performed according to the manufacturer's protocol in a total volume of 20 μ l per single tube reaction. In each assay, DNase-free water was used as nontemplate control and DNA with a known *AS3MT* genotypes were used as a positive control. Assay conditions were 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The SNP assay was set up using the Sequence Detection System (SDS), version 2.1 (Applied Biosystems) as an absolute quantification assay, and after the assay was completed, the plate was read using allelic discrimination settings.

Indicators of As exposure and metabolism

Cumulative As exposure—Each study subject was interviewed to assess individual exposures to As in drinking water. Information about the current and past sources and daily amounts of drinking water was recorder. If the previously used water well was within the study area and the As concentration was known, the As concentration of the previously used well was incorporated into the calculation of the cumulative As exposure or time-weighted exposure (TWE). TWE was calculated for each participant as sum of the products of the amount of water consumed per day (l/day), As concentration in water in each well (mg/l), and time during which water from each well was used (days) (Valenzuela et al., 2005). The TWE is a good indicator of long-term As exposure (Del Razo et al., 1997).

Indicators of As metabolism—The percentages of iAs, MAs, and DMAs in urine were calculated. An alternative method for describing As metabolism employed the primary (MAs/iAs) and secondary (DMAs/MAs) methylation ratios. Moreover, we calculated the percentages of iAs, MAs, and DMAs in trivalent and pentavalent oxidation states.

Statistical analysis—The Hardy–Weinberg equilibrium was evaluated for each SNPs using data from all participants within each group using a Bonferroni-corrected threshold (Duggal et al., 2008). No SNPs were found to violate the Hardy–Weinberg equilibrium. Arsenical values were transformed to a log scale in order to calculate means and range, to perform statistical comparisons between groups and to evaluate potential confounding factors. Differences in the percentages of urinary As species and the species ratios between each genetic polymorphism were evaluated by Student *t*-test. Multiple comparisons between groups were made using one-way repeated-measures ANOVA in case of a normal distribution of the data or using a χ^2 test otherwise. Post hoc analysis was made using the Holm–Sidak *t*-test for pair-wise comparisons. We used unconditional logistic regression analyses to examine associations between each genetic polymorphism and each phenotype.

The comparisons performed were: 1) Non-lesion vs. lesion, 2) stratified by genotype, 3) stratified by haplotypes. Each polymorphism was modeled individually as gene-effects in logistic regression models, and the odds ratios (OR), and 95% confidence intervals were estimated. Pair-wise interactions among all independent SNPs were tested using logistic regression. All analyses were adjusted for age, gender and the TWE, but possible risk factors (alcohol use) was excluded as confounders because they were independent of the studied variables. As shown previously, haplotype information may be more useful than individual polymorphisms in association studies (Brodde and Leineweber, 2005). Therefore, we also performed haplotype analyses for the three *AS3MT* SNPs. Three participants were excluded from the haplotypes association analyses because of the lack of A-477G SNP information. Statistical significance was defined as a *p*-value <0.05. All statistical analyses were performed using STATA for Windows, version 9.0 (Stata, Corp., College Station, TX, USA).

Results

A total of 13 males and 109 females were included in the study. The number of male participants in our study was very low due to the high migration of males to USA. The age range was 18–50 years, and the participants were on average 35 years old. The majority (58.2%) of the study population had As-skin lesions. The main population characteristics, stratified by the presence (*n*=71) or absence (*n*=51) of As-skin lesions are shown in Table 2. The As-skin lesions group had higher levels of TAs in urine [median and range; 94.3 (12.5–1398) vs 40.6 (9.8–62.9) µg/g creatinine; *p*<0.05], TAs in water [104 (29–378) vs 12 (9–45) µg/l; *p*<0.05] than the group without skin lesion.

The effects of two *AS3MT* polymorphisms (A-477G SNPs in promoter region, T35587C SNPs in intron 10, and exonic T14458C) on As metabolism of all study participants are shown in Table 3 and Fig. 1. The median As concentrations in urine and well water of all the individuals studied were 109 µg/g creatinine and 84 µg/l, respectively. It is known that the creatinine level in urine of males is generally higher than that of females because its excretion into the urine is related to mass of muscle. However, no significant difference was observed for creatinine between males and females in this study (data not shown).

