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Association of *CYP27B1* promoter gene variants of vitamin D pathway with pulmonary tuberculosis and vitamin D levels

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ABSTRACT

Cyp27b1 polymorphisms are stated to be associated with different diseases including tuberculosis (TB). Since the gene variants located in the promoter region may have a significant influence on gene transcription/translation and Cyp27b1 enzyme is involved in critical steps in vitamin D metabolism, we aim to study whether *Cyp27b1* gene promoter variants namely −1077 (C/G), −1260 (C/A) and the region immediately 5′ to the promoter −1918 (C/T) have any linkage with pulmonary tuberculosis risk/defence and to determine their influence on vitamin D level in normal healthy controls (HCs) and pulmonary tuberculosis (PTB) patients of the South Indian population. The polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method were used to genotype the genomic DNA after it was extracted using the salting-out approach. The Enzyme-Linked Immunosorbent Assay (ELISA) was used to measure the amount of vitamin D. In the co-dominant model, a significant association was detected with TB liability in the −1077 "GG" genotype [Odds ratio (OR): 2.10(1.18–3.73); p = 0.015]. In addition, a noteworthy linkage was detected with TB protection in the dominant model [GG vs CG + CC, OR: 0.40(0.21–0.75); p = 0.0035]. In the −1918 (C/T) variant, a substantial linkage was detected in the heterozygous −1918 "CT" genotype with TB risk [OR: 1.90 (1.05–3.44); p = 0.046] in co-dominant model, whereas a protective linkage was detected in less recurrent "TT" genotype [OR: 0.42 (0.19–0.94); p = 0.049] with TB. Furthermore, those risky genotypes are substantially linked with more TB risk in males than females. Strong links between −1077 and −1260 variations were revealed by haplotype analysis, and its haplotypes "GC" (−1077G, −1260C) were found to be significantly associated with increased TB risk. Vitamin D deficiency (<20 ng/ml) was detected at a higher frequency in PTB patients than HCs in −1077 "GG", −1260 "CA" and −1918 "CT" risky genotypes. This needs to be confirmed by bigger sample sizes in future research.

1. Introduction

Globally, tuberculosis (TB) continues to be the second leading cause of death. Since WHO began TB monitoring in 1995, the expected number of newly diagnosed cases of TB has increased to 7.5 million in 2022. In 2023, TB will be responsible for 1.25 million fatalities and 10.8 million established TB cases worldwide. India accounted for 26 % of all TB cases worldwide and is ranked first out of 30 countries with a high TB burden [1]. Genetic variables have been linked to either protection or risk for tuberculosis in case-control, twin, family-based, and genome-wide association studies (GWAS) [2–4]. Furthermore, it has been found that TB susceptibility is linked to nutritional inadequacies, including vitamin D [5,6]. Researchers have linked 25(OH)D levels to tuberculosis (TB) sickness through candidate gene association studies in the vitamin D receptor (VDR), vitamin D binding protein (VDBP), and vitamin D pathway genes [7,8]. The synthesis of active vitamin D_3 ($1,25(OH)_2D_3$) is a vital phase in the vitamin D pathway, which acts through the VDR and modulates gene expression through vitamin D-responsible elements (VDREs) [9]. The enzyme involved in the synthesis of active vitamin D_3 is 1α-hydroxylase, which is encoded by the *CYP27B1* gene,

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which is a member of the cytochrome P450 subfamily B, located on chromosome 12q14.1 and comprising 9 exons [10–13]. The translational product is 508 amino acids long, featuring a heme-binding site and a mitochondrial signal sequence in the *N*-terminal [14].

Cyp27b1 polymorphisms are specified to be associated with vitamin D levels and clinical outcome in different diseases [15] including tuberculosis. A study reported the modulation of vitamin D effect on TB sputum conversion by *Cyp27b1* gene variants, whereas a lack of TB association was reported in another study [16–18]. The rare non-synonymous variants of the *Cyp27b1* gene are associated with downregulated levels of active vitamin D and a decrease in the activity of the 1alpha-hydroxylase enzyme [19–21]. It has been suggested that abnormal or hypermethylation in the promoter genes that drive the vitamin D pathway may lower vitamin D levels and be linked to an increased risk of tuberculosis and other diseases [22,23]. Furthermore, the promoter gene variants downregulate vitamin D level by affecting Cyp27b1 expression through interaction with the transcription factors and cause immune dysfunction [24–26]. Globally there were very few or no linkage studies on *Cyp27b1* promoter/upstream gene variations in tuberculosis disease. The most prevalent variant in the *Cyp27b1* promoter gene that was examined was −1260(C/A), which has been linked to type 1 diabetes and autoimmune disorders [27–31]. Other studies stated that the −1260′C' allele has been linked to decreased Cyp27b1 mRNA levels, which in turn lower vitamin D levels in bone disease, diabetes and autoimmune diseases [19,32,33] while the 'A' allele is associated with protection in auto-immune disorders [34]. In addition, the −1077 "GG" genotype has been associated with lower vitamin D levels in lung disease [35].

