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Research paper

Association of *CYP27B1* gene polymorphisms with pulmonary tuberculosis and vitamin D levels

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ABSTRACT

Background and Objectives: Genetic factors are reported to be connected with tuberculosis (TB) infection. Studies have shown that genetic variations in genes involved in the vitamin D pathway influence the levels of vitamin D found in the bloodstream (serum). Cyp27b1 (1α-hydroxylase) is an enzyme that activates the synthesis of bioactive vitamin D_3 by hydroxylation of 25(OH)D_3. The in vitro studies reported rare gene variants of Cyp27b1 such as rs118204011 and rs118204012, associated with loss of Cyp27b1 function and lower serum vitamin D levels. Globally, a critical gap exists in understanding the link between these gene variants with TB and vitamin D levels. Hence, the study objective is to comprehend the association of *Cyp27b1* rs118204009 (G/A), rs118204011 (C/T), and rs118204012 (A/G) with tuberculosis susceptibility/protection and to assess the influence of gene variants on vitamin D levels in both healthy controls (HCs) and those with pulmonary tuberculosis (PTB) in South India.

Methods: Genomic DNA extraction was performed by salting-out procedure and subsequently genotyped through polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method. Vitamin D level was measured by Enzyme-Linked Immunosorbent Assay (ELISA).

Results: In rs118204012 (A/G), a substantial association was found with PTB susceptibility in allele 'A' [Odds Ratio (OR): 1.52 (1.02–2.26); p = 0.044] and 'AA' genotype [OR: 1.69 (1.02–2.81); p = 0.040] through the dominant model. Allele 'G' [OR: 0.66 (0.44–0.98); p = 0.044) was found to be associated with protection against TB. Males were associated with increased susceptibility towards TB compared to females in the rs118204011 "CC" [OR: 3.94 (1.94–7.98); p = 0.002] and rs118204012 'AA' [OR: 4.57 (2.13–9.79); p = 0.0001] genotypes. Vitamin D insufficiency (<30 ng/ml) was more prevalent in PTB patients (66.67 %) with the rs118201012 'AA' genotype compared with healthy controls (57.14 %). This genotype was associated with disease susceptible odds ratio of 1.5.

Conclusion: Cyp27b1 rs118204012 'AA' genotype was found to have association with vitamin D insufficiency and TB susceptibility. In terms of gender, our findings suggest that male individuals are correlated with a higher TB risk. This suggest that the gene variants may be involved in the downstream processing of serum Vitamin D levels and its association with the disease.

1. Introduction

Tuberculosis (TB) remains the second major cause of mortality by a single infectious agent following the COVID-19 pandemic. In 2022, approximately 1.3 million deaths occurred due to TB among individuals who are HIV-negative. The global occurrence of TB in 2022 was estimated to be 10.6 million people and about 7.5 million people were newly diagnosed that year (WHO - Global tuberculosis report, 2023). Multiple factors are involved in TB disease susceptibility, and identifying those factors will be helpful for early diagnosis and the development of a better treatment strategy. Genetic factors are one of those factors stated to be associated with TB illness. The twin, family-based

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linkage studies reported evidence of genetic background with tuberculosis susceptibility or protection (Hill, 2006; Bellamy, 2006; Harishankar et al., 2018).

Research indicates a connection between Vitamin D deficiency and tuberculosis susceptibility (Gibney et al., 2008; Nnoaham and Clarke, 2008). Cytochrome P450 Family 27 Subfamily B Member 1 (Cyp27b1), also known as 1α-hydroxylase, catalyzes the hydroxylation of 25(OH)D3 to produce biologically active 1,25-dihydroxy vitamin D3 (Holick, 2007). The gene *Cyp27b1* comprises nine exons and is situated on chromosome 12q14.1 (Kong et al., 1999). Once activated, vitamin D can influence gene activity through interaction with retinoid-X receptor (RXR) and vitamin D receptor-responsible elements (VDREs) of the vitamin D receptor (McKenna et al., 1999). Vitamin D is reported to enhance the macrophages native immunity by upregulating the antimicrobial peptide cathelicidin expression and promoting autophagy mechanisms, thereby restricting intracellular *M. tuberculosis* (Mtb) growth (Selvaraj et al., 2015).

