HHS Public Access

Author manuscript

Toxicol Appl Pharmacol. Author manuscript; available in PMC 2016 December 15.

Published in final edited form as:

Toxicol Appl Pharmacol. 2015 December 15; 289(3): 381–388. doi:10.1016/j.taap.2015.10.024.

Genetic variation in metallothionein and metal-regulatory transcription factor 1 in relation to urinary cadmium, copper, and zinc

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Abstract

Background—Metallothionein (MT) proteins play critical roles in the physiological handling of both essential (Cu and Zn) and toxic (Cd) metals. MT expression is regulated by metal-regulatory transcription factor 1 (MTF1). Hence, genetic variation in the MT gene family and MTF1 might therefore influence excretion of these metals.

Methods—321 women were recruited in Seattle, WA and Las Cruces, NM and provided demographic information, urine samples for measurement of metal concentrations by mass spectrometry and creatinine, and blood or saliva for extraction of DNA. Forty-one single nucleotide polymorphisms (SNPs) within the MTF1 gene region and the region of chromosome 16 encoding the MT gene family were selected for genotyping in addition to an ancestry informative marker panel. Linear regression was used to estimate the association of SNPs with urinary Cd, Cu, and Zn, adjusted for age, urinary creatinine, smoking history, study site, and ancestry.

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Conflict of interest statement. The authors state that they have no real or potential conflicts of interest that could have influenced this research.

Human subjects. This research was reviewed and approved by institutional review boards at the Fred Hutchinson Cancer Research Center and New Mexico State University. All participants provided written informed consent.

Results—Minor alleles of rs28366003 and rs10636 near the MT2A gene were associated with lower urinary Cd, Cu, and Zn. Minor alleles of rs8044719 and rs1599823, near MT1A and MT1B, were associated with lower urinary Cd and Zn, respectively. Minor alleles of rs4653329 in MTF1 was associated with lower urinary Cd.

Conclusions—These results suggest that genetic variation in the MT gene region and MTF1 influences urinary Cd, Cu, and Zn excretion.

Keywords

metallothionein; metal-regulatory transcription factor 1; cadmium; copper; zinc

INTRODUCTION

Metallothioneins (MTs) are a family of evolutionarily conserved, small cysteine-rich proteins that bind transition metal ions, including with highest affinity zinc (Zn), copper (Cu), and cadmium (Cd) (Bremner and Beattie 1990; Hamer 1986; Isani and Carpene 2014; Vasak and Meloni 2011). MTs are critical to homeostasis of the essential micronutrients Zn and Cu, and for detoxification of the toxic metal Cd, and also likely serve as reactive oxygen species scavengers(Babula et al. 2012). However, uncertainty over the many putative cellular functions of MTs remains(Isani and Carpene 2014; Palmiter 1998). The handling of metal ions by MT has been suggestively implicated in a variety of diseases including Alzheimer's disease(Carrasco et al. 2006; Luo et al. 2013), cancer risk and progression(Goulding et al. 1995; Kelley et al. 1988; Kim et al. 2011), and renal and liver toxicity due to heavy metals(Klaassen and Liu 1997).

In addition to critical roles in handling metals, MTs are transcriptionally regulated in response to metal ions. A key protein in this process is metal-regulatory factor 1 (MTF1), which binds metal responsive elements located upstream of MT genes in the presence of heavy metals (Palmiter 1994). This step may be the critical pathway to the induction of MT in response to metals(Jiang et al. 2003), and thus genetic variation in MTF-1 may modulate expression of MT and thereby influence biological management of metals (Sims et al. 2012).

Earlier studies of genetic variation in MT genes have generally taken a candidate-gene approach in which a few single-nucleotide polymorphisms (SNPs) are analyzed. Nonetheless, these studies have identified suggestive associations with risk of several cancers (Forma et al. 2012; Krzeslak et al. 2012; Starska et al. 2014; Zavras et al. 2010), diabetes (Giacconi et al. 2005; Yang et al. 2008), and other diseases (Chen et al. 2010; Chen et al. 2012; Giacconi et al. 2007; Hayashi et al. 2006) as well as levels of metals in biological matrices such as blood, urine, and kidney tissue(Chen et al. 2012; Kayaalti et al. 2010; Kayaalti et al. 2011; Lei et al. 2012; Raudenska et al. 2014). However, candidate SNP studies such as these—in which a very small number of SNPs are analyzed—may miss other important genetic variation, cannot assess whether the identified associations may be due to additional SNPs in disequilibrium with the candidate SNPs, and require further validation in additional populations. Therefore, to shed further light on the relevance of genetic variation in MT and MTF1 to physiological control of metals, we were motivated to take a more

systematic approach to exploring the associations of MT and MTF1 SNPs with urinary levels of Cd, Cu, and Zn.