The efficiency of As methylation was assessed by calculating the ratio between the urinary concentrations of the product and the substrate; higher values represent a higher methylation capacity (Del Razo et al., 1997). Individuals carrying the G allele in the A-477G variant had the lowest proportion of MAs and the highest proportion of DMAs, which consequently increased the DMAs/MAs ratio (Tables 3 and 4). Evaluation of Met287Thr polymorphism showed that the percentages of iAs and MAs were higher in individuals with the C allele (287Thr variant) than in those with the T allele (Met287). Also, the DMAs proportions were lower in individuals carrying the C allele than in those carrying the T allele (Tables 3 and 4). Our results showed no differences in the MAs/iAs ratio between the evaluated SNPs. A statistically significant difference was observed in the DMAs/MAs ratio for individuals with the Met287Thr polymorphism; individuals with the TC/CC variant had the ratio equal to 9.4, while those with the TT variant had the ratio equal to 6.1. These results indicate that the second methylation step may be less efficient in people carrying the 287Thr variant (Table 3).

We have observed effects of the *AS3MT* polymorphism (A-477G and Met287Thr) on the urinary concentrations of both trivalent and pentavalent As species (Fig. 1; Table 3). No difference was found in the relative proportions of pentavalent arsenicals in relation to the A-477G and T35587C variants. However, individuals with Met287Thr variants showed statistically significant differences in the percentage of MAs^{III+V} in urine (Table 3). For the

trivalent arsenicals, iAs^{III} , MAs^{III} , and $DMAs^{III}$, individuals with the mutant homozygous (GG) genotype in the promoter variant (-477) had a lower proportion of iAs^{III} than homozygotes AA variants and heterozygotes AG variants (Fig. 1). Furthermore, in urine of individuals with the exonic genetic variant (Met287Thr), the percentage of MAs^{III} was significantly higher in mutant homozygote (only one individual) (CC) and heterozygotes (TC) than in normal homozygotes (TT). Individuals heterozygous or homozygous for the Met287Thr variant (C allele) had a lower percentage of $DMAs^{III}$ than wild-type homozygotes ($p < 0.05$). The effects of *AS3MT* polymorphisms on urinary As metabolism observed in total group (Table 3), remained for Met287Thr even after stratifying the groups according to presence of As-skin lesions (Table 4).

The distribution of *AS3MT* polymorphism among individuals with and without skin lesions is shown in Table 5. Marginal differences in the frequencies of the Met287Thr genotype were identified between individuals with and without premalignant skin lesions ($p = 0.055$). These results suggest that the formation of premalignant As-skin lesions could be related to Met287Thr genotype, individuals carrying the C allele (TC+CC) were at risk [OR=4.28; 95%CI (1–18.5)]. The occurrence of A-477G and T35587C SNPs did not differ between the two study groups, suggesting that these polymorphic variant are not associated with the risk of As-skin lesions (Table 5). However, the A-477G polymorphism was associated with differences in the $DMAs/MAs$ ratio in urine (Table 3 and 4).

Haplotypes analyses

The results of the haplotype analyses for *AS3MT* are shown in Tables 6 and 7. Haplotype 3 (ACT) was associated with an increase in the percentage of urinary MAs and decrease in the percentage of $DMAs$ as compared to the haplotypes 1, 2, and 4. However, these differences are mainly associated with the proportion of the methylated trivalent arsenicals (MAs^{III} and $DMAs^{III}$), with the strongest effect for the percentage of MAs^{III} . The percentage of iAs^V followed a pattern similar to that of MAs^{III} percentage for the ACT haplotype. Haplotype 3 had a higher percentage of iAs^V (14.5) than haplotypes 1, 2 and, 4 (9.2%, 8.8%, and 8.0%, respectively). No differences were observed in the MAs/iAs ratios or the $DMAs/MAs$ ratios.