Several candidate gene polymorphisms in vitamin D metabolism are reported to be correlated with vitamin D levels (Bu et al., 2010; McGrath et al., 2010; Wang et al., 2010). Previous reports have indicated the connection of *Cyp27b1* gene variants with tuberculosis (Sadykov et al., 2020; Zhang et al., 2021). The in vitro studies reported that three rare gene variants of *Cyp27b1*, such as rs118204009 (G/A), rs118204011 (C/T), and rs118204012 (A/G), were associated with loss of Cyp27b1 function, lower vitamin D levels, and vitamin D-dependent type 1 rickets in the Canadian population. The nonsynonymous polymorphism rs118204012 at position 189, alters glutamate to glycine, while at location 343, it changes leucine to phenylalanine in rs118204011 (Wang et al., 1998; Wang et al., 2002; Ramagopalan et al., 2011). However, studies are not available on rare gene variants and their interaction with TB disease.

Our formerly studies demonstrated a relationship of *VDR* gene polymorphisms with TB (Selvaraj et al., 2000; Selvaraj et al., 2004; Selvaraj et al., 2008). Moreover, we also studied and described the influence of vitamin D-binding protein (VDBP) and *Cyp2R1* gene variants on pulmonary tuberculosis (Harishankar et al., 2020; Harishankar et al., 2021). Since *Cyp27b1* is responsible for the synthesis of biologically active vitamin D, we hypothesized that polymorphisms in this gene can modulate the vitamin D level and may be linked with susceptibility or protection from pulmonary tuberculosis. In this study, we examined the association of three rare gene variants of *Cyp27b1*: rs118204009 (G/A), rs118204011 (C/T), and rs118204012 (A/G) with tuberculosis susceptibility/protection and to understand their impact on vitamin D levels in the study participants from the South Indian population.

2. Materials and methods

2.1. Study subjects

Healthy volunteers (126 individuals) who were asymptomatic for pulmonary tuberculosis (PTB), with normal x-ray, and absence for interferon-gamma release assay (IGRA), were recruited for the study. Among them 62 were males and 64 were females (mean ± SD: 31.77 ± 10.02). Totally 121 PTB patients were recruited from the clinics of ICMR-NIRT and District TB Centre, Puliyanthope, Chennai. Among them 94 were males and 27 were females (mean ± SD: 39.68 ± 11.76) (Table 1). The inclusion criteria were abnormal x-rays suggestive of active disease, sputum smear and culture positive for Mtb, negative for HIV, immune-suppressive conditions, and other infectious diseases. Blood was collected from treatment-naïve participants, and written approval was attained from all participants who comprised the indigenous population of South Indians residing in and around Chennai. This study has been approved by the ethical committee of ICMR-NIRT, Chennai (NIRT-IEC Number: 1/12/108/IEC/2016).

2.2. Genomic DNA separation

A Simple salting-out technique was carried out to extract the genomic DNA (Miller et al., 1988) and solidified in Tris-EDTA (TE) buffer (pH 8.0). Nanodrop "ND1000" was used to check the pureness and finally stored at − 80°C till usage. The *Cyp27b1* gene polymorphisms rs118204009 (G/A), rs118204011 (C/T), and rs118204012 (A/G) were genotyped by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) technique. PCR products with appropriate markers were checked on a 2 % agarose gel, and digested products were tested on a 2.5 % gel.

2.3. rs118204009 genotyping

The forward primer (FP) 5′-TGTGCTTTGCAACCTAGACTGT-3′ and reverse primer (RP) 5′-GGAAGTTTTCTGGGGCTACTTT-3′ (Table 2) were used to amplify 281 base pair (bp) PCR products. The annealing temperature was 60∘C. The *BssSI* enzyme (New England Biolabs) was employed at 37 °C to break the PCR product. The absence of breaking site in the "AA" genotype produced at 281 bp; the presence of two breaking sites in the "AG" genotype produced three bands of 281, 237 and 44 basepairs. The single breaking site in the "GG" genotype yielded two bands of 237 and 44 basepairs (Agnello et al., 2017).