METHODS

Study populations

For this study, data were pooled from participants recruited for two earlier studies: the Equol, Breast and Bone (EBB) study in Seattle, Washington (Atkinson et al. 2008a) and the New Mexico Metals (NMM) study in the Doña Ana County, New Mexico region (previously unpublished). Data were pooled for three reasons: (1) to increase sample size; (2) to increase the genetic variation included in the study; and (3) increase the ranges of urinary cadmium, copper, and zinc in the study population. All three of these factors would increase statistical power to observe associations.

EBB—The recruitment, exclusion criteria, and clinical protocols of the EBB study have been detailed elsewhere (Atkinson et al. 2008a; Atkinson et al. 2009). Briefly, premenopausal women aged 40–45 years were recruited from a mammographic screening program within Group Health (GH), an integrated healthcare system in Washington State. Based on self-report, peri- and postmenopausal women, women with a history of breast cancer, women with certain digestive conditions and women using antibiotics were also excluded. Study procedures were approved by the Fred Hutchinson Cancer Research Center (FHCRC) and GH Institutional Review Boards.

NMM—Women and men age 40–85 were recruited by *promotoras* (community health workers) employed by the *La Clinica de Familia* primary care clinic network in southern New Mexico. *La Clinica de Familia* is a non-profit organization serving approximately 6,500 low income and rural residents of the Doña Ana County, NM region. Participants were recruited in two time periods, 2011 and 2012. All participants provided written informed consent. Interviews were conducted in either English or Spanish.

Data Collection

Questionnaires—Participants in the EBB and NMM studies completed differing questionnaires that each included demographics and smoking history as well as other dietary, lifestyle, and health topics. Height and weight were measured in the clinic for EBB study participants and self-reported for NMM study participants. EBB study participants completed a self-administered questionnaire; NMM questionnaires were interviewer-assisted.

Urinary Metals—Participants in both studies provided spot urine samples collected in polyethylene collection cups. Samples were refrigerated, aliquoted, and stored at −70C until shipment for trace metals and creatinine analysis.

Urine samples were shipped frozen to the Trace Element Research Laboratory at the Wisconsin State Laboratory of Hygiene (Madison, WI) for assay of a suite of 30 elements, including Cd, Cu, and Zn, quantified using magnetic-sector (high-resolution) inductively-coupled plasma mass spectrometry (SF-ICP-MS). A Finnigan, Element 2, magnetic sector

ICP-MS, interfaced with an ESI FAST (SC-E2-DXS) high efficiency, low-flow nebulizer/autosampler, was employed. The complete analytical system was located within a trace metal clean room. This approach enabled accurate and precise quantification of low levels of elements in complex urine matrices. The signal-to-noise of SF-ICPMS is superior to that of quadrupole ICP-MS, and when operated in medium or high resolution modes, spectral interferences that compromise quantification of many elements in urine by traditional quadrupole ICP-MS, are eliminated. The formation of molybdenum oxide (MoO) is monitored throughout the analytical sequence, and where appropriate, a run position empirical correction is applied to the cadmium data. MoO formation was also evaluated with molybdenum stable isotope spikes in selected participant samples. Urine samples were diluted 1+5:6 and 1+9 with 2% (v/v) high purity 16M nitric acid (containing three internal standards) for analysis. A minimum of three replicate 180-second analyses were performed on each sample after a 60-second uptake and stabilization period. The ESI sample with a long rinse with 2% high purity nitric acid + 0.01% Triton between samples virtually eliminated carry-over. The typical SF-ICP-MS batch included 20 participant samples, 2 sample matrix spikes, 2 blank spikes, 3 certified reference materials (CSRMs, including NIST 2670a, UTAK, and Seronorm), 3 matrix blanks, 2 method blanks, 2 sample duplicates, and a set of check blanks and calibration verification checks run at frequent intervals during the batch sequence.

Urine creatinine was measured at the Fred Hutchinson Cancer Research Center Public Health Sciences Biomarker Laboratory with a Roche Cobas Mira Plus Chemistry Analyzer using creatinine reagent set (cat no C7539, Pointe Scientific, Inc., Canton, MI), following manufacturer's instructions. The Chemistry Analyzer was calibrated with the Pointe Scientific creatinine standard (cat no C7513-STD). Samples were run in duplicates; median duplicate coefficient of variation (CV) was 1.7%. Each batch of 20 participant samples were bracketed by both a low and a high quality control (QC) sample (Pointe Scientific Human urine control set, cat no P7582-CTL).

