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Original Article

Evaluation of Vitamin D Receptor Haplotypes on Modulation of Blood Lead Levels in Occupationally Exposed Workers from North India: A Cross-sectional Study

Himani Rathi[1], Raman Kumar[2], Sarama Saha[3] and Sudip Kumar Datta[4*]

[1]*Department of Biochemistry, Government Medical College, Budaun, U.P., India;* [2]*Department of Biochemistry, All India Institute of Medical Sciences, Jammu, India;* [3]*Department of Biochemistry, All India Institute of Medical Sciences, Rishikesh, Uttarakhand, India;* [4]*Department of Laboratory Medicine, All India Institute of Medical Sciences, New Delhi, India*

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Abstract

Background and objectives: This study evaluates the association of haplotypes of vitamin D receptor (VDR) genes (*BsmI-ApaI-TaqI*) with blood lead (B-Pb) levels.

Method: The VDR polymorphism results for 100 occupationally lead-exposed (LEx) workers in the battery industry and 100 non-lead-exposed controls (controls) from the Delhi-NCR region were analyzed for haplotypes. PCR-RFLP was used to identify three VDR polymorphisms (TaqI, BsmI and ApaI), and the VDR haplotype and linkage disequilibrium (D′) were analyzed in these subjects.

Results: B-Pb, together with the total vitamin D, calcium and phosphorus levels, were reported in the previous study conducted by the investigators. Eight possible haplotypes were observed among the three single nucleotide polymorphisms (BsmI-ApaI-TaqI). Furthermore, significant differences in haplotype frequency were observed between LEx workers and controls for the "baT" haplotype ($X^2 = 4.9$, $p = 0.02$). Importantly, higher B-Pb levels were observed for the "baT" haplotype of the BsmI-ApaI-TaqI polymorphism, and BsmI and ApaI had the highest D′ and r^2 values. However, significant variations in vitamin D, calcium and phosphorus levels were not observed between these haplotypes.

Conclusion: The "baT" haplotype of VDR polymorphisms might be a potential risk factor for lead toxicity, especially in exposed individuals. Hence, this haplotype may be considered during the screening of occupationally exposed individuals, in order to identify those who are susceptible to developing lead toxicity.

Introduction

Lead (Pb) is a widely-prevalent natural constituent of the earth. However, its widespread usage in various industries has made it a major environmental pollutant. In view of its hazardous effects on human health, most developed nations have established strict guidelines for regulating its industrial usage. However, developing countries, such as India, have been lagging in this respect. After the introduction of Pb-free petroleum products, the most common cause of exposure remains occupational, and this most commonly occurs in battery and paint manufacturing plants. Pb is inhaled, ingested, or absorbed through the skin,[1] and this is excreted primarily through the urine and feces, with a small fraction being eliminated via sweat. Urinary excretion mostly represents the blood lead (B-Pb), which is filtered and excreted through the kidneys. However, Pb is also deposited in the bones.[2] In humans, Pb is known to cause a wide range of biological effects, depending on its level and duration of exposure. Both Pb and calcium are divalent cations, and share the same toxico-dynamics and kinetics.[3] In the human body, Pb competes with calcium, and binds to a calcium-binding

Keywords: Vitamin D receptor; Blood lead level; Haplotype; Polymorphism; Occupational exposure.

Abbreviations: B-Pb, blood lead; C, control; HRP, horseradish peroxidase; LD, linkage disequilibrium; LEx, lead-exposed workers; LR, likelihood ratio; PCR, polymerase chain reaction; VDR, vitamin D receptor; Vit D, vitamin D.

***Correspondence to:** Sudip Kumar Datta, Department of Laboratory Medicine, All India Institute of Medical Sciences, New Delhi, India. ORCID: https://orcid.org/0000-0001-5550-663X. Tel: +91-11-26594629, Fax: +91-11-26588641, E-mail: sudip.datta@aiims.edu

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Table 1. Localization of different vitamin D receptor polymorphism variants, base exchange, and pattern of digestion, together with the corresponding digested/undigested base-pair sizes for the different genotypes of each VDR variant

VDR variants	Localization	Base exchange	Genotype	Restriction enzyme digest fragment size (bp)
Bsm I	Intron 8	A > G	GG (mut)	650, 175
			AG (ht)	825, 650,175
			AA (wt)	825
Apa I	Intron 8	G > T	TT (mut)	1,800, 200
			GT (ht)	2,000, 1,800, 200
			GG (wt)	2,000
Taq I	Exon 9	T > C	CC (mut)	1,700, 300
			TC (ht)	2,000, 1,700, 300
			TT (wt)	2,000

bp, base pair; ht, mutated heterozygous; Mut, mutated homozygous; VDR, vitamin D receptor; wt, wild homozygous.

receptor, thereby affecting its metabolism. Furthermore, Pb can modulate the metabolism of calcium and phosphorus through the inhibition of the 1-α-hydroxylase enzyme in the kidneys. Rahman *et al.* reported that Pb impedes vitamin D metabolism by altering the expression of vitamin D metabolizing enzymes.[4] Furthermore, a study conducted on experimental animals revealed that Pb-mediated injury to the kidneys and testes can be mitigated by vitamin D replacement via the immunomodulation and antioxidant effect.[5]

