Glutathione S-transferase gene polymorphisms in association with susceptibility to lead toxicity in lead and cadmium exposed children near an abandoned lead-zinc mining area in Kabwe, Zambia

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Abstract

Interindividual genetic variations determine human's susceptibility to heavy metals-induced toxicity. Thus, we analyzed blood concentrations of lead (Pb) and cadmium (Cd) in 140 lead-exposed children. Genotyping of the glutathione S-transferase (GST) genes, *GSTM1*, *GSTT1*, and *GSTP1* genes, was carried out to investigate their possible association with heavy metal concentrations and the risk of susceptibility to Pb toxicity. Exposure to both heavy metals was prevalent among the children. The blood Pb level ranged from 3.30 to 74.0 μg dL^{-1} with an average value of 26.8 μg dL^{-1} that is five times above its reference level. The average Cd level (0.22 μg L^{-1}) was below its reference level. The metal-gene interaction showed positive correlation between *GSTT1* null genotype and Pb and Cd levels ($\beta = 0.11$; $p = 0.02$ and $\beta = 0.10$; $p = 0.01$, respectively). More pronounced effects ($\beta = 0.19$; $p < 0.01$ and $\beta = 0.25$; $p = 0.04$) were found for the mixture of the three putative genes with blood Pb concentration. The susceptibility analysis using 10 μg dL^{-1} as blood Pb cut-off level showed a high risk of Pb toxicity (OR = 2.54; 95% CI: 1.02–6.32, $p = 0.04$) for children carrying the *GSTP1 Ile/Val* genotype. Further, the combined effect of *GSTP1 Ile/Val* with *GSTT1* null genotype was more pronounced and showed an increased risk of susceptibility to Pb toxicity (OR = 11.7; 95% CI: 1.36–102.1, $p = 0.02$). In summary, this study suggests that *GSTT1* null and *GSTP1 Ile/Val* genotypes are the main genetic factors, and individual and specific combinations of *GSTP1 Ile/Val* with *GSTM1* and *GSTT1* GST polymorphisms are associated with susceptibility to Pb toxicity.

Keywords: Blood, Children, Lead, Cadmium, *GST,* Polymorphism, Kabwe

1. Introduction

Heavy metals like lead (Pb) and cadmium (Cd) have no known biological roles in living organisms and are considered as toxic metals. According to the International Agency for Research on Cancer (IARC), Pb and Cd are classified as probable carcinogenic (group 2A) and known carcinogenic (group 1) compounds, respectively (IARC 2021). Once inside the body, either from natural or anthropogenic sources, they can interact metabolically with essential metals and displace them from their specific cell constituent sites (Jan et al. 2015; Godwill et al. 2019). Generally, heavy metal exposure can cause multiple organ dysfunction that led to toxicity and oxidative stress (Tchounwou et al. 2012; Jan et al. 2015; Godwill et al. 2019). Owing to their developing bodies, children are more prone to numerous adverse effects of heavy metals such as cognitive impairments and central nervous system functional deficits that persist into older age (Jaishankar et al. 2014).

Glutathione-S-transferases (GST), recognized as oxidative stress-related genes, are vital defense enzymes engaged in metal biotransformation and detoxification of reactive oxygen species (Hayes et al. 2005). It catalyzes the conjugations of hydrophobic and electrophilic compounds with reduced glutathione (GSH) and excreted via feces and urine after GSH-metal conjugates formation (Jozefczak et al. 2012). The GST superfamily comprises eight polymorphic genes, and of these, polymorphic variants of the GST-mu 1 (*GSTM1*), theta 1 (*GSTT1*), and pi (*GSTP1*) are the most studied and reported globally (Sharma et al. 2014; Saitou and Ishida 2015). Genetic variations in these genes (deletion in *GSTM1* and *GSTT1* genes and polymorphism in *GSTP1*) contribute to interindividual differences in susceptibility to xenobiotic toxicity (Hollman et al. 2016). Previous studies reported an absence or decreased detoxification ability of GST enzymes and linked it with oxidative stress and health outcomes in different polymorphic GST gene carriers. Individuals with

87 *GSTP1* variant alleles and double-null genotypes of *GSTM1* and *GSTT1* were associated with the
88 induction of oxidative stress (Sirivarasai et al. 2013). In another study, individuals with the *GSTM1*
89 null genotype and *GSTP1* wild allele had lower GSH levels and be at increased risk from exposure
90 to toxicants (Hunaiti and Soud 2000; Khansakorn et al. 2011). Pregnant mothers with combined
91 *GSTM1* and *GSTT1* genetic variants showed an inverse association of blood Pb and birth weight
92 (Lamichhane et al. 2018). However, polymorphisms in these functional genes vary by race and
93 ethnicity (Sharma et al. 2014; Saitou and Ishida 2015).

94 Kabwe, a capital town of the Central Province of Zambia, has a long history of Pb-zinc (Zn) mining
95 that operated from 1902 to 1994 without adequate pollution regulations. Thus, the town is one of
96 the most polluted places in the world. Even though the mine is inactive, the residue left a legacy
97 of contaminated soil with Pb, Zn, and other byproducts like cadmium (Cd). Moreover, illegal
98 mining activities are still ongoing. These create wide-contaminated surroundings and an
99 environment that poses a significant health risk. Recent studies involving a population-wide
100 screening on Pb poisoning in this town reported that approximately 75% of the residents had BLLs
101 higher than 5 μg dL^{-1} (Yabe et al. 2020; Yamada et al. 2020). Nevertheless, only one study
102 investigated gene polymorphism on candidate Pb biomarkers of effect genes delta-aminolevulinic
103 acid dehydratase (ALAD) and vitamin D receptor (VDR) genes in Kabwe (Yohannes et al. 2021).

