

Exploration of divalent metal transporter 1 (*DMT1*) gene intronic IVS4+44C/A polymorphisms in population exposed to cadmium

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ABSTRACT

Background: Cadmium exposure affects the expression of the *DMT1* gene and the function of its transporter protein, impacting the transport and accumulation. This study investigates genetic polymorphisms to understand better the pivotal role of genetic factors in cadmium-related diseases within environmental health research.

Objective: The study sought to examine the intronic IVS4+44C/A polymorphism in the divalent metal transporter 1 (*DMT1*) gene among individuals aged 35-60 residing in regions contaminated with cadmium.

Materials and methods: Blood samples were collected from 306 genetically unrelated individuals (158 females and 148 males). The *DMT1* IVS4+44C/A polymorphism was determined using restriction fragment length polymorphism (RFLP) and Sanger sequencing methods. Urinary cadmium levels were measured with graphite furnace atomic absorption spectrometry (GFAAS). Statistical analyses included Hardy-Weinberg equilibrium testing, analysis of variance (ANOVA), and student's *t*-tests.

Results: The geometric mean of urinary cadmium levels were significantly higher in females (4.03 ± 4.15 $\mu\text{g/gm}$ creatinine) than in males (2.62 ± 2.73 $\mu\text{g/gm}$ creatinine). Remarkably, 85% of females and 66% of males exceeded the reference values for urinary cadmium concentration set by the German Human Biomonitoring (HBM) Commission (HBM I and II). Genotype frequencies were 65.4% homozygote typical (CC), 31.0% heterozygote (CA), and 3.6% homozygote atypical (AA). The C allele frequency was 80.9%, while the A allele frequency was 19.1%. Notably, the *DMT1* IVS4+44C/A polymorphism significantly influenced urinary cadmium levels, with the CA genotype showing higher levels than CC and AA genotypes. Urinary cadmium levels were also statistically increased with the presence of the A allele ($A^+ = CA + AA$) compared to its absence ($A^- = CC$). Furthermore, our analysis revealed that individuals with the CC genotype more frequently surpass the reference values for urinary cadmium in HBM I and II across all age groups despite their overall urinary cadmium levels not being high.

Conclusion: This study indicates that the CA genotype may signify susceptibility to prolonged cadmium exposure, given its association with elevated urinary cadmium levels. Additional research is essential for a thorough grasp of the implications of *DMT1* gene polymorphisms on health outcomes and for establishing monitoring measures for populations residing in cadmium-contaminated areas.

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Introduction

The largest zinc mining site in the country, formerly located in Mae Sot District, Tak Province, operated for over 32 years until its closure in 2017. During 2001-2004,

cadmium (Cd) accumulation as a byproduct of zinc smelting was observed in the environment and the food chain.¹⁻⁵ Natural cadmium occurring contains eight stables and two radioactive isotopes.⁶ Cadmium primarily exists in the divalent cation (+2) oxidation state and exhibits slow oxidation.⁶ Despite the cessation of mining activities, cadmium continues to pose environmental and health concerns in the region. Cadmium has an exceptionally long biological half-life, with significant accumulations in the kidneys (6-38 years) and liver (4-19 years) following initial distribution in the blood (75 to 128 days).^{7,8} The remaining cadmium disperses throughout other tissues (9-47 years), with renal accumulations gradually excreted in the urine, making urinary cadmium levels an indicator of long-term exposure.⁷⁻⁹ Concerning the homeostatic mechanisms governing cadmium regulation, metallothionein (*MT*) and the divalent metal transporter 1 (*DMT1*) genes are key players in the process. Together, they form an intricate system dedicated to maintaining cadmium homeostasis. These components collaborate to mitigate cadmium toxicity by sequestering it within cells, regulating its uptake, and ensuring controlled elimination, thereby safeguarding the body against the adverse consequences of excessive cadmium exposure.¹⁰⁻¹⁵ Examining single nucleotide polymorphisms (SNPs) in genes associated with homeostatic mechanisms is crucial, as these genetic variations may be linked to the development of various diseases.^{11,14-17}

