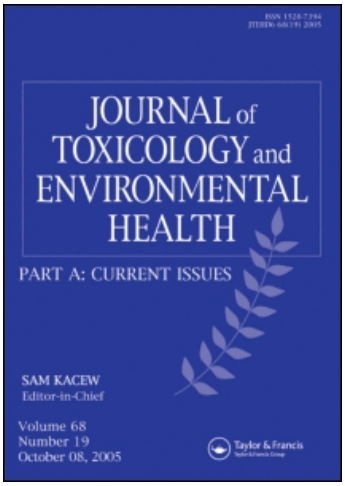

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Genetic Polymorphisms in MTHFR 677 and 1298, GSTM1 and T1, and Metabolism of Arsenic

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Genetic Polymorphisms in MTHFR 677 and 1298, GSTM1 and T1, and Metabolism of Arsenic

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Methylation is the primary route of metabolism of inorganic arsenic in humans, and previous studies showed that interindividual differences in arsenic methylation may have important impacts on susceptibility to arsenic-induced cancer. To date, the factors that regulate arsenic methylation in humans are mostly unknown. Urinary arsenic methylation patterns and genetic polymorphisms in methylenetetrahydrofolate reductase (MTHFR) and glutathione *S*-transferase (GST) were investigated in 170 subjects from an arsenic-exposed region in Argentina. Previous studies showed that subjects with the TT/AA polymorphisms at MTHFR 677 and 1298 have lower MTHFR activity than others. In this study, it was found that subjects with the TT/AA variant of MTHFR 677/1298 excreted a significantly higher proportion of ingested arsenic as inorganic arsenic and a lower proportion as dimethylarsinic acid. Women with the null genotype of GSTM1 excreted a significantly higher proportion of arsenic as monomethylarsonate than women with the active genotype. No associations were seen between polymorphisms in GSTT1 and

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arsenic methylation. This is the first study to report (1) associations between MTHFR and arsenic metabolism in humans, and (2) gender differences between genetic polymorphisms and urinary arsenic methylation patterns. Overall, this study provides evidence that MTHFR and GSTM1 are involved in arsenic metabolism in humans, and polymorphisms in the genes that encode these enzymes may play a role in susceptibility to arsenic-induced cancer.

Millions of people worldwide are exposed to drinking water containing inorganic arsenic (InAs), and exposure to InAs has been associated with cancers of the skin, bladder, and lung (Bernstam & Nriagu, 2000; IARC, 2002; Nordstrom, 2002). Susceptibility to the health effects of arsenic appears to vary widely, and the results of several previous studies showed that interindividual differences in arsenic metabolism may be responsible for some of these susceptibility differences (Chen et al., 2003a, 2003b; Del Razo et al., 1997; Golub et al., 1998; Hsueh et al., 1997; Maki-Paakkanen et al., 1998; Steinmaus et al., 2006; Yu et al., 2000).

The primary metabolic pathway of ingested InAs in humans is methylation (Gebel, 2002; Styblo et al., 2002; Vahter, 2002). Once ingested, InAs is methylated to monomethylarsonic acid [MMA(V)] which is reduced to monomethylarsonous acid [MMA(III)]. MMA(III) is then methylated to dimethylarsinic acid [DMA(V)], which is reduced to dimethylarsinous acid [DMA(III)]. In humans, this process is not complete, and some arsenic remains as InAs and MMA. Urinary excretion is the primary pathway of elimination of arsenic, and the relative distribution of methylated and unmethylated arsenic metabolites in urine is commonly used as a biomarker of the degree to which individuals fully methylate ingested InAs (NRC, 1999). Typically, ingested InAs is excreted as 10–20% InAs, 10–15% MMA, and 60–75% DMA (Hopenhayn-Rich et al., 1993). However, large interindividual variations exist (Vahter, 1999a).

Until recently, methylation of InAs was thought to be primarily a detoxification pathway since the methylated species most commonly measured in human urine samples, MMA(V) and DMA(V), are more water-soluble, more readily excreted, and less acutely toxic than InAs (Buchet et al., 1981a, 1981b; Gebel, 2002; Hughes & Kenyon, 1998; Moore et al., 1997). MMA(III) and DMA(III) are highly unstable in human urine and so have been measured in only a few human studies. However, MMA(III) was shown to be much more toxic in vitro than its pentavalent form, and may even be more toxic than trivalent InAs (Cullen et al., 1989; Lin et al., 1999, 2001; Mass et al., 2001; Petrick et al., 2000; Styblo et al., 1997, 1999, 2000). In addition, several epidemiological studies reported that those who excrete a high proportion of ingested arsenic as MMA (%MMA) have higher risks of arsenic-induced skin lesions, skin cancer, and bladder cancer than those who have lower %MMA levels (Chen et al., 2003a, 2003b; Del Razo et al., 1997; Hsueh et al., 1997; Maki-Paakkanen et al., 1998; Steinmaus et al., 2006; Valenzuela et al., 2005; Yu et al., 2000). As a whole, these studies provide a consistent body of evidence that interindividual differences in arsenic metabolism are correlated with susceptibility to arsenic-induced disease.

