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Individual Differences in Arsenic Metabolism and Lung Cancer in a Case-Control Study in Cordoba, Argentina

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Abstract

In humans, ingested inorganic arsenic is metabolized to monomethylarsenic (MMA) then to dimethylarsenic (DMA), although in most people this process is not complete. Previous studies have identified associations between the proportion of urinary MMA (%MMA) and increased risks of several arsenic-related diseases, although none of these reported on lung cancer. In this study, urinary arsenic metabolites were assessed in 45 lung cancer cases and 75 controls from arsenic-exposed areas in Cordoba, Argentina. Folate has also been linked to arsenic-disease susceptibility, thus an exploratory assessment of associations between single nucleotide polymorphisms in folate metabolizing genes, arsenic methylation, and lung cancer was also conducted. In analyses limited to subjects with metabolite concentrations above detection limits, the mean %MMA was higher in cases than in controls (17.5% versus 14.3%, p = 0.01). The lung cancer odds ratios for subjects with %MMA in the upper tertile compared to those in the lowest tertile was 3.09 (95% CI, 1.08–8.81). Although the study size was too small for a definitive conclusion, there was an indication that lung cancer risks might be highest in those with a high %MMA who also carried cystathionine β-synthase (*CBS*) rs234709 and rs4920037 variant alleles. This study is the first to report an association between individual differences in arsenic metabolism and lung cancer, a leading cause of arsenic-related mortality. These results add to the increasing body of evidence that variation in arsenic metabolism plays an important role in arsenic-disease susceptibility.

Keywords

arsenic; lung cancer; drinking water; metabolism

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Conflict of Interest:
None.

Inorganic arsenic (InAs) occurs naturally in the groundwater and surface water of many parts of the world, and tens of millions of people worldwide are exposed to drinking water containing this known carcinogen (Nordstrom, 2002). Based on epidemiologic evidence from several countries, the International Agency for Research on Cancer (IARC) has concluded that ingestion of inorganic arsenic causes cancer of the bladder, skin, and lung. Other evidence suggests that of all of the various malignant and non-malignant diseases linked to arsenic ingestion, lung cancer is the most common cause of arsenic-related mortality (Smith *et al.*, 1998; IARC, 2002). The excess risks associated with drinking water arsenic may be quite high (Chen *et al.*, 1992; Smith *et al.*, 1992; NRC, 1999; Morales *et al.*, 2000; NRC, 2001). The National Research Council has estimated that the excess cancer risk associated with lifetime exposures to arsenic at the US regulatory drinking water standard of 10 μg/L may be close to 1 in 300 (NRC, 2001). Risks may be even higher in susceptible subpopulations if they exist. These risks are about 30 to 300 times higher than the cancer risks estimated for exposure to all other known drinking water carcinogens at concentrations equal to their current US drinking water standard (Smith *et al.*, 2002).

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 (MMA5) which is reduced to monomethylarsonous acid (MMA3). MMA3 is then methylated to dimethylarsinic acid (DMA5) which is reduced to dimethylarsinous acid (DMA3). In humans, this process is not complete, and some arsenic remains as InAs and MMA (MMA3 and MMA5). Almost all ingested arsenic is excreted through the urine and the relative distribution of arsenic metabolites in urine is commonly used as a biomarker of how well an individual can 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 inter-individual variations exist (Vahter, 1999b).

Until recently, methylation of InAs was thought to be primarily a detoxification pathway since the methylated species most commonly found in human urine, MMA5 and DMA5, are more water soluble, more readily excreted, and less acutely toxic than InAs (Buchet *et al.*, 1981a; Buchet *et al.*, 1981b; Moore *et al.*, 1997; Hughes and Kenyon, 1998; Gebel, 2002). MMA3 and DMA3 are highly unstable in human urine and so have been measured in only a few human studies. However, there is increasing evidence that MMA3 is much more toxic *in vitro* than its pentavalent form, and more toxic than InAs (Cullen *et al.*, 1989; Styblo *et al.*, 1997; Lin *et al.*, 1999; Styblo *et al.*, 1999; Petrick *et al.*, 2000; Styblo *et al.*, 2000; Lin *et al.*, 2001; Mass *et al.*, 2001).

Epidemiological studies have reported associations between individual methylation patterns, specifically the proportion of MMA in urine (%MMA), and the risks of several different arsenic-related diseases including bladder cancer, skin cancer, and arsenic-caused skin lesions (Del Razo *et al.*, 1997; Hsueh *et al.*, 1997; Yu *et al.*, 2000; Chen *et al.*, 2003a; Chen *et al.*, 2003b; Tseng *et al.*, 2005; Steinmaus *et al.*, 2006; Wu *et al.*, 2006; Ahsan *et al.*, 2007; Huang *et al.*, 2007; McCarty *et al.*, 2007; Pu *et al.*, 2007; Huang *et al.*, 2008; Lindberg *et al.*, 2008). These data provide a highly consistent body of evidence linking methylation capacity, and specifically high %MMA, to arsenic-related disease risks. However, to date no study has reported on the potential association between arsenic metabolism and lung cancer.

