Published in final edited form as:

Arch Toxicol. 2014 February ; 88(2): 275–282. doi:10.1007/s00204-013-1146-x.

Differential Methylation of the Arsenic (III) Methyltransferase Promoter According to Arsenic Exposure

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Abstract

Inorganic arsenic is methylated in the body by arsenic (III) methyltransferase. Arsenic methylation is thought to play a role in arsenic-related epigenetic phenomena including aberrant DNA and histone methylation. However, it is unclear whether the promoter of the *AS3MT* gene, which codes for arsenic (III) methyltransferase, is differentially methylated as a function of arsenic exposure. In this study we evaluated *AS3MT* promoter methylation according to exposure, assessed by urinary arsenic excretion in a stratified random sample of 48 participants from the Strong Heart Study who had urine arsenic measured at baseline and DNA available from 1989–1991 and 1998–1999. For this study, all data are from the 1989–1991 visit. We measured *AS3MT* promoter methylation at its 48 CpG loci by bisulphite sequencing. We compared mean % methylation at each CpG locus by arsenic exposure group using linear regression adjusted for study centre, age and sex. A hypomethylated region in the *AS3MT* promoter was associated with higher arsenic exposure. *In vitro*, arsenic induced *AS3MT* promoter hypomethylation and it increased *AS3MT* expression in human peripheral blood mononuclear cells. These findings may suggest that arsenic exposure influences the epigenetic regulation of a major arsenic metabolism gene.

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SUPPLEMENTARY DATA DISCUSSION

The supplemental materials illustrate the raw data used to generate individual % methylation variables at each CpG locus (Supplement 1), the distribution of % methylation values across individuals at each CpG locus (Supplement 2) and mean level of %DNA methylation in a 30-CpG-loci region of the *AS3MT* promoter (Supplement 3).

Keywords

arsenic; epigenetics; DNA methylation; *AS3MT*; arsenic(III) methyltransferase; arsenic metabolism

INTRODUCTION

Inorganic arsenic, a widespread environmental toxicant, is an established carcinogen (IARC, 2004) that may play a role in cardiovascular disease (Agusa et al., 2010; Chen et al., 1996; States et al., 2009) and diabetes (Del Razo et al., 2011; Gribble et al., 2012; Navas-Acien et al., 2008; Navas-Acien et al., 2006). The relationship between arsenic exposure and disease outcomes is thought to be related to arsenic metabolism (Chen et al., 2003; Huang et al., 2008; Huang et al., 2009; Lindberg et al., 2008; Vahter and Concha, 2001). Arsenic (III) methyltransferase (*AS3MT*), coded by the *AS3MT* gene, is a major enzyme in arsenic methylation reactions. The role of *AS3MT* in the metabolism of arsenic has been confirmed by functional genomics (Wood et al., 2006) and experimental assays (Drobna et al., 2010; Drobna et al., 2006). Genetic polymorphisms in the *AS3MT* gene are associated with differences in *AS3MT* gene expression (Engstrom et al., 2011) and with arsenic metabolism (Agusa et al., 2011; Engstrom et al., 2011). Due to the importance of the *AS3MT* gene for arsenic metabolism, a growing body of population genetics research has evaluated population differences in *AS3MT* polymorphism frequencies (Fujihara et al., 2009; Fujihara et al., 2008; Fujihara et al., 2010; Fujihara et al., 2011).

Epigenetic modifications, including DNA and histone methylation and histone acetylation, may alter gene expression and are increasingly recognized as potential mechanisms mediating arsenic health effects (Arita and Costa, 2009; Baccarelli and Bollati, 2009; Hou et al., 2012; Martinez-Zamudio and Ha, 2011). Arsenic exposure has been associated with gene specific promoter methylation of several genes related to tumorigenesis in human-derived cell lines or human epidemiologic samples including Tumour protein P53 (*TP53*) and Ras association domain-containing protein 1 (*RASSF1A*) (Jensen et al., 2009; Lu et al., 2001; Mass and Wang, 1997). However, little research in human populations has evaluated the possible relationship between arsenic exposure and *AS3MT* promoter methylation. Recently, methylation status of a few CpG sites located at the 5' *AS3MT* region was examined in samples from Argentina and Bangladesh using the Illumina Infinium HumanMethylation 450K BeadChip (Engstrom et al., 2013). No studies have measured methylation status of the *AS3MT* promoter region using bisulphite sequencing, a "gold standard" for the measurement of DNA methylation. We hypothesized that arsenic exposure would influence DNA methylation of the *AS3MT* promoter, with possible consequences for arsenic metabolism.