We have examined the linkage between tuberculosis risk and genes related to the vitamin D receptor, vitamin D binding protein, and vitamin D pathway [36–40]. Recently, we have stated the linkage of three infrequent gene variations in the *Cyp27b1* gene such as rs118204009 (G/A), rs118204011 (C/T), and rs118204012 (A/G) with pulmonary tuberculosis (PTB). Specifically, the rs118204012 "AA" genotype is linked with insufficient vitamin D levels and an increased risk of tuberculosis [41]. However, *Cyp27b1* promoter gene polymorphisms have not yet been studied in our study population. Since the gene variants located in the promoter region may have a significant influence on gene transcription/translation and Cyp27b1 enzyme is involved in critical steps in vitamin D metabolism, we aim to study whether *Cyp27b1* gene promoter variants namely rs3782130 −1077 (C/G), rs10877012 −1260 (C/A) and the region immediately 5′ to the promoter −1918 (C/T) have any linkage with pulmonary tuberculosis risk/defence and to determine their influence with vitamin D level in normal healthy controls (HCs) and PTB patients of the South Indian population.

2. Materials and methods

2.1. Sample size

The Sample size was estimated based on the assumption from our previous studies and from others (39, 41, 42) using the online calculator https://riskcalc.org>samplesize or https://openepi.com>samplesize >SSCC. The assumptions were 2-side significance level (α) 5 % or 0.05, power 1-β (probability of detecting a real effect): 0.8, the ratio of case to control: 1, odds ratio: 2.1, probability of exposure in the control group (p_o): 0.4. Hypothetical proportion of cases with exposure (p1): 0.58. The required sample to achieve the above criteria with 80 % power was 116 (using Fleiss formula) and 117 (using Kelsey's formula) in each group.

2.2. Study participants

The study included 124 healthy individuals (55 male and 69 female, mean age ± Standard deviation (SD): 30.30 ± 9.04) and pulmonary tuberculosis patients (85 male and 39 female, mean age ± SD: 38.14 ± 11.43) from Chennai and surrounding areas aged between 18 and below 55 years and the demographic details were shown in Table 1. Individuals who are asymptomatic for TB with no past TB history, normal chest X-ray and found negative for Interferon Gamma Release Assay (IGRA) are categorized as healthy controls. PTB patients were registered at the ICMR-NIRT clinics and the District TB Centre in Puliyanthope, Chennai. PTB patients were recruited based on confirmed positive sputum smear and culture for *M. tuberculosis* (Mtb), along with characteristic clinical or atypical chest x-ray. Before therapy, blood was drawn from PTB patients at their baseline. Samples were excluded if individuals were found to have extrapulmonary TB, viral infections (HIV, HBV, or HCV), diabetes, autoimmune disorders, or psychiatric illnesses. Individuals undergoing anti-TB treatment or receiving immunosuppressive therapy were also excluded from the study. All members were given signed authorization for blood collection. The work was approved by the ICMR-NIRT ethical committee, Chennai (NIRT-IEC Number: 1/12/108/IEC/2016).

Table 1
Demographic details of the study subjects.

	Healthy Controls (HCs)	Pulmonary tuberculosis (PTB) patients
Number of subjects recruited	124	124
Sex (male/female)	55/69	85/39
Median Age (Interquartile range [IQR])	27(12.75)	38(15.25)

2.3. Genomic DNA separation

Genomic-DNA was extracted using a salting-out process from member's blood [43]. The DNA pellet was suspended in Tris-EDTA (TE) buffer, and its purity was evaluated using a Nanodrop "ND1000″ spectrophotometer. Finally, the DNA was kept at −80 °C until usage. The *Cyp27b1* gene variants rs3782130 −1077 (C/G), rs10877012 −1260 (C/A), and rs703842 −1918 (C/T) were genotyped using polymerase chain reaction continued with restriction fragment length polymorphism (PCR-RFLP) [42]. The amplicons and digested products were visualized using agarose gel electrophoresis and DNA ladders (New England Biolabs).

2.4. Cyp27b1 rs3782130 −1077 (C/G) and rs10877012 −1260 (C/A) genotyping

The primers used for both the polymorphisms are forward primer (FP), 5′-GTGTTCCCTAAGTGTTGTCTC-3′ and reverse primer (RP), 5′-GCTGACTCGGTCTCCTCTG-3′ to amplify the 666 base pair (bp) PCR product. The temperature used for the annealing condition was 62 °C. The rs3782130 −1077 (C/G) genotyping was carried out by using *TaqI* restriction enzyme (New England Biolabs), whereas *TfiI* restriction enzyme (New England Biolabs) was used for rs10877012 −1260 (C/A) polymorphism. Both digestion tests were conducted at 65 °C for an overnight incubation period. A 2 % agarose gel was used to separate the digested products [43].

In *Cyp27b1* −1077, the "CC" genotype was generated at 666 bp as an undigested product. The heterozygous "CG" genotype generated four bands at 666 bp, 435 bp, 181 bp, and 50 bp. The mutant homozygous "GG" genotype generated three bands at 435 bp, 181 bp, and 50 bp. The less frequent "AA" genotype of *Cyp27b1* −1260 generated an undigested product at 666 bp. The −1260 "CA" genotype generated three bands at 666 bp, 391 bp, and 275 bp. The sole digestive loci in the −1260 "CC" genotype generated bands at 391 bp and 275 bp.

2.5. Cyp27b1 rs703842 −1918 (C/T) genotyping

The PCR product 164 bp size was magnified using FP, 5′-GACAAGGTGAGAGGAGCCAG-3′ and RP, 5′-CTGGACCTCGTCTCCCAGGAA-3′. The annealing condition was carried out at 58℃. Following

amplification, the PCR product was verified by 2.5 % agarose gel electrophoresis and then further digested using *Tsp509I* enzyme (Thermofisher Scientific) for overnight incubation at 65 °C. A 3 % agarose gel was used to separate the digested products [43].