104 Thus, this study (i) assessed the burden of Pb and Cd concentrations in children blood, (ii)
105 investigated the genetic polymorphism in the GST family; *GSTM1*, *GSTT1,* and *GSTP1*
106 polymorphisms, and (iii) examined the effect of the GST polymorphic variants on blood heavy
107 metal levels and the influence on the risk of susceptibility to Pb toxicity in children living near an
108 abandoned Pb-Zn mining area. We also reported Zn concentration. Thus, the data generated here
109 provide reference data for future studies.

2. Materials and methods

2.1. Study subjects and sampling

A total of 140 healthy children, aged 2–10 years, were recruited from five townships (Chowa, Kasanda, Makululu, Bwacha, and Nakoli townships) in Kabwe, Zambia. Among these children, 102 children live in three townships near the abandoned mine and 38 children from two townships live far away from the mine (Fig. 1). Children were invited to health centers, and parents were asked a short questionnaire on demography and Pb-related clinical symptoms. No evidence or any sign and symptoms of neurotoxicity among the children. Venous blood samples (≈ 3 mL) were obtained from each child by trained nurses into heparinized blood collection tubes in July 2016 (Yohannes et al. 2020). A 500 µL blood sample was kept in a 1.5 mL tube for heavy metal analysis and stored all samples at –20 °C. All analyses were done in the Laboratory of Toxicology, Faculty of Veterinary Medicine, Hokkaido University, Japan.

Fig. 1

2.2. Heavy metals analysis

Blood sample digestion and heavy metal analyses were done as described by Yohannes et al. (2017). Briefly, the 500 µL blood sample was digested with 5 mL 61% HNO_3 and 1 mL 30% H_2O_2 in a speedwave MWS-2 microwave system. Then, samples were made to a final volume of 10 mL using Milli-Q water in a 15 mL tube. Blood Pb, Cd, Zn concentrations were measured using inductively coupled plasma mass spectrometry (ICP-MS 7700 series). Seronorm™ Trace Elements Whole Blood L-2 certified reference material (Sero AS, Norway) was used as quality control. Replicate analyses of this reference material showed recovery rates ranged from 90% to 105% with a detection limit of 0.01 µg L^{-1} for the measured heavy metals.

132 *2.3. GSTs genotyping*

133 Genomic DNA was isolated from whole blood using a NucleoSpin Blood Kit (Macherey-Nagel)
134 and kept at –25 °C until genotyping analysis. A multiplex polymerase chain reaction (PCR) was
135 used for *GSTM1* and *GSTT1* genotyping to detect the presence or absence of the genes with the
136 CYP 1A1 gene as a positive control. A PCR-restriction fragment length polymorphism (PCR–
137 RFLP) method was carried out for *GSTP1 Ile105Val* (rs1695; A > G) polymorphism. PCR was
138 done to a mixture containing 1 μL DNA (>20 ng), 1x PCR buffer, 2.0 mM $MgCl_2$, 200 μM of each
139 dNTP, 0.25 μM of each primer, and 1 U Taq polymerase in a total volume of 10 μL. The sequences
140 of primers and the PCR amplification conditions are displayed in Table 1.

141 Table 1

142 The presence of *GSTM1*, *GSTT1*, and *CYP 1A1* genes were identified by the bands at 219 bp, 480
143 bp, and 312 bp, respectively. The absence of the band describes the gene deletion referred to as
144 null genotype. For *GSTP1* genotyping, the resulting 433 bp PCR product was digested at 55 °C
145 for 4 h with the restriction enzyme *BsmAI* (5 U). The corresponding fragmented product bands
146 were visible with 328 bp and 105 bp fragments (*Ile/Ile* genotype), 328 bp, 222 bp, and 105 bp
147 fragments (*Ile/Val* genotype), and 222 bp and 105 bp fragments (*Val/Val* genotype). The PCR
148 fragments were electrophoresed in a 2% agarose gel with Midori Green direct and visualized under
149 a Blue-LED transilluminator system. Representative gel electrophoresis showed the genotypes of
150 *GSTM1*, *GSTT1*, and *GSTP1* are presented in Fig. 2. For ensuring quality control, random samples
151 (10%) were genotyped twice, and no contamination was confirmed using no-template control
152 samples.

153 Fig. 2

2.4. Statistical analysis

To improve normality and homogeneity of variance of heavy metal concentrations, data were log-transformed before statistical analysis. Blood heavy metals were expressed as mean ± standard deviation and analyzed by the non-parametric Kruskal-Wallis test. Deviation of *GSTP1* genotype distribution from Hardy–Weinberg equilibrium (HWE) was assessed using the Pearson's Chi-squared (χ^2) test. Comparisons of the observed *GSTM1* and *GSTT1* null genotypes and *GSTP1 Val* allele frequencies between this study and global studies were examined using the χ^2 test. We checked the effect of covariates (age and sex) on the statistical analysis, and no significant association was found. Thus, linear regression analysis was performed for assessing the association between polymorphic variants of GST genes and log-transformed blood heavy metal concentrations. Next, crude odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to examine associations between the GST genes genotypes and susceptibility to Pb toxicity using 10 μg/dL as a cutoff level. A $p \leq 0.05$ was considered statistically significant, and a p between 0.051 and 0.100 was marginally significant (Amrhein et al. 2019). The JMP 14.0 software (SAS Institute, Cary, NC, USA) was used to perform all analyses.