Methylenetetrahydrofolate reductase (MTHFR) and glutathione *S*-transferases (GSTs) have been linked to arsenic metabolism in several laboratory and animal investigations (NRC, 1999); but, data from human studies is limited. GSTs may play a role in the reduction of pentavalent arsenic to trivalent arsenic (Aposhian et al., 2004), while MTHFR is a key enzyme in the metabolism of folate which has been linked to both arsenic metabolism and toxicity (NRC, 1999). In this study, the impact of genetic polymorphisms in MTHFR, GSTM1, and GSTT1 on urinary arsenic methylation patterns was assessed in 170 subjects from an arsenic-exposed region in Argentina. Investigating associations between MTHFR, GSTM1, and GSTT1 polymorphisms and arsenic methylation may help determine whether the enzymes encoded by these genes play a role in arsenic metabolism in humans. This work may also provide new information on whether these genetic polymorphisms may be responsible for some of the wide interindividual variability seen in arsenic metabolism. Further, since differences in arsenic metabolism have been linked to differences in arsenic-induced cancer risks, this study could also provide new information on the factors that impact susceptibility to arsenic-associated disease.

MATERIALS AND METHODS

The subjects in this study were recruited as part of an earlier case-control study on arsenic ingestion and bladder cancer (Bates et al., 2004). The study area consisted of Union and Marcos Juarez, two contiguous counties in the eastern part of the Province of Córdoba, Argentina. Bladder cancer cases were identified through rapid case ascertainment involving all pathologists and urologists in the study area. Controls, individually matched to cases by county, gender, and exact year of birth, were selected from computerized voter registration lists. Voter registration lists in Argentina include almost the total population, and all bladder cancer cases identified for this study were found on these lists. Further details on subject recruitment and participation rates are available elsewhere (Bates et al., 2004). This study was approved by institutional review boards in the United States and Argentina, and informed consent was obtained from all participants.

All subjects were visited at their homes and asked to provide a single first morning urine sample. A previous study found that a moderately strong correlation exists between arsenic excretion in single first morning samples and samples collected over 24 h (Calderon et al., 1999). Basic demographic information was collected using a standard questionnaire. All urine samples were immediately placed on ice, then frozen at −20°C upon return to the field laboratory on the same day. Urine samples were transported on dry ice to the University of

Washington, Seattle, for analysis. The urinary concentrations were measured using hydride generation atomic absorption spectroscopy (Crecelius, 1978). The details of the laboratory methods are described elsewhere (Chung et al., 2002). Detection limits for InAs, MMA, and DMA were 0.5, 1, and 2 μg/L, respectively. The corresponding replicate precisions were 15%, 17%, and 11%. Total arsenic, including arsenic in both the organic and inorganic forms, was determined by flow injection analysis/atomic fluorescence spectrometry, and this result was compared with the sum of the species (InAs, MMA, DMA) detected. If a significant amount of arsenic remained undetected, additional digestion or assay for arsenobetaine was performed. Several urine samples had concentrations of InAs, MMA, or DMA below the detection limit. Because the precision of the proportions of arsenic species were very unstable in samples with low arsenic concentrations, this analysis was limited to only those subjects where the sum of InAs, MMA, and DMA was above 5 μg/L. The MMA and DMA measured in this study are the sums of the trivalent and pentavalent forms. Because of their rapid oxidation, MMA(III) and DMA(III) could not be reliably measured in our study (Del Razo et al., 2001; Feldmann et al., 1999; Le et al., 2000). Most samples were stored frozen for 1 to 12 mo before analysis.

Buccal cells were obtained from all subjects as the source of DNA. Methods of sample collection are described in Moore et al. (2004). Samples were transported on dry ice to the United States, where they were centrifuged, and DNA was extracted using the QIAmp DNA Mini kit according to the manufacturer's instructions (catalogue number 51304, QIAGEN, Valencia, CA). PCR was performed with a Gene Amp PCR 9600 thermal cycler (Perkin Elmer). Additional details on PCR and genotyping are described elsewhere (Moore et al., 2004; Zheng et al., 2001). Genetic polymorphisms in GSTM1, GSTT1, MTHFR, and NAD(P)H:quinione oxidoreductase (NQO1) were assessed. Information on NQO1 polymorphisms and bladder cancer risks are reported elsewhere (Moore et al., 2004); however, NQO1 was not included in this analysis since the mechanism by which NQO1 is hypothesized to affect disease risk does not involve arsenic methylation.