The recurrent homozygous genotype "CC" gave rise to one band of size at 164 bp, while the less recurrent homozygous "TT" genotype results in two bands of size at 145 bp and 19 bp. The heterozygous genotype "CT" generated three bands of sizes at 164 bp, 145 bp and 19 bp.

2.6. Serum vitamin D estimation

The circulating vitamin D levels of the enrolled participants were calculated from samples of preserved plasma utilizing the Epitope Diagnostics (EDI) total 25(OH) vitamin D enzyme immunoassay (EIA) kit (EDI, San Diego, CA 92121, USA) in compliance with the manufacturers guidelines. The assay procedure was briefly discussed earlier [41]. 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. Quality control procedures were conducted in accordance with the manufacturer's guidelines. The assay kit includes six calibrator standards—Cal-0 (0 ng/mL), Cal-1 (9.4 ng/mL), Cal-2 (18.8 ng/mL), Cal-3 (37.5 ng/mL), Cal-4 (75 ng/mL), and Cal-5 (150 ng/mL)—as well as two control samples (one positive and one negative). In addition, internal laboratory control samples with known 25-OH vitamin D concentrations were included in each run to ensure assay validity. Inter-assay variability was evaluated using three quality control samples measured across twelve independent assay runs, each performed in duplicate. The coefficient of variation (CV) ranged between 5.5 to 6.5 %, indicating good reproducibility of the 25-OH vitamin D ELISA assay. Softmax Pro was used to create the test sample data. The kit's vitamin D detection limits ranged from 0 to 150 ng/ml. The estimated vitamin D levels were then compared with respective gene variants.

2.7. Statistical approaches

The SNP stats online database was employed to determine the frequencies of alleles, genotypes, and haplotypes. Hardy-Weinberg equilibrium, and sex/age adjusted p-values with odds ratio (OR) using logistic regression in various genetic models [44]. *Epi* Info version 7.2.5 was employed to calculate 95 % confidence intervals (Cis) for the OR and Yates adjusted p-value. The Bayesian information criterion (BIC) and Akaike information criterion (AIC) with the lowest scores were used to choose the best genetic model. Based on this criteria, the best fitting model for −1077 variant was a dominant model (AIC:302.1, BIC:316.1) followed by the co-dominant model (AIC: 303.9, BIC:321.5). In −1918 polymorphism, best model found was a co-dominant model (AIC:301.2, BIC:318.8) followed by the recessive model (AIC:301.8, BIC:315.8) and over dominant model (AIC: 301.8, BIC:315.9). Haploview version 4.2 was used to calculate and construct linkage disequilibrium (D') and blocks. Haploview analysis was utilized to compute haplotype frequencies across the study groups. The normal distribution of the study data was investigated using the Shapiro-Wilk test. Vitamin D levels were assessed using the Wilcoxon signed rank test and the Mann-Whitney *U* test for the same and different groups. A significant linkage was described as a p-value ≤ 0.05.

3. Results

3.1. Linkage of Cyp27b1 promoter gene variants with TB

The *Cyp27b1* promoter −1077 "GG" genotype detects greater frequency (HCs: 50 %; PTB: 41.9 %) followed by "CC" (HCs: 29.8 %; PTB: 23.4 %) and "GG" (HCs: 20.2 %; PTB: 34.7 %) genotypes. A substantial TB hazard was detected with allele 'G' [Odds ratio (OR): 1.52 (1.07–2.17); p = 0.025]. In the co-dominant model, an identical significant association was detected with TB liability in the "GG" genotype [Odds ratio (OR): 2.10(1.18–3.73); p = 0.015]. In addition, a noteworthy linkage was detected by TB defence in the dominant model [GG vs CG + CC, OR: 0.40(0.21–0.75); p = 0.0035] (Table 2).

The allele 'C' (HCs: 73 %; PTB: 77 %) and "CC" genotype (HCs: 63.9 %; PTB: 61.3 %) of *Cyp27b1*-1918 (C/T) gene variants observed greater frequency in both the study participants. The occurrence of "TT" and "CT" genotypes was 17.2 % and 18.9 % in HCs, while 8.1 % and 30.6 % in PTB patients. In the co-dominant model, a substantial linkage was detected in the heterozygous "CT" genotype with TB risk [OR: 1.90 (1.05–3.44); p = 0.046], whereas a protective linkage was detected in the less recurrent "TT" genotype [OR: 0.42 (0.19–0.94); p = 0.049] with TB. In addition, a similar significant defensive and risky linkage was detected with TB in the recessive model (CC + CT vs. TT) [OR: 0.43 (0.18–1.01); p = 0.046] and the overdominant model (CC + TT vs CT) [OR: 1.93 (1.00–3.72); p = 0.047] (Table 2).

In *Cyp27b1*-1260(C/A) gene polymorphism, allele 'C' was detected at greater occurrence (HCs: 54 %; PTB: 53 %) than the 'A' allele (HCs: 46 %; PTB: 47 %), but no linkage was detected with TB in the gene variants (Table 2).