Several studies have also linked folate intake and folate metabolism to arsenic metabolism and arsenic-related disease risks (Gamble *et al.*, 2005; Chen *et al.*, 2007; Gamble *et al.*, 2007; Huang *et al.*, 2007; Kile and Ronnenberg, 2008). The results of these studies have led to the hypothesis that variants in genes that code for folate metabolizing enzymes could account for some inter-individual variation in arsenic metabolism and arsenic-related disease susceptibility. For this reason, we have also performed a preliminary investigation on

whether certain polymorphisms in folate metabolizing genes, such as cystathionine B-synthase (*CBS*), might affect the relationship between arsenic metabolism and lung cancer relative risks.

Methods

The participants of this study were a subgroup of subjects from a case-control study of lung cancer and arsenic in drinking water (publication in progress). The study area for this investigation was the county of Unión in the Province of Córdoba, Argentina, where many private wells are contaminated with arsenic. All newly diagnosed incident cases of primary lung cancer, aged 20 to 80 who were living in Unión, were identified through rapid case ascertainment from 2000–2006 involving all pathologists and pulmonary medicine physicians in the county, and from radiographic services. In the original study, controls, individually matched to cases by sex and exact year of birth, were selected from computerized voter registration lists. The participants in the current study included all cases and controls in the original study who agreed to provide urine samples for arsenic metabolite measurements.

This study was approved by ethical review boards in the US and Argentina, and informed consent was obtained from all participants. All subjects were administered standardized questionnaires in their homes. Information sought included residential history, water sources at each current and past residence, smoking, and occupation. Buccal cell samples for DNA and a single first morning urine sample were also collected by study personnel during the home visits. A previous study has shown that a moderately strong correlation exists between arsenic concentrations in single first morning samples and samples collected over 24 hours (Calderon *et al.*, 1999). Urine samples were kept frozen in the field laboratory at −20° and then transported on dry ice to the University of Washington, Seattle for analysis. The urinary concentrations of inorganic arsenic and its metabolites were measured using hydride generation atomic absorption spectroscopy (Crecelius, 1978). Details of the laboratory methods are described elsewhere (Chung *et al.*, 2002). 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%. The MMA and DMA measured in this study are the sums of the trivalent and pentavalent forms. The trivalent forms, MMA3 and DMA3, are rapidly oxidized during storage and at the time of this study could not be reliably measured in field studies (Del Razo *et al.*, 2001). Most samples were stored frozen for one to four months before analysis.

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). TaqMan® assays were obtained from the Assays-on-Demand service (Applied Biosystems, Foster City, CA) to genotype the SNPs listed below.

Polymorphisms in *CBS* rs234709 and rs4920037; methyltetrahydrofolate (MTHFR) rs1801133 and rs1801131; methionine synthase (MTR) rs1805087; thymidylate synthase (TYMS) rs16430; dihydrofolate reductase (DHFR) rs2618372; and serine hydroxymethyltransferase 1 (SHMT1) rs1979277 were selected *a priori* because they encode enzymes involved in folate metabolism. Polymorphisms in glutathione-S-transferase-1 (GSTO1) rs11509435 and rs4925 were assessed due to their modest associations with urinary %MMA seen in previous studies (Marnell *et al.*, 2003; Meza *et al.*, 2005; Lindberg *et al.*, 2007; McCarty *et al.*, 2007; 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. Genotyping was carried out using TaqMan® SNP Genotyping Assays (Applied Biosystems, Foster City, CA). Amplification reactions (95° C for 10 minutes, then 40 cycles of 95 °C for 15 seconds and 60° C for 1 minute) were performed on the ABI 9700 GeneAmp PCR system and a post-PCR read using the ABI 7700 SDS was performed to determine genotypes.

The proportion of arsenic in each species (%InAs, %MMA, and %DMA) was calculated by dividing the concentration of arsenic in each species by the sum of the concentrations of InAs, MMA, and DMA. At low metabolite concentrations, relatively small inaccuracies in laboratory measurements can cause relatively large errors when calculating metabolite proportions. In addition, the choice of methods used to assign values to subjects with metabolite concentrations below detection can also have large effects on metabolite proportion calculations. For example, in a subject with a total urinary arsenic (InAs + MMA + DMA) of 5 μg/L, assigning values for MMA to subjects with MMA concentrations below detection of either zero, the limit of detection divided by the square root of two, or at the limit of detection (1 μg/L) will give %MMA values of either 0%, 14%, or 20%, which would place the subject in either the lower, middle, or upper tertiles, respectively, of %MMA in this study. For these reasons, we excluded subjects who had InAs, MMA, and DMA concentrations below detection levels. The impact of this was assessed by performing separate analyses where all subjects were used and concentrations below detection were set at ½ the detection level.

Past arsenic exposure was assessed by linking information on residential water sources to arsenic water concentrations, obtained either through historic water records or from water samples we collected from as many current and past residences as possible. The focus of assessing past exposure was on well water, since previous research has shown that arsenic exposure in this area comes almost exclusively from wells (Bates *et al.*, 2004). Arsenic concentrations in some wells used in the past could not be measured since some wells were closed or could not be located. All water samples collected 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 μg/L. Using these data, a year-by-year arsenic exposure profile was created for each subject. Since the focus was on well water, and since previous research suggests that arsenic-related cancer risks are more dependent on arsenic concentration than on cumulative exposure (Lubin *et al.*, 2008), subjects were categorized based on whether or not they ever used well water in the study area and if so, the highest known well water arsenic concentration to which they were exposed.