3. Results and discussion

3.1.Blood Pb, Cd, and Zn concentrations

The subjects of the current study were 65 females and 75 male children with mean ages of 5.72 and 5.69 years, respectively. The majority of children have lived in their respective townships for most of their lifetime. The descriptive statistics of heavy metal concentrations are given in Table 2. The blood Pb concentrations among participants ranged from 3.30 to 74.0 μg dL^{-1} with the mean and median concentrations of 26.8 and 26.3 μg dL^{-1} (interquartile range: 13.6–36.6 μg dL^{-1}), respectively. These mean and median Pb levels were 5 times higher than the blood Pb reference value of 5 μg dL^{-1} (CDC, 2012). Among the 140 participants, 131 (93.5%) children exceeded this blood Pb reference value. The mean/median concentrations of Cd and Zn were 0.22/0.18 μg L^{-1} and 4135/4167 μg L^{-1}, respectively. These concentration values were below their respective reference values (Table 2).

In addition, the measured heavy metals demonstrated some correlations among them and showed Spearman's correlation coefficients $\rho = 0.497$ for Pb–Cd and $\rho = 0.400$ for Pb–Zn. These correlations imply that these elements share the same sources of exposure and children are exposed to both toxic metals. The closed mine is still a source of pollution. As shown in table 2, the concentrations of the measured heavy metals near the mine site were significantly higher than far from the mine site. There were no significant differences between blood heavy metal concentrations and sex, showing equal exposure regardless of sex (data not shown). Owing to their immature defense mechanisms, children are more vulnerable to heavy metals toxicity. Thus, appropriate measures should be taken to prevent or minimize exposure for reducing the toxic effects of Pb and Cd on the community.

197 *3.2.GSTs polymorphism and blood Pb, Cd, and Zn concentrations by GST polymorphic variants*

198 Table 3 shows the distribution rate of *GSTM1*, *GSTT1*, and *GSTP1* genotypes. We found *GSTM1*
199 present genotype as the most prevalent genotype with a frequency of 76.4%, followed by the
200 *GSTT1* present genotype (65.0%) and then the *GSTP1 Ile/Val* genotype (59.3%). The null
201 genotype prevalence was 23.6% and 35.0% for *GSTM1* and *GSTT1*, respectively. The combined
202 polymorphic variants of *GSTM1* and *GSTT1* were 8.6% for null/null, 41.4% for null/present, and
203 50.0% for present/present genotypes. Regarding the *GSTP1* polymorphism, 40 children (28.6%)
204 were *Ile/Ile* homozygous, 83 children (59.3%) were heterozygous *Ile/Val*, and 17 children (12.1%)
205 were *Val/Val* homozygous genotypes (Table 3). The distribution of *GSTP1 Ile*105*Val* showed
206 significant deviation from HWE ($\chi^2 = 6.69$; $p < 0.05$). The analysis revealed an excess number of
207 heterozygous *Ile/Val* genotypes (observed = 83 children vs expected = 68 children). The
208 prevalence of *GSTP1 Ile* and *Val* allele frequencies were 58% and 42%, respectively.

209 The blood heavy metal concentrations per *GSTM1*, *GSTT1*, and *GSTP1* genotypes are depicted in
210 Table 3. None of the measured heavy metals showed significant differences between the genotypes
211 of *GSTM1* and *GSTP1* genes. Whereas the *GSTT1* null group showed significantly higher blood
212 levels for Pb and Cd (Table 3).

213 Table 3

214 GST gene polymorphisms have been studied in various human races with pervasive existence (Fig.
215 3). Globally, the *GSTM1* null genotype frequency ranged from 20–46% in Africans, 42–55% in
216 Europeans, 36–52% in Americans, and 41–60% in Asians (Fig. 3a) (Palma-Cano et al. 2017). The
217 distribution frequency of *GSTT1* null genotype ranged from 30–50% for Asians followed by
218 Africans (20–47%), then Europeans and Americans (11–26%) (Fig. 3a) (Palma-Cano et al. 2017).

Thus, our study *GSTM1* null genotype prevalence rate of 23.6% was found in the lower range in the Africans and lower than the levels reported from other continents. The 35% prevalence rate for the *GSTT1* null genotype in the current study was significantly higher than those reported from European and American countries. Comparing our study GSTP1 *Val* allele frequency (42%) with global *Val* allele frequency based on the new Allele Frequency Aggregator (ALFA) dataset (https://www.ncbi.nlm.nih.gov/snp/rs1695#frequency_tab), we observed apparent variation of the *Val* allele frequencies between Africans and Asians (Fig. 3b). The *Val* allele was more frequent in the Latin American individuals with mostly European and Native American Ancestry (49.7%), followed by Africans (42%), Europeans (32.6%), and then Asians (18.9–28.9%). The chi-squared statistical analysis showed clear out variation between this study and Asians ($p < 0.05$).