3.2. Linkage of Cyp27b1 promoter gene variants between/within the sex

The gene variants were categorized based on sex and examined between/within the sex using SNP stats and EpiInfo version 7.2.5. In general, the male individuals were detected as more vulnerable than the female individuals with TB. In *Cyp27b1* −1077(C/G), a substantial linkage was detected with TB liability in the "GC" [OR: 3.97(1.73–9.13); p = 0.001] genotype of males related to females (Table 3). When the gene variants were examined within the sex, a substantial linkage was detected in female individuals with TB liability in the −1077 "GG" genotype [OR: 2.84(1.22–6.59); p = 0.024], and a similar TB hazard odds ratio [OR: 2.12(0.91–5.00); p = 0.119] was detected in males but not found significant (Table 4).

The genotypes "AC" [OR: 3.99(1.72–9.26); p = 0.002] and "AA" [OR: 3.52(1.21–10.24); p = 0.037] of −1260(C/A) variants were substantially linked with TB liability in males related to females (Table 3). However, no such linkage was detected within the sex **(**Table 4**)**.

In −1918(C/T) polymorphism, a significant linkage was detected with TB in the "CC" genotype [OR: 2.93(1.52–5.66); p = 0.002] of males related to females. In addition, a trend towards TB liability was detected in the heterozygous "CT" genotype [OR: 2.95(0.97–8.95); p = 0.096] of males interrelated with females **(**Table 3**)**. When the gene variants were examined within the sex, a defensive TB linkage was detected in the "TT" genotype of males [OR: 0.30(0.09–0.97); p = 0.045]. A vulnerable odds ratio was detected with TB in the "CT" genotype [male, OR: 1.77 (0.81–3.87); female, OR: 1.58(0.59–4.24)], but an insignificant p-value was attained in both sexes **(**Table 4**)**.

3.3. Linkage of Cyp27b1 haplotype frequencies with TB

Haplotype frequencies of *Cyp27b1* gene polymorphisms were examined by SNP stats online tool to observe any substantial linkage among the three gene variants with TB. The results revealed that the haplotype "CCG" [-1918C, −1260C and −1077G] was substantially linked with TB liability [OR: 10.26 (1.29–81.48); p = 0.017]. In other haplotype combinations no such linkage was detected with TB **(**Table 5**)**.

3.4. Haploview and linkage disequilibrium (LD)

A linkage disequilibrium plot was constructed and analyzed using Haploview software version 4.2. Among the three gene variants, −1077 (C/G) and −1260 (C/A) were found to have a strong linkage

Table 2
Association of Allele/Genotype, genetic models frequencies and vitamin D status of Cyp27b1 promoter polymorphisms in healthy controls (HCs) and pulmonary tuberculosis (PTB) patients.

SNP	Allele/Genotypes	PTB (n=124)	HCs (n=124)	OR (95% CI)	p-value
	Alleles				
rs3782130	G	0.56 (138)	0.45 (112)	**1.52 (1.07–2.17)**	**0.025**
-1077 (C/G)	C	0.44 (110)	0.55 (136)	1	
	Co-dominant model				
	GG	0.347 (43)	0.202 (25)	**2.10 (1.18–3.73)**	**0.015**
	GC	0.419 (52)	0.500 (62)	0.72 (0.44–1.19)	0.251
	CC	0.234 (29)	0.298 (37)	0.72 (0.41–1.26)	0.314
	Dominant model				
	GG	0.347 (43)	0.202 (25)	1	**0.0035**
	CG+CC	0.653 (81)	0.798 (99)	**0.40 (0.21–0.75)**	
	Overdominant model				
	GG+CC	0.581 (72)	0.500 (62)	1	0.055
	CG	0.419 (52)	0.500 (62)	0.58 (0.33–1.02)	
Vitamin D Status among genotypes	**Vitamin D Levels**		**Vitamin D Deficiency**		
	Median [IQR]		**(<20ng/ml)**		
GG	**PTB**	**HCs**	**Median [IQR]**		
GC	35.41[31]	15.22[14]	**8.1 [4.8]**	**11.6 [0.75]**	**1.33(0.20–8.71)**
CC	25.55[28]	16.60[14]	12.5[2.5]	12.6[6]	0.92(0.19–4.54)
	34.02[22]	17.99[10]	17.5[1.1]	16.3[3]	0.86(0.14–5.31)
	Alleles				
rs10877012	C	0.53 (132)	0.54 (134)	1	0.928
-1260 (C/A)	A	0.47 (116)	0.46 (114)	1.03 (0.72–1.47)	
	Genotypes				
	CC	0.323 (40)	0.306 (38)	1.08 (0.63–1.84)	0.891
	AC	0.419 (52)	0.468 (58)	0.82 (0.50–1.36)	0.523
	AA	0.258 (32)	0.226 (28)	1.19 (0.66–2.13)	0.656
Vitamin D Status among genotypes	**Vitamin D Levels**		**Vitamin D Deficiency**		
CC	**Median [IQR]**		**(<20ng/ml)**		
AC	**PTB**	**HCs**	**Median [IQR]**		
AA	35.8[29]	17.5[20]	17.5[1.1]	7.99[5]	0.86(0.14–5.31)
	25.5[28]	16.6[12]	8.1[5]	11.6[4]	1.27(0.26–6.27)
	24.1[28]	17.7[14]	12.5[2.5]	13.7[4]	0.86(0.14–5.3.1)
	Alleles				
-1918 (C/T)	C	0.77 (190)	0.73 (179)	1.19 (0.79–1.79)	0.466
	T	0.23 (58)	0.27 (65)	1	
	Co-dominant model				
	CC	0.613 (76)	0.639 (78)	**1**	
	CT	0.306 (38)	0.189 (23)	**1.90 (1.05–3.44)**	**0.046**
	TT	0.081 (10)	0.172 (21)	**0.42 (0.19–0.94)**	**0.049**
	Recessive model				
	CC+CT	0.919 (114)	0.828 (101)	**1**	**0.046**
	TT	0.081 (10)	0.172 (21)	**0.43 (0.18–1.01)**	
	Overdominant model				
	CC+TT	0.693 (86)	0.812 (99)	**1**	**0.047**
	CT	0.306 (38)	0.189 (23)	**1.93 (1.00–3.72)**	
Vitamin D Status among genotypes	**Vitamin D Levels**		**Vitamin D Deficiency**		
CC	**Median [IQR]**		**(<20ng/ml)**		
CT	**PTB**	**HCs**	**Median [IQR]**		
TT	29.55[32]	14.90[13]	12.92[5]	11.63[6]	0.69(0.13–3.68)
	28.14[18]	14.73[13]	**7.90**[6]	**12.89**[3]	**1.80(0.33–9.89)**
	37.50	29.46[8]	–	17.69	undefined