The relative proportion of arsenic in the form of each species (%InAs, %MMA, and %DMA) in urine was calculated by dividing the concentration of arsenic in each species by the sum of arsenic in the form of InAs, MMA, and DMA combined. The associations of each arsenic species proportion with variables such as age, gender, smoking history, and each genetic polymorphism were first assessed in univariate analyses. Student's *t*-test and the Wilcoxon rank-sum test were used to compare category means. Analysis of variance (ANOVA) was used when three or more means were compared, and a Duncan's multiple range test was performed when the p value for the analysis of variance was below .05. Pearson correlation coefficients (R) were used to compare continuous variables that were approximately normally distributed (%InAs, %MMA, %DMA and MMA/DMA); otherwise, Spearman correlation coefficients were used (total urinary arsenic). Initially, all analyses were done separately for cases and controls. However, because no differences were identified between these groups in the relationships between the genetic polymorphisms and arsenic species proportions, cases and controls are pooled in the results presented here. Because the polymorphisms at nucleotides 677 and 1298 of the MTHFR gene are in negative disequilibrium (Stegman et al., 1999), the relationship between methylation and MTHFR polymorphisms was assessed separately for each possible MTHFR 677 and 1298 combination. Because previous studies showed that subjects with the 677TT and 1298AA combination (TT/AA) have lower MTHFR activity than all other genotypes, the TT/AA genotype was compared to all other genotypes (van der Put et al., 1998; Weisberg et al., 1998).

Linear regression analyses were performed in order to assess the effect of the different genetic polymorphisms on the proportion of urinary arsenic species, while controlling for the potential confounding effect of age (continuous), gender, case status (bladder cancer case versus control), smoking (average number of cigarettes smoked per day), and total urinary arsenic (the sum of arsenic as InAs, MMA, and DMA as a continuous variable). In the model assessing MTHFR, GSTM1 status was entered as a dummy variable (null versus active). In the model assessing GSTM1, each MTHFR 677 and 1298 combination was entered as a separate dummy variable. GSTT1 status had no impact on this analysis and was not entered into the final models. Entering age, smoking (never, former, current), or total urinary arsenic as categorical variables had no impact on the results. Because several previous studies showed that gender differences in GST or MTHFR activity may exist (Egaas et al., 1995; Hoensch et al., 2002; Srivastava et al., 2004; Verma & Rana, 2003), separate analyses were performed in men and women. All analyses were carried out using SAS 8.0e (SAS Institute, Cary, NC).

Several studies showed that seafood can cause large increases in urinary arsenic concentrations, most of which is arsenobetaine or other large organic arsenic species (Edmonds & Francesconi, 1993; Buchet et al., 1994, 1996; Cullen & Reimer, 1989; Foa et al., 1984; Vahter & Lind, 1986; Luten et al., 1982; Yamauchi & Yamamura, 1984). Increases in urinary DMA after seafood consumption may also occur, but are generally much smaller (i.e., usually less than 5% of the total arsenic). These findings suggest that arsenic from food would impact the species proportions reported in this study only in those subjects in which total arsenic intake from food was substantially higher than inorganic arsenic intake from water (about 70% of which is excreted as DMA). The study area for this investigation is hundreds of miles inland and seafood is not a major dietary staple so these effects are not expected to have important impacts in this study. Regardless, the potential impacts of arsenic from seafood was assessed by performing separate analyses removing those subjects in which the levels of arsenobetaine and other large organic species were

greater than the sum of InAs, MMA, and DMA. Although arsenobetaine was only measured in a subset of subjects, the combined concentration of arsenobetaine and other large organic species from seafood or other foods was estimated by subtracting the sum of InAs, MMA, and DMA from the total arsenic level measured using flow injection analysis/atomic fluorescence spectrometry.

RESULTS

Of the 228 subjects recruited for the original case-control study, 215 (94%) provided buccal cell samples from which DNA could be extracted, and 170 (75%) of these had urinary arsenic levels above 5 μg/L. Thirty-one subjects were female (18%), 38 were current smokers (22%), 79 had a history of bladder cancer (46%), and the average age was 68 yr (range, 22 to 84 yr) (Table 1). Women had a significantly lower %InAs and a higher %DMA than men, and increasing age was associated with marked decreasing %InAs (Table 1).

No clear associations were seen between arsenic metabolite proportions and genetic polymorphisms in GSTT1 (Table 2). Women with the null genotype of GSTM1 had a significantly higher mean %MMA and a lower mean %DMA than women with the active genotype. Men with the null genotype for GSTM1 also had a higher quantitative mean %MMA and a lower mean %DMA. Subjects with the TT variant of MTHFR 677 had a marked higher mean %InAs than subjects with the CC or CT polymorphisms. This effect was largely confined to men. Men with the AA polymorphism of MTHFR 1298 had a significantly higher mean %InAs than men with the CC variant. Only one woman had the CC variant of MTHFR 1298.

Table 3 shows the mean proportion of each arsenic species for each combination of MTHFR 677 and 1298 genotype. Subjects with the TT/AA genotype had a significantly higher mean %InAs and a lower mean %DMA than subjects with all other genotypes combined ("Non-TT/AA"). This pattern was seen in both men and women, although the differences were larger in men.