In this study, the intronic region of *DMT1* gene was used to investigate the IVS4 +44C/A polymorphism in the general population residing in cadmium-contaminated areas. This position is in the intron 2 with C/A SNP in the intervening sequence 4+44 (IVS4+44) region.^{10,11} Our study population consisted of 306 subjects (158 females and 148 males) aged 35-60 years from Mae Sot District, Tak Province in western Thailand. The area was reported to have high levels of cadmium in the environment and its accumulation in food chains.¹⁻³ The accumulation of cadmium in the body can be measured through blood or urinary cadmium levels, which are effective biomarkers for exposure. The study employed urinary cadmium levels in the population to indicate long-term exposure. The population was divided according to the German Commission on Human Biomonitoring (HBM) reference values for urinary cadmium exposure.¹⁸ Investigating patterns of *DMT1* gene polymorphisms concerning cadmium exposure helps elucidate individual differences in susceptibility and improves risk assessments. Data on genetic polymorphisms in populations living in cadmium-contaminated areas in Thailand is inadequate. Such data could significantly enhance our understanding of the interactions between genetics and environmental exposures, contributing valuable knowledge for future research. Thus, this study aimed to explore the association between the *DMT1* IVS4+44C/A polymorphism and urinary cadmium levels in a population exposed to cadmium contamination

Materials and methods

Study subjects

In this study, 306 blood samples were collected from genetically unrelated and healthy individuals, including 158 females (mean age 48.84±6.65 years; range 35-60 years) and 148 males (mean age 50.57±6.45 years; range 35-60 years). The study was conducted in the western Thai population living in cadmium-contaminated areas. The exclusion criteria for the subjects included a medical history of renal failure, kidney stones, osteoporosis, and carcinoma. Both genders were divided into 3 different age groups: 35 to 44, 45 to 54, and 55 to 60.

DNA extraction and restriction fragment length polymorphism (RFLP) method

According to the manufacturer's instructions, blood genomic DNA was extracted using the FavorPrep™ Blood Genomic DNA Extraction Mini Kit (Favorgen, Taiwan). DNA was stored at -20 °C until required for polymerase chain reaction (PCR) analysis. PCR amplification was performed using the following forward primer: 5'-GAC ACA TGC AAT ATC TGA CAT TG-3' and reverse primer: 5'-AGG CTA CTA TCC AAC ATG CAG-3'.^{10,11} Each PCR reaction of 25 µL contained 9.5 µL of OnePCR™ master mix with fluorescence dye (GeneDireX®, Taiwan), 2.5 µL of 10 µM of each primer, 1 µL of DNA template, and 9.5 µL of nuclease-free H₂O. The amplification was conducted using SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific, USA) under the following conditions: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. For detection of the *DMT1* IVS4+44 C/A polymorphism (rs224589; GenelD: 4891; accession Number: NC_000012), PCR products were digested with *MnII* restriction enzyme (Thermo Fisher Scientific, USA) by incubating at 37 °C for 4 hr. Each reaction of 20 µL contained 10 µL of PCR product, 2 µL of 10X buffer, 7.75 µL of nuclease-free H₂O and 0.25 µL of *MnII*. Digested PCR products were examined using 3% (w/v) agarose gel electrophoresis. The PCR products of each variant were randomly selected and cleaned using the FavorPrep™ GEL/PCR Purification Mini Kit (Favorgen, Taiwan) for Sanger sequencing. Ten microliters cycle sequencing reactions consisted of 1 µL of BigDye™ Terminator v3.1 (Applied Biosystems, USA), 1 µL of BigDye™ 5X running buffer, 1 µL of 10 pM primer, 5 µL of nuclease-free H₂O and 2 µL of purified PCR product. The cycle sequencing conditions were as follows: 25 cycles of 96 °C for 10 sec, 50 °C for 5 sec, and 60 °C for 4 min. Samples were purified using BigDye™ XTerminator™ purification (Applied Biosystems, USA) and sequenced using the Applied Biosystem 3500xL Genetic Analyzer (Applied Biosystems, USA).