n = number of individuals. IQR- Interquartile range. Numbers in parenthesis indicates number of individuals positive for that genotype. OR & p-value estimated using *epi*-info software v 7.2.5.
In −1077: dominant model (AIC:302.1, BIC:316.1); co-dominant model (AIC: 303.9, BIC:321.5). In −1918: co-dominant model (AIC: 301.2, BIC: 318.8); recessive model (AIC: 301.8, BIC: 315.8); over dominant model (AIC: 301.8, BIC: 315.9).

disequilibrium (D') value of 93 % and absence of LD with *Cyp27b1* −1918(C/T) variant in the 5′ to the promoter. The haplotype "GC" [-1077G; −1260C] is substantially linked with the TB risk odds ratio [OR: 11.39(1.45–89.66; p = 0.000054)] and a chi-square value of 16.3. Moreover, the haplotype "CA" [-1077C; −1260A] is substantially linked with the TB defense odds ratio [OR: 0.23(0.03–2.1); p = 0.008] and a chi-square value of 7.046 **(**Table 6**)**.

3.5. Linkage of Cyp27b1 promoter gene variants with vitamin D deficiency

Similar to our earlier study, vitamin D levels were detected higher in patients (data not shown) when compared with healthy controls [41]. Among the *Cyp27b1* gene variants, genotypes −1077 "GG", −1260 "CA" and −1918 "CT" detected lower vitamin D levels compared with other gene variants in both study groups, while significant differences were absent **(**Fig. 1A, 1B and 1C**)**.

Further, we analysed the frequency of vitamin D deficiency (<20 ng/ml) **(**Fig. 1D**)** and median interquartile range [IQR] among those genotypes **(**Table 2**)**. We detected a higher percentage of vitamin D deficiency in PTB patients with disease risk odds ratio (OR) compared with HCs in −1077″GG" [HCs: 20 %, PTB: 25 %; OR: 1.33(0.20–8.71)], −1260 "CA" [HCs: 44 %, PTB: 50 %; OR: 1.27 (0.26–6.27)] and −1918 "CT" [HCs: 25 %, PTB: 37.5 %; OR: 1.80 (0.33–9.89)]. Similarly, the median [IQR] levels were detected lower in patient groups in −1077"GG" (HCs: 11.6 [0.75]; PTB: 8.1 [4.8]), −1260 "CA" (HCs: 11.6 [4]; PTB: 8.1 [5] and −1918 "CT" (HCs: 12.89 [3]; PTB: 7.90 [6]) genotypes, while no such result was detected in other gene variants

Table 3
Association of Cyp27b1 promoter polymorphisms between the sex in HCs and PTB patients.

Genotypes	Sex	PTB (n = 124)	HCs (n = 124)	OR (95% CI)	p-value
-1077 (C/G)	Female	18	16	1.00	0.130
GG	Male	25	9	2.47 (0.89-6.83)	
GC	Female	11	32	1.00	**0.001**
	Male	41	30	**3.97 (1.73-9.13)**	
CC	Female	10	21	1.00	0.121
	Male	19	16	2.5 (0.91-6.81)	
-1260 (C/A)	Female	16	20	1	0.373
CC	Male	24	18	1.66 (0.68-4.09)	
AC	Female	11	30	1	**0.002**
	Male	41	28	**3.99 (1.72-9.26)**	
AA	Female	12	19	1	**0.037**
	Male	20	9	**3.52 (1.21-10.24)**	
-1918 (C/T)	Female	51	32	1	**0.002**
CC	Male	25	46	**2.93 (1.52-5.66)**	
CT	Female	29	12	1	0.096
	Male	9	11	**2.95 (0.97-8.95)**	
TT	Female	5	9	1	1
	Male	5	12	1.33 (0.29-6.04)	

n = number of individuals.

