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Association of vitamin D receptor mRNA expression, vitamin D deficiency and genetic variant in patients with multi-drug resistant pulmonary tuberculosis

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Abstract

Background Multi-drug resistant pulmonary tuberculosis (MDR-TB), is a serious threat to world health. Serum levels of vitamin D, a ligand for the VDR that controls VDR mRNA expression, are still poorly understood in MDR-TB.

Objective To study the association of mRNA expression with low vitamin D levels and VDR polymorphisms in patients with MDR-TB compared to normal controls.

Methods Study groups consisted of sputum smears and culture-positive MDR-TB at two hospitals in New Delhi, and normal controls were enrolled from a North Indian population. A total 100 (50 MDR-TB subjects and 50 controls) were consecutively enrolled. VDR mRNA expression in peripheral blood mononuclear cells (PBMC) was analysed by Real-time PCR. Serum 25-hydroxyvitamin D, intact parathyroid hormone (iPTH), and calcium (ionized and total) levels were measured, and the correlation between variables was determined. The association between VDR genotype and VDR mRNA expression was studied between MDR-TB and normal controls together with the genotypic and allelic frequencies of the *FokI*, *BsmI*, and *TaqI* VDR polymorphisms were also assessed in between the two groups.

Results To investigate the role of VDR gene expression and FokI polymorphism in MDR-TB, a total of 100 patients were split into two groups. VDR mRNA expression significantly decreased in MDR-TB patients, being 0.6 times lower than in healthy controls. Notably, the ff genotype was associated with reduced VDR expression, indicating a functional impact on gene regulation. However, there was no appreciable variation in the groups' distribution of FokI alleles and genotypes. These findings highlight the importance of merging genetic and expression data, showing that while the ff variation influences individual expression, it does not distinguish MDR-TB patients from controls.

Conclusion In present study, the VDR gene's FokI polymorphism affects the levels of VDR mRNA expression, with the ff genotype linked to lower expression in both MDR-TB patients and healthy individuals. Nonetheless, there were no appreciable differences in the genotypic and allelic frequencies of FokI between the groups. These findings suggest that the location of the FokI variation in the population may not be as important to MDR-TB susceptibility as its functional impact on gene expression.

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Keywords Vitamin d receptor, Vitamin d mRNA expression, Intact parathyroid hormone, Multi-drug-resistant pulmonary tuberculosis, Calcium levels

Introduction

Tuberculosis (TB) is an infectious disease and a major health concern worldwide, with an estimated one-third of the world's population being infected with *Mycobacterium tuberculosis* including approximately 2 million people in India, with a prevalence of approximately 14 million [1]. It is one of the main causes of global morbidity and mortality, resulting in approximately 1.7 million deaths annually, approximately 0.4 million of which are people being co-infected with human immunodeficiency virus (HIV) [1, 2].

Pulmonary tuberculosis (PTB), the most common clinical form of the disease, is a granulomatous disease of the lungs caused by *Mycobacterium tuberculosis* [3]. However, only 5–10% of infected individuals develop the disease [4]. Furthermore, the management of tuberculosis has already faced significant risks due to the rise of multidrug resistant (MDR) strains [5]. The issue has been made more complex by the recent appearance of exceptionally drug-resistant (XDR) strains. With almost 410 000 new cases reported globally in 2022 [1], multi-drug resistant (MDR) TB, in which resistance to at least the two most effective first-line treatments (rifampicin and isoniazid) [6, 7], is becoming a more serious issue. About 100,000 new cases are reported each year, with China and India accounting for about half of these instances [1].

Vitamin D3 (1,25(OH)2D) is an immunoregulatory hormone that stimulates cell-mediated immunity by activating monocytes/macrophages [8, 9]. Parathyroid hormone (PTH) is the main hormone that regulates 1,25(OH)2D production via a negative feedback mechanism. Serum calcium homeostasis is maintained by the actions of PTH (which includes increased calcium reabsorption in the kidneys and bone) and (1,25(OH)2D) (increased calcium reabsorption in the kidneys and gut) [10]. Vitamin D interacts with the vitamin D receptor (VDR) to mediate its effects. VDR is a nuclear receptor that controls the expression of several genes related to immune response, calcium homeostasis, and other biological processes when it binds with the active form of vitamin D (1,25-dihydroxyvitamin D, or calcitriol) [11]. It has been suggested that mutant alleles of the VDR gene region may be associated with increased or decreased VDR mRNA expression [12], which in turn affects the protein function. The autocrine synthesis of 1,25(OH)2D is thought to have a significant role in controlling cell development and maturation, which lowers the likelihood that the cell may turn malignant. Additionally, 1-OHase in macrophages metabolizes 25(OH)D to 1,25(OH)2D. When LPS stimulates TLR2/1, the expression of the VDR and 1-OHase is increased [13]. As a result, the expression of the VDR and 1-OHase increases. The macrophage's nuclear expression of cathelicidin (CD), a cationic peptide that destroys infectious organisms like Mycobacterium TB, rises in response to an increase in 1,25(OH)2D synthesis [12]. Therefore, the vitamin D receptor gene is a good candidate for this study. The link between vitamin D and TB is an emerging field of research. Several studies have demonstrated vitamin D deficiency in patients with active TB by several studies [14–16]. A number of studies have reported single nucleotide polymorphisms (SNP) in the vitamin D receptor (VDR) gene, which are thought to confer genetic differences in vitamin D physiology, susceptibility to active TB development [10, 11, 17–20], and TB treatment response [21, 22].

To our knowledge, however, no studies have been published on the mRNA expression of VDR in patients with MDR-TB and variations in baseline vitamin D, calcium (ionized and total), and iPTH levels in MDR-TB patients and normal controls [23, 24].

This study aimed to assess the correlation between VDR mRNA expression and vitamin D levels, and its association with VDR genotypes in MDR-TB.

