N-6-Adenine-Specific DNA Methyltransferase 1 (N6AMT1) Polymorphisms and Arsenic Methylation in Andean Women

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Inorganic arsenic (iAs) is a toxic and carcinogenic metalloid found at high concentrations in drinking water in many regions around the world [International Agency for Research on Cancer (IARC) 2004]. As a consequence, millions of persons are continuously exposed to arsenic. Individuals who are exposed to iAs in water or food metabolize arsenic by a series of methylation reactions, converting iAs to mono- (MMA) and dimethylated (DMA) metabolites, which are excreted in urine (Marapakala et al. 2012; Vahter 2002). There are major differences in the efficiency of arsenic methylation between individuals and population groups, and less-efficient arsenic metabolism is associated with the increased incidence of arsenic-related health effects such as skin lesions, cardiovascular disease, diabetes, and cancer (Chung et al. 2002; IARC 2004; Leonardi et al. 2012; Lindberg et al. 2008b; Mazumder et al. 2005; Meliker et al. 2007; Navas-Acien et al. 2005). Thus, it is essential to elucidate the factors influencing arsenic metabolism and susceptibility.

Evaluation of the efficiency of arsenic methylation is mainly based on the relative amounts of the different metabolites in urine. Typically, efficient metabolizers of arsenic have > 80% of the total arsenic in urine in the form of DMA and have low percentages of MMA and MMA (Gardner et al. 2011; Lindberg et al. 2008a; Vahter 2002). Poor metabolizers usually have < 60% of the total arsenic in urine as DMA, 20–30% as MMA, and 10–30% as iAs (Vahter 2002).

S-Adenosylmethionine is the main methyl donor for arsenic (Marafante and Vahter 1984). The methyl transfer is accomplished by methyltransferases, in humans mainly the arsenic (+3 oxidation state) methyltransferase (AS3MT) (Engström et al. 2011). Polycombiosms in AS3MT contribute substantially to the variability in the pattern of excreted arsenic metabolites in different population (Engström et al. 2011; Meza et al. 2005). However, As3mt knockout mice are still capable of metabolizing arsenic, albeit to a lesser extent, suggesting the existence of alternative methylation pathways (Chen et al. 2011; Drobna et al. 2009). There is also evidence that other methyltransferases, such as DNA-methyltransferases (DNMT1, DNMT3b), are capable of arsenic methylation (Engström et al. 2011).

In a recent study, Jo et al. (2009) screened yeast deletion mutants to identify genes required for the growth of yeast in the presence of iAsIII and MMAIII and found that an S-adenosylmethionine-dependent methyltransferase, corresponding to the putative N-6-adenine-specific DNA methyltransferase 1 (N6AMT1) in humans, conferred resistance to these arsenic species. In addition, Chen et al. (2011) showed that N6AMT1 could metabolize arsenic in human urothelial cells in vitro and that N6AMT1 selectively metabolized MMA to DMA. The objective of the present study was to assess associations between polymorphisms in N6AMT1 and the efficiency of human arsenic methylation.

Materials and Methods

Study area and population. Participants were all women (n = 188) living on the Andean plateau (~ 3,800 m above sea level) in northern Argentina and exposed to varying levels of arsenic from their drinking water (Concha et al. 2010). The study area has minimal industrial or traffic pollution. Women were recruited in 2008 and 2011 from the village of San Antonio de los Cobres [water arsenic ~ 200 µg/L (Concha et al. 2010)], with about 6,000 inhabitants, and from small surrounding villages with lower arsenic exposure.
We collected blood and urine samples from each individual in the same way in 2008 and 2011. Venous blood samples were collected in K$_2$EDTA tubes (Vacutette$^\text{®}$; Greiner Bio-One GmbH, Greiner, Germany) for DNA extraction and PAX tubes (PreAnalytix GmbH, Hombrechtikon, Switzerland) for gene expression analyses. Spot urine samples were collected in disposable urine collection cups and immediately transferred to 20-mL polyethylene bottles. We collected all the biological samples at the hospital or the local health clinics during the daytime; the project logistics did not allow for fasting before sampling. After a maximum of 24 hr at room temperature after sampling, blood and urine samples were frozen and kept at –20°C until they were transported with cooling blocks to Sweden for analyses. Analyses took place within 2 months of collection.