For our multivariate analyses, we designed one model for each haplotype formed by a combination of the A-477G, Met287Thr and T35587C genetic variants in *AS3MT*. Table 8 shows the results of the multiple logistic regression analyses stratified by the presence of cutaneous signs indicating the relation of haplotypes evaluated with the presence of As premalignant skin lesions. We tested whether As-skin lesions were influenced by polymorphisms in the *AS3MT* gene. Because, gender, age, and TWE are associated with the presence of As-skin lesions, we included these confounding variables in the multivariate analyses. No significant interactions were found between *AS3MT* and TAs in urine or with TWE. The multivariate models for carriers of the haplotypes 3 and 6 showed a high frequency in individuals with skin lesions (9% and 13%, respectively), but possibly due to the small size of each haplotype group. The association was not statistically significant. We only observed a marginal association of haplotype 3 carriers bearing a greater risk of developing premalignant skin lesions from As exposure following adjustments for age, gender and TWE (OR=4.56, $p = 0.09$). The model for haplotype 3 showed that carrying mutant allele C in position (i.e., Thr287) was the most important determinant of presence of As-skin lesions. In contrast, the haplotype 4 carriers of mutant allele of 35587C had a lower risk of arsenic skin lesions (OR=0.65), which is similar to what we observed for haplotype 1 (OR =0.62), although these results were not statistically significant (Table 8).

Discussion

This study including individual exposure assessment and inclusion of trivalent and pentavalent arsenic species in human urine, in order to determine a possible metabolite causal of iAs effects on arsenic-skin lesions. Importantly in this study, we found multivariate associations among the urinary As species with *AS3MT* genotyping after adjusting to age, gender and TWE. Marginal significant differences in the frequencies of the Met287Thr genotype were identified between individuals with and without premalignant skin lesions ($p=0.055$). These results suggest that the formation of premalignant As-skin lesions could be related to Met287Thr genotype.

Although differences in responses among individuals can be attributed to differences in cumulative dosage or the duration of exposure, variations in responses may also reflect inter-individual differences in the kinetic behavior and dynamic effects of the As species that initiate and/or promote disease processes. The variability in the patterns of urinary metabolites from exposed humans may reflect differences in the production or tissue retention of critical metabolites. There is a marked inter-individual variability in the effects of iAs exposure in terms of carcinogenesis and other health outcomes among exposed populations (Hughes et al., 2007).

Various genetic and toxicological endpoints have been used as biomarkers to understand the biological effects of iAs exposure. Recently, evidence from epidemiological studies has consistently demonstrated a relationship between altered urinary arsenic metabolite profiles and polymorphisms in genes implicated in iAs metabolism (Lindberg et al., 2007; Hernandez et al., 2008).

Genetic factors are involved in the development of premalignant skin lesions due to iAs exposure. Recent advances in SNP genotyping methods have enabled the detection of genetic variations associated with increased susceptibility to As-induced skin lesions. Previous studies have identified genetic variations in glutathione S-transferase (*GST*), myeloperoxidase (*MPO*), catalase (*CAT*), tumor suppressor protein 53 (*p53*), xeroderma pigmentosum group D (*XPD*) and X-ray repair cross-complementing group 1 (*XRCC1*) that are associated with As-induced skin lesions (Ahsan et al., 2003; De Chaudhuri et al., 2006; Banerjee et al., 2007; Breton et al., 2007; McCarty et al., 2007). These identified genes are related to cellular glutathione levels, oxidative stress, the nucleotide excision repair pathway, control of cell growth or maintenance of genomic stability. We have examined three SNPs in *AS3MT* gene and found that the Thr287 variant is associated with risk of As-premalignant skin lesions. The frequency of C allele (i.e., Thr287) was higher among the individuals with skin lesions as compared to the individuals without skin lesions. In contrast with our findings are results of an independent study which showed no association between the Thr287 variant and As-skin lesions in an Indian population exposed to iAs in drinking water. Notably, the frequency of heterozygosity (the TC genetic variant) in this population (9%) (De Chaudhuri et al., 2008) resembled the frequency found in our study.

The mechanism by which this polymorphic site affects development of premalignant skin lesions is not clear. However our results together with results of other studies (Lindberg et al., 2007) suggest that the formation of premalignant As-skin lesions is related to iAs metabolism. All human studies that examined the association between As metabolic phenotypes and disease found that individuals with disease manifestations had relatively higher urinary levels or percentage of MAs as compared to individuals without health problems (Del Razo et al., 1997; Valenzuela et al., 2005; Steinmaus et al., 2006). The HG-CT-AAS analytical method used in the present study examined only the HG-active tri- and pentavalent iAs, MAs, and DMAs formed in the process of *AS3MT*-catalyzed methylation.