Single nucleotide polymorphism (SNP) selection—Several approaches were used to select SNPs. First, SNPs described in published reports related to cadmium were included (rs28366003, rs10636, and rs11076161)(Chen et al. 2012; Kayaalti et al. 2010; Kayaalti et al. 2011; Lei et al. 2012). Next we used the Genome Variation Server (GVS v. 7, dbSNP build 137; May 2012, current version: http://gvs.gs.washington.edu/GVS138/index.jsp) (Carlson et al. 2004) to identify tag SNPs, with bin correlation set to $r^2 \geq 0.50$, throughout the MT gene family and MTF1 regions and retaining SNPs with minor allele frequency (MAF) ≥ 15% within the HapMap CEU and MEX populations. We selected SNPs within ~2.5 kbp upstream of MT4A and ~4.2 kbp downstream of MT1X, i.e., ±3 kbp of the ~124 kbp region of chromosome 16 encoding the metallothionein (MT) gene family. MTF1 SNPs were selected from the beginning of the 5′ UTR to the end of the 3′ UTR (~50 kbp total length). A preliminary list of SNPs was optimized for Illumina Golden Gate sequencing technology, resulting in substitution of tag SNPs within the same "bin" to optimize the oligo pool assay (OPA) design score. In this process rs11076160 was substituted for rs11076161 (r^2=1.0 and 0.96 in HapMap-CEU and MEX, respectively). A final list of 41 candidate SNPs comprising 35 tag bins ($r^2 \geq 0.5$) in MT genes and 5 SNPs comprising 5 bins in the MTF1 gene was

generated for genotyping. In addition, participant genotypes of 24 SNPs designed as an ancestry informative marker (AIM) panel suitable for populations in the Americas(Kosoy et al. 2009) were assayed.

DNA preparation and genotyping—For EBB samples, DNA was extracted from the buffy coat fraction of a study blood sample using a Qiagen blood kit as described (Atkinson et al. 2008b; Yong et al. 2010). For NMM samples, saliva collected with OraGene OGR-500 kits (DNA Genotek Inc, Ontario, Canada) and DNA extracted in the FHCRC Specimen Processing Laboratory. Previous studies have demonstrated the concordance of genotypes obtained with DNA from blood and saliva (Abraham et al. 2012).

DNA was genotyped using Illumina's GoldenGate Genotyping assay on the VeraCode Platform (Illumina, San Diego, CA) (Shen et al. 2005) following manufacturer recommendation (VeraCode Assay Guide 11312819 rev A1). In brief, 250 ng of genomic DNA was aliquoted into 96-well plates, processed accordingly and scanned on the BeadXpress reader, using GenomeStudio (v2011.1) software.

We conducted a series of quality control (QC) procedures as follows. Prior to running the study samples, 90 samples representing 30 parent-parent-child CEPH trios (Utah residents with ancestry from northern and western Europe)(Thorisson et al. 2005) were genotyped to assess performance of the Illumina OPA. Assay accuracy was verified by comparing genotypes to publicly available genotype data for these samples from HapMap (http://www.hapmap.org/), 1000Genomes (http://www.1000genomes.org), dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), and by assessing inheritance errors. Two external control samples from the HapMap project were included on each plate to confirm reliability and reproducibility of the genotyping across the study plates. Intra- and inter-plate duplicates comprised >10% of all samples. Laboratory personnel were blinded to all research information about the samples. Other QC procedures included use of barcodes on samples and plates, dedicated materials and working space, and visual review of 96 SNP cluster plots by two laboratory staff members. Samples with weak signals, discordant duplicates, and outliers were repeated at least once.

Sample sequence call rates were >85%. Two SNPs (rs11076160 and rs2018703) failed to cluster and were excluded. Among successfully genotyped SNPs (69 of 71), SNP call rates were >95%. Linkage disequilibrium between SNPs was examined by uploading genotype data to GVS; one pair of SNPs with $r^2>0.8$ and 9 pairs with $r^2>0.5$ not in the same bin during selection were identified and retained for genotyping assays. Of 39 SNPs in the MT region, 38 were in Hardy-Weinberg equilibrium (HWE; P>0.01); rs1599823 was the exception. All 5 SNPs in MTF1 were in HWE (P>0.01). AIM panel SNPs were not tested for HWE.

Statistical analysis

Exclusions—Participants in either study who did not consent to future genetic samples were excluded from genotyping (N=19) as were men in the NMM study, and one woman with unknown smoking history.