Unconditional logistic regression was used to calculate lung cancer odds ratios (OR) comparing subjects with high and low proportions of all three arsenic metabolites (InAs, MMA, and DMA), with our focus being primarily on %MMA. The one to one case-control matching in the original case-control study was not retained since not all subjects in the original study provided urine samples. Category cutoff points for defining low, medium, and high %MMA were based on tertiles. Odds ratios were adjusted for age (≤ 65 versus > 65 years old), gender, smoking (ever versus never), and historical arsenic exposure in drinking water. This last variable was categorized as either (1) never used a well (and therefore presumed to have had low exposure), (2) used a well in the study area, but that well was closed or could not be found (“no measurement”) (3) used a well in the study area and the highest known arsenic concentration among all wells used was below 100 μg/L, or (4) used a well in the study area and the highest known arsenic concentration among all wells used was ≥ 100 μg/L. Entering highest known arsenic concentration in a greater number of categories or as a continuous variable had little impact on results.

Lung cancer ORs for genetic polymorphisms were calculated with logistic regression using the same methods described above. To assess whether a genetic polymorphism might affect the relationship between lung cancer and %MMA, lung cancer ORs were calculated for each percentage point increase in %MMA in analyses stratified by each genetic polymorphism. In preliminary analyses, for each SNP assessed, the associations between genotypes and %MMA, %DMA and %InAs were analyzed using multivariate linear regression, adjusted for age, gender, current smoking status, case-status, and total urinary arsenic. Mean %InAs, %MMA, %DMA levels in subjects with wildtype genotypes were compared to those with heterozygous and variant homozygous genotypes, separately and combined. Data on the impact of SNPs on arsenic methylation-lung cancer risks are only presented for *CBS* in this paper since this was the only gene related to arsenic metabolism (i.e. a linear regression p-value less than 0.05) (These p-values were not adjusted for multiple comparisons since these analyses were exploratory and based on *a priori* hypotheses). For the analyses presented in this paper, *CBS* heterozygotes and homozygous variants were combined into one stratum because of the small sample size. All data analyses were carried out using the SAS statistical program package (Version 8.0e, SAS Institute, Cary, NC). All p-values are two-sided.

Results

Overall, 141 cases and 252 controls were eligible for participation in the case-control study. Of these 109 (77%) of cases and 141 (56%) of the controls were interviewed and provided a urine sample. Eleven cases (8%) and 30 (12%) controls declined participation, and 21 cases (15%) and 81 controls (32%) could either not be located or were too ill to participate. Of those who provided a urine sample, 45 cases (41.3%) and 75 controls (53.2%) had levels of all three arsenic metabolites above the detection levels. Table 1 shows descriptive characteristics and arsenic exposure information of the study subjects. Cases were more likely to be current or former smokers (unadjusted OR = 4.31.; 95% confidence interval (CI), 2.26–8.20). In the analysis of all subjects in the original study (i.e. regardless of whether or not they provided urine samples), increased lung cancer risks were found for those with highest known water arsenic concentrations above 200 μg/L (OR=2.2), but with a wide confidence interval (95% CI, 0.7–7.3). Increased risks were more pronounced in those who smoked and who were exposed to arsenic in drinking water starting more than 40 years before diagnosis (details to be reported in a separate publication).

Table 2 shows the mean relative proportions of each arsenic species stratified by case status, gender, smoking, age, and urinary arsenic. %MMA was higher in cases than controls (17.5 vs. 14.3%, p = 0.01). The mean concentrations (and ranges) of urinary InAs, MMA, and DMA in all subjects with levels above detection were 3.8 μg/L (0.5–17.1 μg/L), 3.8 μg/L (1.0–20.2 μg/L), and 17.1 μg/L (2.0–78.2 μg/L) (not shown in tables). Table 3 displays the unadjusted and adjusted odds ratios for the association between lung cancer and urinary %MMA for those subjects with metabolite concentrations above the detection limits. The adjusted lung cancer OR for subjects with %MMA in the middle and upper tertiles compared to those in the lower tertile were 0.85 (95% CI, 0.29–2.51) and 3.09 (95% CI, 1.08–8.81), respectively. The logistic regression lung cancer OR for %MMA as a continuous variable was 1.106 (p = 0.008) suggesting that each one percentile increase in %MMA (e.g. a %MMA of 15.0% versus a %MMA of 14.0%) is associated with about a 10.6% increase in lung cancer risk. In the analysis using all subjects who provided urine and setting metabolite levels below detection at ½ the detection limit, the adjusted lung cancer OR comparing the upper and lower tertiles of %MMA was 2.14 (95% CI, 0.91–5.06) (not shown in tables). The lung cancer ORs for %DMA, %InAs, MMA/InAs, and DMA/MMA tertiles can be found at http://asrg.berkeley.edu/index.html. %DMA was less strongly associated with lung cancer than was %MMA. The adjusted lung cancer OR comparing subjects in the upper and lower tertiles of %DMA was 0.44 (95% CI, 0.16–1.23).