Fig. 3

In respect to metal–gene interactions, the effect of the three putative genes on blood heavy metal levels showed both direct and inverse significant effects. The regression analysis coefficient for genetic variants of GST genes in association with blood heavy metals is shown in Table 4. Logistic regression analysis using log-transformed concentration as a dependent variable confirmed a positive significant association for the *GSTT1* null genotype with blood Pb ($\beta = 0.11$, $p = 0.02$) and Cd ($\beta = 0.10$, $p = 0.01$) levels. In the combined analysis, the result showed direct association of blood Pb level with *GSTT1* null / *GSTP1 Ile/Val* genotypes ($\beta = 0.19$, $p = 0.002$), with *GSTM1* present / *GSTT1* null / *GSTP1 Ile/Val* genotypes ($\beta = 0.16$, $p = 0.03$), and with *GSTM1* null / *GSTT1* null / *GSTP1 Ile/Val* genotypes ($\beta = 0.25$, $p = 0.04$). For Cd, the double null genotypes showed a significant higher association with beta coefficient of 0.11 ($p = 0.05$). On the other hand, the *GSTP1 Ile/Ile* genotype showed an inverse association with blood Pb level ($\beta = –0.10$, $p =$

0.04), suggesting a protective role of this genotype. The combined effect of this genotype with others also showed negative beta estimates; *GSTP1 Ile/Ile / GSTT1* null (β = –0.17, p = 0.03), *GSTP1 Ile/Ile / GSTM1* present / *GSTT1* null (β = –0.28, p = 0.01), and *GSTP1 Ile/Ile / GSTM1* null / *GSTT1* present (β = –0.32, p = 0.03) in relation with blood Pb levels.

Table 4

To our knowledge, most of the studies on the impact of GST polymorphism are based on case–control studies and only a few had available on healthy people (Khansakorn et al. 2011, 2012). In nonoccupationally exposed populations, Khansakorn et al. (2011) reported an association between GST polymorphism and the level of Cd in blood. The result revealed higher Cd levels for subjects with the *GSTP1 Val/Val* genotype than their counterparts. The combination of *GSTP1* with *GSTM1* and *GSTT1* also showed an association with increased blood Cd levels. In our study, the metal-to-gene interaction showed significant associations between GSTs polymorphism and blood heavy metal concentrations. The study revealed that individual and combined GST gene variants could play substantial roles in heavy metal accumulation in our bodies. The result showed direct positive associations between gene deletion of *GSTT1* and blood Pb and Cd concentration. Moreover, *GSTP1* polymorphism (substitution of *Ile* → *Val*) coupled with the *GSTT1* null genotype showed a higher association with blood Pb concentration. This incidence can reduce the GST enzyme activity and as a result reduce Pb- and Cd-GSH conjugates and excretion. Thus, these toxic metals can accumulate in blood and tissues (Jozefczak et al. 2012). It is known that Pb and Cd can replace essential elements such as calcium, copper, iron, and zinc in several major biological processes, and mimic the actions of essential elements (Tchounwou et al. 2012). Overall, the combined effect of *GSTP1* polymorphism with *GSTM1* and *GSTT1* gene deletions could affect the levels of Pb and Cd in children living around the closed mine.

3.3. The effect of GST genotypes on susceptibility to lead toxicity

The GST genetic variant's impact on susceptibility to Pb toxicity was investigated using a blood Pb level of 10 μg dL^{-1} as a cutoff point. Thus, we categorized the subjects as low exposed (≤ 10 μg dL^{-1}) and high exposed (> 10 μg dL^{-1}) children. The frequencies of the GST genotypes in these groups are depicted in Table 5. The prevalence of *GSTM1* null in high exposed (23.7%) and low exposed (23.1%) groups were similar and susceptibility analysis showed no association between *GSTM1* polymorphism and susceptibility to Pb toxicity (OR = 1.03; 95% CI: 0.38–2.84, $p = 0.94$). For the *GSTT1* polymorphism, the null genotype was marginally higher in the highly exposed group (35.6%) than the low exposed group (19.2%) ($\chi^2 = 3.49$; $p = 0.06$). Analysis of susceptibility to Pb toxicity also showed a marginal association between the *GSTT1* null genotype and risk of lead toxicity (OR = 2.64; 95% CI: 0.92–7.51, $p = 0.06$), suggesting that individuals with *GSTT1* null genotype are at higher risk of susceptibility to Pb toxicity. The combined effects of *GSTM1* present / *GSTT1* null, *GSTM1* null / *GSTT1* present, and *GSTM1* null / *GSTT1* null genotypes were not associated with Pb toxicity ($p = 0.12$, 0.56, and 0.23, respectively).

The *GSTP1* polymorphism deviated from HWE for the high exposed group ($\chi^2 = 7.83$; $p = 0.005$) (Table 5). The *Ile/Val* genotypes showed a higher prevalence of 62.3% in the high exposed group compared with 46.2% in the low exposed group. The *Ile* and *Val* allele percentages were 56% and 44% in the high exposed group, while 67.7% and 32.3% in the low exposed group. These allele frequency differences showed marginal differences ($\chi^2 = 3.18$, $p = 0.07$) between the groups. This result implies that living in precarious conditions might affect the genetic variants. The genetic impact of *GSTP1* on susceptibility to Pb toxicity revealed a significant association of the *GSTP1 Ile/Val* genotype with the susceptibility to Pb toxicity. Individuals with this genotype were at a 2.54-fold higher risk of Pb toxicity (OR = 2.54; 95% CI: 1.02–6.32, $p = 0.04$) than individuals

carrying the *Ile/Ile* genotype. As for the wild *Val/Val* genotype, the OR ratio was greater than one (OR = 3.21) but not significant ($p = 0.15$). The combination of the heterozygous *Ile/Val* and mutant homozygous *Val/Val* genotypes showed a risk of susceptibility to Pb toxicity increased up to 2.63 ($p = 0.02$) times compared with the wild homozygous *Ile/Ile* genotype. The combined effect of all three putative genes showed associations on susceptibility to Pb toxicity. The combination of the *GSTP1 Ile/Val* with *GSTT1* null showed a risk factor of 11.7 (95% CI: 1.36–102.1, $p = 0.02$), while *GSTP1 Ile/Val* with *GSTM1* present and the combination of *GSTP1 Ile/Val* / *GSTM1* present / *GSTT1* null genotypes showed marginal associations with Pb susceptibility (OR = 2.62, 95% CI: 0.91–7.50, $p = 0.07$ and OR = 8.12, 95% CI: 0.89–73.8, $p = 0.06$, respectively) compared to their non-risk genotype counterparts (Table 5). In general, the high prevalence of the Ile/Val genotype might play the main role in Pb susceptibility.