Principal component analysis (PCA) of AIM panel—Genotypes were coded as 0, 1, or 2 for homozygote major allele, heterozygote, and homozygote minor allele respectively. We then conducted PCA on 18 of the 24 AIM panel genotypes to generate 18 orthogonal eigenvectors. Six AIM SNPs were excluded from PCA because the assay failed in some participants (1 AIM SNP failed in 1 participant, 5 failed in 2 participants, 1 failed in 9 participants), which would exclude these participants from further analyses. Comparison of PCA results with 18 or 24 AIM SNPs among participants with data on all 24 AIM SNPs were similar. Scree plots of PCA eigenvectors indicated that the first 3 components were sufficient to capture most variation within the AIM panel; we therefore included 3 components as adjustment covariates in statistical analyses of MT and MTF1 SNPs in association with urinary metals. We also repeated urinary metals analyses with the first 5 PCA components instead of 3.

Association of SNPs with urinary metals—In our primary analysis, data from both studies were pooled and multivariable linear regression with robust standard errors was used to estimate the association of each MT and MTF1 SNP with urinary cadmium (U-Cd), copper (U-Cu), and zinc (U-Zn), adjusted for age, smoking history (never or ever), the three largest components from PCA of the AIMs, urinary creatinine, and study. Genotypes were treated as categorical variables with three levels, and in separate analyses, per-allele trend was evaluated. In all analyses the referent category was the homozygote of the major allele. P-values for the regression coefficient of each urinary metal comparing the homozygote minor allele compared to homozygote major allele (P(mm)), and for the per-allele trend (P(trend)), were obtained from t-tests of the null hypothesis that the linear regression coefficient was zero. Our primary criteria for evaluating association was the value of P(mm).

In additional analyses, the associations of each MT or MTF1 SNP with U-Cd, U-Cu, and U-Zn, were estimated separately in the EBB study and NMM study. To evaluate whether associations differed significantly between the two study samples, for SNPs with significantly associated with any metal in the pooled sample, we repeated analysis in a separate model that included an interaction term between the independent variable describing the per-allele trend in genotype and the variable indicating the study (EBB or NMM). The P-value of this term is denoted P_{int}(study). Hence, this analysis was designed to identify SNPs for which the association between the number of minor alleles and urinary metal(s) differed between studies.

Inclusion of creatinine as a separate variable, as opposed to direct normalization of each participant's urinary metal concentration, has been recommended for heterogeneous study samples such as ours (Barr et al. 2005).

In sensitivity analyses, directly creatinine-normalized urinary metal concentrations were used instead of including creatinine as a separate variable; and log-transformed urinary metals and creatinine were used in place of non-transformed variables.

Nominal statistical significance was initially set to $P<0.05$, and uncorrected P values and confidence intervals estimated. Of 39 MT SNPs evaluated, four were in LD ($r^2>0.8$) with

one or more other SNPs (Supplemental Figure 1), leaving 35 approximately independent tests of association within each metal. None of the 5 MTF1 SNPs evaluated were in LD.

Ethics

This study was reviewed and approved by Institutional Review Board at Fred Hutchinson Cancer Research Center. The EBB study and NMM study were also reviewed by the IRB of GH Research Institute and New Mexico State University, respectively.

RESULTS

A total of 321 women were included in this study from Seattle (EBB study) and New Mexico (Table 1). Mean urinary metals and creatinine were higher in NMM participants than in EBB study participants. NM women were older and had higher BMI than EBB women; the large majority of women in both samples were white, but NM were nearly all Hispanic while EBB women were predominantly non-Hispanic.

Pooling both study samples, the median MAF of SNPs included in the study was 27%. As a result, for each of 24 SNPs, N=20 or more participants were minor allele homozygotes. In addition, the prevalence of 14 MT SNPs and 3 MTF1 SNPs differed significantly between participants from Seattle and New Mexico, but for only 2 SNPs were all minor allele homozygotes from a single study (Table 2).

Several SNPs in the MT gene region were significantly associated with one or more of U-Cd, U-Cu, and U-Zn concentrations adjusted for age, creatinine, study site, AIM principal components, and smoking history. Notably, minor alleles of rs28366003 and rs10636, located within 1 kbp of one another and in weak LD ($r^2<0.2$), were associated with lower urinary concentrations of all three metals.

Minor alleles of rs4784706 were significantly associated with lower U-Cd and U-Cu and associated with lower U-Zn, although the association with U-Zn did not reach statistical significance. rs8044719 was associated solely with lower U-Cd, although homozygosity in the minor allele of nearby rs1599823 (3 kbp) was associated with lower U-Zn.

Minor alleles of rs12444489 were significantly associated with U-Cu and U-Zn but not U-Cd. Finally, homozygosity for minor alleles at rs2301234 and rs8052106 were associated only with lower U-Cu.