Vitamin D is also an important regulator of calcium absorption and metabolism. Vitamin D2 (ergocalciferol, from plant origin) and vitamin D3 (cholecalciferol, from animal origin) are two natural forms of vitamin D. Vitamin D3 (cholecalciferol) is synthesized in human skin from 7-dehydrocholesterol with the help of ultraviolet light. Cholecalciferol is converted into active $1,25(OH)_2D3$ (calcitriol) in two steps in the liver, followed by the kidneys. Calcitriol, which is the active form of vitamin D, plays an important role in calcium metabolism by increasing the intestinal absorption and renal reabsorption of calcium and phosphate during calcium deficiency.[6,7] The function of calcitriol is mediated through the intracellular vitamin D receptor (VDR).[8] The binding of calcitriol with VDR, which is a transcription factor, regulates the genetic expression of calcium-binding receptors and several downstream events. Randomized controlled trials have reported that vitamin D and magnesium supplementation can improve the neuro-developmental disorder-related behavioural and mental health issues in attention deficit hyperactive children.[9] The close association of vitamin D deficiency with various chronic diseases[10] was evidenced by the significant inverse association between dairy consumption and mild hypertension in university students,[11] as well as the alleviation of cardiovascular, arthritic and diabetic disorders in experimental animals with herbal products, such as Nigella Sativa.[12]

In 1988, Baker *et al.* cloned the human-VDR gene, which is localized at chromosome 12ql2-14.[13–15] The VDR gene has 11 exons that span approximately >100 kb of genomic DNA. The 5′ end of the gene comprises of multiple isoforms of exon 1 (from 1A to 1E), while the structural part of the VDR protein is coded by exon 2 to exon 9 of the VDR gene.[16–18] The VDR protein comprises of 427 amino acids with a molecular weight of 48 kDa, and the following multiple functionally active domains: amino terminus A/B domain, C terminal domain with a DNA binding region that consists of 20–90 amino acids, a hinge region or D domain situated between 90 and 130 amino acids, and a final ligand-binding domain or E domain situated from 130 to 423 amino acids.[19] The last domain is the most complex, which involves ligand binding, followed by hetero-dimerization with retinoid X recepto, and finally, the attachment with transcription factors.[20] The variants of VDR polymorphisms are presented in tabular form in Table 1.

VDR ***variants***

Several single nucleotide polymorphisms (SNPs) have been described in the VDR gene. The most reported VDR variants include the following:

- VDR: rs2228570 (T > C, *FokI*): This variant is generated due to shortening of the encoded VDR protein by three amino acids. The reason for this is the change in the T > C base at the translation start site in exon 2 of the VDR gene, which subsequently removes the start site of translation.
- VDR: rs1544410 (A > G, *BsmI*): This variant involves a mutation in intron 8 of the VDR gene.
- VDR: rs7975232 (G > T, *ApaI*): This variant involves a mutation in intron 8 of the VDR gene.

VDR: rs731236 (T > C, *TaqI*): This variant involves a mutation in exon 9 of the VDR gene.

Interestingly, some of these variants are inherited together due to the significant linkage disequilibrium (LD) associated between these, resulting in the description of several haplotypes amongst the variants. LD measures the degree of association of polymorphisms at different loci, which occurs adjacent to each other.[21] On the other hand, haplotypes are blocks of adjacent allelic polymorphisms, in which the length of the block coincides with the strength of the LD across the area. Haplotype alleles may be present around the polymorphic variation within 10–20 kb. Therefore, a massive effort needs to be made to determine the haplotype map of the human genome.[21] The analysis of haplotypes, which are specific combinations of genetic markers within a chromosome cluster location, has been valued as a more powerful approach, when compared to the analysis of single polymorphisms.[22] Knowledge of the LD and haplotype structure of a certain candidate gene is important for the association analyses, in order to understand how polymorphic variations in a gene can contribute to disease risk. Some of the above-mentioned VDR variants were reported to be inherited together due to the significant LD, resulting in the description of several haplotypes amongst the variants, as reported in the Columbian population.[23] However, to date, most studies have concentrated on the analysis of individual SNPs and investigated its effects.[22,24] Gezen AK *et al.* reported that a genotype

study cannot show the significant association, while a haplotype "TaubF" study can show the significant association between Alzheimer's disease and VDR polymorphism. This indicates that haplotype studies can provide more information, when compared to merely the genotype analysis.[25] Hence, the present study evaluated the VDR polymorphism results by determining the association of haplotypes on circulating Pb levels in occupationally Pb-exposed battery workers and non-exposed healthy subjects. The present study attempted to determine the degree of LD between the sites of these SNPs. Furthermore, the haplotype analysis of three common polymorphisms (*BsmI*, *ApaI* and *TaqI*), which are located at the 3′ (tail) end of the VDR gene, was targeted for the present study. The present study was the first to apply the North Indian population to observe the impact of haplotypes on VDR gene studies.