Data on *CBS* rs234709 and rs4920037 polymorphisms were available for 207 and 212 subjects, respectively. No association was seen between lung cancer and *CBS* rs234709 or rs4920037 polymorphisms: the lung cancer OR adjusted for age, gender, smoking, and highest known arsenic exposure comparing *CBS* rs234709 wildtypes to non-wildtypes was 0.89 (95% CI, 0.49–1.59). The corresponding OR for *CBS* rs4920037 was 0.92 (95% CI, 0.50–1.69) (data not shown in tables). No associations with lung cancer were seen with the other genetic polymorphisms we assessed.

Variant genotypes for *CBS* rs234709 and rs4920037 SNPs compared with wild-type homozygotes were associated with 24% (p = 0.01) and 26% (p = 0.02) increases, respectively, in mean %MMA. Table 4 shows the lung cancer odds ratios for each one percentage point increase in %MMA, stratified by the *CBS* polymorphism. All of these analyses involved small numbers of subjects and none of the results was statistically significant. However, for both CBS rs234709 and CBS rs4920037, lung cancer-%MMA associations appear somewhat greater in subjects with non-wildtype alleles than in subjects with wildtype alleles. The lung cancer odds ratios comparing subjects in the upper tertile of %MMA with those in the lower tertile were 0.33 (95% CI, 0.04–2.86) and 3.34 (95% CI, 0.53–20.9) for subjects with *CBS* rs234709 wildtype and non-wildtype genotypes, respectively (not shown in Tables). The corresponding odds ratios for subjects with CBS rs4920037 wildtype and non-wildtype genotypes were 1.43 (95% CI, 0.32–6.35) and 9.48 (95% CI, 0.20–448), respectively.

Discussion

The lung cancer OR of 3.09 (95% CI, 1.08–8.81; p-trend = 0.04) comparing the upper tertile of %MMA to the lower tertile of %MMA, and the lung cancer OR of 1.106 (95% CI, 1.026–1.191; p-value = 0.008) for %MMA as a continuous variable are evidence that subjects who are less effective at methylating MMA to DMA are at greater risks of arsenic-related lung cancer than others. Although the number of subjects in this study is relatively small, the low p-values and the consistency of our results with other human, animal, and laboratory studies suggests that these findings are not due to chance and could represent real effects.

Data on associations between %MMA and increased relative risks of arsenic-related disease in humans are shown in Table 5. A few of the results in this table are for MMA/DMA ratio rather than %MMA. These were included because inter-individual variability in MMA/DMA ratios is more dependent on %MMA than %DMA since inter-individual variability in %MMA is generally much greater than inter-individual variability in %DMA (Buchet *et al.*, 1984; Hopenhayn-Rich *et al.*, 1996; Vahter, 1999a). Thus, variability in MMA/DMA ratios is more likely due to differences in %MMA than differences in %DMA. As seen in Table 5, in every study except for one the odds ratios for arsenic-related disease are higher in those with higher %MMA or higher MMA/DMA ratios. As a whole, these studies provide a fairly large and consistent body of evidence linking %MMA to arsenic-related disease risks.

The study of hypertension by Huang *et al.* (2007) is the only published study of inter-individual differences in arsenic metabolism that did not find a clear association. The reason for this is unknown. It is possible that arsenic does not cause hypertension (the evidence linking arsenic to hypertension is not as strong as it is for the other outcomes assessed in Table 5) or that it causes hypertension by a mechanism that is different from other arsenic-related diseases, although the later is difficult to evaluate since the exact mechanisms of arsenic toxicity are unknown. Interestingly, in the Huang *et al.* study mean %MMA levels were somewhat higher in subjects with hypertension than in those without (14.32% versus 13.07%, n = 871, p = 0.03), and the unadjusted odds ratios for hypertension showed a statistically significant trend in people with low, medium, and high %MMA values [ORs =

1.00; 1.28 (95%CI, 0.90–1.81); 1.47 (95%CI, 1.04–2.07); respectively, p for trend = 0.02]. However, this trend was not seen after adjustment for age, gender, body mass index, smoking, triglycerides, and cumulative arsenic exposure.