One SNP within the MTF1 gene, rs4653329, was associated with lower U-Cd, but no associations between this SNP and other metals were observed (Figure 1).

For each SNP identified as associated with urinary metals in the pooled study sample, analysis was repeated separately in each study population, testing for a difference in association between study sites (Figure 2). Results showed that for all SNPs except rs28366003, associated with U-Cd, and rs1599823, associated with U-Zn, the associations in each study were consistent with the pooled results.

Results were not sensitive to changes in analyses including adjustment for smoking status as never, former, or current rather than never or ever smoking; additional adjustment for body mass index; direct normalization to urinary creatinine by dividing urinary metals by creatinine prior to statistical models; use of log-transformed urinary metals and creatinine; and adjustment for 5 rather than 3 PCA components of the AIM panel. We elected to present non-log-transformed metal concentrations to ease interpretation.

DISCUSSION

In this study we observed several SNPs in the region encoding MT genes that were suggestively associated with urinary concentrations of Cd, Cu, and Zn. In particular the region encoding MT2A, the isoform expressed in a number of tissues in humans, appeared to be a “hot spot” for modulation of urinary Cd, Cu, and Zn. In addition, one SNP in MTF1 was associated with lower urinary Cd but not Cu or Zn.

Previous studies have examined MT gene variation primarily in association with blood or urine levels of Cd, Cu, or Zn using a candidate-SNP approach(Chen et al. 2012; Kayaalti et al. 2011; Lei et al. 2012; Raudenska et al. 2014; Wang et al. 2012). In particular, rs28366003, a A/G substitution in the promoter region of MT2A which may influence expression levels(Kita et al. 2006; Krzeslak et al. 2013), has been examined in several prior studies and associated with elevated metals in kidney (Kayaalti et al. 2010) and inconsistently in blood (Chen et al. 2012; Kayaalti et al. 2011; Lei et al. 2012) but not urine Cd (Chen et al. 2012). Nonetheless, elevated kidney Cd should result in elevated urinary Cd, and our results for MT2A SNPs thus stand in apparent contrast to the earlier study (Kayaalti et al. 2010). The relationship between blood, urine, and kidney tissue Cd is complex and likely depends on MT binding of Cd during filtration (Raudenska et al. 2014; Sabolic et al. 2010). Thus, genetic polymorphisms that may alter MT expression levels may not influence concentrations of metals in different biological matrices in the same manner. Furthermore, our study was conducted among populations in the USA with relatively low exposure in comparison to others (Chen et al. 2012; Lei et al. 2012; Wang et al. 2012), which may modify the influence of MT polymorphisms on blood and urine metal concentrations (Lei et al. 2012).

The minor allele (G) of rs28366003 is rare in the white population of North America (McElroy et al. 2010), and our study included only one homozygote for the minor allele; the association we observed with reduced urinary Cd, Cu, and Zn must be interpreted cautiously. Heterozygotes at rs28366003 also had lower U-Cd than AA homozygotes, although the difference was not statistically significant. However, nearby SNPs rs4784695 and rs10636 were in weak LD with rs28366003 (r^2~0.4 and 0.1 respectively), and also showed suggestive associations with lower urinary metals for homozygotes of the minor allele. Thus, overall, we believe our results are consistent with earlier candidate SNP studies in showing that genetic variation in this region near MT2A influences urinary excretion of metals that bind MT.

Previous reports have also implicated the SNP rs11076161 in MT1A with blood Cd(Chen et al. 2012; Lei et al. 2012), albeit weakly. We attempted to include rs11076160, a SNP in LD

with rs11076161 (r^2~1); unfortunately it failed genotyping. Other SNPs within ±5 kbp of MT1A in our study include those between rs2270836 and rs8044719 (Figure 1), and include two suggestive associations with lower U-Cd and U-Zn.

To our knowledge, genetic variation in MTF1 has not been investigated in association with a measure of exposure to metals such as urinary concentrations. MTF1 is a transcription factor that controls expression of MT and many other genes in response to metal ions including Cd, Cu, and Zn.(Jiang et al. 2003; Sims et al. 2012; Troadec et al. 2010) The SNP we identified, rs4653329, is located in an intron and thus its functional significance is unclear. Nonetheless, our MTF1 results suggest that genetic variation in transcriptional factors that regulate MTs is relevant to understanding physiological response to metals. Notably, the MT2A SNP rs28366003 is in the promoter region of MT2A. Thus, genetic variation in regulation of expression of MTs may emerge as the most important way in which genetic variation influences response to metals, in contrast to variation that directly modulates MT function. Future studies might be aimed at the entire pathway or network that includes MTs as well as their regulatory factors.