In this study we used bisulphite sequencing to evaluate the association of arsenic exposure with differences in *AS3MT* promoter methylation among a sample of adult men and women from Arizona, Oklahoma and North and South Dakota who participated in the Strong Heart Study in 1989–1991. In this population, arsenic exposure in drinking water ranged from low (<10 µg/L) to moderate (50 µg/L), resulting in urine arsenic concentrations that remained stable over the 10-year duration of the Strong Heart Study follow-up examinations (Navas-Acien et al., 2009). In addition, we conducted *in vitro* experiments exposing human peripheral blood mononuclear cells to arsenic to evaluate the role of arsenic in *AS3MT* promoter methylation and gene expression. We also evaluated the impact of *AS3MT* knock-down for the expression and methylation of genes potentially related to arsenic health effects.

MATERIALS AND METHODS

Study Population

The Strong Heart Study is a population-based prospective cohort study of cardiovascular disease and its risk factors in American Indian men and women from Arizona, Oklahoma, and North or South Dakota (Lee et al., 1990). Men and women 45 to 74 years of age listed on tribal rolls in these three areas were invited to participate. A total of 4,549 participants were recruited in 1989–1991, with an overall response rate of 62%. Participants gave informed consent and provided specimens from that visit for analysis of biomarkers of interest for cardiovascular disease risk, including spot urine samples and blood samples. The Strong Heart Study protocol and consent form were approved by local institutional review boards, participating tribes and the Indian Health Service.

To assess arsenic exposure in the population, urine arsenic species including inorganic arsenic, methylarsonate (MMA), dimethylarsinate (DMA), arsenobetaine and other arsenic cations were measured in 3,974 Strong Heart Study study participants with available urine samples using anion-exchange high performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICPMS), as described previously (Scheer, 2012). Strong Heart Study participants were eligible for this study if they had arsenic levels measured in 1998–1991, had available DNA collected in 1989–1991 and 1998–1999, and participated in an ancillary study called the Strong Heart Family Study (North et al., 2003), resulting in a final number of 512 eligible participants. For this study, all data are from the 1989–1991 visit and were collected on the same day for each participant.

To maximize efficiency of this relatively small epigenetic study and reduce exposure misclassification (Stuart and Hanna, 2013; Zubizarreta et al., 2013), we used a stratified random sample to select eight participants with moderate arsenic exposure and eight participants with low arsenic exposure from each region (16 from Arizona, 16 from Oklahoma and 16 from North or South Dakota), resulting in a total of 48 participants. Based on the Strong Heart Study distributions, relatively low and moderate arsenic exposures were defined as urine concentrations < 7.2 µg/L (tertile 1) and 14.0 µg/L (tertile 3), respectively, for the sum of inorganic and methylated arsenic species in 1989–1991. For each participant, detailed socio-demographic and health status information were obtained from detailed in-person interviews, physical examinations and biological specimens.