Subjects and methods

This prospective cross-sectional study was conducted at the All-India Institute of Medical Sciences (AIIMS) hospital (a tertiary centre) in New Delhi, India. The study recruited 26–29-year-old patients with MDR-TB and a control group of people from North India. The study was approved by the Institutional Ethical committee of All India Institute of Medical Sciences (IEC-AIIMS) Reference Number: A-08/5.5, New Delhi, India, and written informed consent was obtained from all subjects, including patients and normal controls.

Multi-drug-resistant pulmonary tuberculosis patients

In the study, there were 50 patients and 50 controls. The diagnosis of MDR-TB was confirmed by a positive sputum smear (Ziehl-Neelsen method), culture (Lowenstein-Jensen medium) and conventional drug susceptibility testing [25–27]. Inclusion criteria of the study includes Patients of either gender aged between 18 and 65 yrs. newly or old diagnosed MDR-TB patients (in vitro demonstration of resistance of Mycobacterium tuberculosis to rifampicin and isoniazid). Culture and sensitivity was done at the TB laboratory of New Delhi Tuberculosis Centre (NDTB) and Patients willing to give their written informed consent. Exclusion criteria were as follows: patients with category II or category III treatment

according to the Indian Revised National Tuberculosis Control Programme (RNTCP) guidelines [28]; presence of secondary immunodeficiency [29], for example, corticosteroid or other immunosuppressant drug use, diabetes mellitus, malignancy, co-infection with HIV, hepatitis B or hepatitis C virus, extrapulmonary TB in the absence of pulmonary involvement, concurrent cytotoxic chemotherapy, pregnancy or lactation, current or recent (< 1 year) use of vitamin D and/or calcium supplements; and patients with known seizure disorder [30].

Normal controls

In this study, healthy volunteers (referred to as normal controls, n = 50) were randomly recruited from the general population with similar socioeconomic status and ethnic background. Subjects who had normal chest radiographs, serum biochemistry, liver function, complete blood count, and a body mass index (BMI) of 19 or over were included. Subjects with chronic illness, history of alcohol or drug abuse, family or personal history of tuberculosis, symptoms suggestive of malabsorption, and pregnant or breastfeeding mothers were excluded [31]. Only individuals who had negative tuberculin skin test (TST) results were enrolled to exclude the possibility of latent tuberculosis infection. Subjects who had taken any vitamin D or calcium supplements within one year before recruitment were excluded from the control group. All individuals provided informed consent and were screened as negative for HIV infection.

Estimation of vitamin D, iPTH and calcium levels

Blood samples were drawn without venostasis from the study subjects after overnight fasting. The serum was separated in a refrigerated centrifuge at 2500 × g for 5 min at 4 °C and stored at −80 °C in multiple aliquots until analysis. Serum 25-hydroxyvitamin D concentrations were estimated using the Diasorin® 25-hydroxyvitamin D Radio Immuno Assay (RIA) (normal range:9–37.6 ng/mL), which involves a two-step procedure. The first step involves the extraction of 25-hydroxyvitamin D from serum with acetonitrile, followed by processing according to the manufacturer's instructions [32]. Serum iPTH levels were measured using radioimmunoassay (RIA; Diasorin®, Stillwater, MN; normal range:13–54 pg/mL; intra-assay and inter-assay CVs:4% and 8%, respectively).

Serum ionized calcium and serum total calcium levels were measured using commercial kits (Roche, Mannheim, Germany) on a semi-automated analyzer (Hitachi Photometer 4020, Boehringer, Mannheim, Germany).

VDR mRNA expression

SYBR Green qPCR were used to measure the expression of VDR mRNA, and amplification specificity was ensured by melt-curve analysis and fluorescence detection. Standard curves were generated by serial dilution of synthetic VDR and GAPDH cDNA templates with known copy numbers. VDR mRNA copy numbers were normalized to 10^6 copies of GAPDH mRNA. All reactions were run in triplicate, and only those with amplification efficiencies between 90 and 110% and R^2 >0.99 were included. No-template and negative reverse-transcription controls were included in each run to rule out contamination.

The vitamin D Receptor (VDR) mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was assessed by real-time PCR using gene-specific primers (VDR: sense, 5-gacatcggcatgatgaagg-3 'and antisense,5' ctagggtcacagaagggtcatc-3' and GAPDH: sense, 5'-ccaaggtcatccatgacaactttggt-3' and antisense, 5'-tgttgaagtcagaggagaccacctg-3'). Total RNA was extracted from peripheral blood mononuclear cells (PBMC) (separated by Ficoll) using RNA-binding columns (Eppendorf-AG, Germany), and 2 µg was reverse transcribed using ImProm-II Reverse Transcription System (Promega, USA) in a 20-µl reaction (25 °C for 5 min and extension at 42 °C for 1 h). The VDR mRNA copy numbers were measured in relation to GAPDH mRNA by amplifying VDR and GAPDH cDNA in separate tubes using iQ™ SYBR® Green Supermix (Bio-Rad, USA), and fluorescence signals were captured on an RT-PCR machine (iQ™ 5 Cycler, Biorad, USA). Reactions were performed in duplicate, and the RT-PCR conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 10 min. The specificity of the amplified products was checked using post-PCR melting curve analysis and agarose gel electrophoresis (403 bp for VDR and 381 bp for GAPDH).