Both oral and written informed consents were provided by all the study participants. The study was approved by the Ministry of Health in Salta, Argentina, and the Regional Ethical Committee at Karolinska Institutet.

Arsenic exposure and metabolism. We assessed exposure to iAs based on the sum of the concentrations of the inorganic arsenic metabolites (iAs + MMA + DMA) in urine (U-As), and we assessed the efficiency of arsenic metabolism based on the relative proportions (percentage of the sum of urinary arsenic metabolites) of iAs metabolites (iAsIII, iAsV), MMA, and DMA in urine (Vahter 2002). Arsenic metabolites in urine (i.e., iAs, MMA, and DMA) were determined using high-performance liquid chromatography (HPLC) (Agilent 1100 series system; Agilent Technologies, Waldbronn, Germany) coupled with hydride generation (HG) and inductively coupled plasma mass spectrometry (ICP-MS: Agilent 7500ce; Agilent Technologies, Tokyo, Japan) (Concha et al. 2010). The HG system was used to introduce only the metabolites of iAs to the ICP-MS. After shaking the urine samples, approximately 0.5 mL of each urine sample was filtered on a 0.20-μm syringe filter and transferred to the HPLC-HG-ICPMS system. For quality control, we analyzed the reference material CRM No.18 (certified DMA concentration of 36 ± 9 μg/L; National Institute for Environmental Studies, Ibaraki, Japan) along with the collected samples. We obtained 43.9 ± 4.7 μg/L (mean ± SD; n = 21), which agreed with previously reported results (Li et al. 2008). In order to compensate for variation in urine dilution, we adjusted the measured concentrations of arsenic in urine to the mean specific gravity of urine (1.020 g/mL) determined by a digital refractometer (EUROMEX RD 712 clinical refractometer; EUROMEX, Arnhem, the Netherlands).

Genotyping and gene expression analysis. DNA was isolated from peripheral blood with the QiaGen DNA Blood Mini kit (QIAGEN, Hilden, Germany). We genotyped five N6AMT1 single nucleotide polymorphisms (SNPs in 5’–3’ order: rs1997605, rs2205449, rs2705671, rs16983411, and rs1048546) by Tagman® SNP genotyping assays on a fast real-time PCR System (7900HT; Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer’s instructions. Blanks and controls for each genotype were included in each run, and genotyping was repeated on 5% of the samples.

Because none of the N6AMT1 SNPs have previously been analyzed in relation to functional impact on gene expression or protein activity, we selected SNPs that tag genetic variation within the gene due to strong linkage disequilibrium with other SNPs in the same region (tagSNPs) (Table 1). TagSNPs for N6AMT1 were selected using Haploview (version 4.1; Barrett et al. 2005) on the HapMap (http://hapmap.ncbi.nlm.nih.gov) CEU population (Utah residents with ancestry from northern and western Europe). We inferred haplotypes from N6AMT1 rs1997605, rs2205449, rs2705671, and rs1048546 by PHASE software (Stephens and Donnelly 2003). We did not include rs16983411 in the final haplotype analysis because the frequency was very low (minor allele frequency of 1%).

Genotyping for A53MT SNPs associated with arsenic metabolism was performed as described previously (Engström et al. 2011). Briefly, eight SNPs were genotyped using Sequenom™ (Sequenom, San Diego, CA, USA) technology. We inferred haplotypes

<table>
<thead>
<tr>
<th>SNP</th>
<th>Type</th>
<th>Base</th>
<th>Position</th>
<th>n</th>
<th>Ancestral study population</th>
<th>HapMap allele frequencies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1997605</td>
<td>Intron</td>
<td>A/G</td>
<td>30257418</td>
<td>188</td>
<td>53/47</td>
<td>85/15 50/50 65/35</td>
</tr>
<tr>
<td>rs2205449</td>
<td>Intron</td>
<td>A/T</td>
<td>30252086</td>
<td>188</td>
<td>38/63</td>
<td>25/75 18/82</td>
</tr>
<tr>
<td>rs2705671</td>
<td>Intron</td>
<td>T/G</td>
<td>3025349</td>
<td>184</td>
<td>58/42</td>
<td>86/14 69/21 94/6</td>
</tr>
<tr>
<td>rs16983411</td>
<td>Intron</td>
<td>A/G</td>
<td>30252047</td>
<td>185</td>
<td>99/1</td>
<td>84/16 87/13 82/18</td>
</tr>
<tr>
<td>rs1048546</td>
<td>3'UTR</td>
<td>G/T</td>
<td>30244877</td>
<td>187</td>
<td>53/47</td>
<td>89/21 37/63 59/41</td>
</tr>
</tbody>
</table>