Materials and methods

Study subjects

The present cross-sectional study was carried out in the Biochemistry Department, SGT Medical College in collaboration with the Department of Lab Medicine, All India Institute Medical Sciences, New Delhi. Ethical clearance was obtained from the Institutional Ethics Committee, SGT University, Haryana, India. For the present study, 100 male occupationally Pb-exposed (LEx) battery workers and 100 age matched non-Pb-exposed male controls (controls) were recruited from the Delhi-NCR region after obtaining the informed consent. These subjects were recruited from battery manufacturing factories and car servicing centres in the Delhi-NCR region. The demographic details, including age, smoking status, alcohol ingestion, duration of employment, nature of duty, and other details, were collected using a pre-validated questionnaire. Furthermore, the dietary intake, food habits and addiction history of all subjects were noted. Subjects who reported any pre-existing medical disorders or were identified to have any obvious chronic medical conditions from the clinical history and examinations were excluded from the study.

Sample analyses

Five millilitres of venous blood sample were collected from the study subjects, and placed in three separate vials. One Ethylenediaminetetraacetic acid vial, which contained 2.0 mL of whole blood collected for B-Pb estimation, was stored at −80 °C until analysis. Furthermore, 1 mL of whole blood was collected in a Ethylenediaminetetraacetic acid vial for DNA extraction and genotyping, and 2 mL of blood was collected in a plain vial for the estimation of biochemical parameters (serum vitamin D, calcium and phosphorous). The serum was separated from the plain vial by centrifugation at 1,500 g for 10 minutes at 4 °C, and stored at −20 °C for further analysis. The serum calcium and phosphorous levels were measured using the Roche Modular P automated chemistry analyzer (Roche diagnostic, Indianapolis, IN).

Estimation of blood lead levels

Whole blood Pb levels were measured using an inductively coupled plasma optical emission spectrometer (ICP-OES; Optima 8000, Perkin Elmer, Waltham, USA). Then, 2.0 mL of whole blood was digested with 2.0 mL of nitric acid and 0.2 mL of hydrogen peroxide using the Microwave Digestive System 3000 (Anton Paar, Graz, Austria), with a specific power, temperature and duration of time. After sample digestion, the final volume was adjusted to 5 mL using Milli-Q water, and analyzed using the ICP-OES.

Estimation of vitamin D

The serum total vitamin D level was measured with the enzyme-linked immunosorbent assay technique using the BioTek spectrophotometer (Winooski, VT, USA) based on the principle of competitive binding. For this process, the vitamin D calibrators, controls and samples were dispensed into pre-designated anti-vitamin D-coated microwells. Then, biotin was dispensed into each well, and vitamin D was allowed to compete with the endogenous vitamin D in the sample, calibrator and control serum for the fixed number of binding sites on the anti-vitamin D antibody. Afterwards, the microwells were washed, and the bounded vitamin D biotin was detected using streptavidin-horseradish peroxidase (HRP). It was observed that the streptavidin-HRP conjugate that bound to the wells decreased as the concentration of vitamin D in the specimen increased. The unbounded streptavidin-HRP conjugate was removed, and the microwells were washed again. Next, tetramethylbenzidine was added and incubated. When blue color developed, the reaction was stopped with the addition of a stop solution. Then, the absorbance was plotted as standard concentration vs. the absorbance. The color intensity was inversely proportional to the amount of vitamin D in the sample.

Genotyping

The genomic DNA was extracted from the whole blood using commercially available DNA extraction kits (Qiagen, Hilden, Germany). The quality and quantity of DNA were determined using the NanoDrop-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific Inc., USA). The VDR gene polymorphisms (TaqI, ApaI and BsmI) were detected by polymerase chain reaction (PCR), followed by restriction fragment length polymorphism (RFLP). The primers were custom-designed, synthesized and procured by Eurofins MWG Operon (Germany).

BsmI polymorphism

The *BsmI* polymorphism restriction site in intron 8 of the VDR gene was amplified by PCR using the following primers: 5′-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA-3′ (FP) and 5′-AAC CAG CGG GAA GAG GTC AAG GG-3′ (RP). The PCR was performed in 30.0 µL of reaction mixture, which contained 15.0 µL of master mix (dNTPs, $Mgcl_2$, Taq polymerase buffer), 0.5 µL of 10.0 µM forward primer, 0.5 µL of 10.0 µM reverse primer, 9 µL of nuclease-free water and 5 µL of genomic DNA. The PCR conditions were, as follows: initial denaturation at 94 °C for four minutes, followed by 35 cycles of denaturation at 94 °C for 45 seconds, annealing at 58 °C for one minute, extension at 72 °C for one minute, and a final extension at 72 °C for five minutes. The PCR product of 825 bp was digested using the *BsmI* restriction enzyme (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA). Then, two fragments of 650 bp and 175 bp were generated after digestion in the presence of the *BsmI* site (Table 1).

Apa I and *TaqI* polymorphisms

The *Apa I* and *Taq I* polymorphism restriction site in intron 8/exon 9 of the VDR gene was amplified by PCR using the following primers: 5′-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA-3′ and 5′-CAC TTC GAG CAC AAG GGG CGT TAG C-3′. PCR was performed in 30.0 µL of reaction mixture, which contained 15.0 µL of master mix (dNTPs, $MgCl_2$, Taq polymerase enzyme, Taq polymerase buffer), 0.5 µL of 10.0 µM forward primer, 0.5 µL of 10.0 µM reverse primer, 9.0 µL of nuclease-free water and 5.0 µL of genomic DNA. The PCR cycle conditions were, as follows: initial denaturation at 95 °C for five minutes, followed

Table 2. General characteristics of the study subjects.