In addition to the studies in Table 5, other data support the hypothesis that %MMA is related to arsenic-disease susceptibility. In Mexico, Del Razo *et al.* reported higher levels of %MMA in subjects with arsenic-related skin lesions than in those without lesions (14.3% versus 9.5%) (Del Razo *et al.*, 1997). In one of the few studies involving MMA3, Valenzuela *et al.* reported higher %MMA3 levels in subjects with arsenic-caused skin lesions (mean %MMA3 = 7.7%, n = 55) than in those without these lesions (mean %MMA3 = 5.9%, n = 21, p = 0.072) (Valenzuela *et al.* 2005). (The Valenzuela and Del Razo *et al.* studies were not included in Table 5 because data were not presented as relative risks). In Finland, Maki-Paakkanen *et al.* found a positive association between lymphocyte chromosomal aberrations and MMA/InAs ratio (Maki-Paakkanen *et al.*, 1998). Additional biologic plausibility comes from laboratory research where several studies have shown that MMA3 is more acutely toxic *in vitro* than MMA5, DMA, and InAs (Cullen *et al.*, 1989; Styblo *et al.*, 1997; Lin *et al.*, 1999; Styblo *et al.*, 1999; Petrick *et al.*, 2000; Styblo *et al.*, 2000; Lin *et al.*, 2001; Mass *et al.*, 2001). These data suggest that MMA, specifically MMA3, may be the primary toxic species of ingested inorganic arsenic. These findings, combined with epidemiologic evidence from Taiwan, Japan, Chile, and Argentina showing clear associations between InAs ingestion and lung cancer risks (IARC, 2002), all support the biologic plausibility of our results linking inter-individual differences in %MMA to increased lung cancer risks.

In our study, MMA was measured as total MMA, that is, MMA3 and MMA5 combined. At the time of this study, it was very difficult to accurately measure MMA3 separately in the field due to its instability in urine. If MMA3 is the primary toxic species, it may be that the total MMA is an accurate surrogate for MMA3, although currently this is unknown. If MMA3 truly is the toxic species, any inaccuracies involved in using total MMA as a surrogate for MMA3 would cause bias towards the null and true relative risks may actually be higher than those found in this study.

As in all of the studies in Table 5, the measurement of urinary methylation patterns was taken after disease diagnosis and assumed to be representative of subject's past methylation patterns. Few studies have assessed changes in methylation patterns in the same individuals over time, but those that have suggest that these patterns remain fairly stable over time (Concha *et al.*, 2002; Steinmaus *et al.*, 2005b). Evidence suggests that stable genetic factors play a more important role in determining inter-individual differences in methylation patterns than do factors that are likely to have greater day to day variability such as diet or smoking (Chiou *et al.*, 1997; Vahter, 1999a; Vahter, 1999b; Vahter, 2000; Chung *et al.*, 2002; Vahter, 2002; Steinmaus *et al.*, 2005a). It should also be noted that although intra-individual variability in methylation patterns could lead to misclassification of past methylation patterns, because we collected and analyzed metabolites from cases and controls using the same protocols, the resulting bias would be non-differential and likely towards the null, not towards the positive associations identified. Similar misclassification might occur as a result of DMA from arsenosugars in seafood. However, the study area is inland with relatively little seafood consumption, and the resulting bias would also likely be non-differential and towards the null.

In our main analyses, we used only samples with metabolite levels above detection limits. While this resulted in a smaller sample size and may have caused some reduction in study power, it likely improved the accuracy of our odds ratio estimates. This is because in samples with metabolite concentrations below detection, laboratory imprecision or

inaccuracies in assigning values to samples below detection (e.g. zero, ½ the detection limit…) can cause relatively large errors when calculating metabolite proportions. These errors would most likely be non-differential and bias any true association towards the null. The decrease in the %MMA-lung cancer OR from 3.09 (95% CI, 1.08–8.81) to 2.14 (95% CI, 0.91–5.06) when we added subjects with metabolite levels below detection is consistent with this effect.

The assessment of methylation after cancer diagnosis also raises concerns about the temporal relationship between disease and methylation capacity. That is, the effects seen in our study and those in the other studies in Table 5 might not be due to the impact of methylation patterns on disease, but rather, due to the impact of disease or disease treatment on methylation patterns. Currently no data are available on the impact of severe chronic disease on arsenic metabolism. However, several of the studies linking %MMA to arsenic susceptibility involve non-melanoma skin cancer, benign skin lesions, or chromosomal aberrations, none of which would be expected to have significant systemic effects on metabolism. The consistency of our findings with these studies and other data on biologic plausibility suggest our results represent the effects of %MMA on lung cancer risks, although the possibility that lung cancer affects %MMA can not be completely ruled out. A longitudinal cohort study might be better able to establish temporality, although this type of study would be incredibly difficult given the 30 to 40 year (or longer) latency of arsenic-caused cancer.

Overall participation rates differed between the cases (77%) and controls (56%) in this study, but it is unlikely this difference had a major impact on our results since our primary exposure variable (%MMA) is probably not strongly related to participation. Most of the major factors that might be associated with both participation and %MMA were adjusted for in our analyses and had little impact on results (e.g. smoking, age, gender). Some dietary variables affect arsenic methylation, but the impacts are mostly small and thus unlikely to have caused the effects identified in this study (Steinmaus *et al.*, 2005a; Li *et al.*, 2008; Heck *et al.*, 2007).