*Allele frequencies for Europeans, Asians, and Africans from HapMap CEU (CEPH, Utah residents from ancestry in northern and western Europe), JPT (Japanese in Tokyo, Japan), and YRI (Yoruba in Ibadan, Nigeria), respectively (Thorisson et al. 2005). From the NCBI dbSNP Single Nucleotide Polymorphisms database (http://www.ncbi.nlm.nih.gov/SNP), genome build 37.3. *Alleles associated with lower %MMA are denoted first. *Chi-square values from the test of Hardy-Weinberg equilibrium were 2.73 for rs1997605, 0.41 for rs2205449, 0.21 for rs2705671, 0.001 for rs16983411, and 2.11 for rs1048546.
from the AS3MT SNPs by PHASE software. In this population we previously found that the major AS3MT haplotype (referred to here as haplotype 2; haplotype frequency = 70%) was GCCATCAC [5´–3´ order of AS3MT SNPs: rs7058104, rs3740400, rs3740393, rs3740390, rs11191439, rs11191453, rs10748835, and rs1067778 (Engström et al. 2011)]. This haplotype was associated with low %MMA and high %DMA, consistent with more efficient arsenic metabolism (Engström et al. 2011). In the 34 women recruited in 2011, we genotyped AS3MT rs3740400, rs3740393, rs11191439, and rs1067778 with ‘Tagma’ SNP genotyping assays according to the manufacturer’s instructions, and inferred haplotype 2 based on the four SNPs only, as we have shown that we obtain very similar inferred AS3MT haplotypes with fewer SNPs compared with a larger number of SNPs (Schiebusch et al. 2013).

To examine gene expression we extracted RNA with the PAXgene Blood RNA kit (PreAnalytiX) and stored the samples at −80°C. We evaluated RNA concentration and purity using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and RNA integrity with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), and the results confirmed high-quality RNA (RNA integrity number > 7.5, where 1 is the worst and 10 the best). For the gene expression analysis, we selected participants who had the best quality and highest quantity of RNA for array analyses. These individuals, with a wide range of urinary arsenic concentrations (10–1,251 µg/L), were classified into low/high U-As concentrations based on median values, and then frequency-matched for age, weight, and BMI so that there were no major differences between the two groups (p > 0.05 for age, weight/BMI), resulting in a total of 63 individuals for the gene expression analyses. For the whole genome gene expression analysis, we used DirectHyb HumanHT-12, version 4.0 (Illumina, San Diego, CA, USA) according to the manufacturer’s instructions, and the analysis was performed at the Swegian Center for Integrative Biology at Lund University. Background signals were filtered by BioArray Software Environment (BASE) (Valлон-Christersson et al. 2009) and results are presented in relative fluorescence units. There were two N6AMT1 transcripts in the HumanHT-12, version 4.0 array: ILMN_2315569, corresponding to mRNA NM_182749.2, which encodes the longer isoform [National Center of Biotechnology Information (NCBI) Nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore)], and ILMN_1754988, corresponding to NM_013240.3, which lacks an alternate in-frame exon, resulting in a shorter protein.

Statistical analysis. We analyzed deviations from Hardy-Weinberg equilibrium using chi-square analysis, and estimated linkage disequilibrium using Haplovew (Barrett 2009).