TMA^sVO also is able to be analyzed using this methodology, but as we found low concentration of TMA^sVO (<8 ng/l) in the urine of only 4 participants; In consequence, TMA^sVO was not considered within the methylation profile of the participants of this study. Other possible HG arsenic form in urine is DMTA (Raml et al. 2007) but it does not significantly interfere with analysis of DMA^sIII and/or DMA^sV (Hernández-Zavala et al., 2008; Matoušek et al 2008). Therefore, the risk of underestimating or misidentifying DMA^s in the present study is minimal.

We examined iAs metabolic phenotypes, including the concentrations and proportions of the trivalent and pentavalent As species in the urine. We found that the Thr287 variant was associated with an increased percentage of MA^sIII and a decreased percentage of DMA^sIII in urine. These results are in a good agreement with studies carried out in European and Chilean populations (Lindberg et al., 2007; Hernandez et al., 2008).

The Met287Thr polymorphism is a nonsynonymous base-pair substitution, which suggests that the alterations at the genomic levels change the biophysical and biochemical properties of the corresponding AS3MT protein. The impact of the Met287Thr polymorphism on the expression and function of AS3MT was recently examined using a primary culture of human hepatocytes exposed to different concentrations of iAs^{III}. The variant Met287Thr heterozygotes (TC variant) had lower percentage of DMA^s and a higher percentage of MA^s than the wild-type (TT variant) when exposed to iAs^{III} (Drobná et al., 2004). Moreover, Wood et al. (2006) have shown that the Thr287 variant expressed in COS-1 cells had higher enzymatic activity and increased protein expression than the Met287 variant.

Additionally, several AS3MT polymorphism in intronic regions have been associated with different urinary arsenic patterns in various ethnic groups, including Argentinean, Mexican and European populations (Lindberg et al., 2007; Meza et al., 2007; Rahman et al., 2007). These studies have shown that the 12390C and 35587C AS3MT variants had lower percentages of MA^s and higher DMA^s/MA^s ratios. To date, the functional role of SNPs in AS3MT introns remains unclear. One possible explanation is that the AS3MT T35587C variant in intron 10 results in an alternatively spliced form of AS3MT mRNA, which loses the capacity for As methylation to the MA^s form resulting in a decreased proportion of urinary MA^s (Meza et al., 2007).

We demonstrated that the A-477G allele frequencies in studied Mexican population is 38%, which is consistent with that of other researchers studying Caucasian-American populations (Wood et al., 2006). No previous study examined this polymorphic site with respect to iAs metabolism. In our study, we found that people carrying the homozygous -477 (GG) mutant alleles had a lower percentage of MA^s, a higher percentage of DMA^s, and consequently had a higher DMA^s/MA^s ratio. The relationship between the As phenotype and the A-477G polymorphism may result from a genetic background effect or from its location in the promoter region of the AS3MT gene. Possibly, A-477G is part of an enhancer site that contributes to the gene expression.

Despite the lack of statistical association between the AS3MT haplotypes and iAs-induced skin lesions in this study, we should not overlook the haplotype 3 (ACT) that can be considered as a biomarker candidate for risk of premalignant skin lesions in populations exposed to iAs. The lack of significant associations and statistical power in our study was in part due to the small sample size in each of the haplotype groups we evaluated. Additionally, it was not possible to perfectly match gender, age, and TWE factors between individuals in with and without skin lesions. Nevertheless, the calculated power analysis to detect a significant association between groups with (58.2%) and without skin lesions (41.8%) at the alpha=0.05 level, with 122 participants, was good (0.73). Some limitations include the

relatively small sample size, and the data for the men were limited, thus precluding us from analyzing whether there were any sex differences in gene–As interactions for risk of As-related skin lesions. Our samples were collected in central Mexico, where the population is represented by a mixture of American Indians, Caucasians and African Americans. Therefore, the genetic background of the study population is less homogeneous than in studies of Asian or European populations. This heterogeneity may have influenced the results of our association analysis. Previously, researchers identified a significant association between the Met287Thr polymorphism and the urinary level of MAs (Hernandez et al., 2008). The high proportions of MAs in urine are known risk factor for several adverse effects and diseases caused by iAs exposure (Ahsan et al., 2007; McCarty et al., 2007; Tseng, 2007). Our findings suggest that along with these risks, there is also an association between 287Thr and As-skin lesions.