Laboratory Methods

AS3MT promoter methylation analysis was performed at the Department of Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health. DNA from whole blood collected in 1989–1991 was solvent-extracted, treated with proteinase K, and stored at the Penn Medical Laboratory of Medstar Health Research, Washington, DC. Genomic DNA (200 ng) was bisulphite-modified using the EZ DNA Methylation kit (Zymo Research, Irvine, CA) prior to PCR. *In silico* analyses and detailed database searches were used to predict the 5'-CGI(s) of *AS3MT* (NC_000010.10: 104626210-104630210) (www.urogene.org/methprimer/). Primers were designed to amplify a 695 bp (–240 to +454) fragment encompassing the predicted 5'-CGI(s) of *AS3MT* (hBS-*AS3MT*-Forward, 5' TGATTTTATTTTAAAAAGGGGAG 3' and hBS-*AS3MT*-Reverse, 5' CAAACCAAACCTTCATTAACCTAAA 3') from bisulphite-modified DNA. Amplicons were generated from individual samples using Platinum Taq DNA polymerase (Life Technologies, Grand Island, New York) and inserted into the pCR2.1 vector (Life Technologies, Grand Island, New York). Six isolates were picked and sequenced for each sample (MacroGen, Rockville, Maryland) to obtain direct measures of DNA methylation at each CpG locus in the *AS3MT* promoter region. An example of the resulting data is shown in

Appendix 1. The DNA methylation data from sequencing were analyzed with the BiQ analyzer. Genomic DNA with 0%, 50% and 100% methylation were used as a quality control to ensure bisulphite conversion quality on sample. Sequences with low conversion rate (<90%) were excluded from further analysis. In one participant, one isolate failed quality control, and so only 5 isolates were used to calculate mean % methylation for that participant.

Statistical Analyses

We analyzed each of the 48 CpG loci in the *AS3MT* promoter region separately using the estimated mean % DNA methylation from 5–6 isolates as the methylation metric of interest per individual at each CpG locus. Histograms were examined to evaluate the distribution of mean % DNA methylation at each CpG locus (Appendix 2) and scatter plots to describe mean % DNA methylation across loci according to study centre, sex, and arsenic methylation profile (i.e. above or below median % MMA, and above or below median %DMA). The scatter plots of % DNA methylation for %DMA are similar to those for %MMA and are not shown. We analyzed the relationship between % DNA methylation at each CpG locus by arsenic exposure group (moderate vs. low) using linear regression adjusting for sex, age and study centre (Arizona, Oklahoma and North or South Dakota). In a sensitivity analysis, we further adjusted for body mass index, smoking status and drinking status. Given the limited number of observations and the departure of residuals from normality, we used a bootstrap to estimate bias-corrected and accelerated 95% confidence intervals, resampling observations from within study centres (DiCiccio and Efron, 1996).

In vitro experiments

To evaluate the potential impact of arsenic on *AS3MT* promoter methylation and gene expression, we exposed human peripheral blood mononuclear cells (Lonza, Walkersville, Maryland) to 200nM non-targeting small interfering RNA (siRNA) control (siCTL) or on-target plus siRNA against *AS3MT* (Dharmacon-ThermoScientific, Pittsburgh, PA) for 3 days prior exposure to 5µM aqueous sodium arsenite for another 48h. RNA and DNA were isolated from all treatment groups. In addition to *AS3MT*, we evaluated gene expression and methylation status of other genes related to arsenic exposure in previous studies, including tumour suppressor genes (*TP53* and *RASSF1A*) and peroxisome proliferator-activated receptor gamma (*PPARG*). The mRNA levels of *AS3MT*, *RASSF1A*, *TP53* and *PPARG* were quantified by TaqMan-based real-time PCR. The delta-delta cycle threshold (Ct) method was used to calculate the relative expression level of transcripts normalized to 18S (Wong and Medrano, 2005). Gene specific methylation of *AS3MT*, *RASSF1A*, *TP53*, and *PPARG* were assayed by MethyLight assay as previously described (Weisenberger et al., 2006). We measured global DNA methylation in genomic DNA (200ng) isolated from each treatment group by 5'-mC enzyme-linked immunosorbent assay (Zymo Research, Irvine, California). Four independent experiments with siRNA treatment were employed. Results of gene expression and methylation analysis were statistically evaluated using GraphPad (GraphPad Software Inc., San Diego, CA). All data groups were analyzed by one-way ANOVA, followed by Tukey's multiple comparison test, and differences between groups were accepted at $\alpha = 0.05$.