TABLE 1
Demographic Variables and Mean Proportions (Standard Deviations) of Arsenic Species in Urine

Variable	N	%	%InAs	%MMA	%DMA	MMA/DMA
All Gender	170	100	15.6 (7.0)	13.9 (6.2)	70.5 (11.0)	0.21 (0.14)
Women	31	18	13.2 (5.5)	12.9 (5.3)	73.9 (9.6)	0.19 (0.10)
Men	139	82	16.2 (7.2)	14.1 (6.4)	69.7 (11.1)	0.22 (0.14)
p- value			.03	.35	.05	.09
Bladder cancer						
Cases	79	46	14.8 (6.4)	13.8 (6.1)	71.4 (10.9)	0.21 (0.14)
Controls	91	54	16.4 (17.4)	13.0 (6.3)	69.7 (11.0)	0.22 (0.14)
p- value			.15	.87	.31	.78
Smoking						
Never	61	36	14.4 (6.2)	13.7 (6.7)	71.9 (11.6)	0.21 (0.14)
Former	71	42	16.0 (6.6)	13.3 (5.8)	70.7 (10.0)	0.20 (0.13)
Current	38	22	17.0 (8.6)	15.3 (6.1)	67.7 (11.3)	0.25 (0.14)
p-value			.16	.25	.17	.29
Age						
<66	62	36	17.8 (7.9)	13.9 (6.0)	68.3 (11.3)	0.22 (0.13)
66–75	70	41	14.0 (5.9)	13.2 (5.8)	72.8 (10.2)	0.20 (0.13)
>75	38	22	15.3 (6.6)	15.1 (7.2)	69.6 (11.1)	0.24 (0.16)
R (*p*-value)[a]			–.20 (.01)	.03 (0.74)	.11 (0.14)	.00 (0.98)
Total urinary arsenic[b]						
Low tertile	57	33	16.2 (7.2)	13.1 (5.9)	70.6 (10.9)	0.20 (0.13)
Medium tertile	57	33	14.9 (6.3)	13.7 (7.0)	71.3 (11.3)	0.21 (0.15)
High tertile	56	33	15.7 (7.4)	14.8 (5.7)	69.5 (10.8)	0.23 (0.13)
R (*p*-value)[c]			–.09 (0.27)	.11 (0.16)	–.01 (0.86)	.10 (0.21)

[a]Pearson correlation coefficient between age and the proportion of arsenic in each species.

[b]This is the sum of arsenic concentrations in the form of InAs, MMA, and DMA. Tertile cutoff points were 7.6 and 18.7 μg/L.

[c]Spearman correlation coefficient between total urinary arsenic (sum of InAs, MMA, and DMA) and the proportion of arsenic in each species.

TABLE 2
Mean Proportion of Each Arsenic Species in Subjects With Different Genetic Polymorphisms in GSTM1, GSTT1, MTHFR 677, and MHFTR 1298

	All subjects					Men					Women				
Variable	*n*	%	%InAs	%MMA	%DMA	*n*	%	%InAs	%MMA	%DMA	*n*	%	%InAs	%MMA	%DMA
GSTM1															
Null	81	48	15.9 (6.7)	14.7 (6.0)	69.3 (10.3)	66	47	16.4 (6.9)	14.7 (6.2)	68.9 (10.7)	15	48	13.7 (4.7)	15.1 (5.3)	71.2 (8.5)
Active	89	52	15.4 (7.3)	13.1 (6.3)	71.5 (11.4)	73	53	16.0 (7.5)	13.6 (6.5)	70.4 (11.5)	16	52	12.6 (6.0)	10.9 (4.7)	76.4 (10.1)
p-value[a]			.61	.09	.20			.72	.33	.43			.57	.03	.04
GSTT1															
Null	20	12	15.5 (7.7)	13.7 (7.1)	70.8 (13.1)	18	13	15.6 (7.8)	13.3 (7.1)	71.0 (13.0)	2	6	14.7 (10.1)	16.8 (9.7)	68.6 (19.7)
Active	150	88	15.7 (6.9)	13.9 (6.1)	70.4 (10.7)	121	87	16.3 (7.2)	14.2 (6.3)	69.5 (10.9)	29	94	13.1 (5.2)	12.7 (5.1)	74.3 (9.1)
p-value[a]			.94	.88	.90			.72	.60	.59			.69	.30	.43
MTHFR 677															
CC	61	36	15.0 (6.1)	12.9 (5.8)	72.1 (9.4)	52	37	15.3 (6.2)	12.7 (5.8)	72.0 (9.3)	9	29	13.6 (5.6)	14.1 (5.6)	72.3 (10.5)
CT	87	51	15.1 (6.7)	14.4 (6.4)	70.5 (11.3)	72	52	15.7 (6.8)	15.0 (6.4)	69.3 (11.1)	15	48	12.1 (5.8)	11.6 (6.0)	76.2 (10.7)
TT	22	13	19.5 (9.0)	14.6 (6.5)	65.9 (12.8)	15	11	21.7 (10.0)	14.7 (7.8)	63.6 (14.9)	7	23	14.8 (3.9)	14.2 (2.7)	70.9 (4.1)
p-value[b]			.02[c]	.31	.08			.007[c]	.14	.03[c]			.54	.43	.42
MTHFR 1298															
AA	80	47	16.9 (8.1)	14.3 (5.8)	68.8 (11.4)	61	44	18.3 (8.5)	14.5 (5.9)	67.3 (11.6)	19	61	12.7 (4.9)	13.5 (5.7)	73.7 (9.5)
AC	78	46	14.7 (5.6)	13.9 (6.8)	71.4 (10.7)	67	48	14.9 (5.5)	14.3 (7.0)	70.8 (10.8)	11	35	13.0 (5.8)	11.7 (5.1)	75.2 (10.0)
CC	12	7	13.4 (5.7)	11.3 (5.0)	75.3 (7.3)	11	8	12.6 (5.2)	11.0 (5.2)	76.5 (6.4)	1	3	22.7	14.4	62.9
p-value[b]			.06	.31	.09			.007[d]	.24	.02[d]			.20	.67	.48

