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Association of genetic variation in cystathionine-β-synthase and arsenic metabolism

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ABSTRACT

Variation in individual susceptibility to arsenic-induced disease may be partially explained by genetic differences in arsenic metabolism. Mounting epidemiological evidence and in vitro studies suggest that methylated arsenic metabolites, particularly monomethylarsonic (MMA3), are more acutely toxic than inorganic arsenic; thus, MMA3 may be the primary toxic arsenic species. To test the role of genetic variation in arsenic metabolism, polymorphisms in genes involved in one-carbon metabolism [methylenetetrahydrofolate reductase (*MTHFR*), methionine synthase (*MTR*), cystathionine-β-synthase (*CBS*), thymidylate synthase (*TYMS*), dihydrofolate reductase (*DHFR*), serine hydroxymethyltransferase 1 (*SHMT1*)] and glutathione biosynthesis [glutathione-S-transferase omega 1 (*GSTO1*)] were examined in an arsenic-exposed population to determine their influence in urinary arsenic metabolite patterns. In 142 subjects in Cordoba Province, Argentina, variant genotypes for *CBS* rs234709 and rs4920037 SNPs compared with wild-type homozygotes were associated with 24% and 26% increases, respectively, in the mean proportion of arsenic excreted as monomethylarsonic acid (%MMA). This difference is within the range of differences in %MMA seen between people with arsenic-related disease and those without such disease in other studies. Small inverse associations with *CBS* rs234709 and rs4920037 variants were also found for the mean levels of the proportion of arsenic excreted as dimethylarsinous acid (%DMA). No other genetic associations were found. These findings are the first to suggest that *CBS* polymorphisms may influence arsenic metabolism in humans and susceptibility to arsenic-related disease.

1. Introduction

Inorganic arsenic (InAs), which occurs naturally in ground water and surface water in many parts of the world, is a known human carcinogen (Cebrian et al., 1983; Focazio et al., 2000; Kurttio et al., 1999; Smith et al., 1998). Millions of people are exposed to elevated concentrations of InAs in their drinking water, putting them at risk of skin, lung and bladder cancers, cardiovascular disease, and developmental and reproductive effects (Guha Mazumder et al., 2005; NRC, 2001). Evidence suggests that susceptibility to arsenic-induced malignancy significantly varies between individuals (Chung et al., 2002; Steinmaus et al., 2006; Vahter, 2002) and may be linked to individual variation in how InAs is metabolized and cleared from the body. A consistent and growing body of evidence has shown that people who metabolize arsenic poorly may be at two to four times the risk of developing certain cancers and other arsenic-related diseases than people who are better metabolizers (Steinmaus et al., 2006).

Methylation is the primary metabolic pathway of ingested InAs in humans (Styblo et al., 2002; Vahter, 2002). Ingested InAs is methylated to monomethylarsonic acid (MMA5) and then reduced to monomethylarsonous acid (MMA3). MMA3 is further methylated and oxidized to dimethylarsinic acid (DMA5), which is reduced to dimethylarsinous acid (DMA3). However, not all ingested arsenic is fully methylated. Some InAs remains unchanged, or is converted only to MMA3 and/or MMA5 (MMA), which is excreted along with DMA3 and DMA5 (DMA) in the urine. In vitro studies have shown that trivalent forms of InAs, particularly MMA3, may be more acutely toxic than InAs suggesting that MMA3 could be the primary toxic arsenic species (Cullen et al., 1989; Lin et al., 1999; Mass et al., 2001; Styblo et al., 1999). Moreover, studies have shown that subjects who excrete higher proportions of MMA relative to InAs and DMA may have

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higher risks of arsenic-associated skin and bladder cancer (Chen et al., 2003a; Huang et al., 2008; McCarty et al., 2007; Steinmaus et al., 2006). These data provide increasing evidence that the extent to which InAs is methylated to the potentially toxic MMA species and to which it is detoxified to DMA may impact one's risk of arsenic-related diseases.

High dietary folate has been associated with improved arsenic methylation and detoxification and reduced risk of arsenic-related diseases. Further, plasma folate has been positively associated with %DMA5 and negatively related to %MMA5 (Chen et al., 2006; Gamble et al., 2005). Previously, we reported that genetic variation in the methylenetetrahydrofolate reductase (*MTHFR*) gene, which catalyzes the transfer of one-carbon groups for methylation processes, and a deletion polymorphism in the glutathione-S-transferase (*GST*) gene influenced arsenic metabolism in a population exposed to high levels of arsenic (Steinmaus et al., 2007). In another study in Argentina, minor effects in arsenic metabolism were found in women with deletion genotypes for GSTM1 and GSTT1 (Schlawicke Engstrom et al., 2007). These studies suggest that variants in genes that code for folate metabolizing enzymes and glutathione biosynthesis could account for some of the inter-individual variation in arsenic metabolism and disease susceptibility. Here, using DNA from a subset of participants from a lung cancer case–control study of arsenic-exposed individuals from the Cordoba Province of Argentina, we further investigated the influence of polymorphisms in *MTHFR* and *MTR* and additional folate metabolizing genes [cystathionine-β-synthase (*CBS*), thymidylate synthase (*TYMS*), dihydrofolate reductase (*DHFR*) and serine hydroxymethyltransferase 1 (*SHMT1*)], as well as glutathione-S-transferase-1 (*GST01*), important in Phase 2 metabolism, on excreted urinary MMA, DMA and InAs levels.