2.4. rs118204011 genotyping

The PCR product size of 279 bp was obtained by using FP-5′-TTCAACATGTTTTTCAGGTGTCC-3′ and RP-5′-TTCTCTGCTATCTCCCTGCTTC-3′ (Table 2). The annealing temperature of 62°C was used. The *BsmI* (New England Biolabs) was employed at 65°C to break the PCR product. The absence of breaking site in the "CC" genotype produced at 279 bp; the presence of two breaking sites in the "CT" genotype produced three bands of 279, 202, and 77 basepairs. The single breaking site in the "TT" genotype produced bands of 202 and 77 basepairs (Agnello et al., 2017).

2.5. rs118204012 genotyping

The 248-bp PCR product was amplified at 65°C annealing temperature by using FP-5′-ATGCGCACTCTCTCCTCAAC-3′ and RP-5′-CTCTGTCCTGGGACTCACCTT-3′ (Table 2). The *MluCI* (New England Biolabs) was employed at 37 °C to break the PCR product. The single breaking site in the "AA" genotype produced two bands at 206 and 42 basepairs; the presence of two breaking sites in the "AG" genotype produced three bands at 248, 206, and 42 basepairs. The absence of

Table 1
Demographic details of the study subjects.

	Healthy Controls (HCs) n = 126	Pulmonary tuberculosis (PTB) patients n = 121
Number of subjects recruited	126	121
Gender (male/female)	62/64	94/27
Median Age (Interquartile range [IQR])	28.5 (14)	40 (18)

Table 2
Primer details.

Sequence	Gene assession number	Location
(FP) 5′-TGTGCTTTGCAACCTAGACTGT-3′ and (RP) 5′-GGAAGTTTTCTGGGGCTACTTT-3′	*rs118204009;* Chr12	Exon 7
FP-5′-TTCAACATGTTTTTCAGGTGTCC-3′ and RP-5′-TTCTCTGCTATCTCCCTGCTTC-3′	*rs118204011;* Chr12	Exon 6
FP-5′-ATGCGCACTCTCTCCTCAAC-3′ and RP-5′-CTCTGTCCTGGGACTCACCTT-3′	*rs118204012;* Chr12	Exon 3

breaking site in the "GG" genotype produced at 248 bp (Agnello et al., 2017).

2.6. Vitamin D level estimation

An enzyme immunoassay (EIA) kit from Epitope Diagnostics (EDI; San Diego, USA) was used for 25(OH)D level estimation from − 80°C stored plasma samples. To summarize, 20 µl of test samples, calibrators, and controls were added to the designated wells. Assay buffer (100 µl) was added and incubated for 1 h under light protection. 25 µl of biotinylated vitamin D analogue was incubated for 30 min and washed with wash buffer. Followed by 100 µl of Streptavidin-HRP and its substrate incubated for 20 min, respectively. The absorbance was measured at 450 nm after the addition of the stop solution. The test sample data was generated using Softmax Pro software. The lower to higher finding limit of the kit is 0–150 ng/ml. The predicted levels were analyzed with variant genotypes of *Cyp27b1* gene polymorphisms.

2.7. Statistical methods

The SNP Stats online program is used for frequency determination of alleles and genotypes and their associations, Hardy-Weinberg equilibrium, and odds ratio (OR) adjusted p-values for gender and age by logistic regression under different genetic models (Sole et al., 2006). EpiInfo version 7.2.5 was used for 2X2 table estimation of the Yates corrected p-value and OR with 95 % confidence intervals. The best-fitting model was identified by the lowest values of the Akaike information criterion (AIC) and the Bayesian information criterion (BIC). The Shapiro-Wilk test was employed to check the normal distribution of the sample. The Wilcoxon signed rank test and the Mann-Whitney *U* test were employed for paired and unpaired comparisons. Significant levels were determined by a p-value ≤ 0.05.

3. Results

3.1. Allele/genotype and vitamin D insufficient frequencies in the study participants

All the individuals were carriers of the wild-type genotype 'GG' in the rs118204009 polymorphism (data not shown).

In rs118204011 (C/T), the allele 'C' was found as a major allele (HCs: 87 %; PTB: 86 %) while 'T' was a minor allele (HCs: 13 %; PTB: 14 %). The genotypes 'CC' and 'CT' were compared between HCs and PTB patients and found not significant. In our population, the homozygous mutant genotype 'TT' was absent (Table 3).