Some limitations to our study should be considered in interpreting the results, including the modest sample size in this exploratory study. In addition, we measured Cd, Cu, and Zn in spot urine samples as opposed to 24-hour collection, which may have introduced more variation and attenuated observed associations(Gunier et al. 2013); however, metals assessment in spot urines compares well to 24-hour samples (Akerstrom et al. 2014). Urinary Cd is generally accepted as a measure of cumulative Cd exposure (Adams and Newcomb 2014; Akerstrom et al. 2013; Lauwerys et al. 1979), and some evidence supports urinary Zn as a measure of intake (Lowe et al. 2009), but the usefulness of urinary Cu as a biomarker of Cu intake or exposure is less clear (Harvey et al. 2009). This may in part explain why our results generally showed weaker associations with urinary Cu than with Cd or Zn.

Finally, for our study we pooled two study samples from different populations that differ in racial and ethnic characteristics, and possibly exposure to Cd, Cu, and Zn. This is a common approach in genetic studies (Rentschler et al. 2013; Rentschler et al. 2014), and although we adjusted for ancestry and source study, we cannot exclude the possibility of residual confounding by unknown factors that differ by the genotypes examined, and between study samples. However, the associations we observed, with two exceptions, were consistent between the very different populations in our study, and this consistency is additional evidence that the associations are not due to differences between the populations.

CONCLUSIONS

In summary, the results of our study suggest that genetic variation in the region encoding functional MT genes may influence urinary levels of metals including Cd, Zn, and Cu. Our study is the first to systematically survey this region of the genome in association with an important toxic metal, Cd, or with Cu and Zn, two metals with critical physiological roles as well as toxicity at high doses. We also observed that variation in MTF1, which regulates transcription of MT as well as other metal-related genes, may influence U-Cd, a measure of

Cd accumulation in the kidneys. Thus our results should motivate future studies of the role of genetic variation in biomarkers of metal exposure, the association of metal exposure with health outcomes, and the interaction of genetic variation and environmental metals exposure in pathological processes (Raudenska et al. 2014).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding. This research was supported by NIH grants U54CA132381, U54CA132383 (New Mexico Metals study), R01CA097366 (Equol, Breast and Bone study), and U01CA63731 (Group Health Breast Cancer Screening Program). The funding sources played no role in the design, conduct, or reporting of the study.

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Highlights

- Genetic variation in metallothionein (MT) genes was assessed in two diverse populations
- Single nucleotide polymorphisms (SNPs) in MT genes were associated with mean urinary Cd, Cu and Zn
- Genetic variation may influence biomarkers of exposure, and associations of exposure with health

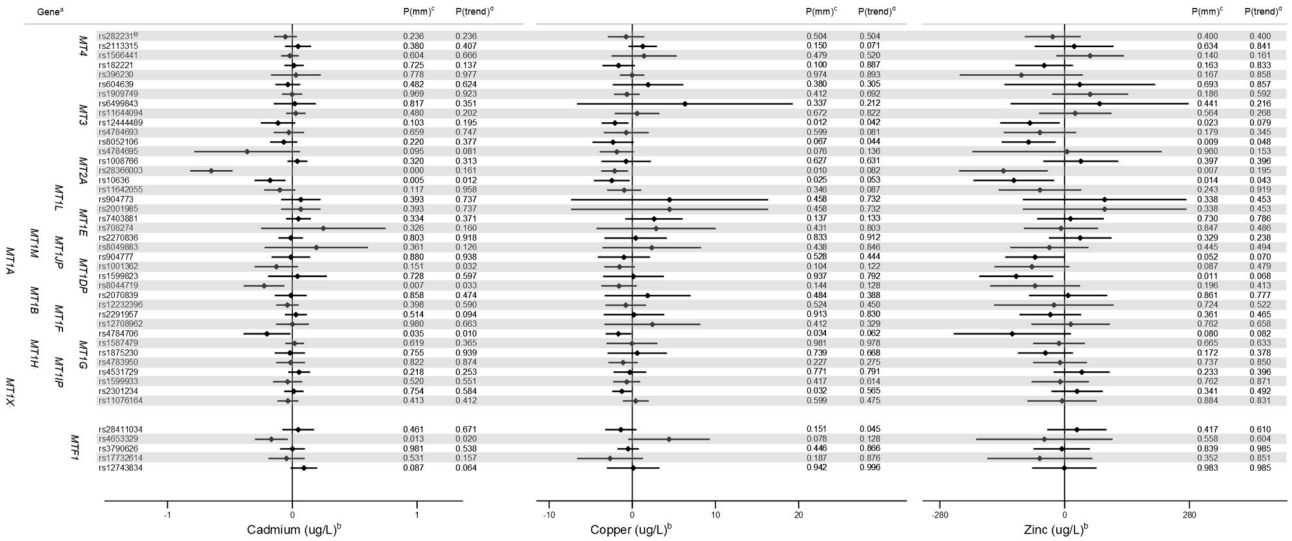

Figure 1.