[a]Student's *t*-test *p* value.
[b]Analysis of variance *p* value.
[c]TT is significantly different from CC and CT on the post hoc Duncan's multiple range test ($p < .05$).
[d]AA is significantly different from CC, but not from AC, on the post hoc Duncan's multiple range test ($p < .05$).

TABLE 3
Mean Proportion of Each Arsenic Species in Subjects With Different Genetic Polymorphism Groups of MTHFR 677 and MTHFR 1298

MTHFR 677/1298	All subjects					Men					Women				
	n	%	%InAs	%MMA	%DMA	*n*	%	%InAs	%MMA	%DMA	*n*	%	%InAs	%MMA	%DMA
CC/AA	16	9	16.7 (8.1)	13.5 (5.3)	69.8 (9.4)	12	9	17.6 (8.8)	12.7 (5.0)	69.8 (9.1)	4	13	14.0 (6.0)	16.0 (6.0)	70.0 (12.0)
CC/AC	33	19	14.9 (5.1)	13.2 (6.2)	72.0 (9.9)	29	21	15.4 (5.1)	13.3 (6.4)	71.3 (10.0)	4	13	11.0 (3.5)	12.0 (6.1)	76.9 (9.3)
CC/CC	12	7	13.4 (5.7)	11.3 (5.0)	75.3 (7.3)	11	8	12.6 (5.2)	11.0 (5.2)	76.5 (6.4)	1	3	22.7	14.4	62.9
CT/AA	44	26	15.7 (7.4)	14.1 (5.7)	70.1 (11.2)	35	25	16.8 (7.5)	14.8 (5.3)	68.4 (10.8)	9	29	11.5 (5.6)	11.7 (6.8)	76.8 (10.9)
CT/AC	43	25	14.5 (6.0)	14.7 (7.2)	70.9 (11.5)	37	27	14.7 (5.9)	15.2 (7.4)	70.2 (11.5)	6	19	13.1 (6.7)	11.5 (5.4)	75.4 (11.4)
TT/AC	2	1	15.9 (6.8)	9.5 (3.6)	74.6 (10.4)	1	1	11.1	6.9	81.9	1	3	20.7	12.1	67.2
TT/AA	20	12	19.8 (9.3)	15.1 (6.6)	65.1 (12.9)	14	10	22.4 (10.0)	15.3 (7.8)	62.3 (14.5)	6	19	13.8 (3.1)	14.6 (2.8)	71.5 (4.1)
Non-TT/AA[a]	150	88	15.1 (6.5)	13.7 (6.2)	71.2 (10.5)	125	90	15.5 (6.5)	14.0 (6.2)	70.5 (10.4)	25	81	13.0 (5.8)	12.5 (5.8)	74.5 (10.5)
p-value[b]			.04	.35	.02			.02	.46	.008			.74	.39	.29

[a]Includes all subjects without the TT/AA genotype in MTHFR 677/1298.

[b]Student's *t*-test comparing arsenic species proportions in subjects with the TT/AA genotype to subjects with all other genotypes ("Non-TT/AA").

Thirty subjects had urinary levels of combined arsenobetaine, arsenocholine, and other large organic species that were greater than the sum of InAs, MMA, and DMA. Removing these subjects increased the magnitude of the associations identified. For example, the difference in %DMA between MTHFR TT/AA subjects and all other subjects increased from 6.1% to 7.4% ($p < .01$), and the difference in %MMA between null and active genotypes of GSTM1 in women increased from 4.2% to 5.5% ($p < .01$).

A linear regression analysis was performed in order to assess the impact of potential confounding variables on polymorphism–methylation associations. Table 4 shows the results of this analysis with the proportion of each arsenic species as the dependent variable and the GSTM1 or MTHFR polymorphisms; age (yr); the sum of InAs, MMA, and DMA (μg/L); history of bladder cancer (case versus control); smoking (average cigarettes smoked in current smokers); and gender as the independent variables. The results of the linear regression analyses are similar to those of the unadjusted univariate analyses. For example, in women, the active genotype of GSTM1 was associated with a 4.5% lower significant %MMA level in the linear regression model (Table 4), and a 4.2% lower %MMA in the unadjusted univariate analysis (Table 2). In men, the TT/AA genotype of MTHFR 677/1298 was associated with a significant 6.5% higher %InAs than all other genotypes combined in the linear regression model (Table 4), and a 6.9% higher %InAs in the unadjusted univariate analysis (Table 3).