We estimated associations between genotypes or haplotypes (independent variables) and proportions of individual metabolites (%iAs metabolites, %MMA, or %DMA as dependent variables) in multivariable-adjusted regression models. All models were adjusted for natural log–transformed total urinary arsenic concentration (lnU-As) because the level of arsenic exposure in itself influences metabolism (Vahter 2002). Genotypes and haplotypes were modeled as categorical variables (for genotypes: zero, one, or two alleles; and for haplotypes: zero, one, or two copies), with the genotype or haplotype associated with low MMA fractions as the reference group for each variant in all models. When the frequency of a homozygote genotype included < 3% of individuals, this group was pooled with the heterozygotes. In the multivariable-adjusted analyses, we considered the AS3MT haplotype to be a potential confounder. We first modeled associations between each SNP genotype or haplotype and each outcome (%iAs, %DMA, or %MMA) in separate models adjusted for lnU-As (model 1), then fit models that also were adjusted for AS3MT haplotype (model 2). We also modeled associations between AS3MT haplotype 2 and arsenic metabolites adjusted for each N6AMT1 variant in individual models. In addition, we modeled the joint effects of the most common N6AMT1 haplotype and AS3MT haplotype 2 by estimating associations for each possible combination of genotypes based on the two haplotypes (i.e., with two copies of each haplotype used as the reference category, two copies of the N6AMT1 haplotype and one copy of the AS3MT haplotype, etc., for a total of eight possible combinations), with adjustment for lnU-As.

We analyzed correlations between total arsenic or fraction of MMA in urine and gene expression data using the Spearman correlation coefficient (r). Gene expression data were normally distributed and, therefore, relations between N6AMT1 SNPs/haplotypes and gene expression for N6AMT1 transcripts were analyzed by analysis of variance (ANOVA).

All calculations were made using IBM SPSS Statistics, version 20.0 (IBM, Chicago, IL, USA). Statistical significance was determined as p < 0.05 (two-tailed).

Results

General characteristics. The characteristics of the study population are presented in Table 2. The studied women (n = 188) were on average 34 years of age with a median lnU-As concentration of 210 µg U-As/L (median urinary fraction of iAs, 12%; MMA, 8.1%; and DMA, 80%).

The N6AMT1 SNPs were situated within 12,451 base pairs (distance between SNP rs1997605 in intron 1 and rs1048546 in 3’ UTR). Genotypes of all SNPs were in Hardy Weinberg equilibrium (Table 1). The allele frequencies for the N6AMT1 SNPs varied between the Andean population and other reference populations from the Hapmap study (Table 1). Rs1048546 was in linkage disequilibrium (LD) with rs2705671 (R² = 0.80) and rs1997605 (R² = 0.94), and the LD between rs2705671 and rs1997605 was R² = 0.85 [see Supplemental Material, Figure S1 (http://dx.doi.org/10.1289/ ehp.1206003)]. Rs2205449 was in weaker LD with these three SNPs (R² between 0.42 and 0.51). Rs16983411 showed a very low minor allele frequency (1%) in Andeans, and it was not in LD with any of the other SNPs.

Table 2. Characteristics of the study population.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Median, %, or n</th>
<th>5th–95th percentiles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age [years (median)]</td>
<td>34</td>
<td>19–64</td>
</tr>
<tr>
<td>Time of residency [years (median)]</td>
<td>25</td>
<td>3.0–53</td>
</tr>
<tr>
<td>Total U-As [µg/L (median)]*</td>
<td>154/24</td>
<td>19–35</td>
</tr>
<tr>
<td>Tobacco users (%)</td>
<td>5.3</td>
<td>0.0–2.0</td>
</tr>
<tr>
<td>Alcohol users (%)</td>
<td>3.7</td>
<td>0.0–2.0</td>
</tr>
<tr>
<td>%MMA (%)</td>
<td>12</td>
<td>4.2–15</td>
</tr>
<tr>
<td>%DMA (%)</td>
<td>8.1</td>
<td>4.2–15</td>
</tr>
<tr>
<td>%N6AMT1 haplotypes (n)</td>
<td>80</td>
<td>65–89</td>
</tr>
<tr>
<td>210</td>
<td>33–502</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>4.8–23</td>
<td></td>
</tr>
<tr>
<td>8.1</td>
<td>4.2–15</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>65–89</td>
<td></td>
</tr>
</tbody>
</table>

*SAC: San Antonio de los Cobres; other villages: Santa Rosa de los Pastos Grandes, Pocitos, Olacapato, Cobres, Rosario de Lerma.