RESULTS

Mean (standard deviation) for the sum of inorganic and methylated arsenic species was 5.2 (1.7) µg/L for participants with low arsenic exposure and 29.2 (23.4) µg/L for participant with moderate arsenic exposure (Table 1). Within the same study centre, participants of low and high arsenic levels were similar in age, body mass index, and education, although in Oklahoma participants with low arsenic were more likely to be smokers than those with high

arsenic and in North and South Dakota participants with low arsenic had higher mean body mass index. Within the same study centre, participants of low and high arsenic levels were not balanced on sex.

Across an approximately 30-CpG-loci region of the *AS3MT* promoter, mean levels of % DNA methylation were lower comparing participants with moderate versus low arsenic exposure and in participants from Arizona and North and South Dakota compared to Oklahoma (Appendix 3). In regression analyses adjusted for sex, age and study centre, hypomethylation of an approximately 30-CpG-loci region in the promoter was observed in participants exposed to moderate versus low arsenic levels (Figure 1). We evaluated mean regional methylation differences over CpG 12 to 42 using the same linear regression bootstrap methods. Participants with moderate arsenic exposure had 16 (95% CI -28, -5) percent lower mean regional methylation after adjustment for study centre and sex. Further adjustment for body mass index, drinking status and smoking status resulted in 17 (95% CI -30, -2) percent lower mean regional DNA methylation in the highest versus lowest arsenic tertile.

In *in vitro* experiments, sodium arsenite exposure induced *AS3MT* and *RASSF1A* gene expression along with a decrease in promoter methylation (Table 2). In contrast, sodium arsenite exposure decreased *TP53* gene expression with an increase in promoter methylation. Arsenite-treated cells showed downregulation of *PPARG* gene expression but no changes in promoter methylation. Knockdown of *AS3MT* by small interfering RNA reversed the arsenic-induced *RASSF1A* promoter hypomethylation and *TP53* hypermethylation (Table 2). Knockdown of *AS3MT* also inhibited arsenic-induced global DNA hypomethylation (Table 3).

DISCUSSION

Arsenic exposure, as measured in urine, was associated with *AS3MT* promoter hypomethylation in a sample of Strong Heart Study participants exposed to low-moderate arsenic exposure. These associations were observed for many CpG loci after adjustment for sex, study centre, age, body mass index, smoking status and drinking status. *In vitro*, we confirmed that arsenic-induced hypomethylation of *AS3MT* increased its gene expression. Knockdown of *AS3MT* altered arsenic-induced gene-specific and global DNA methylation, supporting that *AS3MT* might be an important gene for epigenetic modifications induced by arsenic. While these study findings need to be interpreted with caution given the small sample size and the multiple comparisons, the findings are consistent with our initial hypothesis of arsenic-induced methylation changes in the *AS3MT* promoter. Overall, the study results underscore the need for future work evaluating the relationships and health implications of arsenic exposure, epigenetics, and arsenic metabolism.

AS3MT is an important gene for arsenic metabolism, bioavailability of toxic arsenic species, and toxicological response to arsenic exposure (Drobna et al., 2009; Engström et al., 2010; Thomas, 2009; Thomas et al., 2007). *AS3MT* promoter methylation could be important for arsenic toxicity if arsenic metabolism is a mediator or a modifier of arsenic toxicity. It has been proposed that arsenic metabolism mediates the associations of arsenic exposure with aberrant DNA or histone methylation, perhaps through depletion of the methyl donor S-adenosyl methionine (Reichard et al., 2007) or dysregulation of DNA methyltransferases (Reichard et al., 2007). Over-expression of *AS3MT* via promoter hypomethylation may further deplete S-adenosyl methionine and inhibit DNA methyltransferase and histone methyltransferase activity. This arsenic-mediated epigenetic disruption (Reichard and Puga, 2010) may contribute to global DNA hypomethylation (Benbrahim-Tallaa et al., 2005; Chen et al., 2004) and promoter methylation at specific genes involved in tumorigenesis (Cui et