2. Materials and methods

2.1. Study sample

This paper uses data from a case–control study in Cordoba Province, Argentina. The study included patients aged 20–85 with new-incident cases of lung cancer. The patients, all in Cordoba province, were resident in Union County during 1996–2000 or in adjacent Marcos Juarez County during 1998–2000, and were identified through rapid case ascertainment involving all pathologists in the study area and from radiology services in the study areas. Controls (subjects without lung cancer) were identified from computerized voter registration lists and individually matched to cases by county, gender, and their exact year of birth. The present study includes 142 subjects (Table 1) who provided buccal cell samples. We did not limit the sample to matched pairs with buccal cell samples, as this was unnecessary for our analysis, in which cases and controls were combined into one group. Therefore, the subset of study participants comprised different numbers of cases and controls. Subjects included here were similar to the 250 subjects included in the total population of cases and controls in terms of mean age (all participants vs. participants included in this study, 66.7 vs. 65.6 years old), percent with cancer (43.6% vs. 41.5%), percent females (16.8% vs. 14.8%), and current smoking (30.4% vs. 26.8%).

All living study participants were administered the same standardized questionnaires in face-to-face interviews in their homes. Participants were asked about their water sources at every residence they had lived in for six months or more, volume and types of fluids consumed at home, work, and other places. Smoking histories were obtained including at the time of interview, 20 and 40 years ago. Occupational and medical histories were obtained, years of education, ethnicity, age, weight and other basic personal information. The study was approved by the appropriate institutional review boards in the U.S. and Argentina and informed consent was obtained from all participants. Study personnel visited all subjects at their homes to collect buccal cells and a single, first-morning urine sample.

2.2. Exposure assessment

Water samples, particularly from wells, were collected from the current residence and as many previous residences in the study area as possible. Some wells were inaccessible because they had been closed or could not be located. Then, whenever possible, a sample was collected from a nearby well thought to draw water from the same aquifer (a proxy well). However, these proxy well measurements were excluded from the main analyses because experience showed that two wells that were very close together and supposedly of similar depth could have quite disparate arsenic concentrations. Water samples were frozen at 20 °C, transported to the United States on dry ice, and analyzed for arsenic content using graphite furnace atomic absorption spectroscopy, with a detection limit of 0.5 g/L. Lab personnel were blind to case–control status. We also obtained historical records of arsenic content for community water sources. For each participant, we created a year-by-year exposure profile from questionnaire data on residential history, water and total fluid consumption, and arsenic concentrations for water sources. Water from municipal supplies was assumed to have arsenic concentrations at the medians of the samples taken from each town's supply (collected at homes), unless more relevant historical data were available. Water samples were collected only within the Department of Union. Study participants were assumed not to be exposed to arsenic in drinking water when living outside Union.

Table 1
Sample characteristics by case–control status.

	Cases	Controls	All
Sex (%)			
Female	15.5	14.3	14.8
Male	84.5	85.7	85.2
Age (%)			
30–44	8.6	6.0	7.0
45–59	17.2	27.4	23.2
60–74	51.7	42.9	46.5
≥ 75	22.4	23.8	23.2
Current smoker (%)			
Yes	20.7	31.0	26.8
No	79.3	69.0	73.2
%MMA			
25th percentile	9.0	10.5	9.6
Median	13.3	14.9	13.4
75th percentile	17.2	18.2	17.5
%DMA			
25th percentile	62.3	64.0	63.5
Median	70.5	71.1	71.0
75th percentile	79.3	76.3	76.9
%InAs			
25th percentile	10.7	12.4	11.4
Median	14.7	15.1	15.0
75th percentile	19.3	19.3	19.4
Sample size	58	84	142

2.3. Arsenic analysis

Urine samples were kept frozen in the field laboratories at −20 °C and then transported on dry ice to the University of Washington, Seattle, for analysis. The urinary concentrations were measured using hydride generation atomic absorption spectroscopy and details of the laboratory methods are described in Chung et al. (2002). MMA and DMA measurements presented are the sums of the trivalent and pentavalent forms since the trivalent forms, MMA3 and DMA3, are rapidly oxidized during storage and could not be reliably measured. Most samples were stored frozen for 1–4 months before analysis. The relative proportion of arsenic in each species (%InAs, %MMA, and %DMA) was calculated by dividing the concentration of arsenic in each species by the total concentration of arsenic (i.e., InAs, MMA, and DMA combined).

2.4. DNA extraction, quantification and amplification

DNA was isolated from buccal samples using the PUREGENE™ DNA Purification Kit (Gentra Systems Inc., Minneapolis, MN) and quantified using PicoGreen dsDNA quantitation kits (Molecular Probes, Eugene, OR). All DNA samples were whole genome amplified using GenomiPhi DNA Amplification kits (Amersham BioSciences Corp., Piscataway, NJ).

2.5. Gene and SNP selection

Polymorphisms in the *CBS*, *MTHFR*, *MTR*, *TYMS*, *DHFR* and *SHMT1* genes were selected because they encode enzymes involved in folate metabolism. Two

additional polymorphisms in *GST01* were chosen due to the modest influence of *GST01* SNPs on urinary %MMA in previous reports (Lindberg et al., 2007; Marnell et al., 2003; Meza et al., 2005; Steinmaus et al., 2007). Polymorphisms were selected, especially those with non-synonymous amino acid changes, using the dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) and SNPper (http://snpper.chip.org/) databases (Table 2).