Characteristics	C		LEx	
	n (%)	Mean ± SD	*n* (%)	Mean ± SD
Age (years)	100	34.7 ± 7.9	100	32.6 ± 10.3
20–30 years old	24 (24%)	24.9 ± 2.4	47 (47%)	23.7 ± 3.1
30–40 years old	46 (46%)	33.6 ± 3.1	27 (27%)	34.2 ± 2.9
40–60 years old	30 (30%)	44.1 ± 4.3	26 (26%)	46.8 ± 5.4
Duration of lead exposure (years)	–	–	100	14.8 ± 9.5
>20 years	–	–	33 (33%)	27.4 ± 6.8
>10–20 years	–	–	39 (39%)	13.6 ± 2.4
<10 years	–	–	28 (28%)	5.7 ± 2.5

Lex, lead-exposed workers.

by 35 cycles of denaturation at 94 °C for 45 seconds, annealing at 63 °C for one minute, extension at 72 °C for one minute and 15 seconds, and a final extension at 72 °C for 10 minutes. Then, the PCR product of 2,000 bp was digested with the *TaqI* and *ApaI* restriction enzymes (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA), separately. Afterwards, the fragments of 1,800 bp/200 bp and 1,700 bp/300 bp were respectively generated after digestion when the restriction sites for *TaqI* and *ApaI* were present (Table 1).

All digested products were visualised using 2% agarose gel electrophoresis. The gels were visualized by ethidium bromide ultraviolet illumination, and the image was captured using the gel documentation system (Protein Simple, San Jose, CA, USA).

Statistical analysis

All data were analyzed using Graph-pad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA) and SPSS 23.0 (IBM, Armonk, NY, USA). The haplotype analysis was executed using the Forest Genetics and Forest Tree Breeding software (Georg-August-University Goettingen, version 1.05, available online at www.uni-goettingen.de/en/134935.html), in order to estimate the haplotype frequencies for the study subjects. Log-linear analysis with likelihood ratio (LR) was carried out to determine the LD between alleles using SPSS v.23. The disequilibrium coefficient was calculated based on the analysis accomplished by Lewontin.[26] The association between loci was determined by chi-square test.

Results

The present study evaluated the relationship between VDR haplotypes and B-Pb in 100 occupationally LEx workers and 100 controls. The general characteristics are presented in Table 2. The results for the routine biochemistry parameters are presented in Table 3. The distribution of genotypes and allelic frequencies were based on report of the previous study conducted by the investigators.[27] In that study, it was observed that the *BsmI* "Bb" (0.43%), *ApaI* "Aa" (0.35%), and *TaqI* "TT" genotypes (0.48%) had a higher representation in LEx workers, when compared to the other genotypes, but there was no significant association with the *TaqI* and *ApaI* polymorphisms when LEx workers were compared to controls. Furthermore, there was a statistically significant increase ($p = 0.03$) in *BsmI* "bb" genotype frequency in LEx workers, when compared to controls.

Haplotype frequency

The possible haplotypes for the three VDR polymorphisms (*BsmI*,

Table 3. Biochemistry parameters

Characteristic	NLEC	LEBW	*p*-value
AST(IU/l)	27.3 ± 9.8	34.5 ± 19.6	0.0012*
ALT (IU/l)	30.4 ± 19.1	40.3 ± 30.5	0.0066*
Total protein (gm/dl)	7.3 ± 0.8	7.4 ± 0.4	0.2156
Albumin (gm/dl)	3.9 ± 0.6	4.5 ± 0.5	<0.0001*
ALP(IU/l)	152.5 ± 117.3	179.4 ± 83.6	0.0631
Serum urea (mg/dl)	24.9 ± 7.6	27.4 ± 8.9	0.030*
Serum creatinine (mg/dl)	0.8 ± 0.2	0.9 ± 0.2	0.0029*
Blood lead level (µg/dl)	10.5 ± 12.2	39.5 ± 31.9	<0.0001
Serum vitamin D (ng/ml)	53.5 ± 11.7	18.9 ± 8.9	<0.0001
Serum calcium (mg/dl)	9.1 ± 0.8	8.8 ± 0.5	0.0005
Serum phosphorus (mg/dl)	4.2 ± 1.3	3.8 ± 0.9	0.008

Notes: *$p \leq 0.05$ was considered statistically significant. ALP, Alkaline phosphatases; ALT, Alanine transaminases; AST, Aspartate transaminases; LEBW, Lead exposed battery workers; NLEC, Non lead exposed controls.