In rs118204012 (A/G), the allele 'A' was found as a major allele (HCs: 69 %; PTB: 77 %) and the allele 'G' as a minor allele (HCs: 31 %; PTB: 23 %). The genotype 'AA' found greater frequency, trailed by 'AG' and 'GG' in healthy controls and patient groups. When the allele and genotype occurrences were matched among healthy controls and patients, a substantial association was found with TB risk in allele 'A' [odds ratio (OR): 1.52 (1.02–2.26); p = 0.044] and 'AA' genotype [OR: 1.69 (1.02–2.81); p = 0.040]. A substantial defensive link was found with allele 'G' [OR: 0.66 (0.44–0.98); p = 0.044] (Table 3).

3.2. Cyp27b1 association in different genetic models

The lowest AIC and BIC values from the SNP-Stats software were used to identify the association of the best-fitting model. The results revealed that in rs118204012, a substantial link was found with TB protection in the dominant model ('AA' vs 'AG'+'GG'- 0R: 0.55 (0.32–0.97); p = 0.038), and an analogous trend was observed in the overdominant model ('AA'+'GG' vs 'AG') in the 'AG' genotype [OR: 0.58(0.32–1.04); p = 0.065] (Table 3). In the rs118204011 polymorphism, genetic model association was not found due to the absence of the "TT" genotype.

Table 3
Allele/genotype and vitamin D insufficiency frequencies of rs118204011 and rs118204012 polymorphisms in healthy controls (HCs) and pulmonary tuberculosis (PTB) patients.

SNP	Allele/ Genotypes	PTB (n = 121)	HCs (n = 126)	OR (95 % CI)	p-value
rs118204011 (C/T)	Alleles				
	T	0.140 (35)	0.130 (26)	1	0.781
	C	0.860 (211)	0.870 (176)	1.12 (0.65–1.94)	
	Genotypes				
	CT	0.285 (35)	0.257 (26)	1	0.762
	CC	0.715 (88)	0.743 (75)	1.15 (0.63–2.07)	
	Vitamin D insufficiency (<30 ng/ml)				
	CC	0.750 (9)	0.714 (15)	1.2 (0.24–6.02)	
	CT	0.250 (3)	0.286 (6)	1	
rs118204012 (A/G)	Alleles				
	A	0.770 (186)	0.690 (173)	**1.52 (1.02–2.26)**	**0.044**
	G	0.230 (56)	0.310 (79)	1	
	Genotypes				
	AA	0.628 (76)	0.500 (63)	**1.69 (1.02–2.81)**	**0.04**
	AG	0.281 (34)	0.373 (47)	0.66 (0.38–1.12)	0.16
	GG	0.091 (11)	0.127 (16)	0.69 (0.30–1.55)	0.481
	Dominant model				
	AA	0.628 (76)	0.500 (63)	1	**0.038**
	AG + GG	0.372 (45)	0.500 (63)	**0.55 (0.32–0.97)**	
	Recessive model				
	AA + AG	0.909 (110)	0.873 (110)	1	0.6
	GG	0.091 (11)	0.127 (16)	0.79 (0.32–1.92)	
	Overdominant model				
	AA + GG	0.719 (87)	0.627 (79)	1	**0.065**
	AG	0.281 (34)	0.373 (47)	**0.58 (0.32–1.04)**	
	Vitamin D insufficiency (<30 ng/ml)				
	AA	66.67 % (8)	57.14 % (12)	**1.50 (0.34–6.58)**	
	AG	33.33 % (4)	42.86 (9)	1	

n = number of individuals. Numbers in parenthesis indicates total individuals positive for that genotype.

3.3. rs118204011 (C/T) genotype frequencies between/among the gender

To find any variations between or among the genders, genotype frequencies were stratified and examined by age adjusted. The 'CC' genotype was found to have a significant association with TB susceptibility [OR: 3.94 (1.94–7.98); p = 0.002] in male individuals compared with females. When the genotype frequencies were compared within the genders, no substantial association was found in either gender (Table 4).

3.4. rs118204012 (A/G) genotype frequencies between/among the gender

In males, a significant association was found with susceptibility to TB in the 'AA' genotype [OR: 4.57 (2.13–9.79); p = 0.0001] related to females. When the genotype frequencies were analysed within sex, an analogous noteworthy link was noted with TB susceptibility in 'AA'

Table 4
rs118204011 (C/T) number of individuals positive for various gene variants between/among the gender in HCs and PTB patients.