Table 5

Studies have reported an increased health risk with occupational or environmental Pb exposure among adults with GST polymorphisms. The *GSTM1* and *GSTT1* null genotypes with increased levels on oxidative stress biomarkers such as malondialdehyde and high-sensitivity C-reactive protein (Khansakorn et al. 2011; Sirivarasai et al. 2013), *GSTT1* present genotype with increased Pb-related hypertension (Lee et al. 2012), and *GSTP1* risk genotypes (*Ile/Val* and *Val/Val*) with the increasing effect of Pb on amyotrophic lateral sclerosis disease (Eum et al. 2015) were reported. In another study, a 15 μg g^{-1} tibia Pb concentration was associated with weak cognitive function (a decrement of Mini-Mental State Exam score by 0.24 point) on men with *GSTP1* risk genotype (Eum et al. 2013). In our study, the genetic variants on susceptibility to Pb toxicity showed significant associations with *GSTT1* null and *GSTP1* risk genotypes (*Ile/Val* and *Val/Val* genotypes). The logistic regression analysis showed a risk of > 1 for *GSTT1* null, *GSTP1* risk

genotypes. Moreover, the combined effect of *GSTP1 Ile/Val* and *GSTT1* null showed a more pronounced risk of 11.7 times compared with the non-risk genotypes. Overall, these results indicated that children with deletions of the *GSTT1* gene and *GSTP1 Ile/Val* and *Val/Val* genotypes are at higher risk of Pb susceptibility due to high blood Pb levels. Our findings stress continued prevention programs on lead exposure in younger populations as lead exposure at early ages has harmful impacts on cognitive functions and impair development that can continue throughout the lifespan (Sanders et al. 2009).

We noticed that there are some limitations to this study. i) the small number (26 out of 140) in the low exposed group (< 10 μg dL^{-1}), ii) heavy metal levels were measured at a single point blood sample at which the half-life of heavy metals in the blood is short (especially Pb) compared to bone, and iii) no epidemiological or clinical data for the study subjects. All these factors might inevitably alter determining a true effect incidence and weakened the statistical power on the outcome result. This study, however, still contributes to the understanding of the GSTs' involvement in the development of childhood Pb toxicity in children. Second, in this study, we measured the levels of other heavy metals to highlight their burden on the children residing near the abandoned Pb mine site.

4. Conclusions

Our results stress the significance of genetic factors in determining individual differences in blood heavy metal concentrations. We found a direct association between *GSTT1* null genotype and blood Pb and Cd concentrations. Furthermore, *GSTT1* null genotype and *GSTP1* risk genotypes (*Ile/Val* and *Val/Val*) play more significant roles in modifying the blood Pb and Cd and, as a result, be risk factors associated with Pb susceptibility. Owing to GST's potential role in detoxifying toxic metals, children with more copies of the *GSTP1 Val* allele and gene deletion of *GSTT1* are at higher risk of Pb toxicity. These results suggest that the exposure of heavy metals, especially Pb, may be influenced by genotypic variants of the GST genes. As a preliminary study, the results generated will use as baseline data for future studies. However, replication of this finding using population-based research is warranted.

Ethical approval: Study protocol approval and permission to conduct the research were obtained from the University of Zambia Research Ethics Committee (UNZAREC; REF. No. 012-04-16) and the Ministry of Health Zambia, respectively. Material transfer agreement (MTA, Approval No. E00417) has been issued from the Ministry of Health, Zambia for transporting frozen samples to Japan.

Consent to participate: Participation in this study was voluntary and participants enrolled after getting signed informed consent from the parents.

Consent for publication: Children's' parent signed informed consent before participation and be aware regarding publishing the data. All the coauthors have read the manuscript and agreed for publication.

Competing interests: The authors have declared that no competing interests exist.

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Authors' contribution

YBY: responsible for conceptualization, data analysis, investigation, methodology, and writing both drafting the original paper and reviewing.

SMMN: responsible for conceptualization, analysis, funding acquisition, investigation, and writing, and corresponding author.

JY, HN, HT, AK and KM: participate in sample collection, material preparation, and reviewing the manuscript.

YK: Funding acquisition, and supervision.

KC: Supervision.

MI: responsible for project administration, funding acquisition, supervision, and reviewing the manuscript and corresponding author.

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Tables and Figures

Figure captions

Fig. 1. Gel electrophoresis for representative PCR-RFLP analysis of the VDR SNPs.