Urinary cadmium analysis

Cadmium levels and creatinine urine were measured by graphite furnace atomic absorption spectrometry (GFAAS) (Perkin Elmer, Analyst 600) and the automated

analyzer (Beckman coulter, AU 480 chemistry System) according to our previous protocols.¹⁹ The limit of quantification was 0.5 µg/L. Cadmium level (µg/L) was divided by the determined creatinine amount (gm/L) to obtain creatinine-adjusted urinary cadmium levels (µg/gm creatinine).

Statistical analysis and reference values

The study design was cross-sectional. The frequencies of alleles and genotypes were directly observed, and the departure from the Hardy-Weinberg equilibrium was evaluated by the Chi-Square (χ^2) test. The analysis of variance (ANOVA) or student's *t*-tests was used to identify differences in means $\pm$ SD of cadmium measures across categories of urinary cadmium level, age, and genotype. Urinary cadmium values used in this study were published by the German Human Biomonitoring (HBM) Commission.¹⁸ The German Commission on HBM recommended two reference values, HBM I, and HBM II, for urinary cadmium based on toxicology and epidemiology studies. In adults (>25 years old), urinary cadmium levels below the lower HBM I alert level (2 µg/gm creatinine) are not considered

to be a risk of advanced health effects. In contrast, action levels above HBM II (5 µg/gm creatinine) indicate an increased risk of adverse health effects in susceptible individuals in the general population.

Results

Of the 306 subjects enrolled in the study, the inclusion criteria required that participants have no history of kidney failure, kidney stones, osteoporosis, or carcinoma. The total geometric mean of urinary cadmium level was 4.03 $\pm$ 4.15 µg/gm creatinine in females (ranging from 0.43 to 23.42 µg/gm creatinine) and 2.62 $\pm$ 2.73 µg/gm creatinine in males (ranging from 0.25 to 14.21 µg/gm creatinine). Considering smoking history among individuals, non-smokers had lower urinary cadmium levels than smokers in both genders (Table 1). The female smokers had higher urinary cadmium levels than male smokers. However, there was no statistically significant difference between non-smokers and smokers among the female (*p*=0.406) and male (*p*=0.183) population living in the cadmium-contaminated areas.

Table 1. Baseline characteristics of the study population according to self-reported smoking exposure.

Gender	Variable	N	Age $\pm$ SD (min-max) year	Geometric mean $\pm$ SD of urinary Cd levels (min-max), µg/gm creatinine	<i>p</i> value
Female	Non-smoker	147	49 $\pm$ 6.68 (35-60)	4.02 $\pm$ 4.13 (0.43-23.42)	0.406
	Smoker	11	50 $\pm$ 4.70 (43-58)	4.11 $\pm$ 4.59 (0.95-14.75)	
Male	Non-smoker	73	51 $\pm$ 7.45 (35-60)	2.43 $\pm$ 2.78 (0.24-13.49)	0.183
	Smoker	75	51 $\pm$ 5.35 (37-60)	2.81 $\pm$ 2.67 (0.37-14.21)	

After total genomic DNA was extracted from the blood samples, the *DMT1* intron was amplified, and amplicons of 351 bp were obtained (Figure 1 and Figure 2).

The IVS4+44C/A SNP was determined based on PCR-RFLP technique using *MnII* restriction enzyme with nonpalindromic sequence recognition (Figure 1 and Figure 2).