Genotypes	Gender	PTB	HCs	OR (95 % CI)	p-value
CC	Male	72	40	**3.94 (1.94–7.98)**	**0.0002**
	Female	16	35	1	
CT	Male	23	13	**1.92 (0.68–5.41)**	0.331
	Female	12	13	1	
Among the gender					
CT	Female	12	13	2.02 (0.75–5.40)	0.246
CC		16	35	1	
CT	Male	23	13	0.98 (0.45–2.15)	1
CC		72	40	1	

genotype-positive males [OR: 2.00 (1.04–3.84); p = 0.047]. The absence of a significant association was noted in females (Table 5).

3.5. Vitamin D levels among variant genotypes

When compared to HCs, PTB patients generally had significantly higher vitamin D levels (p = 0.0024) (Fig. 1A). No noteworthy difference was found between the genotypes in HCs and PTB patients in rs118204011 and rs118204012 polymorphisms. However, when the genotypes were matched between the groups, vitamin D levels were significantly higher in rs118204012 'AG' genotype-positive PTB patients compared with HCs (p = 0.0029) (Fig. 1B), while no significant difference was found in the rs118204011 polymorphism (Fig. 1B).

The frequency of vitamin D insufficiency (<30 ng/ml) was compared among the *Cyp27b1* gene variants. In rs118204011, vitamin D insufficiency was found to be higher among 'CC' genotypes (HCs: 71.43 %; PTB: 75 %) compared with 'CT' genotypes (HCs: 28.57 %; PTB: 25 %) in both study groups (Table 3). In rs118204012, 25(OH)D_3 insufficiency was noted to be greater in the 'AA' genotype (HCs: 57.14 %; PTB: 66.67) compared with the 'AG' genotype (HCs: 42.86 %; PTB: 33.33 %) (Figure-1C). Moreover, 25(OH)D_3 insufficiency was noted to be higher in AA' genotype positive patient group with a disease-sensitivity odds ratio of 1.5 compared with healthy controls (Table 3). Due to the low frequency of the 'GG' genotype, we weren't able to compare vitamin D insufficiency in these individuals.

4. Discussion

The prominent global source of illness and death is tuberculosis (TB), which is often associated with host genetic factors and nutritional deficiencies, including vitamin D. Studies reported the relationship of 25 (OH)D_3 deficiency with susceptibility to tuberculosis disease (Gibney et al., 2008; Nnoaham and Clarke, 2008). The gene variants of *VDR*, *VDBP* and the vitamin D pathway have been stated to be linked with 25 (OH)D_3 deficiency and TB risk. (Sadykov et al., 2020; Selvaraj et al., 2000; Selvaraj et al., 2008; Harishankar et al., 2020; Harishankar et al., 2021). The *Cyp27b1* gene, which encodes the 1α-hydroxylase enzyme, mediates the rate-limiting step in the vitamin D metabolism by translating inactive 25-OH vitamin D into biologically active 1,25-dihydroxyvitamin D_3 (Calcitriol; vitamin D3). The dynamic form of vitamin D_3 binds with VDR and modulates gene expression through VDREs in genomic DNA (Kliewer et al., 1992). The study stated that vitamin D_3 modulates the host's innate immunity by upregulating macrophage antimicrobial peptide cathelicidin expression and favoring intracellular killing of Mtb (Liu et al., 2007). In addition, vitamin D down-regulated the Th1 response and up-regulated the anti-inflammatory cytokine response in adaptive immunity. (Vidyarani et al., 2007).

Table 5
rs118204012 (A/G) number of individuals positive for various gene variants between/among the gender in HCs and PTB patients.