Table 4. Estimated haplotype frequency of the vitamin D receptor gene polymorphism in study subjects (total study subjects = 200, LEx workers = 100, controls = 100) using the forest genetics and forest tree breeding software, and the comparison of variables by chi-square test and its significance ($p < 0.05$)

Code	VDR haplotype	LEx	C	Chi^2	*p*-value
Haplotype 1	BAT	1	3	1.02	0.312
Haplotype 2	BAt	18	19	0.03	0.85
Haplotype 3	BaT	00	10	1.01	0.316
Haplotype 4	Bat	2	3	0.21	0.650
Haplotype 5	bAT	4	7	0.87	0.352
Haplotype 6	bAt	9	8	0.06	0.79
Haplotype 7	baT	43	28	4.91	0.026*
Haplotype 8	bat	23	22	0.03	0.86

Notes: *$p < 0.05$. C, controls; LEx, lead-exposed workers; VDR, vitamin D receptor.

ApaI and *TaqI*) are presented in Table 2. Eight possible haplotypes were observed from these three SNPs: H1 (BAT), H2 (BAt), H3 (BaT), H4 (Bat), H5 (bAT), H6 (bAt), H7 (baT), and H8 (bat). The frequency of these haplotypes was as follows: 1%, 18%, 0%, 2%, 4%, 9%, 43% and 23% for LEx workers, respectively; 3%, 19%, 10%, 3%, 7%, 8%, 28% and 22% for controls, respectively. A significant difference in haplotype frequencies was observed between LEx workers and controls for the "baT" haplotype ($X^2 = 4.9$, $p = 0.02$; Table 3). Interestingly, no subject with the BaT haplotype was identified among the LEx workers.

Estimation of blood lead level among the haplotypes

In order to determine any difference in B-Pb between these two groups of participants, in terms of haplotypes, the B-Pb levels in various VDR haplotypes were compared between LEx workers and controls. The B-Pb levels for each haplotype group is presented in Table 4. Haplotype "BAt", "bAT", "bAt", "baT" and "bat" were identified to have significantly higher B-Pb levels in LEx workers, when compared to controls ($p = 0.0003$, 0.04, 0.01, <0.0001 and <0.0001 respectively). The H7 haplotype "baT" was associated with the highest B-Pb level (median and 95% CI = 35.1 [36.8–61.3]) among the eight haplotypes ($p < 0.0001$).

Estimation of vitamin D, serum calcium and serum phosphorus levels among the different haplotypes

The serum calcium, phosphorus and vitamin D levels of the subjects were measured, and the results were analyzed in relation to the different haplotypes, in order to determine the association of these haplotypes with the serum levels for the biochemical parameters (Fig. 1). Among the eight haplotypes, "baT" was significantly higher ($p < 0.0001$), when BLL was compared to BAT and BaT (Fig. 1a). In Figure 1b, the "baT" VDR haplotype had a significantly lower ($p < 0.0001$) serum vitamin D level, when compared to the BaT haplotype. Furthermore, the serum calcium level was significantly low ($p < 0.03$) in the "baT" haplotype, when compared to BaT (Fig. 1c). However, a significant difference was not observed for serum phosphorus among the VDR haplotypes.

Analysis of linkage disequilibrium

The log-linear regression analysis revealed that there was an obvious LD among the three pairs of VDR polymorphisms. The highest degree of LR was observed between the *Bsm1* and *Apa1* polymorphisms (LR = 25.017, $p \leq 0.001$, z = 3.347), followed by *Bsm1* and *TaqI* (LR = 7.278, $p = 0.007$, z = 2.129), and *Apa1* and *TaqI* (LR = 4.787, $p = 0.029$, z = 1.209). These findings corroborate with the D and r coefficients between the above-mentioned pairs of polymorphisms. Based on the LD analysis, for LEx workers, it was observed that *BsmI*, together with *ApaI*, had the highest standardized disequilibrium (D′) and square of the correlation coefficient as a measure of the LD (r^2) value (D′ = 0.859944, $r^2 = 0.417$), followed by *BsmI* together with *TaqI* (D′ = 0.54732, $r^2 = 0.1991$), and *ApaI* together with *TaqI* (D′ = 0.4761, $r^2 = 0.197613$) (Table 5).

Interestingly, it was observed that haplotype "ba" comprised of two mutant alleles of *BsmI* and *ApaI* (which had the highest D′ value). These presented with highest B-Pb level (median and 95% CI = 29.8 [34.9–52.3]), when compared to the other combinations, such as "bt" (median and 95% CI = 25.7 [24.9–42.9]) and "at" (median and 95% CI = 26.1 [23.6–41.8]), which were derived from the mutant alleles of *BsmI* and *TaqI*, and *ApaI* and *TaqI*, respectively (Table 6). Furthermore, it was observed that the 12 haplotypes comprised of two loci of three SNPs with the highest B-Pb levels (aT = 35.1 [36.8–61.30], followed by bT = 32.2 [35.6–58.3]) in combination of the wild allele (T) of *TaqI* and the mutant alleles (b and a) of *BsmI* and *ApaI* (Table 7). Surprisingly, this was also substantiated by the haplotype analysis, which comprised of three loci of the concerned three SNPs. Among the eight haplotypes (Table 5), the highest B-Pb level was detected in "baT" (median and 95% CI = 35.1 [36.8–61.3]). In order to determine which of the eight haplotypes derived from the three loci of three SNPs was the most susceptible to develop Pb toxicity, the odds ratio (OR) was calculated (Table 8). In the table, the "baT" haplotype had the highest OR (OR = 105.0), when compared to the other haplotypes, with the B-Pb level of >10 µg/dL as the cut-off value for poisoning.[28]