Fig 2. Blood lead levels for all genotypes of VDR gene polymorphic variants (*BsmI*, *FokI*, *ApaI* and *TaqI*) in environmental lead exposed children (* = $p < 0.05$; ** = $p < 0.01$; nonparametric Wilcoxon / Kruskal-Wallis tests between aa genotype and other genotypes)

Table 1

Genotype and allele frequency of ALAD and VDR polymorphisms (*BsmI, FokI, ApaI and TaqI*) in children from Kabwe, Zambia in comparison with quartiles of BLLs

Gene	Genotype	N (%)	HWE equation[a]	Quartiles of BLL[b]				
			χ2, *p*-value	Q1, N (%)	Q2, N (%)	Q3, N (%)	Q4, N (%)	*p*-Value *
ALAD	ALAD 1-1	139 (100)		31 (100)	35 (100)	31 (100)	42 (100)	
	ALAD 1-2	0						
	ALAD 2-2	0						
	G	1						
	C	0						
BsmI G > A	BB (AA)	3 (2.2)	1.70, 0.23	1 (3.2)	1 (2.9)	0	1 (2.2)	0.91
	Bb (AG)	49 (35.3)		9 (29.1)	13 (37.1)	13 (41.9)	14 (33.3)	
	bb (GG)	87 (62.6)		21 (67.7)	21 (60.0)	18 (58.1)	27 (64.3)	
	B (A)	0.2						
	b (G)	0.8						
FokI T > C	FF (CC)	92 (66.2)	0.01, 0.89	20 (64.5)	24 (68.6)	20 (64.5)	28 (66.7)	0.89
	Ff (CT)	42 (30.2)		10 (32.3)	11 (31.4)	9 (29.0)	12 (28.6)	
	ff (TT)	5 (3.6)		1 (3.2)	0	2 (6.5)	2 (4.8)	
	F (C)	0.81						
	f (T)	0.19						
ApaI G > T	AA (TT)	63 (45.3)	0.04, 0.92	12 (38.7)	16 (45.7)	17 (54.8)	18 (42.9)	0.03*
	Aa (TG)	62 (44.6)		19 (61.3)	17 (48.6)	11 (35.5)	15 (35.7)	
	aa (GG)	14 (10.1)		0	2 (5.7)	3 (9.7)	9 (21.4)	
	A (T)	0.68						
	a (G)	0.32						
TaqI T > C	TT (TT)	75 (54.0)	2.62, 0.14	14 (45.2)	20 (57.1)	12 (38.7)	29 (69.1)	0.18
	Tt (TC)	59 (42.4)		16 (51.6)	13 (37.1)	18 (58.1)	12 (28.6)	
	tt (CC)	5 (3.6)		1 (3.2)	2 (5.7)	1 (3.2)	1 (3.6)	
	T (T)	0.75						
	t (C)	0.25						

N: number; HWE: Hardy-Weinberg equilibrium

* Chi-square test

[a] HWE equation: χ2 (chi-squared test) < 3.841 and /or *p*-value > 0.05 indicates no deviation from HWE

[b] BLL quartiles (Q1: x ≤ 10 μg/dL; Q2:10 < x ≤ 19 μg/dL; Q3: 19 < x ≤ 25 μg/dL; Q4: x > 25 μg/dL)

Table 2

Comparison of VDR SNPs allele frequencies between Zambian children and different populations

Population	*FokI* (rs2228570)	*BsmI* (rs1544410)	*ApaI* (rs7975232)	*TaqI* (rs731236)	Reference
	C/T	G/A	T/G	T/C	
Zambian children	0.81/0.19	0.8/0.20	0.68/0.32	0.75/0.25	This study
African ancestry	0.83/0.17	0.71/0.29	0.62/0.38	0.75/0.25	Lins et al., 2011
European ancestry	0.52/0.48***	0.52/0.48***	0.57/0.43	0.52/0.48**	″
Asian ancestry	0.64/0.36**	0.92/0.08**	0.35/0.65***	0.93/0.07***	″
Brazil	0.67/0.33*	0.60/0.40**	0.54/0.46*	0.62/0.38	″
Black South Africans	0.84/0.16	0.74/0.26	0.76/0.24	0.66/0.34	Meyer et al., 2017
White South Africans	0.54/0.46***	0.62/0.38**	0.54/0.46*	0.61/0.39*	″
African American	0.77/0.23	0.63/0.37**	0.70/0.30	0.73/0.27	Sarkissyan et al., 2014
China	0.60/0.40**	0.91/0.08**	0.35/0.65***	0.90/0.10***	Yu et al., 2017

* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; Two-tailed Fisher's exact test and chi square test was done using Zambian children as reference value

Table 3

Blood lead levels in environmentally exposed children with different genotypic combinations of VDR gene

Group	Genotype combination	BLL (µg/dL)			
		N	Mean ± SD	*p* value	Range
G1	bbFfAATT	5	37.8 ± 14.9	Ref	24.1 – 60.8
G2	bbFFAATT	6	23.4 ± 10.5	NS	10.5 – 35.1
G3	bbFFAATt	12	19.8 ± 10.2	*	4.7 – 42.2
G4	bbFFAaTT	20	18.0 ± 11.3	*	4.5 – 41.8
G5	bbFFaaTT	11	24.6 ± 7.9	NS	11.1 – 34.8
G6	bbFFAaTt	11	12.7 ± 6.3	**	4.9 – 24
G7	bbFfAaTT	8	15.2 ± 8.5	**	3.4 – 26.2
G8	bbFfAATt	4	14.2 ± 10.1	*	3.9 – 24.5
G9	bbFfAaTt	4	18.8 ± 18.4	NS	5.1 – 45.8
G10	BbFFAATT	4	22.9 ± 13.4	NS	4.4 – 33.5
G11	BbFFAaTT	6	21.0 ± 11.4	NS	10.7 – 40.4
G12	BbFFAATt	9	17.6 ± 6.4	**	5.9 – 23.2
G13	BbFFAaTt	5	20.9 ± 9.1	NS	8.8 – 30.4
G14	BbFFAAtt	5	17.9 ± 10.7	NS	4.7 – 34.1
G15	BbFfAATT	6	13.9 ± 2.9 [b]	**	10.8 – 19.2
G16	BbFfAaTT	3	16.7 ± 12.8	NS	1.65 – 25.1
G17	BbFfAATt	6	17.2 ± 12.6	NS	1.65 – 33.5
G18	BbFfAaTt	3	17.3 ± 6.1	*	10.2 – 21.2
G19	bbFfaaTT	2	30.3 ± 6.6		25.6, 34.9
G20	BbffAATt	2	29.8 ± 6.4		25.3, 34.3
G21	bbffAaTT	1	6.7		
G22	bbffAATt	1	24.8		
G23	bbffAaTt	1	19.3		
G24	bbFFaaTT	1	32.4		
G25	BBFFAATT	1	7.6		
G26	BBFFAATt	1	26.6		
G27	BBFfAATT	1	17		