We found some evidence that the association between %MMA and lung cancer could be related to rs234709 and rs4920037, two intronic polymorphisms in the *CBS* gene. These SNPs may be functionally relevant or may be in linkage disequilibrium with some other functional SNP that may influence *CBS* activity. *CBS* is an important enzyme in the conversion of homocysteine to cystathionine, a precursor to cysteine and glutathione biosynthesis. *CBS* gene variants may influence *CBS* enzyme activity. The exact way this might affect arsenic toxicity is unknown although several possibilities exist (Selhub, 1999). (The relationship between arsenic and homocysteine metabolism is shown in Figure 1.) *CBS* enzyme deficiency can result in increased levels of homocysteine and s-adenosylhomocysteine (SAH), the later being a potent inhibitor of methylation reactions (De Kimpe *et al.*, 1999; Selhub, 1999; Yi *et al.*, 2000). If SAH selectively inhibits the methylation of MMA3 to DMA5, this might lead to increased levels of MMA3 (and a greater MMA3/MMA5 ratio) and thus higher risks of MMA3 associated toxicity. A previous study has identified associations between increased homocysteine and decreased %DMA and increased %MMA, although MMA3 was not specifically measured (Gamble *et al.*, 2005). Another possible mechanism could be related to the involvement of *CBS* in glutathione production. *CBS* deficiencies might lead to decreased glutathione biosynthesis and inhibition of any potential detoxification pathway involving glutathione.

Overall, the results of this study suggest that the association between %MMA and arsenic-related lung cancer may be mediated by genetic variation in *CBS*. These results involve a small number of subjects and need to be confirmed. As such, our *CBS* findings should be

viewed as preliminary exploratory results that may help guide researchers in selecting which genetic factors to be included in future studies.

In conclusion, millions of people are exposed to arsenic worldwide and these exposures may be associated with high cancer risks. Our results add to a gradually expanding body of evidence that inter-individual differences in arsenic metabolism play an important role in arsenic-related disease. Our results are the first to suggest that this may include lung cancer. Although the design of this study prevents us from confirming the temporal relationship between %MMA and lung cancer, the biologic plausibility of our results and their consistency with a variety of other research is evidence that our findings represent a true impact of MMA on lung cancer risks. Data such as these are important in identifying susceptible subpopulations that may need specific regulatory protection. These data may also help elucidate the mechanisms of arsenic-caused disease which are largely unknown. Further research is needed on the potential toxic effects of MMA3 in humans, the genetic and lifestyle factors that influence individual arsenic methylation, and the role of *CBS* genetic variants in arsenic toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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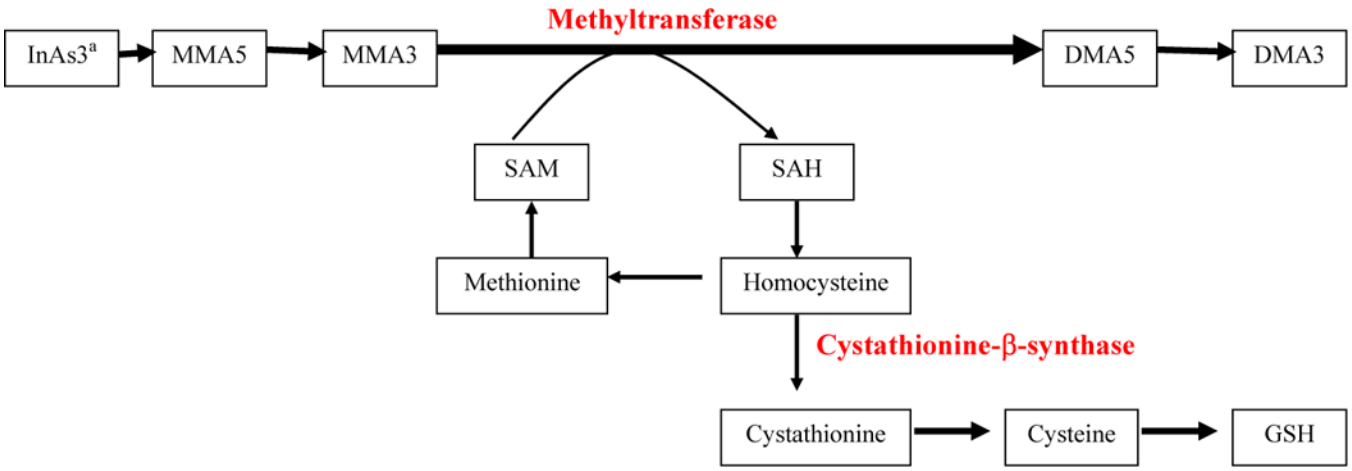

Figure 1.

Involvement of cystathionine β-synthase and homocysteine in the metabolism of methylarsonous acid (MMA3) to dimethylarsinic acid (DMA5) and in glutathione biosynthesis.

Abbreviations: InAs, inorganic arsenic; MMA5, methylarsonic acid; MMA3, methylarsonous acid; DMA5, dimethylarsinic acid; DMA3, dimethylarsinous acid; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; GSH, glutathione

[a]Cystathionine β-synthase and homocysteine are similarly involved in the conversion of InAs3 to MMA5

Table 1

Sociodemographic characteristics of lung cancer cases and controls.