Genotypes	Gender	PTB	HCs	OR (95 % CI)	p-value
AA	Male	62	31	**4.57 (2.13–9.79)**	**0.0001**
	Female	14	32	1	
AG	Male	26	26	2.62 (0.99–6.99)	0.084
	Female	8	21	1	
GG	Male	6	6	2.00 (0.42–9.51)	0.63
	Female	5	10	1	
Among the gender					
AA	Female	14	32	1.04 (0.42–2.57)	1
AG		8	21	0.84 (0.32–2.24)	0.921
GG		5	10	1.20 (0.37–3.93)	1
AA	Male	62	31	**2.00 (1.04–3.84)**	**0.047**
AG		26	26	0.54 (0.28–1.07)	0.109
GG		6	6	0.65 (0.20–2.11)	0.675

The *Cyp27b1* gene (12q 14.1), measures around 6.6 kb in size, and contains 9 exons. The encoded 1α-hydroxylase is a 55 kDa protein that belongs to the cytochrome P450 family and consists of a heme binding site and 507 amino acids with a mitochondrial signal sequence in the N-terminal region (Fu et al., 1997). In vitro studies stated the incidence of rare gene SNPs in the *Cyp27b1* gene, which were associated by a loss of 1α-hydroxylase enzyme activity and reduced activated vitamin D/calcitriol levels (Wang et al., 1998; Wang et al., 2002; Ramagopalan et al., 2011). The rs118204009 *Cyp27b1* variant was first identified by the exome sequencing method in multiple sclerosis (MS) patients, followed by rs118204011 and rs118204012 (Ramagopalan et al., 2011). The rs118204009 is found in exon 7 of the *Cyp27b1* gene, and at position 389, a variation occurs from arginine to histidine (R389H). In rs118204011 polymorphism, at position 343 (L343F), a variation occurs from leucine to phenylalanine in exon 6, and in rs118204012, a variation takes place from glutamate to glycine at position 189 (E189G) in exon 3 of the protein (Wang et al., 1998).

The three *Cyp27b1* gene variants were significantly linked with multiple sclerosis risk in the Canadian population (Ramagopalan et al., 2011). On the contrary, a large cohort study conducted in the European population and another study in Italy didn't find any significant association, and all the study participants were carriers of wild-type alleles for those three gene polymorphisms (Wang et al., 1998; Agnello et al., 2017; Reinthaler et al., 2014). In tuberculosis, it has been stated that VDR gene polymorphisms are linked with faster sputum culture conversion. (Selvaraj et al., 2015). However, the association of *Cyp27b1* rs18204009/011/012 gene variants with tuberculosis was very meager or not yet reported globally.

In this study, we found that all the study participants were positive for the wild-type allele in rs118204009 (data not shown), which is similar to cohort studies conducted in the European population (Wang et al., 1998; Agnello et al., 2017). Contrary to European studies, we found homozygous "CC" as a major frequency genotype, heterozygous "CT" as a minor frequency genotype, and the absence of the "TT" genotype in the rs118204011 polymorphism. In the rs118204012 polymorphism, a frequent allele 'A' and an infrequent allele 'G' were found, with the major frequency genotype "AA", heterozygous genotype "AG," and minor frequency genotype "GG" in all study participants. When the frequencies of allele and genotype were related among the different groups, a significant association was found in allele 'A' [(OR): 1.52 (1.02–2.26); p = 0.044] and "AA" genotype [OR: 1.69(1.02–2.81); p = 0.040] with susceptibility to tuberculosis. Whereas, in the 'G' allele, a significant protective association was observed [OR: 0.66 (0.44–0.98); p = 0.044]. In genetic model analysis, a substantial link was detected towards protection in the dominant model (AA vs. AG + GG) based on the lower AIC and BIC values. However, no such result was found with the rs118204011 polymorphism. It has been reported that *Cyp27b1* gene variants may cause conformational changes in the enzyme, which could disrupt its binding efficiency with its substrate and may lead to a loss of enzyme function (Wang et al., 1998). This suggested that rs118204012 gene variants may interfere with Cyp27b1 enzyme activity, which could lead to susceptibility to tuberculosis.

A gender difference was stated to be linked with tuberculosis, which indicates the prominence of gender as a hazard element for TB (Peer