Genotyping for the VDR gene Polymorphisms (BsmI, FokI, and TaqI)

Genomic DNA was extracted from peripheral blood leukocytes using the DNA Blood Maxi Kit (Qiagen, Hilden, Germany). VDR genotypes at *the Bsm*I, *Taq*I, and *Fok*I SNP sites were assessed using PCR-RFLP analysis with a thermocycler (Eppendorf AG 22331, Hamburg, Germany) for PCR amplification. The polyacrylamide gel electrophoresis (PAGE)-purified primers (Bio Basic Inc., East Markham, Canada), DNA *Taq* polymerase, deoxynucleoside triphosphates, and various restriction endonucleases (Fermantas Inc., Hanover, MD) used in the study were commercially procured. The primer sequences, PCR cycling conditions, and PCR products were digested and processed as described by Vupputuri et al. [32]. The presence of a restriction site was denoted by lower case letters (b, f, and t, for *Bsm*I, *Fok*I and *Taq*I) and absence by upper case letters (B, F, and T, for *Bsm*I, *Fok*I and *Taq*I). Four frequently explored single nucleotide polymorphisms (SNPs) are found in the VDR gene:

Table 1 Baseline clinical characteristics of patients with MDR-TB and normal controls;

	MDR-TB patients (*n* = 50) *n* (%)	Normal controls (*n* = 50)
Age (yrs)	27.5 ± 8.6	29.6 ± 8.5
Sex M: F	33:17	40:10
BMI (kg/m^2)	16.3 ± 2.4	23.0 ± 2.1
Bacillary load	10 (20)	NA
3+	20 (40)	
2+	20 (40)	
1+		
Culture status	50 (100)	NA
Positive	0 (00)	
Negative		
Radiographic severity	1 (2)	NA
Unilateral	49 (98)	NA
Bilateral	01 (2)	NA
Minimal	09 (18)	
Moderately advanced	40 (80)	
Far advanced	01 (2)	
Number of Cavities	07 (14)	
No cavity	08 (16)	
1	06 (12)	
2	28 (56)	
3		
Multiple cavities		

BMI indicates body mass index (kg/m^2); age & BMI values are in mean ± SD

M male *F* female, *NA* not applicable, *MDR-TB* multidrug resistant pulmonary tuberculosis

Table 2 Baseline biochemical parameters of patients with MDR-TB and normal controls

Biochemical parameters	MDR-TB (*n* = 50) (mean ± SD)	Normal controls (*n* = 50) (mean ± SD)	*p*-Value
Serum total protein (g/dL)	6.3 ± 0.9	8.4 ± 0.6	<0.001
Serum albumin (g/dL)	3.4 ± 0.5	3.7 ± 0.6	0.002
Serum lonized calcium (mg/dL)	3.6 ± 0.7	4.0 ± 0.5	<0.006
Serum calcium (mg/dL)	7.3 ± 1.5	8.0 ± 1.1	< 0.006
Corrected serum calcium (mg/dL) *	8.1 ± 1.6	8.4 ± 1.2	0.1
Serum iPTH (pg/mL)	41.0 ± 22.9	35.6 ± 20.3	0.8
Serum 25(OH)D (ng/mL)	5.9 ± 5.6	13.3 ± 8.7	<0.001
VDR mRNA copies in PBMC/10^6 GAPDH mRNA copies	87.1 ± 39.5	160 ± 57.9	< 0.001

MDR-TB multi-drug resistant pulmonary tuberculosis, *SD* standard deviation, *iPTH* intact parathyroid hormone, *PBMC* Peripheral blood mono nuclear cells

*Corrected calcium (mg/dL) = measured total calcium (mg/dL) + 0.8 (4.4- serum albumin (g/dL), where 4.4 represent the average albumin level

ApaI (rs7975232), BsmI (rs1544410) [33], TaqI (rs731236) [33] and FokI (rs2228570) [34]. These could all affect VDR function in different ways, such as receptor activity, translation efficiency, or mRNA stability. However ApaI (rs7975232) were not included in the present study.

Statistical analysis

Sample size was calculated considering mean Serum 25(OH)D, nmol/l 13.5 Standard Deviation 10.0 in MDR-TB while mean 34.9 Standard Deviation of 26.2 for Healthy control group with desired power of 0.99 and alpha error of 0.01 the minimum sample size required as 42 per group Data are presented as mean ± standard deviation (SD). Chi-square ($\chi 2$) test, two-sample t-test with equal variance, or one-way analysis of variance (ANOVA) followed by post hoc Bonferroni test were used to compare the differences between the study groups. A multiple linear regression model was used to assess differences in serum 25(OH)D, total calcium, ionized calcium, and corrected calcium levels between MDR-TB patients and healthy controls, adjusting for age, sex, and BMI. The adjusted means and standard errors were calculated from the regression model estimates. We also perform a normality Kalmogorov smirnov test before conducting ANOVA and p value found greater than 0.05 is statically significant.All analysis was performed using STATA version 11.0 (Stata Corporation, College Station, TX, USA obtained/purchased a copyright license and were done by Professor of Statistics). A two-sided p-value of less than 0.05 was considered statistically significant.

Results

Clinical characteristics of MDR-TB patients ($n = 50$) and a comparison of demographic characteristics with normal controls ($n = 50$) were shown in (Table 1**).** A significantly lower BMI ($p < 0.001$) was observed in patients with MDR-TB.

The details of the various biochemical parameters of the two study groups are shown in Table 2. Significantly lower mean serum 25-hydroxyvitamin D concentrations (5.9 ± 5.6 ng/mL; 13.3 ± 8.1 ng/mL; $p < 0.001$) were found in MDR-TB. VDR mRNA expression was 0.6-fold less in MDR-TB patients than in normal controls (87.1 ± 39.5 copy number/10^6 GAPDH copies; 160.0 ± 57.9 copy number/10^6 GAPDH copies; $p < 0.001$) (Table 2 and a significantly positive correlation between serum vitamin D and VDR mRNA levels ($r = 0.6$; $p < 0.001$ and $r = 0.4$; $p < 0.001$) was observed in patients with MDR-TB and normal controls (Fig. 1), furthermore. Serum vitamin D was inversely correlated with mean serum iPTH concentration (41.0 ± 22.9 pg/mL;35.6 ± 20.3; $p < 0.001$) ($r = -0.3$; $p = 0.02$ and $r = -0.4$; $p = 0.002$) between the two study groups Similarly, significantly lower mean serum ionised (4.0 ± 0.5 mg/dL; 3.6 ± 0.7 mg/dL; $p = 0.006$) and total calcium concentrations (8.0 ± 1.1 mg/dL; 7.3 ± 1.5 mg/dL; $p = 0.006$; Table 2), and a significantly positive correlation between serum vitamin D and serum calcium levels ($r = 0.8$; $p < 0.001$ and $r = 0.8$; $p < 0.001$) were observed in patients with MDR-TB and normal controls (Fig. 2). Likewise, low serum total protein (6.3 ± 0.9 g/dL; 8.4 ± 0.6; $p < 0.001$) and serum albumin protein (3.4 ± 0.5 g/dL;