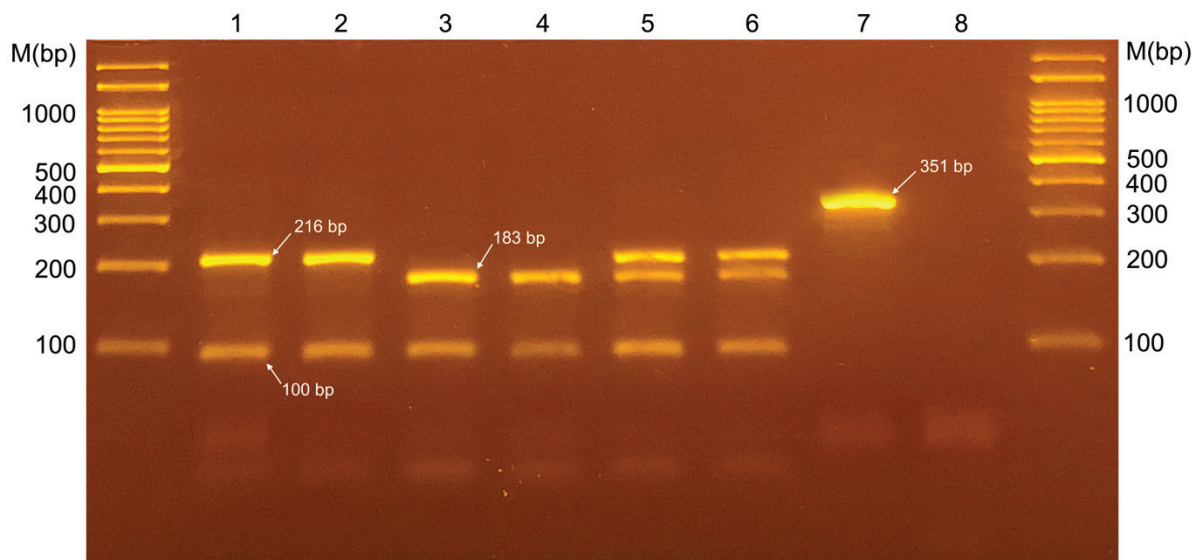


Figure 1. Representative gel image of digested with *MnlI* restriction enzyme and undigested PCR products. (M 100 bp ladder), lanes 1, 2: AA genotype (216, 100 bp), lanes 3, 4: CC genotype (183, 100 bp), lanes 5, 6: CA genotype (216, 183 and 100 bp), lane 7: undigested PCR product (351 bp), lane 8: negative control.