Discussion

Occupational exposure to hazardous materials is presently one of the major challenges to mankind. At present, the study of the underlying genetic predisposition for various observed health effects due to hazardous substances has increasingly become popular. The present study evaluates the association of the haplotypes of VDR gene polymorphisms with B-Pb in the context of occupational exposure. The previous study conducted by the investigators reported the allele frequency and genotype frequency of the concerned SNPs, and the individual associations with the levels of B-Pb, serum vitamin D, serum calcium and serum phosphorus in

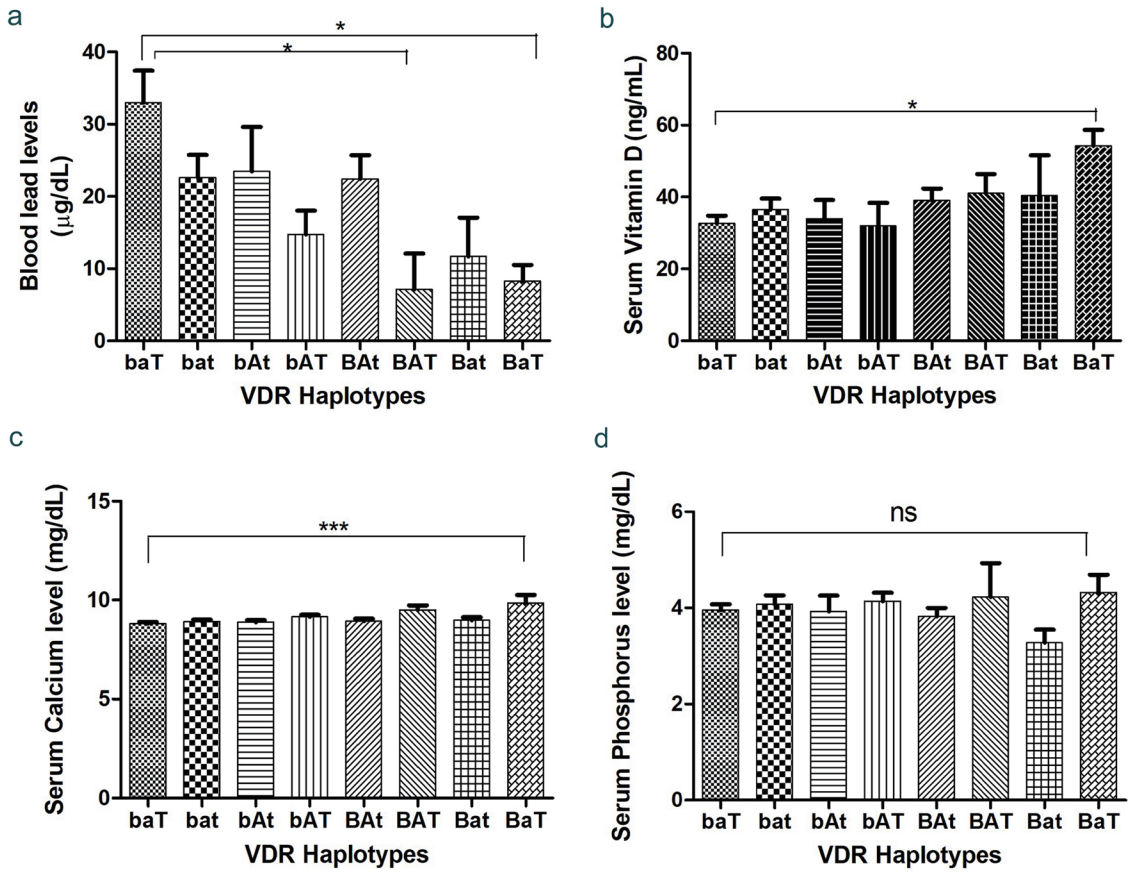

Fig. 1. The histogram demonstrating the comparison of (a) blood lead levels, (b) serum vitamin D levels, (c) serum calcium levels, and (d) serum phosphorous levels among vitamin D receptor (VDR) haplotypes: $^{*}p < 0.05$, $^{***}p < 0.001$; NS, non-significant. Number of study subjects for the different haplotypes: baT = 71, bat = 45, bAt = 17, bAT = 11, BAt = 37, BAT = 4, Bat = 5 and BaT = 10.

LEx workers, when compared controls.[27] The results of this previous study revealed that the B-Pb level was significantly higher in LEx workers, when compared controls, which was expected. However, the serum vitamin D, calcium and phosphorus levels decreased in LEx workers, when compared to controls.[29] This could be due to the 1-α-hydroxylase, which is a crucial enzyme required for the synthesis of calcitriol, which in turn, is inhibited by Pb in renal tubules. Furthermore, after this hormone binds to the VDR, this activates the synthesis of multiple proteins, including calcium-binding proteins (calbindin-D) in the intestine, which are necessary for calcium absorption across the small intestine. In 2012, Pawlas explained that VDR polymorphisms can significantly modulate Pb-induced cognitive behaviours in children.[30] Another study conducted by Weaver *et al.* revealed that VDR genotypes are significantly associated with B-Pb levels in Korean Pb workers.[31]