	All subjects				Subjects with all arsenic metabolites above detection levels			
	Cases		Controls		Cases		Controls	
	No.	%	No.	%	No.	%	No.	%
Total	109	100	141	100	45	100	75	100
Age (years)								
<65	43	39.4	57	40.4	17	37.8	35	46.7
65–75	40	36.7	45	31.9	21	46.7	23	30.7
>75	26	23.9	39	27.7	7	15.6	17	22.7
Gender								
Female	19	17.4	24	17.0	4	8.9	10	13.3
Male	90	82.6	117	83.0	41	91.1	65	86.7
Smoking								
Current	41	37.6	35	24.8	18	40.0	25	33.3
Former	55	50.5	54	38.3	24	53.3	25	33.3
Never	13	11.9	52	36.9	3	6.7	25	33.3
Past drinking water InAs								
Never used a well[a]	54	49.5	66	46.8	21	46.7	36	48.0
Used a well:[a]								
No measurement[b]	33	30.3	44	31.2	9	20.0	20	26.7
< 99 μg/L[c]	6	5.5	16	11.4	3	6.7	9	12.0
100–199 μg/L[c]	7	6.4	10	7.1	5	11.1	6	8.0
≥ 200 μg/L[c]	9	8.3	5	3.6	7	15.6	4	5.3

[a] Drinking water arsenic exposure in the study area comes almost exclusively from well water, so the assessment of past arsenic exposure focused on the use of well water.

[b] These are subjects who reported that they used water from a well in the study area, but an arsenic concentration could not be taken because the well was used in the past and was either closed or could not be located.

[c] Highest known arsenic concentration among all measured wells used by the subject in the study area.

Table 2

Univariate analyses of the mean proportions of each arsenic species (standard deviation).[a]

Variable	N	%	%InAs	%MMA	%DMA
All	120	100	15.7 (5.5)	15.5 (6.7)	68.7 (9.6)
Lung cancer					
Cases	45	37.5	16.0 (6.7)	17.5 (7.3)	66.5 (11.1)
Controls	75	62.5	15.7 (4.7)	14.3 (5.9)	70.1 (8.3)
p-value[b]			0.77	0.01	0.07
Gender					
Women	14	11.7	15.0 (5.9)	14.5 (5.5)	70.5 (9.8)
Men	106	88.3	15.9 (5.5)	15.6 (6.8)	68.5 (9.5)
p-value[b]			0.57	0.54	0.45
Smoking					
Current	43	35.8	15.1 (5.4)	14.7 (6.5)	70.2 (10.3)
Ex-smokers	49	40.8	16.2 (5.9)	16.1 (6.3)	67.7 (8.6)
Never	28	23.3	16.1 (5.0)	15.7 (7.6)	68.2 (10.1)
p-value[c]			0.46	0.25	0.64
Age (years)					
<65	52	43.3	15.9 (4.9)	14.9 (6.5)	69.2 (9.4)
65–75	44	36.7	15.2 (6.2)	16.1 (7.6)	68.7 (10.7)
>75	24	20.0	16.5 (5.6)	15.8 (5.3)	67.7 (8.0)
p-value[d]			0.80	0.36	0.62
Total urinary arsenic[e]					
Low tertile	40	33.3	16.4 (4.7)	16.2 (5.2)	67.3 (8.0)
Medium tertile	40	33.3	15.9 (6.5)	14.7 (6.1)	69.4 (9.5)
High tertile	40	33.3	15.0 (5.3)	15.5 (8.3)	69.5 (11.0)
R (p-value)[f]			−0.11 (0.23)	−0.13 (0.17)	0.14 (0.14)

[a] Includes only subjects with InAs, MMA, and DMA above detection levels

[b] t-test for differences in means

[c] t-test for differences in means comparing ever versus never smokers

[d] t-test for differences in means comparing age < 65 years to age ≥ 65 years

[e] Total urinary arsenic (μg/L) = InAs + MMA + DMA. Total urinary arsenic ranged from 4.8 to 112.3 μg/L, and the tertile cut-off points were 13.3 and 25.4 μg/L

[f] Spearman correlation coefficient and associated p-value between total urinary arsenic as a continuous variable and the proportion of each metabolite as a continuous variable

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Table 3

Lung cancer odds ratios (OR) by tertile of %MMA in subjects with metabolite levels above detection limits.

			Crude		Adjusted[a]		
	Cases	**Control**	**OR**	**95% CI**	**OR**	**95% CI**	**p-value[b]**
Low %MMA[c]	12	28	1.00	Reference	1.00	Reference	
Medium %MMA	12	28	1.00	0.38–2.60	0.85	0.29–2.51	
High %MMA	21	19	2.58	1.03–6.46	3.09	1.08–8.81	p-trend = 0.04
%MMA continuous[d]			1.078	1.015–1.145	1.106	1.026–1.191	p = 0.008

[a] Adjusted for age (≤ 65 versus > 65 years old), gender, smoking (ever versus never), and historic drinking water arsenic exposure (no well use, no measurement, 0–99 μg/L, ≥ 100 μg/L).

[b] p-values for Mantel-Haenszel chi-square test for trend across lung cancer odds ratios by tertiles of %MMA or for the lung cancer odds ratio for %MMA as a continuous variable in the logistic regression analysis.