**Total U-As was adjusted to the mean specific gravity of 1.020 g/mL. *Number of copies associated with lower %MMA are denoted first. **Number of copies that have previously been shown to have an association with lower %MMA are denoted first (Engström et al. 2011).
Table 3. Multivariable regression analyses* of the influence of *N6AMT1* genotypes on fractions of arsenic metabolites.

<table>
<thead>
<tr>
<th>Metabolite/SNP</th>
<th>Genotype</th>
<th>n</th>
<th>Mean (95% CI)</th>
<th>Model 1</th>
<th>Model 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>β (95% CI)</td>
<td>p-Value</td>
<td>β (95% CI)</td>
</tr>
<tr>
<td><strong>iAs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1997605</td>
<td>AA</td>
<td>63</td>
<td>7.7 (7.0, 8.6)</td>
<td>0.67 (-0.44, 1.8)</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>75</td>
<td>8.6 (7.8, 9.2)</td>
<td>1.90 (0.74, 3.1)</td>
<td>0.0017</td>
</tr>
<tr>
<td>rs2205449</td>
<td>AA</td>
<td>30</td>
<td>7.2 (6.0, 8.3)</td>
<td>1.30 (-0.07, 2.7)</td>
<td>0.083</td>
</tr>
<tr>
<td></td>
<td>TA</td>
<td>81</td>
<td>8.6 (7.8, 9.2)</td>
<td>2.10 (0.75, 3.5)</td>
<td>0.0026</td>
</tr>
<tr>
<td>rs270571</td>
<td>TT</td>
<td>77</td>
<td>9.2 (8.6, 10.0)</td>
<td>0.84 (-0.24, 1.9)</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>70</td>
<td>8.7 (7.9, 9.4)</td>
<td>2.00 (0.73, 3.2)</td>
<td>0.0020</td>
</tr>
<tr>
<td>rs16983411</td>
<td>AA</td>
<td>181</td>
<td>8.5 (8.0, 9.0)</td>
<td>3.30 (0.06, 6.6)</td>
<td>0.046</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>4</td>
<td>11.6 (8.6, 15.0)</td>
<td>1.10 (0.01, 2.2)</td>
<td>0.048</td>
</tr>
<tr>
<td>rs1048546</td>
<td>GG</td>
<td>60</td>
<td>7.5 (6.7, 8.4)</td>
<td>2.30 (1.1, 3.5)</td>
<td>0.0004</td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>77</td>
<td>8.9 (7.9, 9.9)</td>
<td>0.16 (-2.2, 2.5)</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>50</td>
<td>9.7 (8.9, 10.7)</td>
<td>1.50 (-4.2, 3.4)</td>
<td>0.32</td>
</tr>
<tr>
<td><strong>MMA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1997605</td>
<td>AA</td>
<td>63</td>
<td>73.2 (73.3, 80.9)</td>
<td>0.08 (-2.6, 2.4)</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>75</td>
<td>78.9 (77.4, 80.7)</td>
<td>1.50 (-4.2, 3.4)</td>
<td>0.32</td>
</tr>
<tr>
<td>rs2205449</td>
<td>AA</td>
<td>30</td>
<td>79.0 (76.4, 81.6)</td>
<td>0.032 (-3.0, 3.1)</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>TA</td>
<td>81</td>
<td>79.0 (77.5, 80.7)</td>
<td>0.81 (-3.9, 2.3)</td>
<td>0.60</td>
</tr>
<tr>
<td>rs270571</td>
<td>TT</td>
<td>77</td>
<td>78.3 (76.6, 79.8)</td>
<td>-1.10 (-3.6, 1.4)</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>70</td>
<td>78.4 (76.8, 80.1)</td>
<td>-1.20 (-4.0, 1.6)</td>
<td>0.40</td>
</tr>
<tr>
<td>rs16983411</td>
<td>AA</td>
<td>181</td>
<td>78.8 (77.7, 79.8)</td>
<td>0.60 (-3.0, 2.3)</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>4</td>
<td>78.3 (70.9, 85.3)</td>
<td>0.21 (-2.9, 3.3)</td>
<td>0.90</td>
</tr>
<tr>
<td>rs1048546</td>
<td>GG</td>
<td>60</td>
<td>79.4 (74.8, 81.1)</td>
<td>1.40 (-4.7, 7.5)</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>77</td>
<td>78.8 (77.3, 80.6)</td>
<td>-0.31 (-2.8, 2.2)</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>50</td>
<td>77.8 (75.6, 79.8)</td>
<td>-1.70 (-4.4, 1.1)</td>
<td>0.23</td>
</tr>
</tbody>
</table>