2.6. Genotyping

Genotyping was carried out using TaqMan® SNP Genotyping Assays (Applied Biosystems, Foster City, CA). Amplification reactions (95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min) were performed on the ABI 9700 GeneAmp PCR system and a post-PCR read using the ABI 7700 SDS was performed to determine genotypes.

2.7. Statistical analyses

Detection limits for InAs, MMA, and DMA were 0.5, 1.0, and 2.0 μg/L, respectively. The corresponding replicate precisions were 15%, 17%, and 11%. Accuracy was achieved by using NIST-traceble calibrants for inorganic arsenic and by cross-validating gravimetric standard solutions for individual organic arsenic species to the NIST-traceable standard solutions using both species-specific (HPLC/ICP/MS) and total arsenic (ICP/MS) response. Accuracy was assessed for actual samples by analysis of a NIST-traceable calibrant at least once per 10 actual samples, in the concentration range of 10–100 ppb. For the 21 check samples run interspersed with study samples, the average absolute error was 9%. Subjects with concentrations below the detection limit were omitted from the final sample since small errors at estimating low concentrations can have marked effects on calculating metabolite proportions.

For each SNP, the associations between genotypes and %MMA, %DMA and %InAs were analyzed with multivariate linear regression analysis. We controlled the false discovery rate (FDR) using the Benjamini–Hochberg procedure and we report "*q*-values" to aid the reader in interpreting the *p*-values in the context of many tests (Benjamini and Hochberg, 1995). A *q*-value reports the minimum FDR that occurs when the result may be considered statistically significant. In the regression models, we adjusted for age, gender, current smoking status and total level of excreted arsenic defined as the sum of MMA, DMA and InAs in the same sample from which %MMA, %DMA and %InAs were measured. We also adjusted for case–control status in our final models; however, results did not differ when case–control status was not included in the regression models for comparison.

For these analyses, we modeled the relationship between genotypes and %MMA, %DMA and %InAs two ways. The first model treated genotype as categorical and thus allowed for different relationships between heterozygous vs. wild-type and variant homozygous vs. wild-type. The second model combined the heterozygous and variant homozygous genotypes into one category, thereby estimating the relationship between a variant vs. wild-type. All analyses were performed with the statistical computing software R, version 5.2.1, using glm and the multtest package for multiple test adjustments (Pollard et al., 2005; R Development Core Team, 2008).

3. Results

Results for associations between genetic polymorphisms and %MMA, %DMA and %InAs are listed in Tables 3–5. We found statistically significant associations between *CBS* rs4920037 and rs234709 genotypes and %MMA excreted in urine. Specifically, the *CBS* rs4920037 and 234709 variant alleles were associated

Table 2
Primers and TaqMan™ probes.

Gene	rs Number	Location		Probe/primer or ABI assay ID	5′ – 3′ Sequence (or nucleotide sequence surrounding the probe)
GSTO1	rs4925	Exon 5	C > A, 140Ala > Asp	C_11309430_10	AGAAGCCAAAATAAAGAAGACTATG[A/C]TGGCCTAAAAGAAGAATTTCGTAAA Forward (probe binds to the same strand as forward primer)
GSTO1	rs11509435	Intron 2	GGC/–	F R GGC Deletion	GGAGTCAGCCAGGAGCTT GCTCCGACCCGTTCCC VIC-CCGGCGGCATGTT 6FAM-CCCCCGGCATGTT
MTHFR	rs1801131	Exon 9	1298A > C, 429Glu > Ala	F R A C	CCCGAGAGGTAAAGAACAAAGACTT GGAGGAGCTGCTGAAGATGTG VIC-CAAAGACACTTTCTTC 6FAM-AGACACTTGCTTCACT
MTHFR	rs1801133	Exon 6	677C > T, 222Ala > Val	F R C T	CGGTGCATGCCTTCACAA CTGACCTGAAGCACTTGAAGGA VIC-TGATGAAATCGGCTCC 6FAM-ATGATGAAATCGACTCC
TYMS	rs16430	5′UTR	CTTTAA/–	F R AAGTTA Deletion	AGCTGAGTAACACCATCGATCATG GGACGAATGCAGAACACTTCTTTA VIC-TGGTTATGAACTTTAAAGTT 6FAM-TGGTTATGAACTTTATAGTTG
SHMT1	rs1979277	Exon 13	C > T, 474Leu > Phe	F R T C	CAGAGCCACCCTGAAAGAGTTC AGTGGGCCCGCTCCTTTA VIC-CGCCTCTTTCTTC 6FAM-CGCCTCTCTCTTC
MTR	rs1805087	Exon 27	2756A > G, 919Asp > Gly	F R A G	GAATACTTTGAGGAAATCATGGAAGA TCTGTTTCTACCACTTACCTTGAGAGACT VIC-AGACAGGACCATTATG 6FAM-ACAGGGCCATTATG
CBS	rs234709	Intron 5	C > T	C_1605447_10	CCCTACAGCCTCCCATTGTCCTCCT[C/T]CCCC TGTGGGGCGTCACCTGCCCGG Forward
CBS	rs4920037	Intron 12	G > A	C_1605440_1	GAGTCCCCAGAGGTCTAGATCAACT[A/G]TGGT CCCTACAGGGCCTCTGCTGCC Reverse (probe binds to the same strand as reverse primer)
DHFR	rs2618372	Intron 6	C > A	C_16035252_10	TTCTCCACTGAGGAATCTTCTGACC[A/C]TTCC AGCCTCCAATGAACCCCAATC Forward

Table 3
Association between genotype[a] and %MMA.