(Fig. 1D **and** Table 2**).**

4. Discussion

Numerous genetic research, including case-control, GWAS, and twin studies, have demonstrated the connection between genetic variants and tuberculosis defense/vulnerability in different ethnic communities [2–4]. A person's nutritional status, including their level of vitamin D, is one of the elements that influences their vulnerability to tuberculosis. Variants in vitamin D-related genes in VDR, VDBP and pathway genes have been found to be linked to deficit vitamin D levels and the risk of PTB [7,8,39,40]. One of the most important stages of active vitamin D production involves the enzyme 1α-hydroxylase, which is encoded by the *Cyp27b1* gene on chromosome 12q14.1 and consists of 9 exons [10,11,13]. The active vitamin D [1, 25-dihydroxyvitamin D3] enhances macrophages antibacterial capacity and destroys intracellular Mtb [45]. Furthermore, vitamin D boosted the anti-inflammatory cytokine response and decreased the T helper 1 (Th1) reaction in acquired immunity [46].

Variants in the *Cyp27b1* gene have been linked with various diseases [15] including tuberculosis. An investigation found that, the *Cyp27b1* intronic gene mutation accelerated the impact of vitamin D on TB patients sputum conversion [16]. We recently found that the non-synonymous mutation rs118204012 'AA' genotype is linked with insufficient vitamin D levels and tuberculosis risk [29]. Rare non-synonymous variations of the *Cyp27b1* gene are also associated with downregulated levels of active vitamin D and a decrease in the activity of 1alpha-hydroxylase enzyme [19–21]. Besides, *Cyp27b1* promoter variants have been linked with autoimmune diseases and type 1 diabetes [27–31]. However, linkage studies on *Cyp27b1* promoter/upstream gene variations in tuberculosis disease were either rare or non-existent. Since promoters are crucial regulatory sites for transcription/translation by binding with regulatory proteins and have an impact on gene function [47], we want to look into the relationship between *Cyp27b1* gene promoter variants and pulmonary tuberculosis risk/defense, as well as their effect on levels of vitamin D.

The findings of our investigation showed a significant association with TB risk in the co-dominant model for the −1077G allele [OR: 1.52

Table 4
Association of Cyp27b1 promoter polymorphisms within the sex in HCs and PTB patients.

Sex	Genotypes	PTB n = 124	HCs n = 124	OR(95% CI)	p-value
-1077(C/G)	GG	18	16	**2.84 (1.22–6.59)**	**0.024**
Female	CG	11	32	0.45 (0.19–1.05)	0.099
	CC	10	21	0.78 (0.33–1.90)	0.758
Male	GG	25	9	2.12 (0.91–5.00)	0.119
	CG	41	30	0.77 (0.40–1.53)	0.578
	CC	19	16	0.70 (0.32–1.52)	0.484
-1260 (C/A)	CC	16	20	1.70 (0.75–3.88)	0.288
Female	AC	11	30	0.51 (0.22–1.19)	0.172
	AA	12	19	1.17 (0.49–2.77)	0.892
Male	CC	24	18	0.81 (0.39–1.68)	0.706
	AC	41	28	0.90 (0.45–1.77)	0.892
	AA	20	9	1.57 (0.66–3.76)	0.419
-1918 (C/T)	CC	25	46	0.89 (0.39–2.03)	0.953
Female	CT	9	11	1.58 (0.59–4.24)	0.51
	TT	5	12	0.70 (0.23–2.15)	0.725
Male	CC	51	32	0.98 (0.49–1.98)	1
	CT	29	12	1.77 (0.81–3.87)	0.214
	TT	5	9	**0.30 (0.09–0.97)**	**0.045**

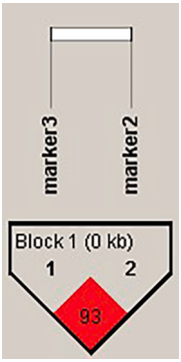
n = number of individuals.

Table 5
Association of −1918(C/T), -1260(C/A) and −1077(C/G) haplotypes with pulmonary tuberculosis.

−1918	−1260	−1077	Controls n = 118	Patients n = 124	Odds Ratio (95 % CI)	p-value
C	A	G	0.3802 (45)	0.3749 (46)	1.00	–
C	C	C	0.3313 (39)	0.3146 (39)	0.93 (0.54–1.59)	0.897
T	C	C	0.1939 (23)	0.1168 (14)	0.52 (0.25–1.08)	0.111
T	A	G	0.0560 (7)	0.1049 (13)	1.86 (0.71–4.83)	0.293
C	C	G	0.0087 (1)	0.0766 (10)	**10.26 (1.29–81.48)**	**0.017**
T	A	C	0.0171 (2)	0	0.00 (inf-inf)	0.456
C	A	C	0.0128 (1)	0	0.00 (inf-inf)	0.980
T	C	G	0	0.0121 (2)	0.00 (inf-inf)	0.499

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

Table 6
Linkage disequilibrium among −1077(C/G) and −1260(C/A) promoter gene variants and its haplotypes association with pulmonary tuberculosis.



