Delta-aminolevulinic acid dehydratase (ALAD) and vitamin D receptor (VDR) genes polymorphisms in children residing in an abandoned lead-zinc mine area

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Abstract

Lead is a ubiquitous environmental pollutant that poses serious health problems to humans, especially to children. However, genetic variability in individuals varies their susceptibility to lead poisoning. One possible factor is genetic polymorphism. Thus, this study aimed to investigate the association between blood lead level (BLL), and polymorphisms in the delta-aminolevulinic acid dehydratase (ALAD) *MspI* (rs1800435) and vitamin D receptor (VDR) *FokI* (rs19735810), *BsmI* (rs15444410), *ApaI* (rs7975232) and *TaqI* (rs731236) genes in children exposed to lead. A total of 140 children (aged 2 - 10 years) were recruited in areas living closer to and far away from an abandoned lead-zinc mine in Kabwe, Zambia. Blood samples were collected from each child for BLLs and polymorphisms analysis.

All children were homozygous for the ALAD 1 allele, indicating there might be bioavailable lead in the children's blood which can transfer to the soft tissues and the brain. The distribution of the VDR gene polymorphisms showed major alleles prevalence's of 81%, 80%, 68%, and 75% for *FokI*, *BsmI*, *ApaI*, and *TaqI* polymorphisms, respectively. The aa genotype of VDR *ApaI* showed significantly higher BLL compared to other genotypes of the VDRs polymorphism. The *TaqI* - TT genotype was associated with an increase of lead exposure risk in female children (OR = 2.06; 95% CI:1.04 – 4.06, $p = 0.03$). The haplotype analysis showed 10 haplotypes with a frequency above 1%, and the *FbAt* haplotype showed a protective role against lead toxicity. In conclusion, the children, especially female children, which exposed to lead mainly from the abandoned lead-zinc mine might be at a higher risk of developing lead poisoning. Further, larger scale sample sizes are needed to corroborate the role of ALAD and VDR genetic variants on the implications of lead toxicity in the general population, particularly in children.

Keywords: Lead; Gene polymorphism; ALAD; VDR; Children

1. Introduction

Lead is a ubiquitous environmental contaminant that caused adverse health problems and widespread environmental contamination. To date, no safe blood lead-exposure threshold has been identified. Children compared to adults are particularly susceptible, and at a greater risk to the neurotoxic effects of lead (Sanders et al., 2010). Lead can cause damage to children's cognitive functioning that persist into adulthood and in some cases irreversible neurological damage (Flora et al., 2012; Lanphear et al., 2005;). There is an inter-individual variation in susceptibility to the adverse health effects of lead and molecular evidence has indicated that genetic factors such as gene polymorphism can modify lead toxicity or might be protective from lead poisoning (Kim et al., 2014). The delta-aminolevulinic acid dehydratase (δ-ALAD) and vitamin D receptor (VDR) have shown potential as candidate genes for lead susceptibility in humans (Onalaja and Claudio, 2000).

Aminolevulinic acid dehydratase, the second enzyme in the heme biosynthesis, is a polymorphic enzyme with two codominant alleles called ALAD 1 and ALAD 2 (Wetmur et al., 1991a). The difference between these two polypeptides is a single nucleotide polymorphism (SNP) results in the substitution of neutral asparagine for positively charged lysine at position 177 of the coding region (rs1800435). The biological explanation to this substitution makes the ALAD 2 enzyme more electronegative than the ALAD 1 and may have a greater affinity for lead ions (Battistuzzi et al., 1981; Wetmur et al., 1991a). Several studies reported higher blood lead concentrations in the ALAD 2 allele carriers than the ALAD 1 allele carriers (Montenegro et al., 2006; Wetmur et al., 1991b), suggesting the individual variation in susceptibility to lead exposure.

Lead can substitute calcium in many biological systems since both are divalent cations. Thus, lead can exert toxic effects by mimicking the actions of calcium and interact with proteins (Tchounwou et al., 2012). The high binding capacity of lead to calcium-binding proteins suggested that at low

calcium intake lead absorption can increase in our body. Bruening et al. (1999) reported significantly lower dietary calcium intake in children with elevated blood lead levels. The vitamin D endocrine system and the VDR are vital parts for calcium homeostasis. And in humans, several VDR SNPs have been identified (Uitterlinden et al., 2004a). Among these several SNPs, the *ApaI* - rs7975232, *BsmI* - rs1544410, *FokI* - rs2228570, and *TaqI* - rs731236 were investigated intensively for their association with altered calcium metabolism and human traits (Uitterlinden et al., 2004b, Valdivielso and Fernandez, 2006).