al., 2006; Marsit et al., 2006; Okoji et al., 2002) and diabetes (Bailey et al., 2013). In addition to being a mediator, arsenic metabolism may also be an effect modifier. By changing chemical forms of arsenic, and thus bioavailable doses, arsenic metabolism could modify risk of toxicities specific to arsenic species (Chen et al., 2011). For example, arsenic is a powerful oxidant (Jomova et al., 2011), with differing redox catalytic potentials across different arsenic compounds. Therefore, population heterogeneity in arsenic metabolism could result in varying relationships between inorganic arsenic exposures and oxidative damage. Additional studies are needed to determine the impacts of gene regulation of *AS3MT* on overall arsenic toxicity.

To evaluate potential transcription factors binding sites, we performed *in silico* analysis (TRANSFAC®, Biobase Biological Databases, Beverly, Massachusetts) of the 5' *AS3MT* promoter region. The putative transcription factor binding sites include hepatocyte nuclear factor (HNF), CCAAT-enhancer binding protein (C/EBP), GATA-1, -2-3, Sp1, transcription factor family E2F and homeobox protein NKX2. Interestingly, in previous studies, inorganic arsenic altered expression of *C/EBP* (Lin et al., 2005), *SP1* (Jutooru et al., 2010), *GATA-3* (Yao et al., 2008) and *E2F* (Yamamoto et al., 2008). Nevertheless, how site-specific methylation of *AS3MT* contributes to the binding with these transcription factors needs further investigation.

In this study, while arsenic exposure was associated with DNA hypomethylation of the *AS3MT* promoter, the pattern of arsenic metabolites in urine, as measured by %MMA or %DMA, was not associated with differences in DNA methylation. If arsenic exposure affects *AS3MT* promoter methylation and this promoter's methylation status is functionally important, we would expect an association between the pattern of arsenic metabolites (i.e. %MMA) and DNA methylation of the *AS3MT* promoter. It is possible that either DNA hypomethylation in this promoter is not functionally important for *AS3MT* expression, or that increased *AS3MT* expression is not relevant for the pattern of arsenic metabolites in urine. Alternatively, the association of arsenic with *AS3MT* promoter hypomethylation in our relatively small study could be a spurious finding. A recent array-based study evaluating arsenic exposure levels and methylation status of *AS3MT* in populations from Bangladesh (N=127) and Argentina (N=103) found no association with a few CpG sites at 5' *AS3MT* (Engstrom et al., 2013). Our study, however, focused precisely on the *AS3MT* promoter (30 CpG sites on a 695 base pair (-240 to +454) fragment that encompasses the predicted 5' CpG islands of *AS3MT*) and used bisulphite sequencing to access methylation status of each CpG site. However, the lack of apparent association between *AS3MT* promoter methylation and arsenic metabolism in our study may also be due to limitations of our measurements. First, arsenic metabolite patterns measured in urine are an imperfect proxy for internal metabolite patterns, and a subtle impact on internal metabolite patterns may not be apparent from the urine measures. Although less is known about blood arsenic species than urine arsenic species, there is some evidence that the two compartments may differ in the relative proportion of arsenic species (Hall et al., 2007). The differences between blood and urine could be related to differences in renal elimination of different arsenic species (Buchet et al., 1981; Kala et al., 2004; Vahter and Concha, 2001). Second, DNA samples came from blood rather than the cells primarily involved in arsenic metabolism processes (Styblo et al., 2002). Third, measurement error in arsenic speciation could result in some misclassification of %MMA, making the contrast between groups less distinct. Moreover, dichotomized %MMA might be an inadequate summary of population-level differences in arsenic metabolism. Another possible explanation is that one or more of the yet-to-be-identified arsenic methyltransferases (Drobna et al., 2006) is affected in the opposite way from *AS3MT*, leading to no apparent change in arsenic metabolism. Additional data including arsenic species in blood and the methylation status of other arsenic metabolism genes, as well as an increased sample size, are needed to clarify the relationships between

gene-specific promoter methylation and arsenic metabolism in human populations. More detailed experimental studies will also be important to further evaluate the relevance of *AS3MT* promoter methylation and gene expression for arsenic metabolism.