[c] Low, medium and high are based on %MMA tertiles in all subjects with metabolite levels above the detection limit. The upper and lower tertiles are 17.2 and 11.8%. The range for the low, medium, and high %MMA tertiles are 3.8–11.81%, 11.84–17.22%, and 17.28–42.5%, respectively.

[d] Logistic regression where the dependent variable is lung cancer case status and the independent variable is %MMA as a continuous variable. The values given are the odds ratio and its 95% confidence interval for each 1% increase in %MMA.

Table 4

Lung cancer adjusted odds ratios (OR)[a] for each percentage point increase in %MMA, in analyses stratified by cystathionine synthase (*CBS*) polymorphism

Polymorphism	N	OR[b]	95% CI
CBS rs234709 CC (wildtype)	45	0.89	0.74–1.07
CBS rs234709 CT or TT (non-wildtype)	60	1.10	0.99–1.22
CBS rs4920037 GG (wildtype)	68	1.04	0.94–1.15
CBS rs4920037 GA and AA (non-wildtype)	38	1.14	0.91–1.42

[a] Logistic regression odds ratio where the dependent variable is lung cancer case status and the independent variable is %MMA as a continuous variable. The values given are the odds ratio and its 95% confidence interval for each 1% increase in %MMA.

[b] Adjusted for age (≤ 65 versus > 65 years old), gender, smoking (ever versus never), and highest level of past known arsenic exposure (no well use, no measurement, 0–99 μg/L, ≥ 100 μg/L). Only includes subjects with arsenic metabolite concentrations above the detection limit.

Table 5

Epidemiologic studies of arsenic metabolism and relative risks of arsenic-related disease.

		Low %MMA			High %MMA			
Study (Country)	**Outcome**	**Definition**	**OR**	**95% CI**	**Definition**	**OR**	**95% CI**	**Strata**
Current study (Argentina)	Lung cancer	%MMA < 11.8%	1.00	Ref	%MMA > 17.2%	3.09	1.08–8.81	All
Chen et al., 2003 (Taiwan)	Bladder cancer	MMA/DMA < 0.21	1.12	0.26–4.77[a]	MMA/DMA > 0.21	4.23	1.12–16.01	CAE > 12 mg/L-yr
Steinmaus et al., 2006 (Argentina)	Bladder cancer	%MMA < 16.7%	1.00	Ref	%MMA ≥ 16.7%	2.17	1.02–4.63	Smokers[b]
Steinmaus et al., 2006 (US)	Bladder cancer	%MMA < 14.9%	1.00	Ref	%MMA ≥ 14.8%	2.70	0.39–18.6	Intake > 100 μg/day
Pu et al., 2007 (Taiwan)	Bladder cancer	%MMA ≤ 3.0%	1.00	Ref	%MMA > 9.2%	2.8	1.6–4.8	All
Huang et al., 2008 (Taiwan)	Bladder cancer	%MMA < 11.40%	1.5	0.4–5.9[a]	%MMA ≥ 11.40%	3.7	1.2–11.6	CAE ≥ 20 mg/L-yr
Hsueh et al., 1997 (Taiwan)	Skin cancer	%MMA ≤ 26.7%	8.35	1.07–65.0[a]	%MMA > 26.7%	23.9	2.55–225	CAE > 20 mg/L-yr
Yu et al., 2000 (Taiwan)	Skin cancer	%MMA ≤ 15.5%	1.00	Ref	%MMA > 15.5%	5.50	1.22–24.81	All
Chen et al., 2003 (Taiwan)	Skin cancer	MMA/DMA < 0.2	1.89	0.60–6.01[a]	MMA/DMA > 0.20	7.48	1.65–33.99	CAE > 15 mg/L-yr
Ahsan et al., 2007 (Bangladesh)	Skin lesions	%MMA < 9.8%	1.00	Ref	%MMA > 16.4%	1.57	1.10–2.26	All
McCarty et al., 2007 (Bangladesh)	Skin lesions	na	1.00	Ref	10X MMA/InAs[c]	1.50	1.00–2.26	All
Lindberg et al., 2008 (Bangladesh)	Skin lesions	%MMA ≤ 7.9%	1.00	Ref	%MMA > 12%	2.8	1.9–4.2	All
Tseng et al., 2005 (Taiwan)	PVD	%MMA ≤ 11.42	2.64	0.56–12.4[a]	%MMA > 11.42%	4.57	1.01–20.61	CAE > 0
Wu et al., 2006 (Taiwan)	Atherosclerosis	%MMA < 13.4%	1.7	0.6–4.5[a]	%MMA ≥ 13.4%	2.7	1.0–7.8	CAE > 1.7 mg/L-yr
Huang et al., 2007 (Taiwan)	Hypertension	%MMA < 8.14%	1.00	Ref	%MMA ≥ 15.55%	1.04	0.66–1.62	All

Abbreviations: CAE, cumulative arsenic exposure; CI, confidence interval; OR, odds ratio; PVD, peripheral vascular disease; Ref, reference group

[a] Reference groups (where OR = 1.00) are those with low %MMA or MMA/DMA ratio and low CAE.

[b] No statistically significant effect seen in non-smokers.

[c] Relative risk for a 10-fold increase in MMA/InAs ratio.