Lead exposure represents a significant contributor to adverse health effects in developing countries. Children's deaths due to lead poisoning were reported from the recycling of used lead-acid battery in Senegal (Haefliger et al., 2008) and artisanal gold mining in Nigeria (Dooyema et al., 2011). Because of the abandoned lead-zinc mine in the town which operated for almost a century, Kabwe - Zambia present in a list of the top ten world's most polluted places (Blacksmith Institute, 2013). Yabe et al. (2015) has reported blood lead levels up to 427.8 µg/dL in children residing close to the closed mine in Kabwe. Besides, previous studies reported very high lead concentrations in different biological and environmental matrices (Nakayama et al., 2011; Toyomaki et al., 2020; Yabe et al., 2011, 2013, 2020;). However, regardless of the high burden of lead exposure in Kabwe town, gaps still exist in the knowledge of lead exposure and its impact on children. To our knowledge, there is only one study that investigated the methylation status of ALAD and p16 genes in children exposed to environmental lead from Kabwe, Zambia (Yohannes et al., 2020). Nevertheless, no data exist concerning the impacts of lead on genetic variants among the people from Kabwe.

In this study, we aimed to investigate the associations of ALAD *MspI* (rs1800435), VDR – *ApaI* (rs7975232), *BsmI* (rs1544410), *FokI* (rs2228570), and *TaqI* (rs731236) polymorphisms with blood lead concentration in children living closer to or/and distance away from an abandoned lead-zinc

mine in Kabwe, Zambia. As there is no information about gene polymorphism in association with the lead exposure in Kabwe, the present study is one of the first efforts and the result of this study will serve as baseline information for future researches.

2. Materials and Methods

2.1. Study subjects and sampling

The sampling area information and demographic data of the recruited children have been published in our previous study (Yohannes et al., 2020). Participation in this study was voluntary. The study recruited a total of 140 randomly chosen children from five townships called Bwacha, Chowa, Kasanda, Makululu, and Nakoli in Kabwe town, Zambia. The children's parents or guardians signed written informed consent forms before the enrollment.

The study was conducted in July 2016. From each child, 3 mL of the venous blood sample was collected into vacuum tubes containing heparin. The BLLs were measured using a portable LeadCare II analyzer after blood collection that same day as described by Yohannes et al. (2020). For genetic analysis, the blood samples were immediately stored at –20 °C after lead analysis. After obtaining a material transfer agreement (MTA, Approval No. E00417) from the Ministry of Health, Zambia, the frozen samples were transported to Japan. All the genetic analyses were carried out in the Laboratory of Toxicology, Faculty of Veterinary Medicine, Hokkaido University, Japan.

2.2. *Genetic* analyses

Genomic DNA was isolated from whole blood using the NucleoSpin Blood Kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions. The concentration and quality of genomic DNA were assessed using a NanoDrop 1000 UV/Vis Spectrophotometer (Thermo Fisher Scientific Inc., USA). The purified DNA was kept at –20 °C until genotyping was conducted.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed for the genotyping of ALAD - *MspI* (G/C), VDR - *BsmI* (G/A), *FokI* (T/C), *ApaI* (G/T), and *TaqI* (T/C). The ALAD polymorphism (917 bp) was performed using primers: 5′-AGACAGACATTAGCTCAGTAGAGG-3′ and 5′-GGCAAAGACCACGTCCATTCAC-3′. Genomics for the *BsmI* polymorphism (831 bp) was determined using primers: 5′-GACCTGTGGCAACCAAGACT-3′ and 5′-AACCAGCGGAAGAGGTCAAG-3′; *FokI* polymorphism (267 bp) using primers: 5′-AGCTGGCCCTGGCACTGACTCTGGCT-3′ and 5′-ATGGAAACACCTTGCTTCTTCTCCCTC-3′; and *ApaI* and *TaqI* polymorphisms (832 bp) using primers: 5′-CAGAGCATGGACAGGGAGCAA-3′ and 5′-AGGCAGCGGTGGAGGCATCTCT-3′. PCR reaction was performed in a 20 μL reaction mixture containing 0.3 μmol/L of each primer, 500 ng of genomic DNA and 10 μL EmeraldAmp PCR Master Mix (2x premix, TaKaRa Bio, Japan). PCR conditions consisted of an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for ALAD and *BsmI*, and 68 °C for *FokI*, *ApaI* and *TaqI* for 30 s and extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min. The DNA bands were visualized under a Blue-LED transilluminator system and photographed.