Because trivalent methylated arsenicals rapidly oxidize, storage conditions may significantly affect results of urine speciation (Del Razo et al., 2001; Gong et al., 2001). Future studies evaluating methylated trivalent As species in the urine should consider an immediate analysis of fresh urine samples, preventing the oxidation of unstable methylated As^{III}-containing metabolites.

This is the first study that indicates an association between *AS3MT* polymorphism and As-induced skin lesions. Our findings indicate that *AS3MT* polymorphism contributes to the etiology of premalignant skin lesions associated with chronic exposure to iAs. However, further studies are needed to clarify this association.

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Fig. 1.

The relative proportions of trivalent arsenic metabolites in urine of individuals with different *AS3MT* SNPs: A477G ($n=119$), Met287Thr and T35587C polymorphisms sites, stratified by genotype ($n=122$). (AA) homozygous wild-type, (AG) heterozygous, (GG) mutant homozygous in the case of A-477G genetic variant. (TT) homozygous wild-type, (TC) heterozygous, (CC) mutant homozygous for Met287Thr and T35587C SNPs. Values are expressed as the mean $\pm$ SEM of urinary proportion of iAs^{III} (A), MAs^{III} (B), and DMAs^{III} (C). * $p<0.05$ using the Student *t*-test.

Table 1

TaqMan primers and probes used for *AS3MT* genotyping.

SNP ID ^a	rs number ^b	Region	Nucleotide change	Primer (5'-3') ^c	Probe ^d
A-477G	rs7085104	Promoter	A>G	TTTGGTCTCCGTTTGGTGATGTAT-F	VIC-TTCTGTGAACTCTGGCAA
4602				CCGTGAAATCCATCTTCTTCAGTGT-R	FAM-TGTGAACTCCGGCAAA
M287T	rs11191439	Exon 9	T>C	AATGGAGGAAATTACAGGACATGAAAAAGA-F	VIC-ATTGGCATCAAAACATTAGT
14458				AGAAAAGAATACCAAGTCATGGAAATTGT-R	FAM-TGGCATCAAAACGTTAGT
T35587C	rs11191453	Intron 10	T>C	AGGTATCTCATTTGGAGGTGAAAGGA-F	VIC-AGACTGCTGTATTATT
35587				GCCAGACAGATCTCAGAGTAGAGTA-R	FAM-ACTGCTGTGTTTATT

^aSNP ID indicates the SNP identification number relative to the nucleotide location in the consensus sequence: AY817668 (in <http://ncbi.nlm.nih.gov>).

^brs numbers were cited from NCBI SNP data base (<http://www.ncbi.nlm.nih.gov/projects/SNP/>).

^cF, forward primer; R, reverse primer.

^dAllelic changes in the probe are shown in boldface.

Table 2

Demographic and descriptive statistics of the study population.

Variable	Non skin lesion	With skin lesion
	N (%)	N (%)
<i>Age (years)</i>		
<35	24 (47)	36 (51)
≥35	27 (53)	35 (49)
<i>Gender</i>		
Male	4 (8)	9 (13)
Female	47 (92)	62 (87)
<i>Drinking status</i>		
Alcohol consumption		
Drinker	7 (14)	14 (20)
Non-drinker	44 (86)	57 (80)
<i>TAs in water (µg/l)</i>		
≤10	34 (67)	8 (11)
>10	17 (33)	63 (89)
<i>TAs in urine (µg/g creatinine)</i>		
≤50	25 (49)	17 (24)
>50	26 (51)	54 (76)
<i>Time-weight of exposure (mg/years)</i>		
≤1	35 (69)	3 (4)
>1	16 (31)	68 (96)
<i>Arsenic-skin lesions</i>		
Melanosis		54 (76)
Keratosis		58 (82)

Table 3

The effects of *AS3MT* polymorphisms on the distribution of As metabolites in urine ($n=122$).