In the present study, the haplotype frequency of three VDR

Table 5. Comparison of blood lead levels measured by ICP-OES for vitamin D receptor haplotypes (*BsmI ApaI-TaqI*) based on three loci between lead-exposed workers and controls, where total LEx workers = 100, controls = 100, with a significant value and the highest B-Pb

VDR haplotype	LEx, B-Pb (*n*), Median (95% CI)	C, B-Pb (*n*), Median (95% CI)	*p*-value
BAT	20.9 (1)	Below detection limit (3)	NC
Bat	28.4 (18), (21.6–43.7)	12.3 (19), (7.7–17.7)	0.0003
BaT	(0)	9.5 (10), (2.2–13.4)	NC
Bat	23.8 (2), (−50.4–98.2)	5.3 (3), (−4.1–11.2)	NC
bAT	22.3 (4), (14.8–33.9)	5.8 (7), (0.64–17.8)	0.04
bAt	25.4 (9) (11.3–58.9)	11.8 (8) (7.2–13.4)	0.01
baT	35.1 (43), (36.8–61.3)	8.3 (28), (4.3–12.3)	<0.0001
Bat	26.1 (23), (23.6–43.4)	8.5 (22), (6.8–15.6)	<0.0001

B-Pb, blood lead; C, controls; *n*, number; NC, not calculable; LEx, lead-exposed workers; VDR, vitamin D receptor.

Table 6. Estimation of linkage disequilibrium and likelihood ratios for vitamin D receptor SNPs in lead-exposed subjects using SPSS v.23 (total lead-exposed workers = 100)

Total (100, lead exposed)	LR	*P*	Z value	D′	R^2	Chi^2	*p*
Bsm I* Apa I	25.017	<0.001	3.347	0.859944	0.417726	41.7726	<0.001
Apa I* taq I	4.787	0.029	1.209	0.4761	0.197613	19.76132	<0.001
Bsm I* taq I	7.278	0.007	2.129	0.54732	0.199104	19.91043	<0.001

D′, standardized disequilibrium measure; LD, linkage disequilibrium; LR, likelihood ratio; r^2, square of the correlation coefficient as a measure of LD; z, strength of association/effect.

Table 7. Blood lead levels for vitamin D receptor haplotypes (*BsmI-ApaI-TaqI*) based on two loci between the two study subjects (total study subjects = 200, lead-exposed workers = 100, controls = 100; highest B-Pb)

	Lead exposed workers		Controls	
	n	B-Pb median (95% CI)	*n*	B-Pb median (95% CI)
Bsm1 and Apa1				
BA	19	27.9 (21.6–42.5)	22	9.6 (6.7–15.9)
Ba	2	23.8 (−50.5–98.2)	13	5.4 (3.2–11.2)
bA	13	24.3 (16.1–47.5)	15	9.9 (6.1–13.5)
Ba	66	29.8 (34.9–52.3)	50	8.5 (6.7–12.4)
Apa1 and Taq1				
AT	5	21.0 (16.9–30.4)	10	5.6 (1.2–13.3)
At	27	27.9 (23.7–43.3)	27	12.3 (8.5–15.6)
aT	43	35.1 (36.8–61.3)	38	9 (5.2–11.4)
At	25	26.1 (23.6–41.8)	25	7.2 (6.3–14.3)
Bsm1 and Taq1				
BT	1	20.9	13	7.7 (2.8–11.1)
Bt	20	28.4 (21.8–41.7)	22	9.5 (6.9–16.0)
bT	47	32.2 (35.6–58.3)	35	7.7 (5.1–11.9)
Bt	32	25.7 (24.9–42.9)	30	9.0 (7.7–14.2)

B-Pb, blood lead.

polymorphisms (*BsmI*, *ApaI* and *TaqI*), the degree of LD, and the effect on the susceptibility to develop Pb toxicity was estimated. It was observed that the haplotype (based on two SNPs) "ba", which comprised of mutated alleles of *BsmI* and *ApaI*, had the highest B-Pb, when compared to "aa" and "tt". This was corroborated by the findings of the LD analysis, in which the maximum D′ and least "r^2", and the highest LR were observed for *BsmI* and *ApaI* in Pb poisoning cases. This indicates that the coinheritance of these two

Table 8. Calculation of the odds ratio of vitamin D receptor haplotypes based on three loci (BsmI-ApaI-TaqI) using Graph-pad prism 6.0. (total study subjects = 200, lead-exposed workers = 100, controls = 100)

Group	baT		bat		bAt		bAT		Bat	
	+	−	+	−	+	−	+	−	+	−
Lex	42	1	21	2	7	2	3	1	17	1
Control	8	20	9	13	5	3	2	5	11	8
Total	50	21	30	15	12	5	5	6	28	9
X^2	38.8, 1		12.85, 1		0.47, 1		2.2, 1		6.7, 1	
p-value	<0.0001		0.0003		0.49		0.13		0.009	
Odds ratio	105.0		15.2		2.1		7.5		12.3	
95% CI	12.3–898.3		2.8–81.5		0.25–17.6		0.45–122.8		1.35–113.1	

Notes: (+): respective haplotype with blood lead level of >10 µg/dL, (−): respective haplotype with blood lead level of <10 µg/dL. LEx, lead-exposed workers.