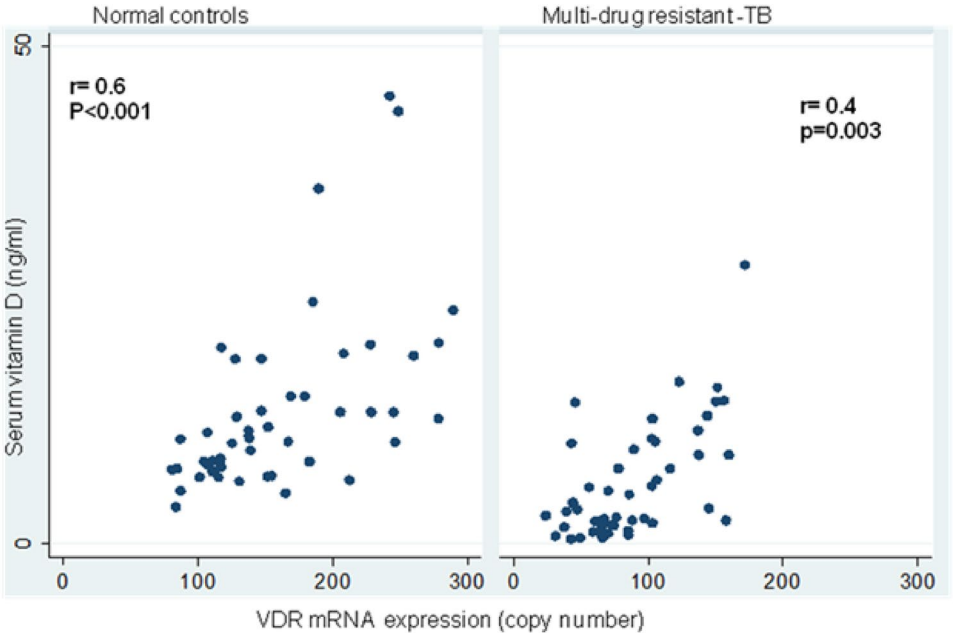


Fig. 1 Scatter plot showing correlation between serum vitamin D and mRNA levels in patients with MDR-TB and normal controls

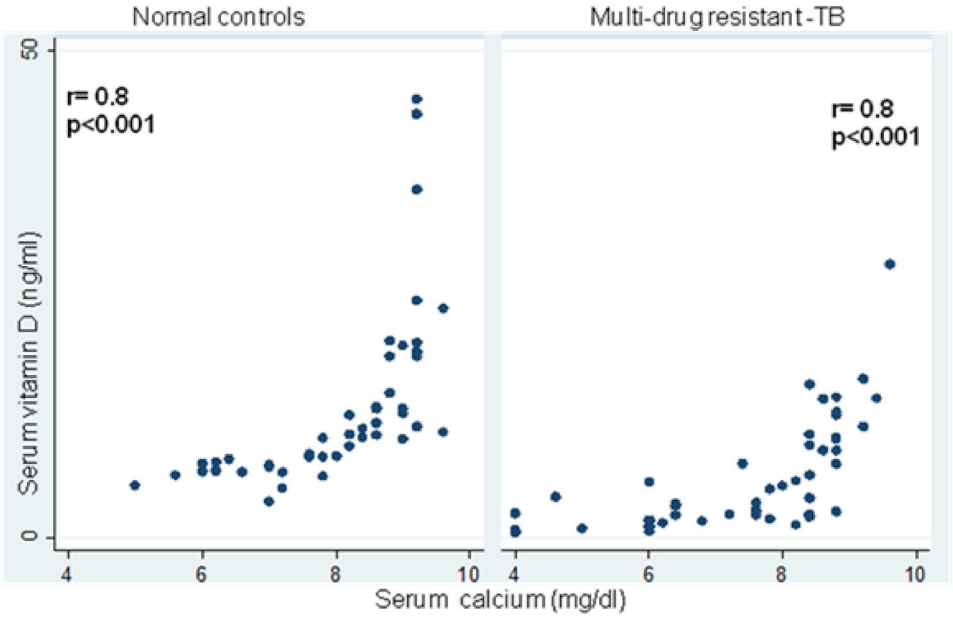


Fig. 2 Scatter plot showing correlation between serum vitamin D and serum calcium levels in patients with MDR-TB and normal controls

3.7 ± 0.6; $p < 0.02$) were observed in patients with MDR-TB as compared to normal controls Table 2.