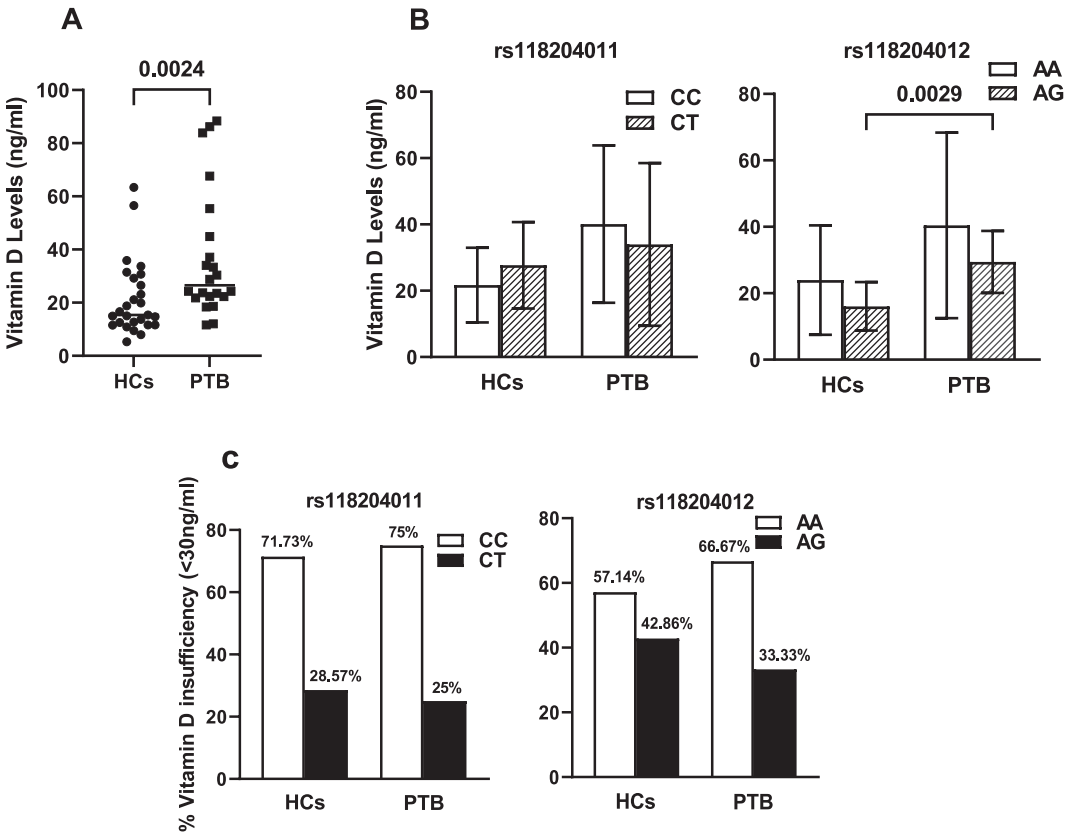

Fig. 1. A. Vitamin D levels among healthy controls (HCs) and pulmonary tuberculosis (PTB) patients Results are expressed as mean ± standard deviation (SD) and p value analyzed by the Mann-Whitney *U* test. B. Vitamin D levels among variant genotypes of Cyp27b1 gene rs118204011 and rs118204012 polymorphisms in HCs and PTB patients Results are expressed as mean ± SD and p value analyzed by the Mann-Whitney *U* test. C. Frequency of vitamin D insufficiency (<30 ng/ml) among variant genotypes of rs118204011 and rs118204012 polymorphisms in HCs and PTB patients.

et al., 2022). To find out if there were any differences between or among the genders, we stratified and examined the genotype frequencies by age adjusted. The findings showed that, when comparing males to females, the rs118204011 "CC" genotype was substantially associated with tuberculosis susceptibility in males [OR: 3.94 (1.94–7.98); p = 0.0002]. Likewise, in males, a noteworthy association was found with susceptibility to tuberculosis in the rs118204012 "AA" genotype when related to females [OR: 4.57 (2.13–9.79); p = 0.0001] and among the males [OR: 2.00 (1.04–3.84); p = 0.047]. These results were similar to our earlier study of *Cyp2r1* polymorphisms in the South Indian population (Harishankar et al., 2021). In addition to genetic factors, it has been suggested that social contacts, HIV comorbidity, and other hazard elements such as tobacco and liquor intake increase the probability of being susceptible to tuberculosis in males (Narasimhan et al., 2013).