SNPs would make an individual more vulnerable to develop Pb poisoning. Furthermore, it was observed that when the mutant alleles of *BsmI* and *ApaI* pairs with the wild allele of the *TaqI* polymorphism (aT and bT), these would have higher Pb levels, when compared to the "ba" haplotype. This was substantiated by the findings, in which the haplotypes of three SNPs were considered at the same time. Furthermore, it was observed that the most common haplotype for the VDR gene polymorphism, "baT", was associated with the highest B-Pb level and the lowest calcium level, when compared to the other haplotypes. These findings were validated through the calculation of ORs, in which the "baT" haplotype was identified to have the highest OR for the development of Pb toxicity in LEx workers. This implies that "baT" may play a crucial role in Pb toxicity in occupationally exposed individuals. Hence, this may be contemplated as a biomarker for identifying populations at risk of developing Pb toxicity, especially in the setting of chronic exposure.

The cellular actions of vitamin D depend on the VDR that regulates the production of calcium-binding proteins. Some studies have reported a strong LD between *BsmI-ApaI-TaqI* at the 3′ end. Furthermore, a strong LD was also observed between *BsmI* and the polyadenosine A (poly A) variable number of the tandem repeat at the 3′ untranslated region (UTR), which may affect the VDR-mRNA stability.[15] In addition, studies have reported that VDR gene polymorphism modulates the Pb absorption, that is, the toxicity.[30] Multiple variants of the VDR gene are known for affecting its expression. However, merely the genotype and allele frequency analysis would not be able to explain all molecular effects of the VDR gene polymorphism. This presents the importance of performing the haplotype analysis. To the best our knowledge, the present study is the first study conducted in India that reported the impact of VDR haplotypes on B-Pb. Hence, it was not possible to compare the present findings with other previous findings conducted in the Indian setting. However, some studies have been conducted, to date, which established an association between VDR haplotypes and B-Pb levels outside India. Rezende conducted a study in Brazil, and observed that the "fab" haplotype of VDR has lower Pb-P, Pb-B and %Pb-P/Pb-B in exposed subjects. This is, in part, compliant with the present findings, in which the coexistence of mutated alleles of *BsmI* and *ApaI* makes a person susceptible to Pb poisoning.[32] Subsequently, in 2010, Rezende *et al.* reported that the "fab" haplotypes of the VDR gene (*FokI*, *ApaI* and *BsmI*) are associated with low B-Pb levels in pregnant women.[33] In 2010, Chen *et al.* carried out a study on Han children, and concluded that the "Atb" and "AtB" haplotypes have a decreased association with Pb poisoning, while haplotypes "aTb" and "ATb" of the VDR gene significantly increased in the Pb poisoning group, which to some extent is in agreement with the results of the present study.[34] However, the differences in age and ethnicity need to be considered before a firm conclusion can be accepted.

Future directions

Occupational hazard has long been considered as one of the risk factors for disease occurrence. Thus, prolonged exposure of Pb can cause various metabolic derangements, including vitamin D metabolism, which induces various skeletal and extra-skeletal functions in the body. Furthermore, previous studies have reported the association between various VDR SNPs and B-Pb levels. The present study revealed that the "baT" haplotype of VDR polymorphisms might be a potential risk factor for Pb toxicity, especially in Pb-exposed individuals. It was further suggested that this haplotype may be considered for the screening of occupationally exposed individuals to identify those who are susceptible to develop Pb toxicity. However, the present study warrants the further functional validation of these haplotypes, which should be conducted at the molecular level, in order to identify the actual mechanism of action.

Conclusions

In summary, it was identified that the VDR gene haplotype, which involves the *BsmI*, *ApaI* and *TaqI* polymorphisms, plays an important role in the modulation of B-Pb levels in the setting of chronic occupational exposure. Furthermore, it was found that the "baT" haplotype of *BsmI-ApaI-TaqI* is significantly associated with high B-Pb levels, when compared to the other seven haplotypes. Therefore, it can be inferred that the "baT" haplotype might be a potential risk factor for Pb toxicity in exposed individuals. Hence, this may be considered in the screening of occupationally exposed individuals, in order to identify those who are susceptible to develop Pb toxicity.

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Conflict of interest

The authors declare that they have no competing interests, and that the manuscript was approved by all authors for publication.

Author contributions

SKD conceived the idea for the manuscript. HR participated in the data acquisition, drafting and preparation of the manuscript, including the reference selection, interpretation of the findings from the cited sources, critical revision of the content, and the preparation of the figures for the manuscript. RK took part in the drafting and preparation of the manuscript, including the reference selection, interpretation of the findings from the cited sources, and the critical revision of the content. SS participated in the preparation of the manuscript, including the interpretation of the findings from the cited sources, and the critical revision of the content. All authors made a significant contribution to the study, and approved the final manuscript.

Data sharing statement

All data generated and analyzed in the study are included in the manuscript. No additional data are available.

Ethics statement

The study was approved by the Institutional Ethical Committee (IEC), SGT University, Haryana, India.

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