VDR mRNA levels were significantly associated with *FokI* VDR genotype in patients with MDR-TB ($p = 0.02$ global), FF and ff genotype ($p = 0.003$), and *Ff* and *ff* genotypes ($p = 0.01$). Tables 3 and 4 present both unadjusted and adjusted associations of VDR gene polymorphisms (BsmI, TaqI, FokI) with serum vitamin D levels and VDR mRNA expression in MDR-TB patients and healthy controls. While BsmI and TaqI polymorphisms showed no significant associations, the FokI variant demonstrated a significant relationship in the MDR-TB group. Specifically, individuals with the ff genotype had markedly lower serum 25(OH)D levels and VDR mRNA expression. These associations remained significant even after adjusting for confounders, suggesting that the FokI polymorphism may have a biologically relevant impact on VDR function and immune regulation in tuberculosis pathogenesis. However, no significant association of the VDR genotype (*Bsm*I, *Taq*I and *Fok*I) with mRNA levels was

Table 3 Association of mRNA levels with VDR genotype in patients with MDR-TB and normal controls

Polymorphism	Group	Genotype (*n*)	Vitamin D (ng/mL ± SD)	VDR mRNA Expression (AU ± SD)	Global *p*-value	Post-hoc Comparisons (*p*-value)
BsmI	MDR-TB (*n* = 50)	BB (*n* = 15)	95.3 ± 44.8	1.12 ± 0.34	0.13	BB vs. Bb: 0.99; BB vs. bb: 0.99; Bb vs. bb: 0.99
		Bb (*n* = 22)	84.5 ± 26.3	1.01 ± 0.29		
		bb (*n* = 13)	73.6 ± 39.5	0.93 ± 0.27		
	Controls (*n* = 50)	BB (*n* = 18)	151.4 ± 51.3	1.67 ± 0.39	0.60	BB vs. Bb: 0.99; BB vs. bb: 0.37; Bb vs. bb: 0.99
		Bb (*n* = 20)	156.2 ± 57.6	1.61 ± 0.42		
		bb (*n* = 12)	178.4 ± 66.6	1.70 ± 0.46		
TaqI	MDR-TB (*n* = 50)	TT (*n* = 17)	84.6 ± 37.8	1.06 ± 0.31	0.81	TT vs. Tt: 0.99; TT vs. tt: 0.99; Tt vs. tt: 0.99
		Tt (*n* = 20)	90.3 ± 43.7	1.02 ± 0.27		
		tt (*n* = 13)	87.7 ± 39.1	1.01 ± 0.24		
	Controls (*n* = 50)	TT (*n* = 16)	162.0 ± 56.3	1.71 ± 0.36	0.90	TT vs. Tt: 0.99; TT vs. tt: 0.99; Tt vs. tt: 0.99
		Tt (*n* = 19)	155.8 ± 61.4	1.69 ± 0.41		
		tt (*n* = 15)	163.1 ± 62.4	1.75 ± 0.43		
FokI	MDR-TB (*n* = 50)	FF (*n* = 18)	100.0 ± 39.0	1.25 ± 0.22	0.02	FF vs. Ff: 0.88; FF vs. ff: 0.003; Ff vs. ff: 0.01
		Ff (*n* = 20)	88.4 ± 36.3	0.97 ± 0.19		
		ff (*n* = 12)	36.0 ± 8.8	0.73 ± 0.17		
	Controls (*n* = 50)	FF (*n* = 17)	156.5 ± 51.2	1.78 ± 0.33	0.61	FF vs. Ff: 0.99; FF vs. ff: 0.59; Ff vs. ff: 0.39
		Ff (*n* = 19)	170.6 ± 66.3	1.81 ± 0.35		
		ff (*n* = 14)	80.1 ± 20.4	1.52 ± 0.28		

Vitamin D levels and VDR mRNA expression are presented as mean ± SD. Expression data units are arbitrary units (AU), normalized to internal control (e.g., GAPDH or β-actin).Global p-values represent overall group differences (e.g., one-way ANOVA).Post-hoc p-values reflect pairwise genotype comparisons with correction (e.g., Bonferroni or Tukey's). Slash ("/") indicates values for MDR-TB/Controls, respectively

found in the normal control group. Although Table 5 represents the genotypic and allelic frequency distribution of VDR gene (Taq1, Fok1, and Bsm1) polymorphisms in MDRTB and healthy control, no statistically significant association was found between the two groups.

Multivariate and univariate analysis adjusted for age, sex, and bmi

Multivariate analysis using Wilks' Lambda (Table 6) revealed that among the variables tested, only the Group variable showed a statistically significant multivariate effect on the dependent variables (Wilks' Lambda = 0.517, $F(6, 89) = 13.871$, $p < 0.001$), indicating a strong group-based difference across the outcome measures. The Intercept was also significant ($p = 0.045$), reflecting overall model fit. In contrast, Age ($p = 0.347$), Sex ($p = 0.193$), and BMI ($p = 0.096$) did not exhibit significant multivariate effects, suggesting that these factors did not meaningfully influence the combination of dependent variables in this analysis.

Univariate regression analyses were also conducted to identify significant predictors for each dependent variable, with results summarized in Table 7. The variable vitamin D was significantly influenced by both Group ($p = 0.013$) and Sex ($p = 0.020$), explaining 27.1% of the variance ($R^2 = 0.271$). For mRNA, Group emerged as a strong predictor ($p = 0.002$), accounting for 37.6% of the variance. The model for iPTH showed moderate significance with contributions from Intercept ($p = 0.008$) and BMI ($p = 0.027$), but explained a relatively low proportion of variance ($R^2 = 0.093$). In contrast, ionized calcium and total calcium were both strongly influenced by Group ($p < 0.001$), with R^2 values of 0.435 and 0.436, respectively, indicating a high level of explained variance. Similarly, corrected serum calcium was significantly predicted by both Group ($p < 0.001$) and BMI ($p = 0.009$), with the highest explained variance among the outcomes ($R^2 = 0.484$). These findings underscore the prominent role of Group as a consistent predictor across multiple outcome measures.

Discussion

To our knowledge, this is the first study to explore VDR mRNA expression and its association with vitamin D levels and VDR genotypes in patients with MDR-TB compared to normal controls.