SNP	*N* variant (%)	Coefficient[b]	95% CI[b]	*p*-Value[b]	*q*-Value (FDR)[c]
CBS rs4920037 G > A (*N*=124)					
GA vs. GG	50 (40.3)	0.042	(0.015,0.069)	0.003	0.030
AA vs. GG	15 (12.1)	0.032	(−0.007,0.071)	0.108	0.943
GA, AA vs. GG	65 (52.4)	0.038	(0.012,0.065)	0.005	0.032
CBS rs234709 C > T (*N*=125)					
CT vs. CC	38 (30.4)	0.035	(0.009,0.061)	0.010	0.049
TT vs. CC	4 (3.2)	0.020	(−0.019,0.058)	0.318	0.943
CT, TT vs. CC	42 (33.6)	0.034	(0.011,0.058)	0.006	0.032
MTHFR rs1801133 Ala > Val (*N*=125)					
Ala/Val vs. Ala/Ala	57 (45.6)	−0.014	(−0.042,0.014)	0.330	0.868
Val/Val vs. Ala/Ala	19 (15.2)	−0.028	(−0.090,0.033)	0.368	0.943
Ala/Val, Val/Val vs. Ala/Ala	76 (60.8)	−0.006	(−0.034,0.018)	0.678	0.988
MTHFR rs1801131 Glu > Ala (*N*=125)					
Glu/Ala vs. Glu/Glu	42 (33.6)	−0.013	(−0.042,0.016)	0.367	0.868
Ala/Ala vs. Glu/Glu	15 (12.0)	0.018	(−0.033,0.069)	0.493	0.943
Glu/Ala, Ala/Ala vs. Glu/Glu	57 (48.6)	−0.008	(−0.033,0.020)	0.577	0.988
DHFR rs2618372 C > A (*N*=123)					
CA vs. CC	49 (39.8)	0.002	(−0.024,0.029)	0.868	0.868
AA vs. CC	6 (4.9)	0.003	(−0.068,0.073)	0.943	0.943
CA, AA vs. CC	54 (44.7)	0.003	(−0.022,0.029)	0.838	0.988
SHMT1 rs1979277 Leu > Phe (*N*=125)					
Leu/Phe vs. Leu/Leu	46 (36.8)	0.008	(−0.019,0.035)	0.574	0.868
Phe/Phe vs. Leu/Leu	11 (8.8)	−0.013	(−0.060,0.033)	0.576	0.943
Leu/Phe, Phe/Phe vs. Leu/Leu	57 (45.6)	0.004	(−0.022,0.029)	0.774	0.988
MTR rs1805087 Asp > Gly (*N*=122)					
Asp/Gly vs. Asp/Asp	30 (24.6)	0.005	(−0.026,0.035)	0.767	0.868
Gly/Gly vs. Asp/Asp	6 (4.9)	0.009	(−0.032,0.051)	0.665	0.943
Asp/Gly, Gly/Gly vs. Asp/Asp	36 (29.5)	−0.001	(−0.031,0.026)	0.952	0.988
GSTO1 rs11509435 GGC/− (*N*=122)					
+/− vs. +/+	52 (42.6)	−0.003	(−0.030,0.025)	0.854	0.854
−/− vs. +/+	12 (9.8)	0.003	(−0.042,0.048)	0.888	0.888
+/−, −/− vs. −/−	64 (52.4)	0.000	(−0.027,0.024)	0.912	0.988
*TYMS*_rs16430 CTTTAA/− (*N*=121)					
+/− vs. +/+	56 (46.3)	0.006	(−0.022,0.034)	0.664	0.868
−/− vs. +/+	15 (12.4)	−0.010	(−0.053,0.032)	0.637	0.943
+/−, −/− vs. −/−	71 (58.7)	0.003	(−0.026,0.027)	0.832	0.977
*GSTO1*_rs4925 Ala > Asp (*N*=122)					
Ala/Asp vs. Ala/Ala	57 (46.7)	0.000	(−0.030,0.024)	0.829	0.868
Asp/Asp vs. Ala/Ala	9 (7.4)	0.023	(−0.054,0.066)	0.846	0.943
Ala/Asp, Asp/Asp vs. Ala/Ala	66 (54.1)	0.038	(−0.025,0.026)	0.977	0.977

[a] Sample sizes vary by SNP due to missing genotype values.
[b] Obtained from multivariate linear regression, adjusting for age, gender, current smoking status and total level of excreted arsenic.
[c] The false discovery rate (FDR) was controlled using the Benjamini – Hochberg procedure.

with 3.8 and 3.4 percentage point increases in %MMA (*CBS* rs4920037: *p*-value=0.005, *q*-value=0.032; *CBS* rs234709: *p*-value=0.006, *q*-value=0.032) (Table 3). We found that at the mean level of %MMA (14.4%), the *CBS* rs4920037 variant compared to wild-type genotypes was associated with an overall 26% increase in %MMA (from 14.4%MMA to 18.2%MMA). Similarly, the rs234709 variant compared to wild-type genotype was associated with a 24% increase in %MMA (from 14.4%MMA to 18.8%MMA). No other associations were found between genetic polymorphisms and %MMA (Table 3).