Marker 3: −1077(C/G); Marker 2: −1260(C/A)
Haploview analysis

Haplotype	Freq.	Case, Control Ratio Counts	Case, Control Frequencies	Odds Ratio (95 % CI)	Chi Square	p- Value
CC	0.476	106.8: 141.2, 123.8: 112.2	0.431, 0.525	0.67(0.41–1.12)	4.287	0.0384
GA	0.458	118.8: 129.2, 102.8: 133.2	0.479, 0.436	1.19 (0.72–1.98)	0.915	0.3388
GC	0.05	22.2: 225.8, 2.2: 233.8	0.090, 0.009	**11.39 (1.45–89.66)**	16.293	**5.43E-05**
CA	0.015	0.2: 247.8, 7.2: 228.8	0.001, 0.030	**0.23 (0.03–2.1)**	7.046	**0.008**

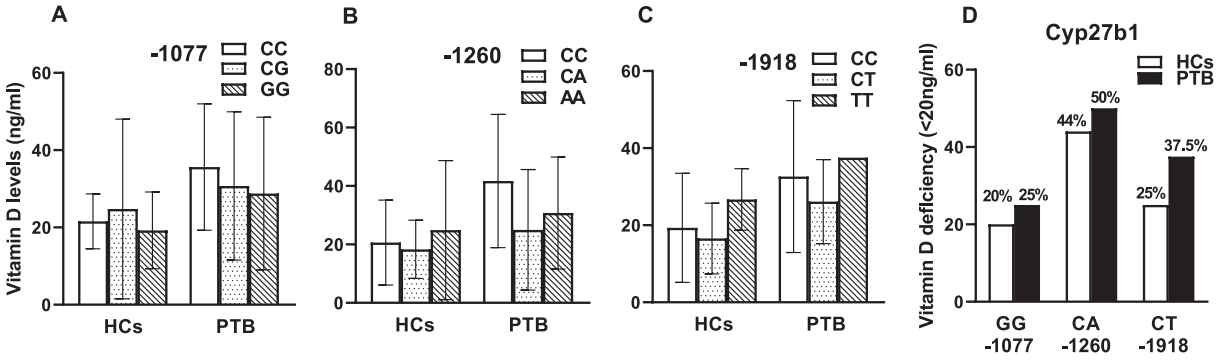


Fig. 1. A–C. Vitamin D levels (ng/ml) among *Cyp27b1* gene polymorphisms in healthy controls (HCs) and pulmonary tuberculosis (PTB) patients. Results are expressed as mean ± standard deviation (SD). **D**. Frequency of vitamin D deficiency (<20 ng/ml) among variant genotypes of *Cyp27b1* polymorphisms in HCs and PTB patients.

(1.07–2.17); p = 0.025] and the −1077 "GG" genotype [OR: 2.10 (1.18–3.73); p = 0.015]. Furthermore, in the dominant model, a substantial protective association was found [GG vs CG + CC, OR: 0.40 (0.21–0.75); p = 0.0035] (Table 2). The heterozygous "CT" genotype in the −1918 variation was significantly associated with TB risk in both the over-dominant model [CC + TT vs. CT, OR: 1.93 (1.00–3.72); p = 0.047] and the codominant model [OR: 1.90 (1.05–3.44; p = 0.046]. However, in the −1918 "TT" genotype, a substantial defensive linkage was found in the recessive model [CC + CT vs. TT, OR: 0.43 (0.18–1.01); p = 0.046] and codominant model [OR: 0.42 (0.19–0.94); p = 0.049] (Table 2). A study found that the −1077 "GG" genotype was associated with reduced Cyp27b1 expression compared to the −1077 "CC" genotype [35] and this variation is an interactive location for the nuclear factor-1 (NF-1) transcription factor, which interacts with other co-activators to control target gene transcription [24,25]. The −1260 "C" allele, another promoter variant, has been implicated with significant functional variation in 1α-hydroxylase activity and immunological activities. This allele modifies the consensus sequence in the *CYP27B1* promoter from TATTT to TCTTT, which is the binding location for the transcription factor caudal type homeobox 2 (CDX2) [26]. Another study stated that the −1260 "CC" genotype contributes to immunological dysfunction by lowering calcitriol levels in *T*-cells and monocytes [48]. In our study, we found a lack of linkage in this promoter variation. A similar absence of linkage was found in diabetes patients of South Asians and Europeans [49]. The substantial risk/defensive linkage found in *Cyp27b1* promoter gene variants in this study might be connected to the varying expression of the *Cyp27b1* gene, which would alter immunological functions.

Haplotype analysis showed that the combination of "CCG" (−1918 "C," −1260 "C," and −1077 "G") was associated with a higher risk of tuberculosis (OR: 10.26 (1.29–81.48); p = 0.017) (Table 5). Additionally, haploview analysis detected a strong link between the −1077 and −1260 promoter variants, with a linkage disequilibrium (D') score of 93. The haplotypes "GC" (−1077 'G', −1260 'C') and "CA" (−1077 'C', −1260 'A') were associated with higher risk [OR: 11.39 (1.45–89.66); p = 5.43E-05] and defensive odds ratios for TB [OR: 0.23 (0.03–2.1); p = 0.008] (Table 6). In addition to TB, the studies stated the strong linkage disequilibrium of *Cyp27b1* haplotypes and its combination with *VDR* gene variants with respiratory, auto-immune and cardiovascular disease risk [43,50,51]. It has been stated that the risk promoter haplotypes may cause significant functional differences in *Cyp27b1* expression and variation in 1α-hydroxylase activity [43]. Moreover, −1260C may cause changes in the CDX2 transcription factor binding site which could play an important role in intestine-specific *VDR* gene expression and immune functions [26].