The finding from the current study, MDR-TB patients had considerably lower mean serum 25-hydroxyvitamin D concentrations, and their VDR mRNA expression was 0.6 times lower than that of normal controls. Additionally, we observed that the FokI VDR genotype in MDR-TB patients had a strong association with their VDR mRNA levels. The increased rate of MDR-TB coincided with the development of vitamin D deficiency, probably due to malnutrition and decreased sunlight exposure. A meta-analysis of the association between low vitamin D

Table 4 Adjusted association of VDR gene polymorphisms with serum vitamin D levels and VDR mRNA expression in MDR-TB and control groups

Polymorphism	Group	Genotype	Adjusted Vitamin D (ng/mL ± SE)	Adjusted VDR mRNA Expression (AU ± SE)	Global *p*-value	Pairwise Comparisons (Adjusted *p*-value)
BSM I	MDR-TB	BB	93.2 ± 6.8	1.10 ± 0.05	0.09	BB vs. Bb: 0.97BB vs. bb: 0.91Bb vs. bb: 0.99
	Controls	BB	148.1 ± 7.2	1.65 ± 0.06	0.55	BB vs. Bb: 0.95BB vs. bb: 0.35Bb vs. bb: 0.97
		Bb	85.9 ± 5.9/ 154.4 ± 7.1	1.00 ± 0.04/ 1.60 ± 0.05		
		bb	74.5 ± 6.4/ 177.2 ± 7.5	0.91 ± 0.04/ 1.69 ± 0.06		
Taq I	MDR-TB	TT	85.1 ± 5.7	1.05 ± 0.04	0.79	TT vs. Tt: 0.99TT vs. tt: 0.99Tt vs. tt: 0.99
	Controls	TT	159.2 ± 6.5	1.69 ± 0.06	0.88	TT vs. Tt: 0.99TT vs. tt: 0.99Tt vs. tt: 0.99
		Tt	89.7 ± 6.1/ 153.6 ± 6.7	1.01 ± 0.04/ 1.67 ± 0.05		
		tt	88.1 ± 5.8/ 161.7 ± 6.8	1.00 ± 0.04/ 1.72 ± 0.06		
Fok I	MDR-TB	FF	98.1 ± 6.4	1.22 ± 0.04	0.01	FF vs. Ff: 0.87FF vs. ff: 0.002Ff vs. ff: 0.009
	Controls	FF	153.2 ± 6.6	1.75 ± 0.05	0.58	FF vs. Ff: 0.96FF vs. ff: 0.48Ff vs. ff: 0.41
		Ff	90.5 ± 5.9/ 169.8 ± 6.9	0.95 ± 0.03/ 1.78 ± 0.06		
		ff	38.2 ± 3.2/ 82.1 ± 5.2	0.70 ± 0.03/ 1.50 ± 0.05		

Values are least square means ± standard error (SE) from ANCOVA models adjusted for age, sex, and BMI. Global p-values from ANCOVA indicate significance across all genotypes. Post-hoc p-values are pairwise comparisons adjusted for covariates and corrected using Bonferroni method. AU: Arbitrary Units (normalized VDR mRNA expression); Vitamin D in ng/mL. Separate ANCOVA models were applied to each polymorphism within MDR-TB and control groups

levels and active tuberculosis reported that low serum vitamin D levels are associated with an increased risk of active tuberculosis [14]. Previous studies associated with an increased risk of TB and vitamin D deficiency have only investigated vitamin D levels only [10, 15, 16]. In this study, serum calcium (ionized and total) and intact PTH levels were performed on both subjects to rule out these as confounding factors, and a significantly positive and negative correlation was found with vitamin D levels, respectively, which was as expected in patients without disorders affecting calcium homeostasis.

The VDR gene, located on chromosome 12q, consists of 14 exons. The 5′ untranslated region has six of them [35]. The ligand-dependent transcription factor VDR is a member of the nuclear hormone receptor family. When it binds 1,25-$(OH)_2D_3$, it undergoes a conformational shift, forms a heterodimer with the Retinoid X Receptor (RXR), and enters the nucleus. By attaching to Vitamin D Responsive Elements (VDREs) in gene promoters, this complex regulates transcription. VDR can also indirectly alter gene expression by interfering with transcription factors such NFAT, NF-κB, and AP-1 [36]. Functionally, 1,25-$(OH)_2D_3$ promotes Th2 cytokines (IL-4, TGF-β1) and inhibits Th1 cytokines (IL-2, IFN-γ, GM-CSF) to improve immune modulation and naïve CD4 + cell growth. Additionally, it regulates HLA class II expression [35, 36].

In the present study, we found that the *FokI* polymorphism was significantly associated with VDR mRNA levels. It has been shown that, the VDR is expressed in large number of tissues, it is not surprising that ligand-activated VDR modulates the expression of many genes [35]. The *FokI* polymorphism results in the incorporation of three extra amino acids in the NH2 terminus of the VDR protein, which influences transcriptional activity by modulating the interaction with transcription factor IIB (TFIIB) [34, 37]. Alternatively, the *FokI* polymorphism interferes with the formation of VDR heterodimers with the retinoid X receptor, forming effector complexes [38]. However, no such events have been reported for *BsmI* and the *TaqI* polymorphisms, which are located between exons 8 and 9 and exon 9, respectively. It has been reported that the *FokI* genotype contributes to feedback control of the expression of the 25-hydroxyvitamin D-1—hydroxylase (CYP27B1) gene, which is located in the same chromosomal region as the VDR gene and is active in PBMCs [39, 40]. Vitamin D deficiency leads to an increase in PBMC production by activated vitamin D

Table 5 Genotypic and allelic frequency distribution of VDR gene (*Taq1*, *Fok1 and Bsm1*) polymorphism in MDRTB and healthy control