Negative associations were observed between *CBS* genotypes and urinary %DMA where the *CBS* rs4920037 and rs234709 variant alleles were associated with 4.8 and 5.0 percentage point decreases in %DMA (CBS rs4920037: *p*-value=0.020, *q*-value=0.108; CBS rs234709: *p*-value=0.010 and *q*-value=0.098) (Table 4). At the mean level of %DMA (69.7%) when comparing *CBS* variant to wild-type genotypes, our findings indicate 7% decreases from 69.7%MMA to 64.7%MMA associated with the *CBS* 4920037 SNP and from 69.7%DMA to 64.9%DMA associated with the *CBS* rs234709. No other associations were found between genetic polymorphisms and %DMA (Table 3).

No associations were seen between *MTHFR*, *MTR*, *TYMS*, *SHMT1* and *GST01* genotypes and urinary %MMA or %DMA. No associations were observed between any polymorphisms investigated and %InAs (Table 5). The mean levels and mean %MMA, DMA and InAs by genotype are listed in Table 6.

4. Discussion

Here we present the first study to report that genetic variation in the *CBS* gene influences urinary MMA and DMA levels. Specifically, we found that there were 26% and 24% higher mean %MMA levels associated with the *CBS* rs234709 and rs4920037 variants compared to wild-type carriers in this arsenic exposed population. As the urinary %MMA in our population ranged from near zero to 40%, the magnitude of the change in the mean levels of %MMA associated with these *CBS* variants could be considered

Table 4
Association between genotype[a] and %DMA.

SNP	N variant (%)	Coefficient[b]	95% CI[b]	*p*-Value[b]	*q*-Value (FDR)[c]
CBS rs4920037 G > A (*N*=124)					
GA vs. GG	50 (40.3)	−0.049	(−0.091, −0.007)	0.025	0.123
AA vs. GG	15 (12.1)	−0.027	(−0.085,0.032)	0.376	0.740
GA, AA vs. GG	65 (52.4)	−0.048	(−0.089, −0.008)	0.022	0.108
CBS rs234709 C > T (*N*=125)					
CT vs. CC	38 (30.4)	−0.052	(−0.092, −0.013)	0.011	0.111
TT vs. CC	4 (3.2)	−0.040	(−0.101,0.02)	0.192	0.740
CT, TT vs. CC	42 (33.6)	−0.050	(−0.087, −0.013)	0.010	0.098
MTHFR rs1801133 Ala > Val (*N*=125)					
Ala/Val vs. Ala/Ala	57 (45.6)	0.001	(−0.041,0.044)	0.959	0.959
Val/Val vs. Ala/Ala	19 (15.2)	0.000	(−0.092,0.091)	0.994	0.994
Ala/Val, Val/Val vs. Ala/Ala	76 (60.8)	−0.006	(−0.046,0.034)	0.782	0.875
MTHFR rs1801131 Glu > Ala (*N*=125)					
Glu/Ala vs. Glu/Glu	42 (33.6)	0.024	(−0.02,0.067)	0.294	0.924
Ala/Ala vs. Glu/Glu	15 (12.0)	0.031	(−0.039,0.102)	0.385	0.740
Glu/Ala, Ala/Ala vs. Glu/Glu	57 (48.6)	0.021	(−0.019,0.062)	0.305	0.875
DHFR rs2618372 C > A (*N*=123)					
CA vs. CC	49 (39.8)	0.006	(−0.035,0.047)	0.768	0.959
AA vs. CC	6 (4.9)	0.014	(−0.049,0.078)	0.657	0.740
CA, AA vs. CC	54 (44.7)	0.005	(−0.034,0.045)	0.787	0.875
SHMT1 rs1979277 Leu > Phe (*N*=125)					
Leu/Phe vs. Leu/Leu	46 (36.8)	0.001	(−0.04,0.042)	0.950	0.959
Phe/Phe vs. Leu/Leu	11 (8.8)	0.017	(−0.06,0.094)	0.666	0.740
Leu/Phe, Phe/Phe vs. Leu/Leu	57 (45.6)	0.007	(−0.031,0.046)	0.718	0.875
MTR rs1805087 Asp > Gly (*N*=122)					
Asp/Gly vs. Asp/Asp	30 (24.6)	−0.018	(−0.062,0.025)	0.437	0.924
Gly/Gly vs. Asp/Asp	6 (4.9)	0.028	(−0.036,0.093)	0.389	0.740
Asp/Gly, Gly/Gly vs. Asp/Asp	36 (29.5)	−0.010	(−0.054,0.033)	0.642	0.875
GSTO1 rs11509435 GGC/− (*N*=122)					
+/− vs. +/+	52 (42.6)	0.013	(−0.029,0.054)	0.554	0.924
−/− vs. +/+	12 (9.8)	0.030	(−0.063,0.123)	0.524	0.740
+/−, −/− vs. −/−	64 (52.4)	0.014	(−0.025,0.053)	0.488	0.875
*TYMS*_rs16430 CTTTAA/− (*N*=121)					
+/− vs. +/+	56 (46.3)	−0.008	(−0.051,0.034)	0.707	0.959
−/− vs. +/+	15 (12.4)	0.020	(−0.048,0.088)	0.570	0.740
+/−, −/− vs. −/−	71 (58.7)	−0.001	(−0.041,0.04)	0.969	0.969
*GSTO1*_rs4925 Ala > Asp (*N*=122)					
Ala/Asp vs. Ala/Ala	57 (46.7)	0.014	(−0.027,0.055)	0.509	0.924
Asp/Asp vs. Ala/Ala	9 (7.4)	−0.042	(−0.151,0.067)	0.452	0.740
Ala/Asp, Asp/Asp vs. Ala/Ala	66 (54.1)	0.014	(−0.025,0.054)	0.480	0.875