*Model 1: Arsenic metabolite = α + β genotype + γ × U-As (In transformed). Model 2: Arsenic metabolite = α + β genotype + γ × U-As (ln) + λ × AS3MT haplotype. Allele genotypes associated with lower %MMA are denoted first. *Mean values and 95% CIs are adjusted values based on model 1.
association is generally consistent with additive effects of each haplotype, i.e., $\beta = 2.1; 95\% \text{ CI: } 0.75, 3.5$ for no copies versus two copies of N6AMT1 haplotype 1 (Table 4), and $\beta = 3.3; 95\% \text{ CI: } 1.8, 4.7$ for no copies versus two copies of AS3MT haplotype 2 [see Supplemental Material, Table S3 (http://dx.doi.org/10.1289/ehp.1206003)]. In general, mean %MMA increased as the number of copies of AS3MT haplotype 2 copies increased and as copies of N6AMT1 haplotype 1 decreased, and vice versa, although %MMA was highest among 8 women with either one or two copies of N6AMT1 haplotype 1 and no copies of AS3MT haplotype 2 (11.1; 95% CI: 9.0, 13.2). This pattern of associations was only seen for %MMA.

**N6AMT1 expression analyses.** N6AMT1 expression in whole blood (ILMN_2315569 median = 121 fluorescence units, range 96–106; and ILMN_1754988 median = 112 fluorescence units, range 94–131) was above the overall median level of expression for all transcripts on the array (109.3 fluorescence units, with median expression levels for individual transcripts ranging from 82.9 to 22,445). There were no statistically significant correlations between N6AMT1 expression and total arsenic concentrations or %MMA (data not shown); the strongest correlation found was between U-As and ILMN_1754988 ($r_c = -0.17; p = 0.18$). In general, ILMN_2315569 expression was lowest in women with genotypes and haplotypes associated with the lowest %MMA (used as reference group in Table 6), although in all but two cases (rs2205449 and haplotype 1) heterozygous carriers had the highest expression (i.e., there was not a monotonic association according to numbers of alleles). For rs2705671 and N6AMT1 haplotype 9, there were statistically significant differences among all three genotypes or haplotypes as a group ($p$-values 0.047 and 0.029, respectively), but post hoc analyses showed that the difference for rs2705671 was significant only between TT and GT ($p = 0.040$, compared with $p = 0.87$ for TT and GG); and for haplotype 9 between no and one copies ($p = 0.027$ compared with $p = 0.94$ for 0 and two copies). An allele–dose effect of the expression of N6AMT1 was observed for some SNPs/haplotypes (Table 6).

**Discussion**

In our study population of Andean women, variation in the relative amount of MMA in urine was associated with genetic variation in N6AMT1 in an allele–dose dependent manner, a finding that supports the hypothesis that N6AMT1 is involved in human arsenic metabolism. Associations between %MMA and N6AMT1 variants persisted when adjusted for a common AS3MT haplotype and copy number of AS3MT and N6AMT1. This result is consistent with previous studies showing an association between N6AMT1 expression and urinary MMA. In our study population of Andean women, urinary MMA was associated with genetic variation in N6AMT1.