DISCUSSION

The results of this study suggest that subjects with the TT/AA genotype of MTHFR 677/1298, and women with the null genotype of GSTM1, are less effective metabolizers of ingested arsenic than others. These results provide evidence that the GSTM1 and MTHFR enzymes are involved in arsenic methylation in humans. These findings also suggest that polymorphisms in the genes that encode these enzymes may explain some of the interindividual variability seen in this metabolic process. This analysis involves relatively small numbers of subjects and multiple statistical comparisons, and it is possible that some of these findings could be due to chance. However, the genotypes that were analyzed in this study were selected based on a priori hypotheses, and our results are consistent with other research linking GST and MTHFR to arsenic metabolism and arsenic-related health effects (Brouwer et al., 1992; Buchet & Lauwerys, 1987; Crandall & Vorce, 2002; Csanaky & Gregus, 2005; Davison et al., 2003; Hirata et al., 1990; McDorman et al., 2002; Mitra et al., 2004; Nemeti & Gregus, 2004; Oya-Ohta et al., 1996; Vahter & Marafante, 1987). This consistency suggests that the associations identified in this study actually represent true effects and not chance occurrence.

MTHFR is a key enzyme in folate metabolism and catalyzes the synthesis of 5-methyltetrahydrofolate, which is involved in the methylation of homocysteine to methionine, a potential methyl donor in arsenic methylation. The C → T substitution at nucleotide 677 and the A → C substitution at nucleotide 1298 have both been linked to decreased and increased risks of certain cancer types (Curtin et al., 2004; Lin et al., 2004; Singal et al., 2004; Ulvik et al., 2004; Zhang et al., 2004). Several studies reported associations between folate and methionine deficiencies and decreased arsenic excretion, diminished arsenic methylation, and increased arsenic toxicity (Brouwer et al., 1992; Crandall & Vorce, 2002; McDorman et al., 2002; Mitra et al., 2004; Spiegelstein et al., 2003;

TABLE 4
Linear Regression Analysis With the Proportion of Each Arsenic Species as the Dependent Variable and Age, Gender, Case Status, Total Urinary Arsenic,[a] GSTM1, and MTHFR Polymorphisms as the Independent Variables

	All subjects				Men				Women			
	B[b]	SE	*p*	R^2	*B*	SE	*p*	R^2	*B*	SE	*p*	R^2
GSTM1 (active versus null)												
%InAs	–0.6	1.1	.60	.16	–1.0	1.2	.44	.20	–0.2	2.6	.94	.45
%MMA	–1.6	1.0	.11	.09	–0.9	1.2	.45	.09	–4.5	2.7	.11	.42
%DMA	2.2	1.7	.21	.12	1.8	2.0	.35	.13	4.7	4.9	.35	.39
MTHFR 677/1298 (TT/AA versus all other subjects)												
%InAs	4.9	1.6	.003	.14	6.5	2.0	.001	.15	1.9	2.5	.46	.32
%MMA	1.1	1.5	.46	.06	0.9	1.8	.62	.05	3.6	2.5	.16	.33
%DMA	–6.0	2.6	.02	.09	–7.4	3.2	.02	.09	–5.6	4.5	.23	.31

[a]This is the sum of arsenic concentrations in the form of InAs, MMA, and DMA.

[b]Regression coefficient. Increase in %InAs, %MMA, or %DMA associated with the genetic polymorphism group. For example, in men, %InAs is 6.5% higher in subjects with the TT/AA genotype than in subjects with all other genotypes combined.

Wlodarczyk et al., 2001). For example, in one study, erythrocyte micronuclei levels in arsenic-exposed folate-deficient mice were approximately twofold higher than those in arsenic-exposed folate-sufficient mice (McDorman et al., 2002). In another study, urinary excretion of arsenic was 20% lower in arsenic-exposed rabbits fed a low-methionine diet compared to arsenic-exposed rabbits fed a normal diet (Vahter & Marafante, 1987). Several human studies showed that the MTHFR 677AA/1298TT genotype is associated with a lower MTHFR enzyme activity than all other genotypes (van der Put et al., 1998; Weisberg et al., 1998). For example, van der Put et al. (1998) reported MTHFR activity levels (in nmol CH_2O/mg protein-h) for TT/AA, CT/AA, CC/AA, CT/AC, CC/AC, and CC/CC genotypes of 6.5 (±2.6), 17.5 (±5.3), 26.2 (±6.7), 12.5 (±3.7), 21.8 (±5.1), and 16.0 (±4.2), respectively. These results are consistent with our findings. The higher levels of %InAs and lower levels of %DMA that were found with the TT/AA genotype suggest that subjects with this genotype are less effective methylators of ingested InAs than others.