The resulting amplified products were subjected to restriction digestion using their respective enzymes obtained from New England Biolabs (NEB). The samples were incubated for 4 h at 65 °C for *BsmI* and *TaqI*, and heat inhibited for 20 min at 80 °C; incubated for 4 h at 37 °C for *ApaI* and *FokI*, and heat inhibited for 20 min at 65 °C; incubated for 4 h at 37 °C for *MspI* (ALAD) with no heat inactivation step. Digestion of the ALAD amplified product by *MspI* produces fragments of 584 bp and 158 bp for the ALAD 1-1, 584 bp, 513 bp and 158 bp for ALAD 1-2, and 513 and 158 bp for the ALAD 2-2 genotypes. The VDR Alleles were designated by capital letters A, B, F, and T in the absence of restriction site and by small letters a, b, f, and t for the presence of restriction site for *ApaI*, *BsmI*, *FokI*, and *TaqI* polymorphisms, respectively. The genotypes and fragment lengths were 832 bp for AA, 832 bp, 615 bp and 217 bp for Aa, and 615 bp and 217 bp for aa genotypes; 831 bp for BB, 831 bp, 655 bp and 176 bp for Bb, and 655 bp and 176 bp for bb genotypes; 267 bp for FF, 267 bp, 197 bp and 70 bp for Ff, and 197 bp and 70 bp for ff genotypes; 494 bp and 338 bp for TT, 498 bp, 338 bp, 293 bp and 201 bp for tt, and 338 bp, 293 bp and 201 bp for tt genotypes. The details of nucleotide sequence, primer sets, and genotypes with fragment sizes after RFLP are presented as **Supplementary Material**. Digested products were visualized after agarose gel electrophoresis under a Blue-LED transilluminator system. Gel electrophoresis showed the identification of genotypes of each VDR SNPs has been presented in Figure 1.

Fig. 1.

2.3. Statistical analysis

The JMP Pro 14 (SAS Institute, Cary, NC, USA) software was used for statistical analysis, and a *p*-value < 0.05 was considered significant. The distribution of genotypes and allele frequencies among the studied children was analyzed for Hardy-Weinberg equilibrium (HWE) by the chi-square test ($\chi 2$), and the genotype and allele frequencies were compared in relative to BLL quartiles. The comparison of allele frequencies between the studied subjects and other studies with different populations was performed by pairwise chi-square test. The odds ratios (ORs) with 95% confidence interval (CI) were calculated to compare the risk of lead toxicity in low and high BLL level based on the first quartile BLL, and between females and males. Due to low frequencies of aa, BB, ff, and tt genotypes, we grouped *ApaI* genotype as AA and (Aa + aa) groups; *BsmI* genotype as (BB + Bb) and bb groups; *FokI* genotype as FF and (Ff + ff) groups, and *TaqI* genotype as TT and (Tt + tt) groups. SNPStats software (available online at http://bioinfo.iconcologia.net/SNPstats).was used to perform haplotype analysis and analyze the association with BLL.

3. **Results**

A total of 140 children, 75 males and 65 females aged between 2 and 10 years, were engaged in the present study (Yohannes et al., 2020). The mean ages of children were 5.7 ± 1.78 years for females and 5.6 ± 1.88 years for males. The average BLL was 19.4 ± 10.6 μg/dL and ranged from 1.65 to 60.8 μg/dL among the children. There was no significant difference in BLL between sexes (p = 0.236) with mean values of 20.5 ± 12.4 μg/dL and 18.4 ± 8.82 μg/dL in female and male children, respectively.

3.1. Prevalence of ALAD and VDR SNPs, and haplotype frequencies of VDR genes

The alleles and genotypes frequencies (%) of ALAD and VDR genes SNPs among the studied children from Kabwe, Zambia are summarized in Table 1. All loci follow the Hardy-Weinberg equilibrium (p > 0.05). Molecular analysis revealed that all the children tested for the ALAD genotype were 100% ALAD 1-1. The overall prevalence of the VDR SNPs allele frequencies of A/a, B/b, F/f, and T/t for the *ApaI*, *BsmI*, *FokI*, and *TaqI* polymorphisms were 0.68/0.32, 0.20/0.80, 0.81/0.19, and 0.75/0.25, respectively. For VDR-*ApaI*, a significantly higher percentage of allele a (39%) was observed at the Q4 group with BLL > 25 μg/dL (Table 1).

Table 1

204 3.2.Evaluation of VDR SNPs genotypes distribution between this study children and other studied
205 populations

206 The distribution of the four VDR SNPs data was available from other studies and the International
207 HapMap project populations (http://hapmap.ncbi.nlm.nih.gov/). A pairwise chi-square test was
208 conducted to compare the alleles frequencies of the present study with different populations. The
209 distribution of VDR SNPs alleles showed no significant difference between the present studied
210 subjects and the black race population. On the other hand, the white race population and Chinese
211 people showed significant differences in the allele frequencies of the VDR SNPs compared to the
212 present study as shown in Table 2.

213

214 **Table 2**

215

216 3.3. BLLs among the four VDR SNPs

217 Figure 1 shows the association between VDR SNPs and BLLs among the studied children. The *ApaI*
218 polymorphism exhibited a higher mean BLL (25.9 μg/dL) in aa genotype and a lower BLL (16.9
219 μg/dL) in the Aa genotype ($\chi 2 = 11.3$, DF = 2, $p = 0.003$). No variations were observed in BLLs in
220 *TaqI*, *BsmI*, and *FokI* SNP genotypes (Fig. 2). Further, we examined the different VDR SNPs
221 genotype combinations and revealed the highest mean BLL (37.8 ± 14.9 μg/dL) in AA/TT/bb/Ff
222 combination (Table S1). The highest BLL (60.8 μg/dL) was also observed in this group (Fig. 2).