Vitamin D levels were estimated in both study individuals and correlated with the gene variants. Similar to our earlier studies, 25(OH)D_3 levels were noted to be greater in PTB patients than in controls (Figure-1A; p = 0.0024). In addition, Taiwanese studies and those in Asian countries like Indonesia, China, South Korea, and Afghanistan reported similar results in patient groups (Harishankar et al., 2020). It has been stated that *M. tuberculosis*-infected antigen-presenting cells induced the expression of the *Cyp27b1* gene, thereby enhancing the production of the active vitamin D3 (Liu et al., 2006). Another reason might be the release of stored vitamin D from adipose tissue into the patient's blood (Sharma, 2000). As we reported earlier, down-regulated VDR expression in PTB patients may lead to defective signalling (Selvaraj et al., 2009). Since the anti-microbial peptide cathelicidin is up-regulated through VDR (Liu et al., 2006), the efficient clearance of the Mtb bacilli was ineffective in PTB patients.

In patients, higher 25(OH)D_3 levels were found when correlated with gene variants related to HCs. (Fig. 1B). In the rs118204012 "AG" genotype, a significantly higher level was found than in HCs (Fig. 1B; p = 0.0029). Further, the percentage of vitamin D insufficiency (<30 ng/ml) among the gene variants was analysed in both study groups. The results revealed that in the rs118204011 "CC" genotype, a greater proportion of 25(OH)D_3 insufficiency was noted in PTB patients (75 %), related to healthy controls (71.73 %), with an odds ratio (OR) of 1.2. Similarly, a higher vitamin D insufficiency was found with the rs118204012 "AA" genotype in PTB patients (66.67 %) than controls (57.14 %) with an OR of 1.5 and significantly linked with TB risk (Table 3). The latest study stated that vitamin D levels were considerably decreased in patients when related to the IFN-γ positive/negative groups and acted as a defensive cause for TB development. Moreover, the levels were significantly correlated with pro- and anti-inflammatory cytokines such as IFN-γ, TNF-α, IL17A, and IL-4 in PTB and IL-6 and G-CSF in the controls, which might be a probable biomarker for PTB and treatment observation (Moideen et al., 2023). It has been reported that DNA methylation is significantly linked with vitamin D levels in serum. Between TB and controls, there were 55 distinct CpG sites were found with varying levels of methylation; 41.5 % were in the *CYP27B1* gene. Moreover, 5.7 % of CpG sites were significantly associated with treatment outcomes (Wang et al., 2018). This suggests that the lower 25(OH)D_3 levels linked with rs118204011 "CC"/rs118204012 "AA" might have been hypermethylated related to "CT" and "AG" genotype-positive individuals in the CpG sites. The observational studies revealed that vitamin D scarcity is connected with reduced anti-TB response, treatment relapse, and a negative correlation with sputum culture conversion. However, discrepancies were found between clinical trials and studies using

observational methods because of inadequate vitamin D dosage and a small sample size (Rathored et al., 2012; Sato et al., 2012; Mehta et al., 2013). This study has limitations such as the absence of rs118204011 "TT" and the lower frequency of rs118204012 "GG" in our population. This could be due to ethnic variation among different populations. Moreover, a limited sample size could hamper the revealing of minor associations that need to be ruled out with a greater size to endorse the study findings. Due to the limited sample size, we were unable to categorize different vitamin D levels for analysis, instead, we clubbed together below sufficient levels (<30 ng/ml). A future study will be conducted with a bigger population size to rule out the limitations.

5. Conclusion

The rs118204012 "AA" genotype is significantly linked with susceptibility to tuberculosis. In the dominant model ("AA" vs. "AG + GG"), a substantial defensive link was noted with "AG" and "GG" related to the "AA" genotype. In addition, a higher vitamin D insufficiency was observed with the "AA" genotype in patients (66.67 %) than healthy controls (57.14 %), with a susceptible odds ratio of 1.5. This suggest that the gene variants may be involved in the downstream processing of serum Vitamin D levels and its association with the disease. Among the genders, males are found to be significantly more susceptible than females in the rs118204011 "CC" and rs118204012 "AA" genotypes.

CRediT authorship contribution statement

Murugesan Harishankar: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pavithra Sampath:** Methodology, Data curation. **A. Vamsi Kumar:** Methodology. **R. Srividhya:** Methodology. **Veerasamy Athikesavan:** Methodology. **Uma Devi Ranganathan:** Writing – review & editing, Resources. **Paramasivam Selvaraj:** Writing – review & editing, Conceptualization. **Ramalingam Bethunaickan:** Writing – review & editing, Validation, Supervision, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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