N: Number of children; BLL: Blood lead level; Ref: Reference; NS: Not significant
* = $p < 0.05$; ** = $p < 0.01$; Nonparametric Wilcoxon analysis for comparison for each pair for sample numbers with greater than three

Table 4

Distribution of VDR genotypes in low vs high BLL subjects and associations with lead toxicity

SNP	Genotype / Allele	Low BLL children[#] (N = 31)		High BLL children[#] (N = 108)		HWE equation	OR [95% CI], *p*-value
		N	%	N	%	X^2, *p*	
BsmI	BB	1	3.2	2	1.9	0.81, 0.66	(BB + Bb) vs bb
	Bb	9	29	40	37		1.33 [0.57 – 3.12], 0.50
	bb	21	67.7	66	61.1		
	B	11	18	44	20		1.19 [0.57 – 2.46], 0.64
	b	51	82	172	80		1
FokI	FF	20	64.5	72	66.7	0.08, 0.95	FF vs (Ff + ff)
	Ff	10	32.3	32	29.6		1.1 [0.47 – 2.54], 0.82
	ff	1	3.2	4	3.7		
	F	50	81	176	82		1.05 [0.52 – 2.16], 0.88
	f	12	19	40	18		1
ApaI	AA	12	38.7	51	47.2	6.89, 0.03*	AA vs (Aa + aa)
	Aa	19	61.3	43	39.8		1.42 [0.63 – 3.20], 0.40
	aa	0	0	14	13		
	A	43	69	145	67		0.90 [0.49 – 1.66], 0.74
	a	19	31	71	33		1
TaqI	TT	14	45.2	61	56.5	1.37, 0.50	TT vs (Tt + tt)
	Tt	16	51.6	43	39.8		1.58 [0.71 – 3.52], 0.26
	tt	1	3.2	4	3.7		
	T	44	71	165	76		1.32 [0.70 – 2.49], 0.38
	t	18	29	51	24		1

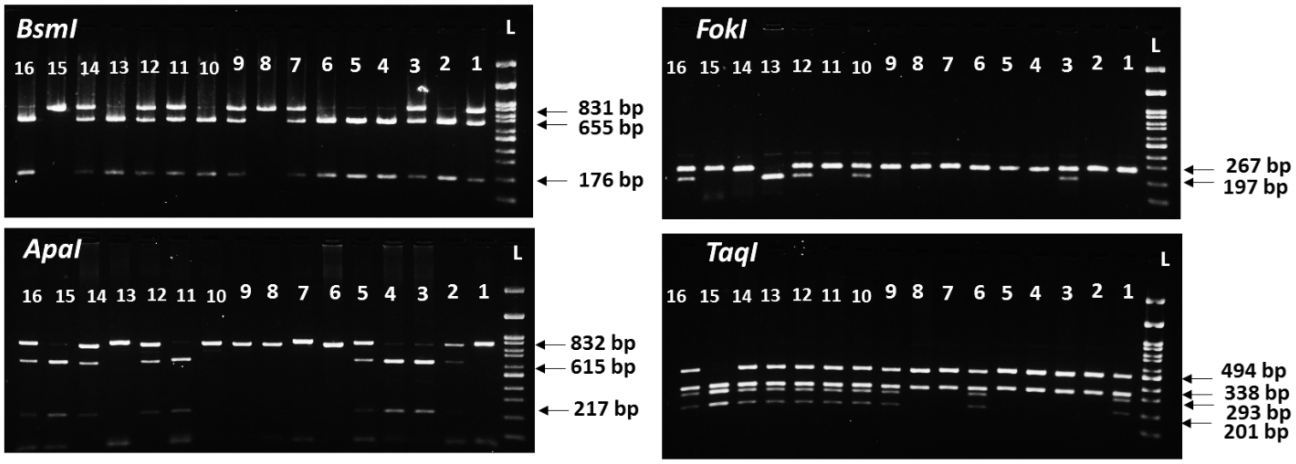
N: Number of children; %: Frequency; BLL: Blood lead level; HWE: Hardy-Weinberg equilibrium
OR: Odds ratios; 95% CI: 95% Confidence interval
[#] Low BLL children: BLL ≤ 10 μg/dL; High BLL children: BLL > 10 μg/dL
***** Result is significant at $p < 0.05$