<table>
<thead>
<tr>
<th>Table 4. Multivariable regression analyses* of the influence of N6AMT1 haplotypes on fractions of arsenic metabolites.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 5. Multivariable regression analyses* of haplotype × haplotype interaction between AS3MT (haplotype 2) and N6AMT1 (haplotype 1) and fractions of arsenic metabolites.</td>
</tr>
</tbody>
</table>

\*Model: Arsenic metabolite (%As or %DMA or %MMA) = $\alpha + $ β N6AMT1 haplotype + γ U-As (ln transformed). Model 2: Arsenic metabolite = $\alpha + $ β AS3MT haplotype + γ U-As (ln transformed) + $\lambda $ AS3MT haplotype 2. Number of copies of each haplotype associated with lower %MMA are denoted first. $^a$Mean values and 95% CIs are adjusted values based on the presented statistical model.
that was also associated with %MMA. When homozygous carriers of variant of each gene were compared, associations between \textit{N6AMT1} variants and %MMA were not as strong as associations with the \textit{AS3MT} haplotype. When estimated according to combined copy numbers of \textit{N6AMT1} and \textit{AS3MT} haplotypes associated with low %MMA, associations were consistent with an additive effect of variants in the two genes, such that women with two copies of the two haplotypes had the lowest mean %MMA (6.2; 95% CI: 4.6, 7.8), in contrast with a mean of 10.4 (95% CI: 8.7, 12.0) \((\beta = 4.2; 95\% \text{ CI: 1.9, 6.5})\) among women with no copies of either haplotype. Although there were few individuals in some of the combined haplotype groups, the differences observed could be sufficient to increase the risk of arsenic-related disease, as higher %MMA in urine [mostly MMA(V)] is related to increasing risk of several adverse health effects (Chung et al. 2002; IARC 2004; Leonard et al. 2012; Mazumder et al. 2005; Meliker et al. 2007; Navas-Acien et al. 2005; Valter 2002). All \textit{N6AMT1} SNPs except one (rs16983411) were common in the study population (minor allele frequencies > 40%) and in HapMap reference populations (Thorisson et al. 2005), and apart from rs2205449, more than half of the participants were carriers of alleles that were associated with lower %MMA. For \textit{AS3MT}, we previously showed a strong overrepresentation of the haplotype associated with low MMA and high DMA (i.e., an efficient and less toxic metabolism) in this Andean population, compared with all other studied populations worldwide (Schlawicke Engström et al. 2007).

The gene \textit{N6AMT1} was recently identified in humans (Ratel et al. 2006) and is located on 21q12.1 [NCBI HomoloGene database (http://www.ncbi.nlm.nih.gov/homologene)]. It is conserved in chimpanzee, dog, cow, mouse, rat, chicken, zebras, fruit fly, mosquito, \textit{Caenorhabditis elegans}, \textit{Saccharomyces pombe}, \textit{Arabidopsis thaliana}, and rice (NCBI HomoloGene database). The mouse homolog of \textit{N6AMT1} has been shown to methylate glutamine in the translation termination factor eRF1 and to be crucial for embryological development (Li et al. 2009). \textit{N6AMT1} has about 25% amino acid sequence similarity with \textit{AS3MT} in the S-adenosylmethionine binding domain of class I (Ajees et al. 2012; see also NCBI Conserved Domains database (http://www.ncbi.nlm.nih.gov/HHpred)), a structural fold shared by most methyltransferases (Schubert et al. 2003). However, the structural similarity between \textit{N6AMT1} and \textit{AS3MT} does not include the three cysteine residues (C72, C174, and C224) that bind inorganic 