[a] Sample sizes vary by SNP due to missing genotype values.
[b] Obtained from multivariate linear regression, adjusting for age, gender, current smoking status and total level of excreted arsenic.
[c] The false discovery rate (FDR) was controlled using the Benjamini–Hochberg procedure.

substantial. There was also a modest inverse association between these *CBS* variants and urinary %DMA, where we found a 7% decrease in mean levels of %DMA in association with the *CBS* rs4920037 and rs234709 SNPs when compared to wild-type genotypes. The differences in %MMA and %DMA associated with genetic variation in *CBS* in this study is equal to or larger than corresponding differences seen with other factors shown to influence arsenic metabolism including gender, certain dietary variables, and smoking (Hsueh et al., 2003; NRC, 1999; Steinmaus et al., 2007, 2005; Vahter, 1999). These differences are also within the range of differences in %MMA associated with arsenic-associated cancer and other diseases. For example, in several studies of %MMA and arsenic-related diseases, the differences in %MMA between cases and controls ranged between 1–5% which is similar to the magnitude we found associated with the CBS variants (Ahsan et al., 2007; Pu et al., 2007; McCarty et al., 2007; Steinmaus et al., 2006; Huang et al., 2008). These findings suggest that genetic variation in the CBS gene has a significant impact on arsenic metabolism and could affect arsenic-related disease risks.

CBS is an important enzyme in the transsulfuration pathway (Vahter and Marafante, 1987) that catalyzes the conversion of homocysteine (HCY) to cystathionine, a substrate for glutathionine synthesis. Perturbations in the transsulfuration pathway such as *CBS* deficiency result in severe hyperhomocysteinemia, while *CBS* gene variants may moderately influence *CBS* enzyme activity and circulating HCY levels (Fredriksen et al., 2007). Elevated circulating HCY is associated with genome hypomethylation and a reduction in S-adenosylmethionine levels (Jiang et al., 2007). The latter is an important methyl donor for arsenic metabolism and other methylation processes (Vahter, 2002). Furthermore, the influence of plasma HCY and folate status on urinary and/or blood MMA and DMA metabolites (Gamble et al., 2007; Hall et al., 2007) and on arsenic-related disease (Huang et al., 2008) further highlight the importance of folate in arsenic metabolism and toxicity. Both *CBS* rs234709 and rs4920037 SNPs

Table 5
Association between genotype[a] and %INAS.