Table 5

Genotype frequencies of VDR SNPs and association with gender

SNP/ Genotype		All children		HWE equation	OR [95% CI], *p*-value
		Female N (%)	Male N (%)	χ^2, *p*	Female vs Male
BsmI	BB	1 (1.5)	2 (3)	0.33, 0.84	(BB + Bb) vs bb
	Bb	24 (37)	25 (34)		1.08 [0.54 – 2.16], 0.81
	bb	40 (61.5)	47 (63)		
FokI	FF	44 (68)	48 (65)	2.39, 0.30	FF Vs (Ff + ff)
	Ff	21 (32)	21 (28)		1.13 [0.56 – 2.29], 0.72
	ff	0 (0)	5 (7)		
ApaI	AA	29 (45)	34 (46)	0.16, 0.92	AA vs (Aa + aa)
	Aa	30 (46)	32 (43)		0.94 [0.48 – 1.85], 0.87
	aa	6 (9)	8 (11)		
TaqI	TT	41 (63)	34 (46)	10.1, 0.006**	TT vs (Tt + tt)
	Tt	19 (29)	40 (54)		2.01 [1.02 – 3.97], 0.04*
	tt	5 (8)	0 (0)		

N: Number of children; %: Frequency; HWE: Hardy-Weinberg equilibrium
OR: Odds ratios; 95% CI: 95% Confidence interval
* Result is significant at $p < 0.05$



L, 100 bp DNA ladder; 1-16, sample #.

For BsmI: #8 and 15 (831 bp) are BB homozygotes; #1, 3, 7, 9, 11, 12 & 14 (831, 655 and 176 bp) are Bb heterozygotes; #2, 4-6, 10, 13 & 16 (655 and 176 bp) are bb homozygotes. For FokI: #1, 2, 4-9, 11, 14 & 15 (267 bp) are FF homozygotes; #3, 10, 12 & 16 (267 and 197 bp) are Ff heterozygotes; #13 (197 and 70 bp) is ff homozygote. For ApaI: #1, 6-10 & 13 (832 bp) are AA homozygotes; #2, 12, 14 & 16 (832, 615 and 217 bp) are Aa heterozygotes; #3, 4, 11 & 15 (615 and 217 bp) are aa homozygotes. For TaqI: #2-5, 7 & 8 (494 and 338 bp) are TT homozygotes; #1, 6, 9-14 & 16 (494, 338, 293 and 201 bp) are Tt heterozygotes; #15 (338, 293 and 201 bp) is tt homozygote.

Fig. 1.

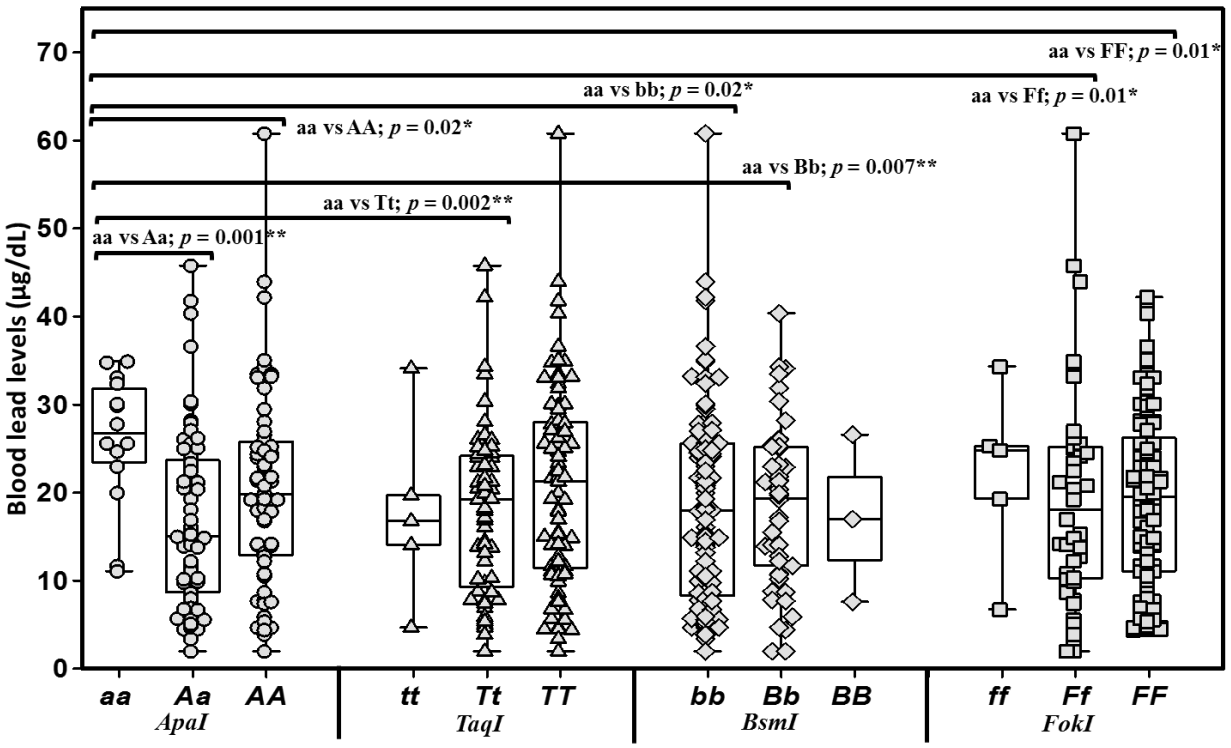

aa vs FF; p = 0.01*
aa vs bb; p = 0.02*
aa vs Ff; p = 0.01*
aa vs AA; p = 0.02*
aa vs Bb; p = 0.007**
aa vs Tt; p = 0.002**
aa vs Aa; p = 0.001**
Blood lead levels (µg/dL)
0
10
20
30
40
50
60
70
aa
Aa
AA
ApaI
tt
Tt
TT
TaqI
bb
Bb
BB
BsmI
ff
Ff
FF
FokI

Fig 2.