SNPs	% iAS ^{III+V}	% MAS ^{III+V}	% DMA ^{III+V}	MAS ^{III+V} /iAS ^{III+V}	DMA ^{III+V} /MAS ^{III+V}	% iAS ^V	% MAS ^V	% DMA ^V
<i>A-477G</i>								
Genotype frequency	18	11	71	0.8	8.7	9.2	3.3	23.9
AA/AG (0.85)	13*	9**	78**	0.7	10.8**	8.0	2.1*	23.8
GG (0.15)								
Allelic frequency								
A (0.62)	18	11	71	0.8	8.7	9.2	3.3	23.9
G (0.38)	17	10	73	0.7	10.0**	8.8	2.5	23.8
<i>Met287Thr</i>								
Genotype frequency								
TT (0.88)	17	10	73	0.7	9.4	8.5	3.1	23.5
CT/CC (0.12)	21*	15**	64*	0.7	6.1*	13.4**	2.7	26.7
Allelic frequency								
T (0.934)	17	11	72	0.7	9.0	9.1	3.1	23.5
C (0.066)	21	15**	64**	0.7	6.1*	13.4**	2.7	26.7
<i>T35587C</i>								
Genotype frequency								
TT (0.58)	18	12	70	0.7	8.5	9.9	3.1	22.8
CT (0.42)	16	10	74	0.8	9.8	8.1	3.2	25.5
Allelic frequency								
T (0.79)	17	11	72	0.7	9.0	9.1	3.1	23.9
C (0.21)	16	10	74	0.8	9.8	8.1	3.2	25.5

Student *t*-test.

Significance is shown in footnote of corresponding tables ($p<0.05$).

* $p<0.1$.

** $p<0.05$.

Table 4
The effects of *AS3MT* polymorphisms on the distribution of As metabolites in urine according the presence of As-skin lesions.

SNPs Allelic (n)	% iAs ^{III+V}	% MAs ^{III+V}	% DMAs ^{III+V}	MAs ^{III+V} /iAs ^{III+V}	DMAs ^{III+V} /MAs ^{III+V}	% iAs ^{III}	% MAs ^{III}	% DMAs ^{III}	% iAs ^V	% MAs ^V	% DMAs ^V
<i>A-477G</i>											
Non-skin lesions											
A(21)	18.0	11.4	70.6	0.74	8.6	8.1	7.6	47	9.9	3.8	23.5
G(29)	18.4	9.9*	71.7	0.74	10.5**	8.9	7.3	46.4	9.5	2.6*	25.2
As skin lesions											
A(26)	16.0	12.2	71.8	0.84	6.8	7.3	7.8	47.4	8.7	4.3	24.4
G(43)	15.9	10.4**	73.7	0.72	9.4**	7.5	7.9	50.8	8.4	2.4**	22.8
<i>Met287Thr</i>											
Non-skin lesions											
T(47)	17.4	10.0	72.6	0.75	10.3	8.4	6.9	49	9.0	3.0	23.6
C(4)	25.6*	16.1**	58.3*	0.63	4.6*	9.4	13.0**	25.6*	16.2	3.1	32.7
As skin lesions											
T(60)	15.8	10.5	73.6	0.76	8.7	8.1	7.3	50.0	8.1	3.2	23.5
C(11)	19.1	15.3**	65.5*	0.81	6.6*	12.3**	12.7**	40.9	12.4*	2.6	24.5
<i>T35587C</i>											
Non-skin lesions											
T(30)	18.4	11.0	70.6	0.70	9.5	8.3	7.7	48.6	10.1	3.3	22.0
C(21)	17.6	9.7	72.6	0.79	10.3	8.7	7.0	45.1	8.9	2.7	27.5
As skin lesions											
T(41)	17.5	12.1	70.4	0.72	7.7	7.8	9.2	46.9	9.7	2.9	23.4
C(30)	14.8*	10.0*	75.1*	0.83	9.5*	7.3	6.6*	51.0	7.5*	3.5	24.1

Student *t*-test.

Significance is shown in footnote of corresponding tables (*p*<0.05).

*
p<0.1.

**
p<0.05.

Table 5

Analysis of association between AS3MT polymorphisms and the presence of arsenic skin lesions.