223

224 **Fig. 2.**

3.4. Association between VDR SNPs and susceptibility to lead toxicity

To examine the impact of BLLs on the VDR genotyping frequency and their association with risk of lead toxicity, we categorized the subjects based on the lowest quartile of blood lead (Q1) into two groups as low BLL children (BLL ≤10 μg/dL) and high BLL children (BLL >10 μg/dL). Table 3 shows the associations of VDR SNPs genotype distributions with the risk of lead toxicity in low and high BLL groups.

Table 3

Results showed that the only *ApaI* genotype distributions were different in the two studied groups (χ^2 = 6.68, p = 0.03). Significantly higher aa genotype among high BLL children was found compared to low BLL children. The frequencies of *ApaI* genotypes determined from low BLL children were 100% for (AA + Aa) and 0% for aa genotype while in high BLL children the frequency of (AA + Aa) and aa was present in a percentage of 87% and 13%, respectively. No statistically significant correlation was observed between the VDR genotypes and susceptibility to lead toxicity. However, the BB+Bb (OR = 1.36), FF (OR = 1.07), AA (OR = 1.44) and TT (OR = 1.54) genotypes showed an OR value of greater than 1 (Table 3). This result suggests the possibility of a higher risk of lead toxicity to children with high frequencies of TT, AA, BB, and FF genotypes than the tt, aa, bb, and ff genotypes carrier children, respectively.

Table 4 shows VDR genotype distributions and the risk of lead toxicity in association with gender. Genotypic frequencies of 63% and 29% in females and 45% and 55% in males were observed for TT and Tt genotypes, respectively. On the contrary, the tt genotype was found only in females with a frequency of 8%. The *TaqI* genotype distribution between female and male subjects was statistically different ($\chi^2 = 13.0$, $p = 0.001$). We found that the TT groups for *TaqI* SNP showed an increased risk of lead toxicity in female children (OR = 2.06, 95% CI:1.04 – 4.06, $p = 0.03$). For the other VDR SNPs, we did not observe a significant association but, an OR > 1 in the presence of one or mixed alleles was observed.

Table 4

3.5. haplotype analysis of VDR genes

Haplotype analysis performed in the four-marker haplotype alleles (VDR-*FokI*, *BsmI*, *ApaI*, and *TaqI*) was presented in Table 5. It was observed 10 haplotypes with a frequency above 1% in our analysis. The three most represented haplotype were *FbaT* (28.7%), followed by *FbAT* (25.7%) and *FbAt* (12.1%). Compared between the children, the frequency of *FbAt* was significantly higher in low BLL children (21.9%) compared to children with high BLL (9.9%). The association analysis between the haplotype and susceptibility to lead toxicity showed that the *FbAt* haplotype was associated with a statistically decrease risk of lead toxicity (OR = 0.33, 95% CI:0.11 – 1.00, $p = 0.04$), indicating a protective role against lead toxicity.

Table 5

4. Discussion

Lead exposure can cause adverse health effects, particularly in children. To our knowledge, this is the first study to investigate the ALAD and VDR genes SNPs in association with lead toxicity in children living closer to or/and far away from an abandoned lead-zinc mine in Kabwe, Zambia. Considering 5 µg/dL, the current CDC blood reference value, 92% of the children's blood samples exceeded this value in the present study. Thus, the findings from this study may have some implications for lead toxicity and support the ongoing efforts to reduce childhood lead exposure in a region where only levels of lead in human and biota samples were investigated without any further genetic analysis. Thus, in the current study, we aimed to assess the impact of lead exposure in children through the biomarkers of susceptibility genes such as ALAD and VDR genes polymorphisms (Sakai, 2000a).

In the current study, we found all children were ALAD 1-1 genotypes. This finding was in accordance with other African population studies from Liberian populations (Benkmann et al., 1983), Ghanaian, Ovambos (Bantusin of Namibia), and Xhosas (Cape Town of South Africa) (Fujihara et al., 2009) that showed 100% ALAD 1-1 genotype, with neither ALAD 1-2 nor ALAD 2-2 genotypes. Although the more electronegative ALAD 2 allele could bind more lead easily and maintain higher BLLs than the ALAD 1 allele, the latter allele could be more prone to the detrimental effects of lead because of the easy transfer of the bioavailable lead from blood to the soft and hard tissues (Kelada et al., 2001). Previous studies reported that individuals with ALAD 1-1 genotypes had higher bone lead levels, which suggested higher body burdens of lead and could be at greater risk of the long-term health effects of lead (Wan et al., 2014; Yang et al., 2012). Kim et al. (2004) also reported higher hematological effects of lead for ALAD 1 homozygote subjects and they are more likely to be anemic. Another study on male Japanese lead workers by Sakai et al. (2000b) showed high

aminolevulinic acid (ALA), which is neurotoxic to the brain, in plasma for ALAD 1-1 genotype. These results suggest that the ALAD 1 homozygotes may be at greater risk due to the bioavailable lead in the blood that can transfer and accumulate in the tissues and brain. Also, we reported significant hypermethylation of the ALAD gene in these lead-exposed children with an odd ration of greater than one for anemia (Yohannes et al., 2020). Thus, the people, particularly infants and children in Kabwe might be at high risk of lead poisoning.