\begin{table}[h]
\centering
\caption{\textit{N6AMT1} gene expression as expressed relative fluorescence units, stratified by \textit{N6AMT1} SNPs and haplotypes.}
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{N6AMT1 SNP or haplotype$^a$} & \textbf{Genotype or no. of copies} & \textbf{ILMN\_23155669} & \textbf{ILMN\_1765088} \\
\hline
rs1997805 & AA & 24 & 117.2 (111.3, 123.0) & 111.0 (108.0, 114.0) \\
 & AG & 24 & 126.2 (119.8, 133.9) & 114.7 (111.9, 117.8) \\
 & GG & 15 & 122.9 (112.6, 133.2) & 115.2 (111.3, 119.1) \\
 & \textit{p} = 0.12 & & \textit{p} = 0.10$^b$ & \\
rs2205449 & AA & 11 & 114.7 (107.3, 122.1) & 113.9 (107.8, 119.1) \\
 & TA & 24 & 120.3 (114.4, 126.3) & 112.3 (109.9, 114.7) \\
 & TT & 28 & 126.3 (119.5, 134.2) & 114.2 (111.1, 117.5) \\
 & \textit{p} = 0.09$^b$ & & \textit{p} = 0.60$^a$ & \\
rs2705971 & TT & 28 & 116.8 (111.3, 120.7) & 111.9 (108.9, 114.9) \\
 & GT & 24 & 127.3 (120.8, 135.1) & 114.6 (111.7, 117.8) \\
 & GG & 11 & 119.6 (107.7, 131.8) & 115.7 (111.8, 119.6) \\
 & \textit{p} = 0.04$^b$ & & \textit{p} = 0.22$^b$ & \\
rs1048546 & GG & 21 & 115.6 (109.3, 121.8) & 111.0 (107.5, 114.6) \\
 & GT & 28 & 126.1 (120.3, 132.9) & 114.3 (111.8, 117.0) \\
 & TT & 14 & 122.7 (111.6, 133.8) & 115.5 (113.3, 119.6) \\
 & \textit{p} = 0.073$^b$ & & \textit{p} = 0.15$^a$ & \\
Haplotype 1 & 2 copies & 11 & 114.7 (107.3, 122.1) & 113.9 (108.7, 119.1) \\
 & 1 copy & 24 & 120.3 (114.4, 126.3) & 112.3 (109.9, 114.7) \\
 & 0 copies & 28 & 126.3 (119.5, 134.2) & 114.2 (111.2, 117.5) \\
 & \textit{p} = 0.60$^a$ & & \textit{p} = 0.15$^a$ & \\
Haplotype 3 & 1 and 2 copies & 21 & 125.7 (117.6, 133.7) & 111.8 (108.2, 115.4) \\
 & 0 copies & 42 & 120.5 (115.6, 125.4) & 114.3 (112.2, 116.4) \\
 & \textit{p} = 0.24$^b$ & & \textit{p} = 0.20$^b$ & \\
Haplotype 9 & 0 copies & 27 & 117.2 (111.9, 121.5) & 117.1 (108.8, 114.7) \\
 & 1 copy & 26 & 128.1 (121.9, 135.5) & 114.1 (111.3, 117.2) \\
 & 2 copies & 10 & 118.1 (105.7, 132.4) & 116.1 (118.9, 120.4) \\
 & \textit{p} = 0.029$^b$ & & \textit{p} = 0.20$^b$ & \\
\hline
\textit{a}The genotype/haplotype associated with lower %MMA is denoted first and used as reference group. rs16863411 was excluded from the analysis because there were so few carriers with variant genotypes \((n = 3)\). Due to low frequency of carriers of two copies of haplotype 3, carriers of one and two copies were merged. \textit{b}Values from ANOVA.
\end{tabular}
\end{table}

A limited range of expression of \textit{N6AMT1} in blood may be one of the reasons why we did not find any association between \textit{N6AMT1} expression in whole blood and total arsenic in urine; alternatively, the expression of \textit{N6AMT1} may not be induced by arsenic. Genotypes associated with low %MMA were associated with the lowest \textit{N6AMT1} expression, although in most cases expression did not change monotonically according to allele or haplotype copy numbers. The direction in expression is similar to observations for \textit{AS3MT}: For carriers of the haplotype associated with more proficient arsenic metabolism and less MMA in urine, reduced \textit{AS3MT} expression was found in blood (Engström et al. 2011). Still, the relation between genotype–gene expression and metabolite pattern should be further explored, ideally in tissues where \textit{N6AMT1} is highly expressed, such as the adrenal and parathyroid glands and the kidneys (http://www.proteinatlas.org) (Ren et al. 2011; Uhlen et al. 2005).

Although the associations with %iAs or %DMA may be influenced by %MMA, the findings for \textit{N6AMT1} suggest different effects of \textit{N6AMT1} on the two methylation steps. Findings for \textit{AS3MT}, on the other hand, suggest that it may affect both steps to about the same extent (Engström et al. 2011).

Conclusions

Polymorphisms in \textit{N6AMT1} significantly predicted the %MMA in urine in a population of women from the Argentinean Andes, suggesting additional pathways and methyltransferases involved in the metabolism of arsenic. This emphasizes the need to consider \textit{N6AMT1} in future studies of populations exposed to arsenic.
N6AMT1 variants and arsenic methylation


References