SNP	*N* variant (%)	Coefficient[b]	95% CI[b]	*p*-Value[b]	*q*-Value (FDR)[c]
CBS rs4920037 G > A (*N*=124)					
GA vs. GG	50 (40.3)	0.007	(−0.018,0.031)	0.589	0.654
AA vs. GG	15 (12.1)	0.027	(−0.026,0.08)	0.836	0.929
GA, AA vs. GG	65 (52.4)	0.010	(−0.014,0.034)	0.419	0.524
CBS rs234709 C > T (*N*=125)					
CT vs. CC	38 (30.4)	0.018	(−0.006,0.041)	0.145	0.606
TT vs. CC	4 (3.2)	−0.035	(−0.079,0.009)	0.121	0.623
CT, TT vs. CC	42 (33.6)	0.015	(−0.007,0.037)	0.171	0.524
MTHFR rs1801133 Ala > Val (*N*=25)					
Ala/Val vs. Ala/Ala	57 (45.6)	0.013	(−0.012,0.037)	0.308	0.606
Val/Val vs. Ala/Ala	19 (15.2)	−0.024	(−0.06,0.012)	0.201	0.623
Ala/Val, Val/Val vs. Ala/Ala	76 (60.8)	0.011	(−0.012,0.034)	0.336	0.524
MTHFR rs1801131 Glu > Ala (*N*=125)					
Glu/Ala vs. Glu/Glu	42 (33.6)	−0.010	(−0.035,0.015)	0.427	0.606
Ala/Ala vs. Glu/Glu	15 (12.0)	−0.018	(−0.058,0.022)	0.382	0.637
Glu/Ala, Ala/Ala vs. Glu/Glu	57 (48.6)	−0.014	(−0.037,0.01)	0.252	0.524
DHFR rs2618372 C > A (*N*=123)					
CA vs. CC	49 (39.8)	−0.008	(−0.032,0.015)	0.484	0.606
AA vs. CC	6 (4.9)	0.007	(−0.027,0.041)	0.686	0.857
CA, AA vs. CC	54 (44.7)	−0.008	(−0.031,0.015)	0.485	0.539
SHMT1 rs1979277 Leu > Phe (*N*=125)					
Leu/Phe vs. Leu/Leu	46 (36.8)	−0.009	(−0.033,0.014)	0.446	0.606
Phe/Phe vs. Leu/Leu	11 (8.8)	0.018	(−0.017,0.054)	0.664	0.857
Leu/Phe, Phe/Phe vs. Leu/Leu	57 (45.6)	−0.011	(−0.033,0.011)	0.334	0.524
MTR rs1805087 Asp > Gly (*N*=122)					
Asp/Gly vs. Asp/Asp	30 (24.6)	0.011	(−0.014,0.036)	0.310	0.606
Gly/Gly vs. Asp/Asp	6 (4.9)	0.039	(−0.024,0.103)	0.228	0.623
Asp/Gly, Gly/Gly vs. Asp/Asp	36 (29.5)	0.011	(−0.014,0.036)	0.377	0.524
GSTO1 rs11509435 GGC/− (*N*=122)					
+/− vs. +/+	52 (42.6)	−0.010	(−0.034,0.014)	0.411	0.606
−/− vs. +/+	12 (9.8)	−0.018	(−0.055,0.019)	0.341	0.637
+/−, −/− vs. −/−	64 (52.4)	−0.012	(−0.035,0.01)	0.280	0.524
*TYMS*_rs16430 CTTTAA/− (*N*=121)					
+/− vs. +/+	56 (46.3)	0.002	(−0.022,0.026)	0.880	0.880
−/− vs. +/+	15 (12.4)	−0.002	(−0.055,0.051)	0.945	0.945
+/−, −/− vs. −/−	71 (58.7)	−0.002	(−0.025,0.021)	0.859	0.859
*GSTO1*_rs4925 Ala > Asp (*N*=122)					
Ala/Asp vs. Ala/Ala	57 (46.7)	−0.011	(−0.034,0.012)	0.364	0.606
Asp/Asp vs. Ala/Ala	9 (7.4)	−0.023	(−0.062,0.016)	0.249	0.623
Ala/Asp, Asp/Asp vs. Ala/Ala	66 (54.1)	−0.014	(−0.037,0.009)	0.225	0.524

[a] Sample sizes vary by SNP due to missing genotype values.
[b] Obtained from multivariate linear regression, adjusting for age, gender, current smoking status and total level of excreted arsenic.
[c] The false discovery rate (FDR) was controlled using the Benjamini–Hochberg procedure.

are in linkage disequilibrium (LD) in HapMap CEU with a *CBS* 699C > T SNP (rs234706, r^2=0.69 and 0.54, respectively) shown to exert modest effects on circulating HCY levels. Thus, the observed increase in %MMA and decrease in %DMA associated with *CBS* rs234709 and rs4920037 variants in our study population may be due to their linkage with rs234706 or another *CBS* functional SNP that influences HCY and S-adenosylmethionine production. Validation studies will be needed to determine the relevance of *CBS* gene variants in independent populations and fine mapping studies will be necessary to determine potentially causal *CBS* variants that may influence MMA:DMA ratios and arsenic toxicity.

We found no association with the combined *MTHFR* 677TT/1298AA genotype and urinary arsenic metabolite proportions as we reported in a previous study (Steinmaus et al., 2007). However, the relatively small sample size in the current analyses may have restricted our ability to detect differences in arsenic metabolism between combined *MTHFR* genotypes.

There is increasing evidence that MMA3 is more toxic than its pentavalent form and may be more toxic than InAs or DMA (Cullen et al., 1989; Lin et al., 1999; Mass et al., 2001; Petrick et al., 2000; Styblo et al., 1999). In a recent study in an arsenic-exposed region in Mexico, %MMA3 levels were higher in subjects with arsenic-induced skin lesions (mean %MMA3=7.7%, n=55) than in exposed subjects without skin lesions (mean %MMA3=5.9%, n=21, p=0.072) (Valenzuela et al., 2005). Based on this and in vitro studies showing MMA3 is highly acutely toxic, it is plausible that MMA3 is the primary toxic species and its relative proportion in humans plays a role in disease susceptibility. Since MMA3 is highly unstable in human urine, we present our findings for total MMA (MMA3 and MMA5 combined). As such, we do not know if our *CBS* findings represent effects of MMA3, MMA5, or both. If they are due to MMA3, basing our analyses on total MMA would likely have attenuated the effect estimate, since total MMA is most likely not an accurate marker for MMA3.

Table 6
%MMA, %DMA and %INAS by genotype.