This study had a number of strengths but also several limitations. Strengths include the high quality laboratory methods for arsenic speciation and for the DNA methylation. The study was conducted in humans instead of animal models which metabolize arsenic differently (Wang et al., 2002). We were able to detect an association between arsenic exposure and *AS3MT* promoter methylation over multiple CpG loci in a biologically plausible pattern (i.e. adjacent CpG often showed similar signals despite being analyzed separately). Our study compared extremes of arsenic exposure in the Strong Heart Study population, reducing potential biases from exposure misclassification (Stuart and Hanna, 2013; Zubizarreta et al., 2013). The major limitation was the sample size, which limited the study power and limited our ability to control for confounders. Also, the study was cross-sectional, and we cannot discard the possibility that *AS3MT* promoter methylation affects arsenic concentrations in urine. Another limitation is the measurement of DNA methylation in whole blood. Because arsenic affects multiple tissues in the body, whole blood DNA is a likely relevant tissue. Moreover, despite cell heterogeneity blood DNA is increasingly used to evaluate determinants of epigenetic modifications and also the association of epigenetic modifications with chronic diseases (Baccarelli et al., 2010; Seow et al., 2012; Toperoff et al., 2012). Finally, another limitation was the lack of gene expression data on participants at the time of the study because only DNA samples from the 1989–1991 baseline visit were available for this analysis. However, we evaluated gene expression of *AS3MT* *in vitro*.

In conclusion, this study found statistically significant DNA hypomethylation of the *AS3MT* promoter with moderate arsenic exposure, after adjusting for socio-demographic factors, body mass index, smoking status and drinking status, suggesting that arsenic exposure may affect the epigenetic regulation of a major arsenic metabolism gene. It is possible that dysregulation of arsenic methyltransferases in response to arsenic may contribute to epigenetic changes throughout the genome. Future research in larger studies is needed to clarify the importance of this association for arsenic speciation in blood and at target organs, and how arsenic exposure, arsenic metabolism, and DNA methylation may relate to disease risks.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

FUNDING

Supported by grants from the National Heart Lung and Blood Institute (R01HL090863 and by Strong Heart Study grants HL41642, HL41652, HL41654 and HL65520) and the National Institute of Environmental Health Sciences (R01ES021367, R00ES016817 and P30ES03819). M. Gribble was supported by T32 training grants from the National Heart, Lung, and Blood Institute (5T32HL007024) and the National Institute of Diabetes and Digestive and Kidney Diseases (5T32DK062707-10).

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Figure 1. Difference in mean % DNA methylation at each *AS3MT* promoter CpG locus after adjustment for study centre, age and sex

Bias-corrected and accelerated 95% confidence intervals were calculated from 1000 bootstrap replicates, except for CpG loci 30, 47 and 48 where only 999 replicates yielded estimable parameters. Observations were resampled from within each study centre. The gray line represents the hypomethylated region. The difference in mean regional methylation comparing moderate vs. low arsenic was -0.16 (95% CI $-0.28, -0.05$) percent.

Table 1

Participant characteristics by study centre and arsenic exposure levels

Summary statistics are for the observed sample from which bootstrap samples redrawn.