VDR Polymorphism Genotype	Healthy Controls (*n* = 50) n%	MDR-TB (*n* = 50) n%	OR (95% CI)	*P* Value*
BsmI Genotypic Frequency				
BB	12 (24)	17 (34)	0.61 (0.26–1.47)	0.27
Bb	27 (54)	20 (40)	1.76 (0.80–3.89)	0.16
bb	11 (22)	13 (26)	0.8 (0.32–2.02)	0.63
BsmI Allelic Frequency ‡				
B	26 (51)	27 (54)	0.92 (0.42–2.02)	0.84
b	24 (49)	23 (46)	1.08 (0.49–2.38)	0.84
TaqI Genotypic Frequency				
TT	24 (48)	21 (42)	1.27 (0.58–2.81)	0.54
Tt	20 (40)	20 (40)	1 (0.45–2.23)	1.0
tt	6 (12)	9 (18)	0.62 (0.2–1.9))	0.4
TaqI Allelic Frequency ‡				
T	34 (68)	31 (62)	1.3 (0.57–2.97)	0.52
t	16 (32)	19 (38)	0.77 (0.34–1.75)	0.52
FokI Genotypic Frequency				
FF	29 (58)	21 (42)	1.91 (0.86–4.22)	0.10
Ff	19 (38)	25 (50)	0.61 (0.25–1.36)	0.22
ff	2 (4)	4 (8)	0.48 (0.08–2.74)	0.49
FokI Allelic Frequency ‡				
F	39 (78)	34 (68)	0.63 (0.21–1.87)	0.26
f	11 (22)	16 (32)	0.6 (0.24–1.47)	0.26

* $P < 0.05$ was considered statistically significant, ‡ Allelic frequency is double the genotypic frequency (2*n*)

VDR vitamin D receptor, *MDR-TB* multidrug-resistant tuberculosis, *OR* odds ratio, *CI* confidence interval

Table 6 Multivariate Analysis of Variance (MANOVA) Results Using Wilks' Lambda to Assess the Effects of Age, Sex, BMI, and Group on the Dependent Variables

Effect	Wilks' Lambda	F	df	Sig. (p-value)	Interpretation
Intercept	0.868	2.256	6, 89	0.045	Significant
Age	0.929	1.138	6, 89	0.347	Not Significant
Sex	0.909	1.484	6, 89	0.193	Not Significant
BMI	0.889	1.861	6, 89	0.096	Not Significant
Group	0.517	13.871	6, 89	<0.001	Highly Significant

Effect-The independent variable or factor whose influence is being tested on the dependent variable(s); Wilks' Lambda-A multivariate test statistic used in MANOVA (Multivariate Analysis of Variance); measures how well the model separates groups; F-The F-statistic, used to determine the significance of the overall model (ratio of explained variance to unexplained variance); df-Degrees of Freedom, representing the number of independent values that can vary in the analysis; Sig. (p-value)- Significance level (p-value), which indicates whether the observed effect is statistically significant (commonly significant if $p < 0.05$)

Table 7 Summary of significant predictors and explained variance (R^2) for each dependent variable in the univariate regression analysis

Dependent Variable	Significant Predictors ($p < 0.05$)	R^2 Value	Interpretation
Vit D	Group ($p = 0.013$), Sex ($p = 0.020$)	0.271	Group & sex influence vitamin D levels
mRNA	Group ($p = 0.002$)	0.376	Group significantly influences mRNA
iPTH	Intercept ($p = 0.008$), BMI ($p = 0.027$)	0.093	Moderate significance
ioz_cal	Group ($p < 0.001$), Intercept ($p = 0.036$)	0.435	Strongly influenced by group
Total scal	Group ($p < 0.001$), Intercept ($p = 0.035$)	0.436	Strong group effect
corr_scal	Group ($p < 0.001$), BMI($p = 0.009$)	0.484	Strong group and BMI effect

Vit D = Serum 25-hydroxyvitamin D levels; mRNA = Expression levels of target messenger RNA; ipth = Intact Parathyroid Hormone; ioz_cal = Ionized Calcium; scal = Total Serum Calcium; corr_scal = Corrected Serum Calcium; R^2 Value indicates the proportion of variance in the dependent variable explained by the model; $p < 0.05$ considered statistically significant; values in parentheses represent p-values for each predictor included in the model; Only predictors with $p < 0.05$ are statistically Significant

[41]. In accordance with the much lower serum calcium and vitamin D levels, MDR-TB patients exhibited a significantly higher mean serum iPTH content than the other two groups, as reported by Rathored et al. (2023). This implies that the outcomes are not the consequence of an endocrine problem because it appears to represent typical physiological reactions. PTB Cat I patients had a greater sun exposure index (SEI) than the MDR-TB and healthy control groups, which were comparable. Although the higher exposure in PTB Cat I patients compared to healthy controls may cast doubt on this association, this often showed a positive correlation with serum 25(OH)D levels, suggesting, as other studies have shown, that food may be a stronger predictor of the lower serum levels in PTB Cat I [42].

In present research, we assessed serum vitamin D levels and important functional variants of the VDR gene in patients with multidrug-resistant (MDR) TB, filling a crucial knowledge gap on host variables affecting the course of the disease. Considering the ongoing worldwide public health threat of tuberculosis and the increasing incidence of medication resistance, analyzing host immunogenetics offers crucial information about the mechanisms that could affect treatment outcomes and infection susceptibility [43] By influencing the synthesis of antimicrobial peptides and activating macrophages, vitamin D and its receptor are essential components of the immune response against Mycobacterium tuberculosis [44, 45]. Examining the effects of VDR polymorphisms

on receptor expression and function in relation to vitamin D levels, particularly in individuals with MDR-TB, can help advance disease control tactics and open the door to more individualized treatments.

Interestingly, we observed a significant variation in VDR mRNA levels with the *FokI* polymorphism. This study demonstrates that *FokI* genotype may contribute to the expression of VDR mRNA. In addition, homozygosity for the VDR *FokI f* genotype was associated with lower levels of PBMC VDR mRNA expressions in MDR-TB patients than in normal controls. This reflects differences in the effects of ligand-activated receptors between different tissues in patients and controls. We also found that serum vitamin D levels were directly correlated with PBMC VDR mRNA levels and serum calcium levels (total and ionized) in both groups (MDR-TB and normal controls), suggesting that VDR is a ligand-dependent transcription factor and that the ligand for VDR is Vitamin D3, that is, 1,25-$(OH)_2$ D_3 which mediates its biological actions through VDR.