The genetic variants of VDR have been identified as potential factors to influence the lead toxicokinetic in the human body, and the effects of VDR SNPs such as *FokI*, *BsmI*, *ApaI*, and *TaqI* have been reported (Onalaja and Claudio, 2000; Rezende et al., 2008). In the present study, the observed genotype frequencies of the four VDR SNPs are conformed to the Hardy-Weinberg equilibrium. The A, b, F, and T alleles were the dominant alleles with incidences of 68%, 80%, 81%, and 75% in *ApaI*, *BsmI*, *FokI*, and *TaqI* polymorphisms, respectively. The most common genotype for each polymorphism was bb (62.1%) for *BsmI*, FF (65.7%) for *FokI*, both AA and Aa (45%) for *ApaI*, and TT (53.6%) for *TaqI*. The general distributions of these VDR SNPs were consistent with other population studies of black South Africans (Meyer et al., 2017), African Americans (Sarkissyan et al., 2014), and African ancestry studies (Lins et al., 2011). On the contrary, there were significant differences in allele distribution between this study subjects and white South Africans (Meyer et al., 2017), European ancestry, Asian ancestry and Brazilians (Lins et al., 2011), and Chinese population (Yu et al., 2017). So, our results offered additional evidence to the distribution specificity of VDR gene polymorphisms based on ethnicity.

In the present study, we found an association of high BLLs in *ApaI* polymorphism variants. The prevalence of aa genotype increases from 0% (Q1) to 22% (Q4), and the aa genotype of ApaI variants had significantly increased mean blood lead levels than those of the other genotypes (aa vs AA, p =

0.02; aa vs Aa p = 0.001). This finding corroborates with previous studies reported that *ApaI* aa genotype had higher BLLs than the Aa and AA genotypes in workers exposed to lead due to their workplace (Chuang et al., 2004; Himani et al., 2020). Further analysis among the SNPs showed that the aa genotype had significantly higher BLL than FF, Ff, Bb, bb, and Tt genotypes ($p < 0.05$). No significant associations of BLLs within *BsmI*, *FokI*, and *TaqI* variants were observed.

The influences of VDR SNPs to different traits have been investigated in adults with occupational lead exposure (Chuang et al., 2004; Himani et al., 2020; Shaik et al., 2019; Wananukul et al., 2012) or patients with different types of diseases such as cancer (köstner et al., 2009), diabetes (Ahmed et al., 2019) and autism (Cie´sli´nska et al., 2017). In the current study, we investigated the correlation between the VDR SNPs and environmental lead exposure in children. The results showed no statistical associations between the genotypes at the VDR polymorphic sites and susceptibility to lead toxicity. Nevertheless, the BB+Bb, FF, AA, and TT genotypes showed OR values of greater than one, but not statistically significant. Previous studies showed the association of these VDR genotypes with bone mineral density. Haynes et al. (2003) showed increased bone density in the VDR-*FokI* FF genotype while the AA genotype in *ApaI* and BB genotype in *BsmI* were associated with lower bone mineral density (Li et al., 2012). In another study by Pawlas et al. (2012), the *BsmI* B allele showed an inverse correlation against cognitive function with a steeper slope (β = –0.08), suggesting that lead has a negative influence on children's performance IQ. In the present study, the highest BLL (60.8 µg/dL) was detected in these genotypes. Thus, children with high frequencies of those genotypes might be at higher risk of calcium absorption and IQ problem. We also found an association between gender and *TaqI* polymorphism. In the present study, female children with TT genotypes showed higher susceptibility to lead toxicity (OR = 2.06, p = 0.03) than males. Chakraborty and his coworkers (2008) examined the effect of lead on the neuromotor response in

association with *TaqI* genotypes in 82 children and observed adverse effects of lead exposure on postural balance response in association with genetic polymorphisms of VDR at the *TaqI* site. A study from the National Health and Nutrition Examination Survey also investigated the relationship between the children's cognitive function and blood lead concentration in association with VDR SNPs and suggested that the *TaqI* variants can alter the children´s central nervous system toxicity of lead (Krieg Jr et al., 2010). These results suggest that the children in Kabwe might be at higher risk for the biological effects of lead. In the current study, haplotype structure revealed 10 haplotypes with >1% frequency. There was a trend of increased frequency of *FbAt* haplotype in low BLL children compared to High BLL children, suggesting that this haplotype could be protective against lead toxicity.