Group	N	Genotype frequency (%)			Allele frequency (%)		
A-477G		AA	AG	GG	A	G	
As-skin lesion	69	26 (38)	33 (48)	10 (14)	85 (62)	53 (38)	
Non-lesion	50	21 (42)	21 (42)	8 (16)	63 (63)	37 (37)	
		$X^2_i=0.04$, $p=0.83$			$X^2_i=0.01$, $p=0.90$		
		OR=1.60 ^a 95%CI (0.6-4.3)					
Met287Thr		TT	TC	CC	T	C	
As-skin lesion	71	60 (84.5)	10 (14.1)	1 (1.4)	130 (91.5)	12 (8.5)	
Non-lesion	51	47 (92)	4 (8)	0	98 (96)	4 (4)	
		$X^2_i=3.22$, $p=0.055$			$X^2_i=1.32$, $p=0.25$		
		OR ^b =4.28 ^a 95%CI (1-18.5)*					
T35587C		TT	TC	CC	T	C	
As-skin lesion	71	41 (58)	30 (42)	0	112 (79)	30 (21)	
Non-lesion	51	30 (59)	21 (41)	0	81 (79)	21 (21)	
		$X^2_i=0.02$, $p=0.88$			$X^2_i=0.1$, $p=0.90$		
		OR=0.76 ^a 95%CI (0.3-1.9)					

The genotype and allele distributions of the AS3MT polymorphisms in individuals exposed to inorganic arsenic are shown for groups with and without skin lesions.

Two-sided X^2_i test.

^a Logistic regression models adjusted by age, gender and TWE.

^b Comparison between TT and TC+CC;

* $P=0.05$.

Table 6

AS3MT haplotypes and frequencies in the Study population.

Haplotypes	Frequency
1 –477A/287T/35587T (ATT)	0.84
2 –477G/287T/35587T (GTT)	0.60
3 –477A/287C/35587T (ACT)	0.076
4 –477A/287T/35587C (ATC)	0.28
5 –477A/287C/35587C (ACC)	0.008
6 –477G/287C/35587T (GCT)	0.11
7 –477G/287C/35587C (GCC)	0.042

Table 7

Arsenic metabolism and extended AS3MT haplotype frequencies in the study population.

Haplotypes	1(ATT)	2(GTT)	3(ACT)	4(ATC)	5(ACC)	6(GCT)	7(GCC)
N	100	72	9	34	1	13	5
% iAs ^{III+V}	17.7	16.9	25	16.6	19.9	21	14.3
% MAs ^{III+V}	11.3 ^a	10.2 ^a	19	10.2 ^a	9.2	16	9
% DMA ^S ^{III+V}	71 ^a	72.9 ^a	56	73.2 ^a	70.1	63	76.7
MAs ^{III+V} /iAs ^{III+V}	0.77	0.74	0.77	0.89	0.46	0.76	0.66
DMA ^S ^{III+V} /MAs ^{III+V}	8.7	10	4.5	9.8	7.7	6	8.8
% iAs ^V	9.2 ^{a,b}	8.8 ^{a,b}	14.5	8.0 ^{a,b}	15	12.6	10.3
% iAs ^{III}	8.5	8.1	10.2	8.6	5	8.4	4
% MAs ^V	3.3	2.5	3.3	3.6	2	2.2	1.6
% MAs ^{III}	8 ^a	7.7 ^a	15.5	6.6 ^a	7	13.5	7.3
% DMA ^S ^V	23.4	23.8	30.5	25	12.4	22.4	20.6
% DMA ^S ^{III}	47.6 ^a	49.1 ^a	26	48.2 ^a	58.6	40.9	56.2

Values are a geometric mean of each arsenic metabolite.

^a Compared with 3(ACT) haplotype.

^b Compared with 6(GCT) haplotype, significant at $p < 0.05$, using ANOVA and the Holm-Sidak test.

Stratified analyses of the *AS3MT* gene haplotypes for the A-477G, Met287Thr and T35587C SNPs and the risk of arsenic-skin lesions.

Table 8

Haplotype	Non-lesion No. (%)	As-skin lesion No. (%)	Adjusted OR ^a	95% CI	P
1 (ATT)	42 (84)	58 (84)			
2 (GTT)	29 (58)	43 (62)	1.61	0.6–4.3	0.34
3 (ACT)	3 (6)	6 (9)	4.56	0.8–26.4	0.09
4 (ATC)	14 (28)	20 (29)	0.65	0.2–1.9	0.43
6 (GCT)	4 (8)	9 (13)	3.01	0.6–13.9	0.16
7 (GCC)	1 (2)	4 (6)	2.02	0.2–22.7	0.56

^a Logistic regression analyses adjusted by age, gender and TWE.