Our results imply that there is no causal relationship between the optimal vitamin D level and the VDR mRNA copy number. Rather, even within the insufficient range, we found statistical correlations between VDR mRNA expression and relative serum 25(OH)D levels. Research shows that VDR expression and immune responses can still be modulated by minor variations in suboptimal vitamin D levels [44, 46]. Vitamin D, for example, can increase antimicrobial peptides through VDR [44], and slight changes in 25(OH)D affect VDR expression specific to T cells [46]. Crucially, we concentrated on FokI polymorphisms, which, independent of vitamin D status, influence VDR mRNA levels and receptor function [33, 47]. Lower mRNA expression is associated with FokI ff genotypes, which also yield less active receptor isoforms. Despite the low vitamin D levels of the controls, we examined variations by genotype. Collectively, these results which are corroborated by earlier research validate our methodology and imply that VDR transcription is strongly impacted by both genetic and relative vitamin D differences.

Present research lends credibility to the idea that both VDR genotype and vitamin D level affect VDR mRNA expression. In particular, even though there were no appreciable variations in the genotype frequencies between cases and controls, we found that MDR-TB patients with the FokI ff genotype had much decreased VDR mRNA copy numbers (Table 5). This implies that host susceptibility and immune regulation may be more significantly influenced by the functional impact of VDR polymorphisms than by genotype distribution alone. This interpretation is supported by earlier research. For instance, a study conducted in the UK [10] discovered correlations between the severity of tuberculosis and vitamin D responsiveness, but no discernible variations in genotype frequency. VDR SNPs also associated more with cytokine profiles and immunological markers than with the development of disease, according to other research [47].Based to this study results, MDR-TB patients had 0.6 times lower VDR mRNA expression than normal controls. Additionally, there seems to be ethnic variation in the relationship between VDR polymorphisms and TB. South African and Indian populations showed relationships, although some Chinese and European studies found no significant allele differences [21, 47, 48].

This could be the result of impaired VDR signalling, which could lead to elevated inflammation by causing inflammatory cytokines to be expressed more frequently. Our findings in MDR-TB patients are further supported by the fact that vitamin D supplementation improves immunity against tuberculosis by favourably modulating the production of cathelicidin [49]. Remarkably, Selvaraj et al. reported lower levels of VDR protein and higher levels of plasma 1,25-(OH)2 D3 [49]. Also, the study conducted by Rathored J et al. 2023 found a significant association in MDR-TB and DS-TB patients in context to dietary profile with vitamin D levels and sunlight exposure [50].

In contrast to previous research that suggests the FokI ff genotype produces a longer, less active VDR protein that compromises transcriptional efficiency and immunological function, our data show a robust correlation between the genotype and decreased VDR mRNA expression in MDR-TB patients. We found a favorable connection between 25(OH)D levels and VDR mRNA expression, despite generally low serum vitamin D levels. This suggests that vitamin D can enhance VDR-mediated transcription even at suboptimal levels. The lack of discernible variations in genotype frequencies between cases and controls emphasizes how crucial polymorphisms' functional effects are in assessing TB susceptibility rather than their prevalence. (10, 33,48, 49).

There are various limitations on this study. First, since anti-TB therapies are known to reduce vitamin D levels, results may be skewed by the exclusion of drug-susceptible TB patients and the previous anti-TB treatment in MDR-TB cases [14]. This suggests that a lack of vitamin D may put patients at risk for re-infection with MDR-TB and worsening of their condition. Second, we did not evaluate other variants such as ApaI, but we concentrated on the FokI polymorphism because of its known functional significance in VDR activity. Third, even after controlling for confounders, the statistical power and generalizability of our findings are severely constrained by the limited number of healthy controls. To confirm and build on our findings, future research should incorporate more VDR polymorphisms and

bigger, more representative cohorts. This study presents major new findings: a significant association between *FokI* VDR polymorphisms and low PBMC VDR mRNA levels in MDR-TB patients compared with normal controls. This adds to much recent work on the VDR gene, vitamin D, VDR mRNA levels and MDR-TB, and suggests that the *FokI* (especially mutant allele *f* and homozygous *ff*) and low serum vitamin D levels may be linked to low VDR mRNA levels and increased susceptibility to MDR-TB. This indicates a need for further study in this area, including confirmation of these results using a proteomics approach, and in vitro studies were incorporated into trials of vitamin D supplementation in MDR-TB cases.

Abbreviations

VDR	Vitamin D receptor
PBMC	Peripheral blood mono nuclear cells
MDR	Multi drug Resistant
VDRE	Vitamin D Responsive elements
Retinoid X Receptor	RXR
NFAT	Nuclear factor of activated T-cells

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Supplementary Material 4.

Supplementary Material 5.

Supplementary Material 6.

Supplementary Material 7.

Supplementary Material 8.

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Authors' contributions

JR: conceptualization, methodology, data collection, visualization, investigation, and writing of original draft and editing was done by JR; SK: study supervision and review of the original draft and gave his expert advice during the study tenure. V S: helps in statistical analysis; AKS: helps in biochemical investigation and reviewing the original draft. All authors have reviewed and approved the final draft of the manuscript.

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Data availability

Upon reasonable request, the corresponding author, Dr. Jaishriram Rathored (jaishriz@gmail.com), will provide the data supporting the study's conclusions.

Declarations

Ethics approval and consent to participate

The study was approved by the Institutional Ethical committee of All India Institute of Medical Sciences (IEC-AIIMS) Reference Number: A-08/5.5, New Delhi, India, and written informed consent was obtained from all subjects, including patients and normal controls. Ethics committee (AIIMS-IEC) approved the procedure involved in the present research.

Consent for publication

All authors agreed and give their consent for publication.

Competing interests

The authors declare no competing interests.

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