Previous studies also identified associations between glutathione or GSTs and arsenic metabolism or arsenic-related adverse health effects (NRC, 1999; Aposhian et al., 2004). GSTs are a large family of enzymes that catalyze the conjugation of glutathione to a variety of compounds including several known carcinogens. Carriers of homozygous deletions in GSTM1 and GSTT1 genes ("null" genotypes) have an absence of activity in certain GST enzymes (Seidegard & Pero, 1988) and studies in humans reported associations between the GSTM1 null genotype and increased risks of some cancer types (Engel et al., 2002; Johns & Houlston, 2000). Glutathione and glutathione *S*-transferases seem to be involved in the reduction of the pentavalent arsenic metabolites to their corresponding trivalent species (Aposhian et al., 2004). Several laboratory and animal studies identified associations between glutathione depletion and diminished arsenic methylation, decreased arsenic excretion, and increased arsenic toxicity (Buchet & Lauwerys, 1985, 1987; Csanaky & Gregus, 2005; Davison et al., 2003; Hirata et al., 1990; Nemeti & Gregus, 2004; Oya-Ohta et al., 1996; Shimizu et al., 1998). In addition, glutathione *S*-transferase omega 1-1 has been shown to be involved in the reduction of pentavalent inorganic arsenic (InAsV), MMAV, and DMAV in human liver cells (Aposhian et al., 2004). GSTM1 could have a similar activity, or it may be involved directly or indirectly in some other step of the methylation process. To date, its exact role in arsenic methylation is not known.

Few studies assessed the role of genetic polymorphisms in GSTM1 and T1 on urinary arsenic methylation patterns in humans. In a study of 115 men and women from a highly exposed region of Taiwan, subjects with the null genotype of GSTM1 had %InAs levels that were 3.8% significantly higher, and %MMA levels that were 4.1% lower, than subjects with the active genotype (Chiou et al., 1997). These results are inconsistent with our findings of higher %MMA levels in women with the null GSTM1 genotype. The reason for this inconsistency is unknown, although it could be due to chance. Other explanations could relate to the differences between the two study populations. For example, in our study, a statistically significant difference was only seen in women. In the Taiwan study, gender-stratified analyses were not presented. Another major difference between the two populations was that arsenic exposures were much higher in the Taiwan study than in our study. In the Taiwan study, 56% subjects had urinary arsenic levels above 50 μg/L, and 28% had levels above 300 μg/L. In our study, these numbers were 18% and 3%, respectively. Certain nutritional deficiencies that were relatively common in some arsenic-exposed regions of Taiwan have been shown to impact arsenic metabolism, and these may have also played a role in the Taiwan results (Engel & Receveur, 1993; Hopenhayn-Rich et al., 1996; Yang & Blackwell, 1961). In the Argentina study area, nutrition was generally good. All of these factors, as well as other ethnic variations (Vahter, 1999a), have all been linked to differences in arsenic methylation patterns, and could account for some of the discrepancy between our study and the Taiwan results.

Our findings linking GSTM1 and MTHFR polymorphisms to arsenic metabolism are consistent with other evidence linking inherited genetic traits to arsenic methylation (Chung et al., 2002; Concha et al., 2002; Vahter, 1999b, 2000, 2002). In a study of 11 families in Chile, researchers found that the correlation in %MMA between siblings (whose genetic makeup is likely very similar) was much greater than that between parents (who would not necessarily share the same genetic traits) [intraclass correlation coefficient (ICC) = 0.69, $p < .01$ in sibling–sibling pairs; ICC = 0.01, $p = .97$ in mother–father pairs] (Chung et al., 2002). Two other studies showed that individual arsenic methylation patterns remain fairly stable over time, which is also consistent with a strong genetic influence (Concha et al., 2002; Steinmaus et al., 2005). Other studies reported that arsenic methylation patterns may vary by ethnicity (Vahter, 2000, 2002), and inheritance was found to be a major factor in the individual variation of the activity of several other human methyltransferases (Weinshilboum, 1988, 1992). Additional studies have identified associations between arsenic methylation patterns and genetic polymorphisms in human glutathione *S*-transferase omega 1 (hGSTO1) and arsenic 3-methyltransferase (AS3MT) (Marnell et al., 2003; Meza et al., 2005; Schmuck et al., 2005). Combined with the results of our study, these findings provide a gradually emerging body of evidence that genetic factors play an important role in determining interindividual differences in arsenic metabolism.