Mean difference in urinary Cd, Cu, and Zn concentrations (μg/L) associated with selected metallothionein (MT) gene region and metal-regulatory transcription factor 1 (MTF1) single nucleotide polymorphisms. Shading is for clarity.

[a] Approximate relative location of MT genes (chromosome 16) and MTF1 gene (chromosome 1). The MT region is ~125 kbp. The MTF1 region is ~50 kbp.

[b] Mean difference in urinary metal comparing homozygote minor to homozygote major alleles (referent), adjusted for age, study site, urinary creatinine, smoking (never/ever), and the largest three principal components of the ancestry informative marker panel. Difference >0 indicates homozygote minor alleles is associated with higher mean urinary metal.

[c] P(mm) is P-value for comparison of mean urinary metal between homozygote major to homozygote minor alleles.

[d] P-value for trend in urinary metals with number of minor alleles.

[e] Comparing heterozygotes to homozygotes because no homozygotes with minor alleles were included in the study sample.

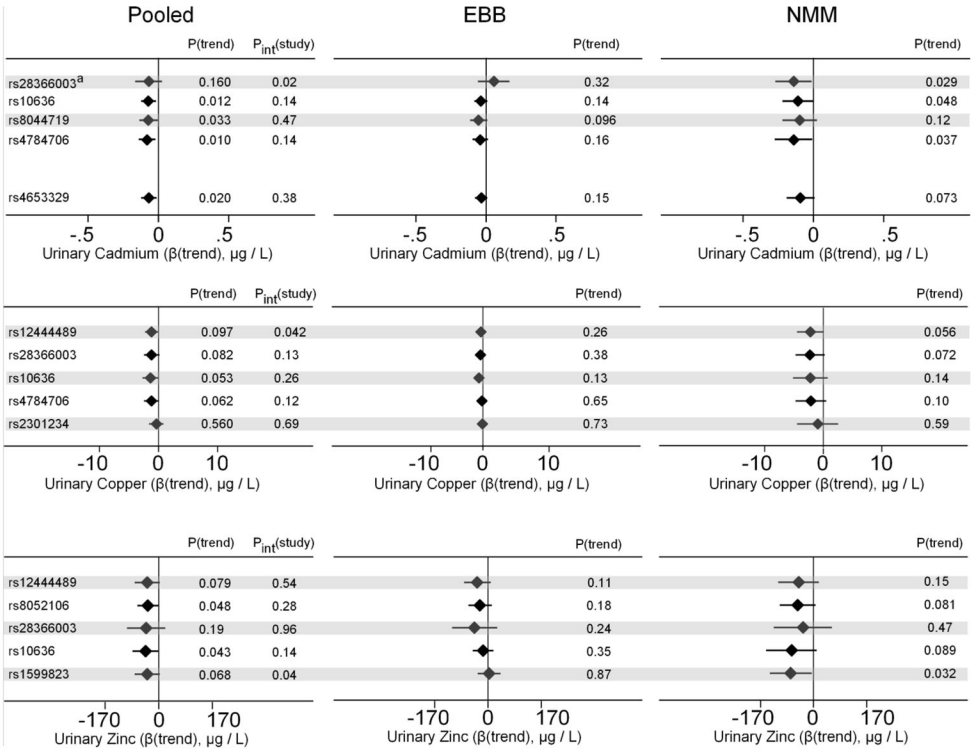

Figure 2.

Pooled results and results from each study (Equol, Breast and Bone (EBB) and New Mexico Metals (NMM)), showing the association of trend in minor alleles with urinary metals (β(trend)), for SNPs significantly associated with one or more urinary metal concentrations in the primary analysis (Figure 1). P_{int}(study) is the P-value of the interaction between study and the SNP, in association with urinary metals; P_{int}(study)>0.05 indicates no significant difference between studies. Shading is for clarity.

a Results for heterozygotes are plotted because of small number of homozygotes of the minor allele

Table 1

Selected participant characteristics according to study site, Equol, Breast, and Bone study (EBB) or New Mexico Metals study (NMM). All participants are women.