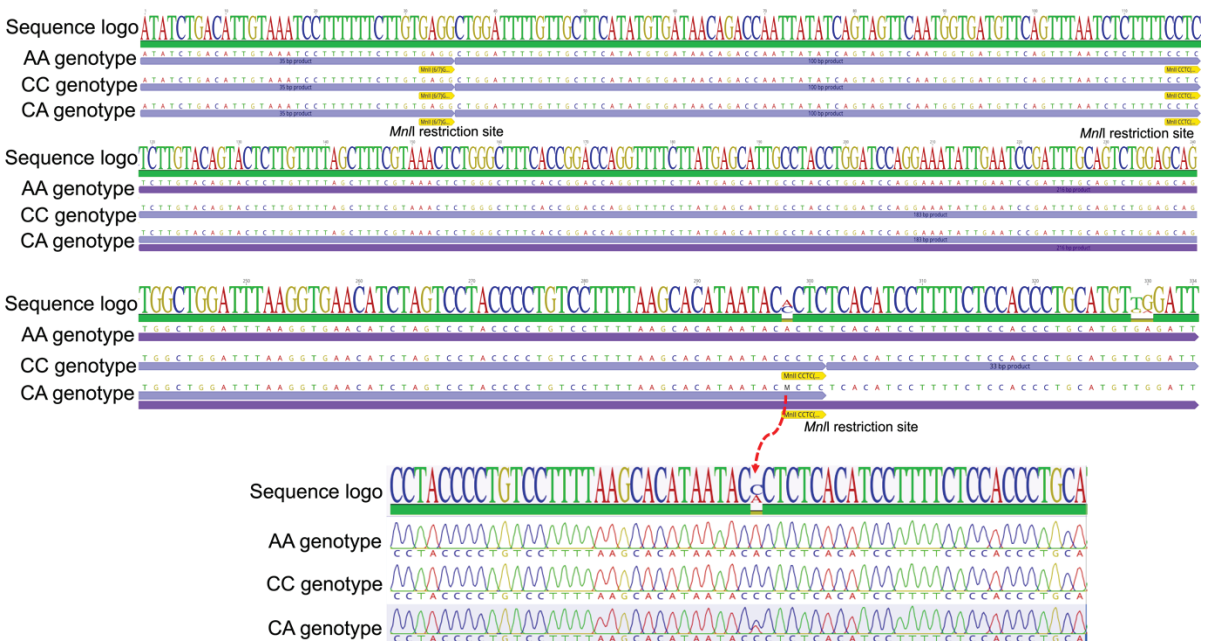


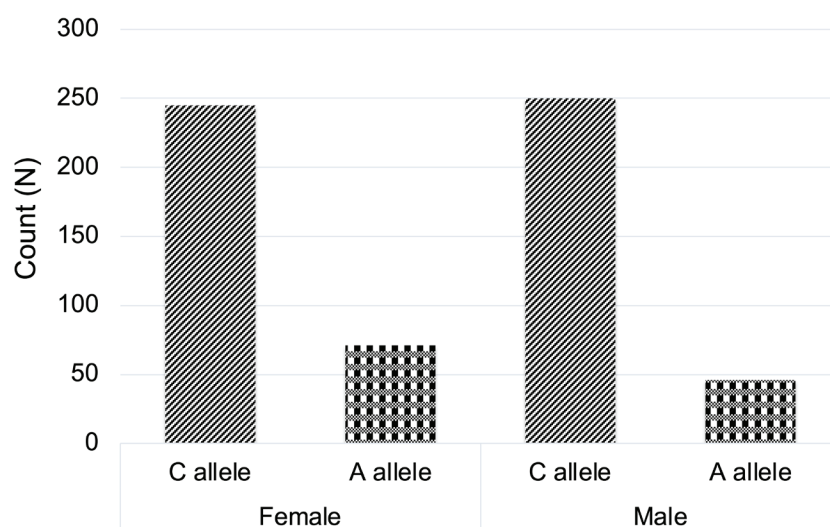
Figure 2. DNA sequence and cutting of the 351 bp amplicon with *MnlI* for the presence of A and C alleles.

Genotype frequencies among 306 subjects were 65.4% homozygote typical for CC (N=200), 31.0% heterozygote for CA (N=95), and 3.6% homozygote atypical for AA (N=11) (Table 2). The frequency of the C allele was 80.9%, while that of the A allele was 19.1%. The genotype and allele frequencies in both female and male individuals

were consistent with the Hardy-Weinberg equilibrium ($p=0.936$ for females and $p=0.938$ for males) (Table 2). The frequency of the A allele in the female individuals was higher than in the male individuals; however, the results were not statistically significant ($p=0.6812$) (Figure 3).

Table 2. Genotype and allele frequencies of *DMT1* IVS4+44C/A polymorphism in female and male individuals.

Gender	Polymorphism	Observed frequency of genotypes		Predicted frequency by Hardy-Weinberg equilibrium		p value
		N	%	N	%	
Female	IVS4+44C/C	93	58.9	94.98	60.1	0.936
	IVS4+44C/A	59	37.3	55.05	34.8	
	IVS4+44A/A	6	3.8	7.98	5.1	
	IVS4+44C allele	245	77.5	-	-	
	IVS4+44A allele	71	22.5	-	-	
Male	IVS4+44C/C	107	72.3	105.6	71.3	0.938
	IVS4+44C/A	36	24.3	38.9	26.3	
	IVS4+44A/A	5	3.4	3.6	2.4	
	IVS4+44C allele	250	84.5	-	-	
	IVS4+44A allele	46	15.5	-	-	
Total	IVS4+44C/C	200	65.4	200.2	65.4	0.946
	IVS4+44C/A	95	31.0	94.6	30.9	
	IVS4+44A/A	11	3.6	11.2	3.7	
	IVS4+44C allele	495	80.9	-	-	
	IVS4+44A allele	117	19.1	-	-	

**Figure 3.** Distribution of C and A allele frequencies in the female and male individuals.

Individuals with the CA genotype had higher geometric mean urinary cadmium levels than the CC and AA genotypes. This study revealed that the cadmium levels were significantly higher in the combined male and female population with the presence of the A allele ($A^+ = CA+AA$) compared to those without the A allele ($A^- = CC$) ($p=0.0007$). Regarding gender, it was shown that female subjects with the CA genotype and male subjects with the AA genotype had higher urinary cadmium levels than those

with the CC genotype. Females exhibited a statistically significant difference in cadmium levels ($p=0.0001$), while males did not show a statistically significant difference ($p=0.0789$). The values of urinary cadmium concentration based on HBM I (2 $\mu\text{g/gm}$ creatinine) and HBM II (5 $\mu\text{g/gm}$ creatinine) for adults older than 25 years old were exceeded by 73% for CC, 80% for CA, and 82% for AA genotypes (Table 3).