	Overall			Arizona			Oklahoma			North or South Dakota		
	Moderate As	Low As	N	Moderate As	Low As	N	Moderate As	Low As	N	Moderate As	Low As	N
N	24	24	24	8	8	8	8	8	8	8	8	8
Mean Age (SD)	55.9 (6.8)	54.0 (7.5)		53.9 (5.1)	53.8 (5.5)		55.2 (6.1)	53.0 (8.5)		58.7 (8.7)	55.0 (8.8)	
% Male	29.2	33.3		25	37.5		50	25		12.5	37.5	
Mean BMI (SD)	30.6 (6.0)	31.5 (5.8)		33.1 (8.3)	29.2 (4.7)		29.4 (4.8)	30.6 (5.1)		29.2 (3.6)	35.1 (6.7)	
Mean Education (SD)	11.7 (3.3)	11.4 (2.9)		10.9 (3.7)	9.6 (2.3)		12.3 (2.8)	13.4 (1.4)		11.9 (3.6)	11.3 (3.4)	
% Current Smoker	20.8	37.5		12.5	12.5		12.5	50		37.5	50	
Mean % MMA (SD)	14.9 (5.5)	14.5 (4.4)		13.2 (3.2)	13.9 (6.0)		17.0 (7.9)	14.2 (3.0)		14.6 (4.1)	15.6 (3.9)	
Mean Urine Arsenic* (SD)	29.2 (23.4)	5.2 (1.7)		32.5 (31.9)	5.6 (1.5)		17.9 (3.4)	5.5 (1.1)		37.3 (23.0)	4.5 (2.2)	

* Sum of inorganic and methylated arsenic species.

Moderate arsenic refers to urine arsenic concentrations in the highest tertile ($> 14.0 \mu\text{g/L}$) and low arsenic to concentrations in the lowest tertile ($< 7.2 \mu\text{g/L}$).

Table 2

Gene expression and promoter methylation for *AS3MT*, *RASSF1A*, *PPARG* and *TP53* in human peripheral blood mononuclear cells under controlled conditions, exposed to arsenite (5 μ M) and with *AS3MT* knockdown*

Treatment group	Gene expression Relative expression ratio mean (min, max)	p-value**	Promoter methylation % methylation reference mean (min, max)	p-value**
<i>AS3MT</i>				
siCTL	0.90 (0.70, 1.00)		25.44 (22.57, 30.94)	
As+siCTL	1.93 (1.41, 2.27)	0.02	5.58 (5.33, 5.83)	0.02
As+siAS3MT	0.57 (0.43, 0.70)	0.02	49.08 (48.90, 49.15)	0.01
<i>RASSF1A</i>				
siCTL	0.94 (0.81, 1.00)		59.88 (50.08, 69.69)	
As+siCTL	3.56 (2.78, 4.56)	0.05	25.97 (20.80, 35.70)	0.05
As+siAS3MT	0.91 (0.90, 0.92)	0.04	54.20 (51.27, 56.46)	0.05
<i>PPARG</i>				
siCTL	1.12 (1.00, 1.35)		5.33 (2.92, 6.98)	
As+siCTL	0.22 (0.19, 0.27)	0.02	6.07 (4.44, 5.45)	0.4
As+siAS3MT	0.25 (0.16, 0.34)	0.07	9.52 (5.05, 14.00)	0.43
<i>TP53</i>				
siCTL	1.07 (1.00, 1.22)		2.60 (2.47, 2.68)	
As+siCTL	0.51 (0.38, 0.49)	0.04	7.26 (6.36, 8.34)	0.02
As+siAS3MT	0.82 (0.78, 0.89)	0.07	3.48 (2.21, 4.91)	0.05

* Human peripheral blood mononuclear cells were exposed to 200nM of non-targeting small interfering RNA control (siCTL) or on-target plus siRNA against *AS3MT* (siAS3MT) for 3 days prior exposure to 5 μ M aqueous sodium arsenite for another 48h. 4 independent sets of experiments were performed.

** For each gene, the first p-value is for As+siCTL compared to siCTL, and the second one is for As+siAS3MT vs. As+siCTL, based on one-way ANOVA followed by Tukey's multiple comparison test.

AS3MT: Arsenic (III) methyltransferase gene

RASSF1A: Ras association domain-containing protein 1 gene

PPARG: Peroxisome proliferator-activated receptor gamma gene

TP53: Tumour protein P53 gene

Table 3

Knockdown effect of AS3MT on global DNA methylation in response to sodium arsenite

	global 5-methylcytosine ng, Mean (Min, Max)	p-value
siCTL	12.54 (12.22, 12.74)	
As+siCTL	6.38 (5.48, 7.13)	0.01 vs. siCTL
As+siAS3MT	10.08 (9.70, 10.37)	0.01 vs. As+siCTL