There are limitations to our study. First, this study is a cross-sectional study that collected samples at a single point in time, and thus it is difficult to determine incidence. Second, the small sample size may weaken the statistical power. Third, the current study depends on lead levels using blood samples at which the half-life of lead is about 35 days in blood, which may inevitably affect the result to some degree. Reliable bone lead measurements are needed. Thus, our findings need to be replicated in other long-term follow-up studies with large scale samples among the population exposed to both environmental and occupational lead, and bone lead measurement with the prevalence of disease as VDR SNPs have been linked to human traits.

5. Conclusions

This study provides insight into the association of ALAD and VDR polymorphic variants in association with BLL in children exposed to environmental lead. Our finding suggests that all subjects were ALAD 1 homozygotes at which they might be at risk due to the bioavailable lead in the blood that can transit to soft tissues and the brain. Regarding the VDR SNPs, the *ApaI* homozygous mutant genotype showed association with BLLs in environmentally lead-exposed children. The TT-genotype in *TaqI* (rs731236) site was allied with lead toxicity in female children. In haplotype analysis, the haplotype *FbAt* showed a protective role against lead toxicity compared to the most frequent haplotype (*FbaT*). Overall, further larger-scale samples alongside bone lead measurements are warranted to assess the ALAD and VDR SNPs in association with their implications on lead toxicity manifestations in the general population.

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Declaration of Competing Interest:

None.

Authors Contribution

Yared B. Yohannes: Conceptualization, Data curation, Formal analysis, Methodology, Writing - original draft, Writing - review & editing; **Shouta M.M. Nakayama** and **Yoshinori Ikenaka**: Funding acquisition, Investigation, Writing - review & editing; **John Yabe, Hokuto Nakata, Haruya Toyomaki, Andrew Kataba** and **Kaampwe Muzandu**: Investigation, Writing - review & editing; **Kennedy Choongo**: Supervision; **Mayumi Ishizuka**: Funding acquisition, Project administration, Supervision

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Ethical approval

The study protocol was approved by the University of Zambia Research Ethics Committee (UNZAREC; REF. No. 012-04-16), and permission was granted by the Ministry of Health through the Zambia National Health Research Ethics Board and the Kabwe District Medical Office.

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Tables and Figures

Figure captions

Fig 1. PCR-RFLP analysis for VDR gene- FokI, BsmI, ApaI and TaqI polymorphisms. M: 100 bp DNA marker; Lane 1: ff homozygote, Lane 2: Ff heterozygote, Lane 3: FF homozygote; Lane 4: bb homozygote, Lane 5: Bb heterozygote, Lane 6: BB homozygote; Lane 7: aa homozygote, Lane 8: Aa heterozygote, Lane 9: AA homozygote; Lane 10: tt homozygote, Lane 11: Tt heterozygote, Lane 12: TT homozygote.

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)

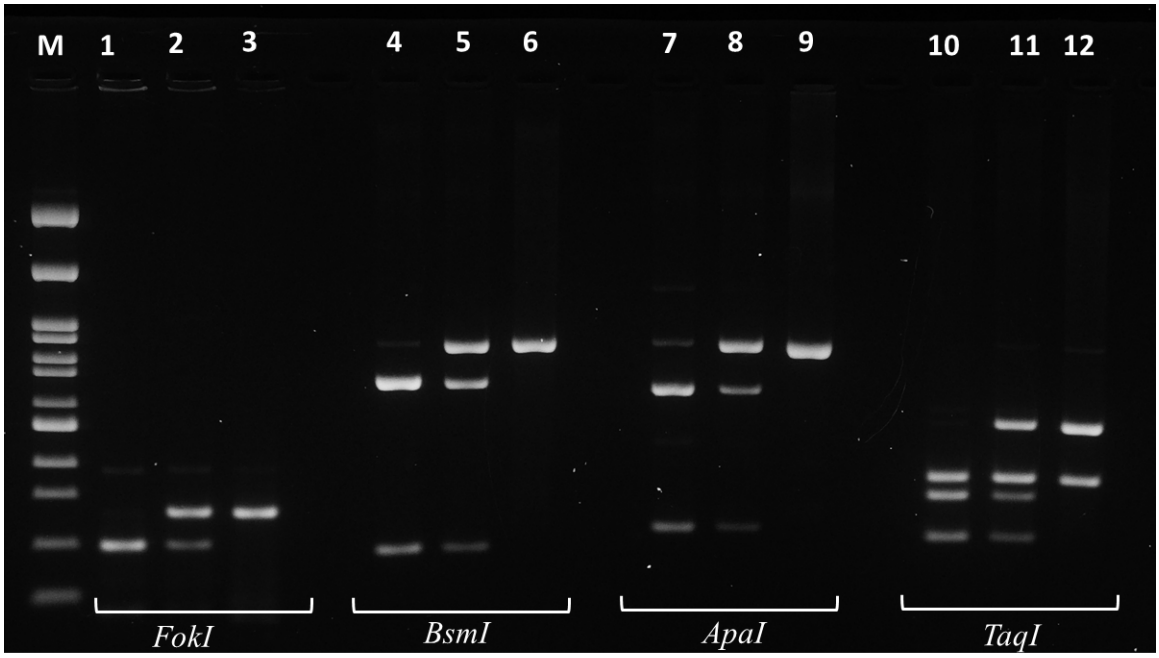
M
1
2
3
4
5
6
7
8
9
10
11
12
FokI
BsmI
ApaI
TaqI

Fig 1.