Table 3. DMT1 IVS4+44C/A polymorphism and urinary cadmium (U-Cd) level.

Gender	Polymorphism	N	Geometric mean±SD of urinary Cd levels (µg/gm creatinine)	Human Biomonitoring (HBM) value Frequency, N (%)			p value
				<HBM I	HBM I (2 µg/gm creatinine)	HBM II (5 µg/gm creatinine)	
Female	CC	93	3.82±3.63	14 (15)	46 (50)	33 (35)	0.0001 (<i>p</i> <0.01)
	CA	59	4.45±4.79	9 (15)	23 (39)	27 (46)	
	AA	6	2.89±4.77	1 (17)	3 (50)	2 (33)	
	A(-)(CC)	93	3.82±3.63	14 (15)	46 (50)	33 (35)	
	A(+)(CA+AA)	65	4.43±4.78	10 (15)	26 (40)	29 (45)	
Male	CC	107	2.57±2.66	40 (37)	49 (46)	18 (17)	0.0789 (<i>p</i> >0.05)
	CA	36	2.67±2.64	10 (28)	13 (36)	13 (36)	
	AA	5	3.34±4.54	1 (20)	2 (40)	2 (40)	
	A(-)(CC)	107	2.57±2.66	40 (37)	49 (46)	18 (17)	
	A(+)(CA+AA)	41	2.75±2.88	11 (27)	15 (36.5)	15 (36.5)	
Total	CC	200	3.09±3.24	54 (27)	95 (48)	51 (25)	0.0007 (<i>p</i> <0.01)
	CA	95	3.72±4.24	19 (20)	36 (38)	40 (42)	
	AA	11	3.04±4.46	2 (18)	5 (46)	4 (36)	
	A(-)(CC)	200	3.09±3.24	54 (27)	95 (48)	51 (25)	
	A(+)(CA+AA)	106	3.64±4.26	21(20)	41 (38)	44 (42)	

A comparison between age ranges, genotype patterns, and levels of exposure revealed that both females and males showed high levels of cadmium exposure according to age (Figure 4). The increase in cadmium level showed a statistically significant difference with age and genotypes in both females (ANOVA, *p*=0.0020) and males (ANOVA

p=0.0180). Focusing on the number of exposures to cadmium that exceeded the urinary cadmium reference values for HBM I and II across all ages, the results showed that individuals with the homozygote typical CC genotype more frequently exceeded these reference values (Figure 5).