When the analysis was done within the sex, the −1077 "GG"

genotype was associated with a disease risk odds ratio in males [OR: 2.12 (0.91–5.00)], while a substantial strong linkage was found in females [OR: 2.84 (1.22–6.59); p = 0.024]. On the other hand, males with the −1918 "TT" genotype showed a strong defensive linkage [OR: 0.30 (0.09–0.97); p = 0.045] and a similar trend in females [OR: 0.70 (0.23–2.15)] **(**Table 4**)**. According to sex-stratified analysis among the sex, males were more at risk than females in −1077 "GC" [3.97 (1.73–9.13); p = 0.001], −1260 "AC" [3.99 (1.72–9.26); p = 0.002] & "AA" [OR: 3.52(1.21–10.24); p = 0.037] and −1918 "CC" [2.93 (1.52–5.66); p = 0.002] & "CT" [2.95 (0.97–8.95)] genotypes **(**Table 3**)**. This observation was similar to our earlier studies on *Cyp2r1* and rare nonsynonymous *Cyp27b1* gene variants in TB [28,29]. Besides genetic factors, other aspects that increase the risk of tuberculosis in men include lifestyle choices, social contacts, co-existence of HIV and intake of liquor and tobacco [52].

As we previously reported, vitamin D levels were found to be higher in the patient group [40,41]. Various studies conducted in Taiwanese and Asian countries stated the similar result in patients [39]. According to research findings, vitamin D deficiency has been linked to tuberculosis vulnerability in healthy individuals [6]. When an active disease develops in an individual, Mtb infection may increase the expression of Cyp27b1 through the toll-like receptor in antigen-presenting cells, thereby raising vitamin D levels [53]. Furthermore, patients may have higher blood vitamin D levels due to excretion of accumulated vitamin D from adipose tissue [54]. Our previous study [55] indicated that patients had reduced expression of VDR, which may result in impaired generation of antimicrobial cathelicidin and ineffective clearance of Mtb by macrophages [53].

Promoter gene variants correlation with vitamin D levels not found significant variations among the genotypes. However, the levels were detected lower in the risky genotypes such as −1077″GG", −1260″CA" and −1918″CT" **(**Fig. 1A-1C**)** in both the study groups. Further, we analysed the percentage of vitamin D deficiency (<20 ng/ml) among the genotypes in healthy and patient group **(**Fig. 1D**)**. The results revealed a higher percentage of vitamin D deficiency in patients in those risky genotypes [-1077″GG": HCs: 20 %, PTB: 25 %; −1026″CA": HCs: 44 %, PTB: 50 %; −1918″CT": HCs: 25 %, PTB: 37.5 %] with disease risk odds ratio. Similar result was detected in median [IQR] levels (−1077"GG" (HCs: 11.6 [0.75]; PTB: 8.1 [4.8]), −1260 "CA" (HCs: 11.6 [4]; PTB: 8.1 [5] and −1918 "CT" (HCs: 12.89 [3]; PTB: 7.90 [6]), while no such result was observed in other gene variants **(**Table 2**)**. Other studies stated that the −1260'C' allele has been linked to decreased Cyp27b1 mRNA levels, which in turn lower vitamin D levels [19,32,33]. Moreover, the −1077 "GG" genotype has been associated with lower vitamin D levels than the "CC" genotype in lung disease [35]. The molecular mechanisms explored that the promoter gene variants downregulate vitamin D levels by affecting Cyp27b1 expression through interaction with the transcription factors and cause immune dysfunction [24–26]. Furthermore, *Cyp27b1* promoter hypermethylation reported to be connected with lower vitamin D levels [22,23]. A study revealed 55 differently methylated cytosine-phosphate-guanine (CpG) spots among TB cases and controls, 41.5 % were found to be in the *Cyp27b1* gene. In addition, 5.7 % of CpG locations were associated with TB treatment outcome [22]. However, the study design, limited sample size, and variable in vitamin D intake were the reasons for the discrepancy between observational and clinical investigations [56–58]. Limitations of this study includes the lower frequency of the 1918 "TT" genotype and the short sample size, which may impede tiny links. Future work with a higher sample size will be supportive to overcome this constraints.

5. Conclusion

In conclusion, the promoter variations of the −1077 "GG" and −1918 "CT" genotypes were found to be significantly associated with TB risk in the co-dominant model and to be linked to a higher level of vitamin D deficiency in patients. Additionally, such genotypes were associated with a higher risk of tuberculosis in men than in women. Strong links between −1077 and −1260 variations were revealed by haplotype analysis, and its haplotypes "GC" (−1077G, −1260C) were found to be significantly associated with increased TB risk. This implies that variants in the *Cyp27b1* promoter gene may contributes to the downstream processing of vitamin D levels and are linked to an increased risk of tuberculosis.

Ethical approval

This study was approved by the Institutional Ethics Committee ICMR-NIRT, Chennai, Reference no. 1/12/108/JMI/IEC/2016. Informed consent was obtained from all participants. Institutional manuscript committee approval ID no: NIRT-RIC-25-23, 21-04-2025.

CRediT authorship contribution statement

Harishankar Murugesan: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pavithra Sampath:** Methodology, Data curation. **Karthikeyan Ramamurthy:** Methodology. **Aarti Muralitharan:** Methodology. **Dhanyaa Muthukumaran:** Methodology. **Athikesavan Veerasamy:** Methodology. **Uma Devi Ranganathan:** Writing – review & editing, Resources, Funding acquisition. **Selvaraj Paramasivam:** Writing – review & editing. **Ramalingam Bethunaickan:** Writing – review & editing, Visualization, Supervision, Investigation, Conceptualization.

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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.

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Data availability

The data that has been used is confidential.

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