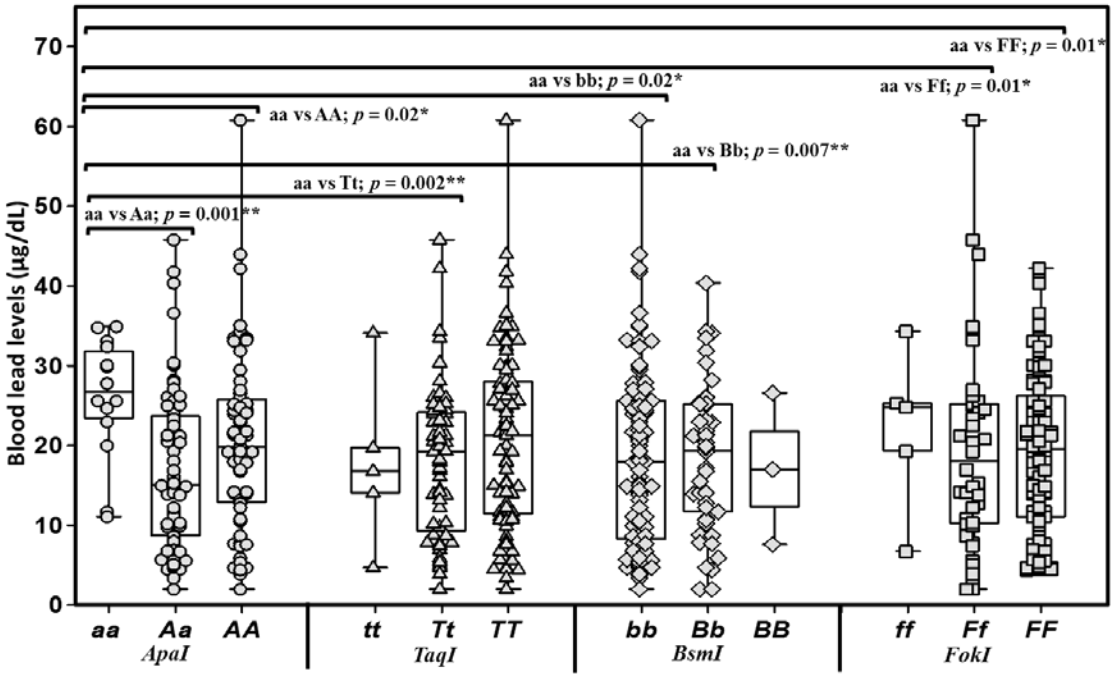
Blood lead levels (µg/dL)
0
10
20
30
40
50
60
70
aa vs FF; p = 0.01*
aa vs bb; p = 0.02*
aa vs Ff; p = 0.01*
aa vs AA; p = 0.02*
aa vs Bb; p = 0.007**
aa vs Tt; p = 0.002**
aa vs Aa; p = 0.001**
aa
Aa
AA
ApaI
tt
Tt
TT
TaqI
bb
Bb
BB
BsmI
ff
Ff
FF
FokI

Fig 2.

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]				
			$\chi 2$, *p*-value	Q1, N (%)	Q2, N (%)	Q3, N (%)	Q4, N (%)	*p*-Value *
ALAD	ALAD 1-1	140 (100)		31 (22.1)	36 (25.7)	32 (22.9)	41 (29.3)	
	ALAD 1-2	0						
	ALAD 2-2	0						
	G	1						
	C	0						
FokI T > C	FF (CC)	92 (65.7)	0.001, 0.99	20 (64.5)	24 (66.7)	21 (65.6)	27 (65.8)	NS
	Ff (CT)	43 (30.7)		10 (32.3)	12 (33.3)	9 (28.1)	12 (29.3)	
	ff (TT)	5 (3.6)		1 (3.2)	0	2 (6.3)	2 (4.9)	
	F (C)	81						
	f (T)	19						
BsmI G > A	BB (AA)	3 (2.1)	1.88, 0.17	1 (3.2)	1 (2.8)	0	1 (2.4)	NS
	Bb (AG)	50 (35.7)		9 (29.1)	14 (38.9)	13 (40.6)	14 (34.1)	
	bb (GG)	87 (62.1)		21 (67.7)	21 (58.3)	19 (59.4)	26 (63.4)	
	B (A)	20						
	b (G)	80						
ApaI G > T	AA (TT)	63 (45.0)	0.09, 0.76	12 (38.7)	16 (44.4)	17 (53.1)	18 (43.9)	0.01*
	Aa (TG)	63 (45.0)		19 (61.3)	18 (50.0)	12 (37.5)	14 (34.1)	
	aa (GG)	14 (10.0)		0	2 (5.6)	3 (9.4)	9 (22.0)	
	A (T)	68		69	69	72	61	
	a (G)	32		31	31	28	39	
TaqI T > C	TT (TT)	75 (53.6)	2.85, 0.09	14 (45.2)	20 (55.6)	13 40.6)	28 (68.3)	NS
	Tt (TC)	60 (42.8)		16 (51.6)	14 (38.9)	18 (56.3)	12 (29.3)	
	tt (CC)	5 (3.6)		1 (3.2)	2 (5.5)	1 (3.1)	1 (2.4)	
	T (T)	75						
	t (C)	25						