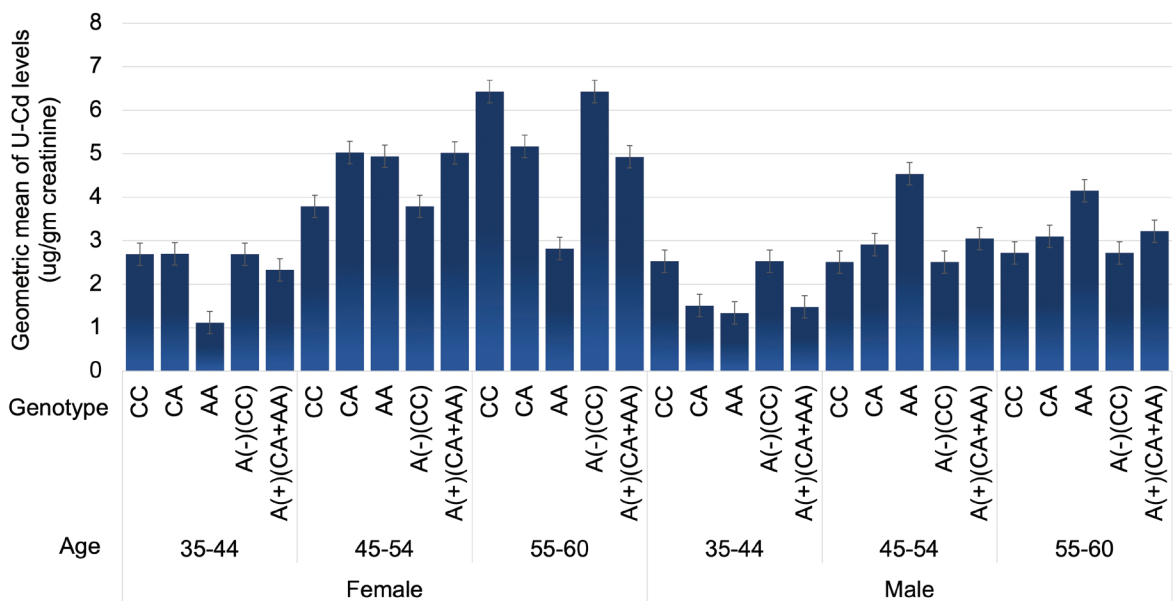


Figure 4. Distribution of genotype and urinary cadmium levels in female and male subjects.

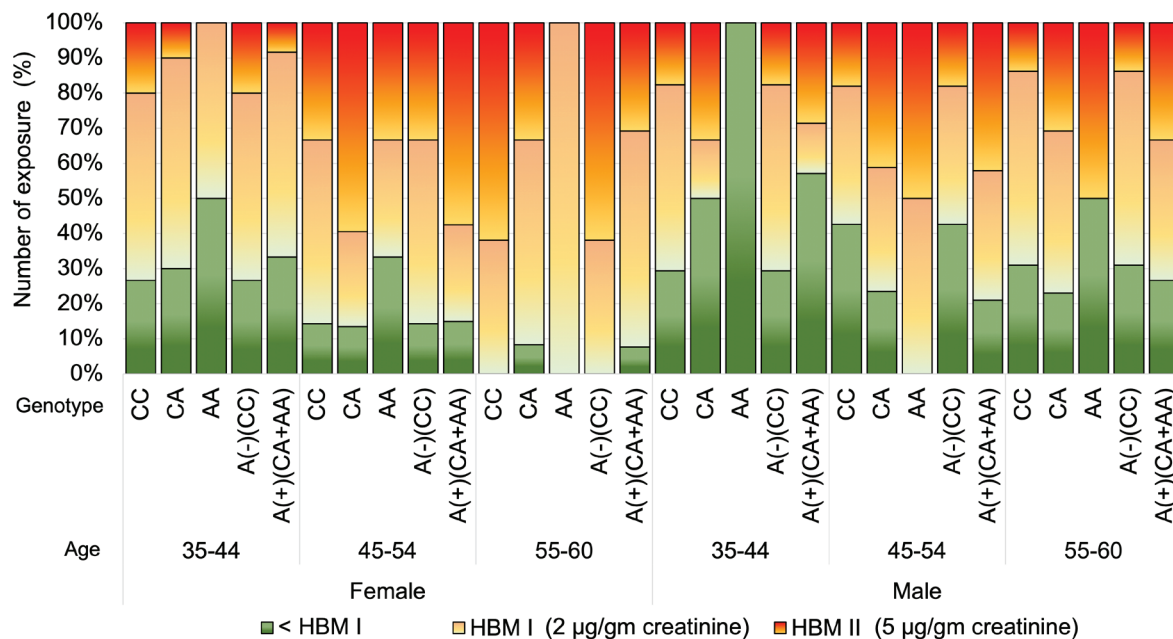


Figure 5. Distribution of genotype and guideline values for urinary cadmium levels in female and male subjects.