SNP	MMA		DMA		INAS	
	Mean	%	Mean	%	Mean	%
CBS rs4920037 G > A (*N*=124)						
GG	3.2	13.5	16.8	71.5	3.5	15.0
GA	3.5	17.5	13.2	66.7	3.1	15.8
AA	5.0	14.5	22.9	66.7	6.5	18.8
GA/AA	3.6	17.2	14.1	66.7	3.4	16.1
CBS rs234709 C > T (*N*=125)						
CC	2.8	12.8	16.1	72.6	3.2	14.5
CT	4.0	16.4	16.3	67.4	3.9	16.2
TT	3.3	16.1	14.2	68.3	3.2	15.6
CT/TT	3.8	16.3	15.8	67.6	3.8	16.1
MTHFR rs1801133 Ala > Val (*N*=125)						
Ala/Ala	4.3	15.3	19.9	70.1	4.1	14.6
Ala/Val	2.7	13.7	14.0	70.3	3.2	16.0
Val/Val	3.0	16.9	11.8	67.8	2.7	15.3
Ala/Val, Val/Val	2.8	14.5	13.4	69.7	3.0	15.8
MTHFR rs1801131 Glu > Ala (*N*=125)						
Glu/Glu	2.9	15.0	13.2	69.0	3.1	16.0
Glu/Ala	3.8	14.0	19.3	71.1	4.0	14.8
Ala/Ala	4.4	16.4	18.9	70.1	3.6	13.5
Glue/Ala, Ala/Ala	4.0	14.6	19.2	70.9	3.9	14.5
DHFR rs2618372 C > A (*N*=123)						
CC	3.0	14.5	14.5	69.7	3.3	15.8
CA	3.8	14.7	18.4	70.3	3.9	15.0
AA	2.7	14.9	12.8	70.2	2.7	14.9
CA, AA	3.7	14.8	17.8	70.3	3.8	15.0
SHMT1 rs1979277 Leu > Phe (*N*=125)						
Leu/Leu	3.3	14.7	15.7	69.5	3.6	15.8
Leu/Phe	3.9	15.5	17.3	69.6	3.7	14.9
Phe/Phe	1.9	12.9	10.7	72.9	2.1	14.2
Leu/Phe, Phe/Phe	3.4	15.0	16.1	70.3	3.4	14.7
MTR rs1805087 Asp > Gly (*N*=122)						
Asp/Asp	3.7	15.0	17.2	69.9	3.7	15.1
Asp/Gly	2.8	15.1	12.6	68.4	3.0	16.5
Gly/Gly	2.1	12.0	12.6	72.9	2.6	15.1
Asp/Gly, Gly/Gly	2.7	14.6	12.6	69.2	3.0	16.2
GSTO1 rs11509435 GGC/− (*N*=122)						
+/+	3.4	14.9	15.8	69.1	3.7	16.0
+/−	3.4	14.4	16.8	70.5	3.6	15.0
−/−	2.6	14.9	12.4	71.2	2.4	13.9
+/−, −/−	3.3	14.5	16.0	70.7	3.3	14.8
*TYMS*_rs16430 CTTTAA/− (*N*=121)						
+/+	3.8	14.7	18.0	69.7	4.0	15.6
+/−	3.0	15.3	13.8	69.1	3.1	15.7
+/−	2.7	13.6	14.6	72.7	2.8	13.7
+/−, −/−	3.0	14.9	13.9	69.8	3.0	15.2
*GSTO1*_rs4925 Ala > Asp (*N*=122)						
Ala/Ala	3.4	14.9	15.9	69.0	3.7	16.1
Ala/Asp	3.4	14.5	16.6	70.3	3.6	15.2
Asp/Asp	3.3	16.4	14.2	70.7	2.6	12.9
Ala/Asp, Asp/Asp	3.4	14.8	16.3	70.4	3.5	14.9

A limitation of this study is that only one urine sample was collected from each subject. Previous studies found that methylation patterns remain fairly stable over time, although some day-to-day variability exists (Concha et al., 2002; Steinmaus et al., 2005), This variability, along with laboratory imprecision could have led to some misclassification of methylation status. Because samples were collected, stored, and analyzed independent of genetic status, these factors would most likely have had non-differential effects, biasing our results towards the null, and would not have produced the positive associations identified.

Another consideration is whether the proportions of urinary arsenic metabolites, which comprise the primary route of arsenic excretion (Buchet et al., 1981) are directly relevant to levels at the target organ sites. Experimental studies showed that chemically inhibiting methylation reactions in vivo results in increased tissue retention of arsenic, an increase in the proportion of arsenic excreted in the urine as InAs, and a decrease in the proportion excreted as DMA (Marafante and Vahter, 1984; Marafante et al., 1985). Furthermore, several epidemiologic studies have reported that subjects with high urinary %MMA values have higher risks of arsenic-associated disease (Chen et al., 2003a; Del Razo et al., 1997; Hsueh et al., 1997; Maki-Paakkanen et al., 1998; Valenzuela et al., 2005). These studies provide some evidence that the proportion of urinary arsenic metabolites reflect internal arsenic metabolism and are related to the toxic effects of arsenic at the target organ sites.

5. Conclusion

Our findings suggest that *CBS* SNPs may influence arsenic metabolism in humans. This may account for some of the inter-individual variability seen in the arsenic metabolic process. Because variances in arsenic metabolism have been linked to differences in arsenic-induced cancer risks (Chen et al., 2003a, 2003b; Hsueh et al., 1997; Yu et al., 2000), these findings provide new evidence that *CBS* SNPs may affect susceptibility to arsenic-induced disease. Although this study was relatively small, the results are important in that they can help guide the selection of factors that should be further investigated in future larger studies. In addition to work on *CBS*, future research is also needed to investigate the role of MMA3 and to help identify other factors that may control arsenic metabolism and confer arsenic-disease susceptibility. This type of research may help to identify subpopulations that are particularly susceptible to arsenic-associated disease and could provide important new insights into the carcinogenic mechanisms of this common drinking water contaminant.

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