N: number; HWE: Hardy-Weinberg equilibrium

* Chi-square test

[a] HWE equation: $\chi 2$ (chi-squared test) < 3.841 and /or *p*-value > 0.05 indicates no deviation from HWE

[b] BLL quartiles (Q1: $x \leq 10$ μg/dL; Q2: $10 < x \leq 19$ μg/dL; Q3: $19 < x \leq 25$ 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	0.80/0.20	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: Distribution of VDR genotypes in low vs high BLL subjects and associations with lead toxicity

SNP	Genotype / Allele	High BLL children[#] (N = 109)		Low BLL children[#] (N = 31)		HWE equation	OR [95% CI], *p*-value
		N	%	N	%	X^2, *p*	
BsmI	BB	2	1.8	1	3.2	0.91, 0.63	(BB + Bb) vs bb
	Bb	41	37.6	9	29		1.36 [0.58 – 3.18], 0.46
	bb	66	60.6	21	67.7		
FokI	FF	72	66.0	20	64.5	0.05, 0.97	FF vs (Ff + ff)
	Ff	33	30.3	10	32.3		1.07 [0.46 – 2.46], 0.87
	ff	4	3.7	1	3.2		
ApaI	AA	52	46.8	12	38.7	6.68, 0.03*	AA vs (Aa + aa)
	Aa	43	40.4	19	61.3		1.44 [0.64 – 3.26], 0.37
	aa	14	12.8	0	0		
TaqI	TT	61	56.0	14	45.2	1.25, 0.54	TT vs (Tt + tt)
	Tt	44	40.4	16	51.6		1.54 [0.69 – 3.44], 0.28
	tt	4	3.6	1	3.2		

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 4: 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)	0.26, 0.87	(BB + Bb) vs bb
	Bb	24 (37)	26 (34)		1.04 [0.54 – 2.08], 0.89
	bb	40 (61.5)	47 (63)		
FokI	FF	44 (68)	48 (64)	4.51, 0.11	FF Vs (Ff + ff)
	Ff	21 (32)	22 (29)		1.17 [0.58 – 2.38], 0.64
	ff	0 (0)	5 (7)		
ApaI	AA	29 (45)	34 (45)	0.11, 0.94	AA vs (Aa + aa)
	Aa	30 (46)	33 (44)		0.92 [0.47 – 1.79], 0.81
	aa	6 (9)	8 (11)		
TaqI	TT	41 (63)	34 (45)	13.0, 0.001**	TT vs (Tt + tt)
	Tt	19 (29)	41 (55)		2.06 [1.04 – 4.06], 0.03*
	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 $p < 0.05$

Table 5: Haplotypes frequency of vitamin D receptor gene polymorphisms in high and low BLL children

FokI	*BsmI*	*ApaI*	*TaqI*	Total	High BLL children[#]	Low BLL children[#]	OR (95% CI)
F	*b*	*a*	*T*	0.287	0.295	0.258	1
F	*b*	*A*	*T*	0.257	0.274	0.188	1.58 (0.60 - 4.13)
F	*b*	*A*	*t*	0.121	0.099	0.219	0.33 (0.11 - 1.00) *
F	*B*	*A*	*T*	0.083	0.079	0.069	1.29 (0.29 - 5.68)
f	*b*	*A*	*T*	0.071	0.064	0.108	0.41 (0.10 - 1.61)
F	*B*	*A*	*t*	0.065	0.068	0.071	0.76 (0.17 - 3.46)
f	*b*	*a*	*T*	0.034	0.030	0.048	0.73 (0.10 - 5.37)
f	*B*	*A*	*T*	0.030	0.036	0.036	0.62 (0.06 - 6.19)
f	*b*	*A*	*t*	0.028	0.029	0	
f	*B*	*A*	*t*	0.020	0.022	0	

[#] Low BLL children: BLL ≤ 10 µg/dL; High BLL children: BLL > 10 µg/dL
* Result is significant at $p < 0.05$