Discussion

Divalent metal transporter 1 (*DMT1*) gene is known as the natural resistance-associated macrophage protein 2 (*NRAMP2*) gene or the solute carrier family 1 member 2 (*SLC1A2*) gene on human chromosome 12q13.^{10,11} This gene encodes a member of a family of solute transporter proteins which are ubiquitously expressed in human tissues, and responsible for iron uptake in duodenal enterocytes and kidneys,¹¹ iron-regulatory in the placenta,²⁰ and iron homeostasis in brain.²¹ The *DMT1* is not only responsible for the uptake and translocation of iron from endosomes during the transferrin cycle but also absorbs and transports the other divalent cation metals, including manganese, copper, zinc, and some toxic metals such as lead and cadmium.^{11,22,23} In humans, metal excretion relies on homeostasis mechanisms rather than specific pathways. Cadmium absorption starts in the gastrointestinal tract, where intestinal metallothionein (MT) is protective to mitigate cadmium toxicity.^{12,13} The cadmium-metallothionein (Cd-MT) complex from the liver enters the bloodstream and is filtered by the kidneys into urine.^{12,13,24} However, if the amount of cadmium absorbed by the body exceeds the metallothionein ability to form complexes, cadmium accumulates.^{13,24} Additionally, the divalent metal transporter 1 (1) protein, responsible for iron absorption and divalent metal transport, including cadmium, can facilitate the transport of toxic heavy metals into cells.^{10,11,14,15} This process increases oxidative stress, inflammation, and DNA damage.^{14,15} Cadmium disrupts cellular function by interfering with essential metal transport processes, increasing oxidative stress, and promoting inflammation, ultimately contributing to cellular dysfunction and disease.^{14-15,22,25-28} The *DMT1* gene polymorphisms have been linked to various diseases, including Parkinson's disease,²² Wilson's disease,²⁵

hypertension,²⁶ iron deficiency anemia,²⁷ and age-related macular degeneration (AMD).²⁸

In this study, we observed the *DMT1* IVS4+44 C/A polymorphism in a population historically contaminated by cadmium. Analysis of cadmium levels in urine, adjusted by urine creatinine, revealed a significantly higher geometric mean of urinary cadmium levels in females than males. Furthermore, urinary cadmium levels of non-smokers and smokers were not significantly different between females and males living in areas with a history of cadmium contamination. The genotype frequencies in 306 subjects were highest for the CC genotype (65.4%), followed by the CA genotype (31.0%) and the AA genotype (3.6%). The frequency of the C allele was higher than the A allele. This distribution was similar to studies conducted on the Turkish population.^{10,11}

Limited studies have explored the impact of *DMT1* IVS4+44 C/A polymorphism on heavy metal levels. A survey by Kayaalti *et al.* suggested that individuals with the CC genotype might be more susceptible to increased blood iron, lead, and cadmium levels than those with the AA and CA genotypes.¹¹ Blood cadmium levels are reliable indicators of recent exposure,⁷ implying that the CC genotype may heighten susceptibility to short-term cadmium exposure, particularly in the general population. Our results indicate that individuals with the CA genotype exhibited higher geometric mean urinary cadmium levels than those with the CC and AA genotypes. Furthermore, although the overall urinary cadmium levels were not high, the highest frequency of the CC genotype among 200 subjects exceeded urinary cadmium reference values for HBM I and II across all age groups. Thus, our findings suggest that *DMT1* IVS4+44 C/A polymorphisms contribute to inter-individual variations in urinary cadmium levels. The CC genotype may signify susceptibility to recent

cadmium exposure in the general population. In contrast, the CA genotype may indicate susceptibility to long-term cadmium exposure in populations residing in cadmium-contaminated areas.

Conclusions

This marks the initial investigation delving into the connection between the *DMT1* IVS4+44 C/A polymorphism and urinary cadmium levels among genetically unrelated individuals residing in cadmium-contaminated regions. These findings could have important implications for identifying susceptible individuals to cadmium toxicity and developing effective prevention strategies. However, this study has limitations. First, there is limited population size, which must be replicated in other populations. Second, a lack of blood cadmium detection indicates recent exposure, allowing a comparison with previous studies.

Ethical approval

This study was performed in line with the principles of the Declaration of Helsinki, which was approved by the Research Ethics Committee for Research in Human Subjects at the Department of Medical Sciences, Ministry of Public Health, Thailand (Study code. 4/2561, date of approval: 17 February 2023).

Conflict of interest

The authors declare that there is no conflict of interest.

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