The associations identified between arsenic metabolism and GSTM1 and MTHFR polymorphisms in this study were mostly gender specific. Some of this could be related to chance and

the relatively small number of women in the study. However, gender differences in the activity levels of GST and MTHFR, and in GST–disease and MTHFR–disease associations, have also been reported in other studies (Brown et al., 2004; Chango et al., 2000; Dresler et al., 2000; Habif et al., 2001; Hoensch et al., 2002; Saadat et al., 2004; Sgambato et al., 2002; Shankar et al., 2004; Tang et al., 1998; van Rijnsoever et al., 2005; Wu et al., 2001). For example, in a study of 291 subjects from France, statistically significant differences in homocysteine levels (a biomarker of MTHFR activity) between subjects with the 677CC and subjects with the 677TT MTHFR polymorphisms were identified in men, but not in women (Chango et al., 2000). In another study, the lung cancer odds ratio (OR) for the null genotype of GSTM1 compared to the active genotype was higher in females (OR = 2.5; 95% CI: 1.09–5.72) than in males (OR = 1.40; 95% CI 0.58–3.38) (Tang et al., 1998). Although the difference between these ORs was not statistically significant, similar gender differences were reported in other studies (Dresler et al., 2000; Sgambato et al., 2002; Shankar et al., 2004). Hypothesized mechanisms for these differences involve differences in hormone levels, or gender differences in related enzymatic pathways (Tang et al., 1998). To date, however, the exact reasons for these gender specific effects are unknown.

Although associations between arsenic methylation patterns and polymorphisms were identified in GSTM1 and MTHFR in this study, no clear interactions were found between arsenic and these polymorphisms in producing bladder cancer (Moore et al., 2004). One possible reason for this might be related to the relatively low arsenic exposures in the study area. The median time-weighted drinking-water arsenic concentration in the original case-control study was below 10 μg/L (Bates et al., 2004). In addition, because the long latency associated with arsenic-induced cancer was not known at the time of this study, detailed information was not collected on arsenic exposures from 40 yr or more before cancer diagnoses. Exposure metrics based on drinking water arsenic concentrations in water sources used by the study subjects from 5 to 40 yr before cancer diagnosis were not associated with bladder cancer risk. However, the use of well water (the primary source of high arsenic exposures in this area) versus surface water more than 40 yr before cancer diagnosis was associated with some increase in risk in smokers (Bates et al., 2004). Regardless, the relatively low arsenic exposures, and the lack of detailed arsenic exposure data from 40 yr or more before cancer diagnosis, may have limited the ability of this study to identify interactions between genetic polymorphisms and arsenic in producing bladder cancer. In a recent lung cancer case-control study from an arsenic-exposed region in northern Chile, subjects who had both the 2A genotype in CYP1A1 (Msp1) and the null genotype of GSTM1 had greater odds of lung cancer than other subjects (OR = 2.51; 95% CI 1.07–5.40) (Adonis et al., 2005). In the analysis of GSTM1 alone, females with the null genotype of GSTM1 (regardless of CYP1A1 genotype) had a quantitative higher odds ratio than males with the null genotype (1.83 versus 0.86, $n = 51$ cases), although these results were not statistically significant.

A limitation of this study is that only a single urine sample was collected from each subject. Previous studies found that methylation patterns remain fairly stable over time, although some day to day variability does 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 nondifferential effects. As such, these effects would have most likely biased our results toward the null, and would not have produced the positive associations identified.

Another issue is whether or not the proportions of the arsenic metabolites in urine are directly relevant to what is occurring internally at the target organ sites. Urination is the primary route of arsenic excretion and most ingested arsenic is excreted through the urine (Buchet et al., 1981b). Experimental studies showed that chemically inhibiting methylation reactions internally results in increased tissue retention of arsenic as well as an increase in the proportion of arsenic excreted in the urine as inorganic arsenic and a decrease in the proportion excreted as DMA (Marafante & Vahter, 1984; Marafante et al., 1985). In addition, several epidemiologic studies reported that subjects with high urinary %MMA values have higher risks of arsenic-associated disease (Chen et al., 2003a, 2003b; Del Razo et al., 1997; Hsueh et al., 1997; Maki-Paakkanen et al., 1998; Steinmaus et al., 2006; Valenzuela et al., 2005; Yu et al., 2000). While these studies do not tell us exactly how arsenic exerts its toxic effects, they provide some evidence that the proportion of arsenic metabolites in urine do reflect internal arsenic metabolism and are related to the toxic effects of arsenic at the target organ sites.

In conclusion, the data presented here suggest that GSTM1 and MTHFR may be involved in arsenic metabolism in humans, and polymorphisms in the genes that code for these enzymes may account for some of the interindividual variability seen in this metabolic process. Because differences in arsenic metabolism have been linked to differences in arsenic-induced cancer risks in other studies (Chen et al., 2003a, 2003b; Hsueh et al., 1997; Steinmaus et al., 2006; Yu et al., 2000), the findings of this study provide some evidence that polymorphisms in MTHFR and GSTM1 genes may affect susceptibility to arsenic-induced disease. Because these analyses involved a relatively small number of subjects, especially women, our findings should be confirmed in a larger study. Future research is also needed to help identify the other factors that may control arsenic metabolism and confer arsenic-disease susceptibility. This type of research may help to identify subpopulations who are particularly susceptible to arsenic-associated disease and therefore could be useful in helping establish safe and effective drinking-water regulation.

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