	EBB (Seattle, WA) (N=170)	NMM (Las Cruces, NM) (N=151)
	Mean (SD) or N (%)	**Mean (SD) or N (%)**
Age (y)	42.4 (1.4)	52.5 (9.1)
Ever-smoker	54 (32%)	38 (25%)
BMI (kg/m^2)		
<25	86 (51%)	22 (15%)
25–29.9	52 (31%)	65 (43%)
30-	32 (19%)	63 (42%)
Hispanic[a]		
No	164 (96%)	<10
Yes	<10	145 (96%)
Race[a]		
White	151 (89%)	139 (92%)
Asian / Pacific Islander	13 (8%)	0
Other[b]	<10	11 (7%)
Urinary cadmium	0.25 (0.30)	0.46 (0.54)
Urinary copper	5.7 (5.2)	11.1 (13.3)
Urinary zinc	183.5 (222.8)	440.2 (388.5)
Urinary creatinine	62.4 (53.0)	112.9 (69.5)

[a] Numbers do not sum to total due to missing values.

[b] African-American, American Indian / Alaska Native, or self-identified Other or “mixed”.

Table 2

Genotype frequencies in study participants according to study. Major allele (M) and minor allele (m) were assigned based on frequency in the combined study samples.

			EBB (N=170)			NMM (N=151)			
			MM	Mm	mm	MM	Mm	mm	
SNP	M	m	%	%	%	%	%	%	P[a]
MT gene region									
rs282231	A	G	85	15	0	83	17	0	0.64
rs2113315	A	G	35	45	19	38	48	14	0.42
rs1566441	G	T	24	52	24	32	46	21	0.20
rs182221 [b]	G	C	63	32	5	69	29	2	0.31
rs396230	A	G	69	28	2	72	28	1	0.47
rs604639	A	G	55	40	5	60	35	5	0.66
rs1909749	G	A	44	44	12	64	28	7	0.00
rs6499843	G	A	72	26	2	53	39	8	0.00
rs11644094	A	G	33	49	18	45	46	9	0.02
rs12444489	C	T	61	34	5	60	33	7	0.76
rs4784693	C	T	78	19	3	72	27	1	0.10
rs8052106 [b]	A	G	49	41	10	45	40	15	0.37
rs4784695	G	T	75	25	0	75	24	1	0.32
rs1008766	G	C	23	47	30	41	40	19	0.00
rs28366003	A	G	88	12	0	84	15	1	0.37
rs10636	G	C	54	42	4	62	34	4	0.33
rs11642055 [b]	G	A	72	25	3	78	21	1	0.22
rs904773	A	C	66	31	2	54	36	9	0.01
rs2001985	T	G	66	31	2	54	36	9	0.01
rs7403881	G	C	27	48	25	23	59	18	0.14
rs708274	G	T	67	29	4	72	26	1	0.26
rs2270836	C	T	39	47	14	26	52	21	0.04
rs8049883	G	A	67	28	5	72	26	2	0.36
rs904777	C	G	32	54	14	38	50	12	0.59

			EBB (N=170)			NMM (N=151)			
			MM	Mm	mm	MM	Mm	mm	
SNP	**M**	**m**	**%**	**%**	**%**	**%**	**%**	**%**	**P[a]**
rs1001362	G	A	74	22	4	72	26	2	0.57
rs1599823 [b]	G	A	34	60	6	37	54	10	0.44
rs8044719	G	T	75	22	3	72	27	1	0.40
rs2070839	C	T	44	46	10	31	51	18	0.03
rs12232396	C	T	62	34	4	77	22	1	0.01
rs2291957	G	T	54	39	7	48	46	5	0.37
rs12708962	G	A	46	44	10	30	54	15	0.01
rs4784706	G	A	74	21	5	74	23	3	0.59
rs1587479 [b]	G	T	43	47	10	38	54	7	0.38
rs1875230	C	A	55	39	6	52	46	2	0.13
rs4783950	T	C	38	46	16	19	54	28	0.00
rs4531729	T	C	33	42	25	46	46	8	0.00
rs1599933	T	G	34	49	17	25	50	25	0.10
rs2301234	G	T	39	50	11	50	46	4	0.03
rs11076164	T	C	28	42	29	35	52	13	0.00
MTF-1									
rs28411034	G	A	50	39	11	68	29	3	0.00
rs4653329	G	T	54	40	6	47	46	7	0.44
rs3790626	T	C	46	39	14	34	54	12	0.03
rs17732614	A	G	81	18	1	74	25	1	0.32
rs12743834	C	T	34	46	19	19	60	21	0.01

[a] P for difference between EBB and NMM study samples (Pearson's χ^2 test)

[b] SNP failed genotyping in <5% of study participants who were therefore excluded for this SNP.