J Clin Immunol (2009) 29:470–478
DOI 10.1007/s10875-009-9277-9

Plasma 1,25 Dihydroxy Vitamin D_3 Level and Expression of Vitamin D Receptor and Cathelicidin in Pulmonary Tuberculosis

P. Selvaraj · S. Prabhu Anand · M. Harishankar · K. Alagarasu

Received: 14 October 2008 / Accepted: 26 January 2009 / Published online: 14 February 2009

Abstract
Introduction Vitamin D_3, which exerts its effect through vitamin D receptor (VDR), is known for its potent immunomodulatory activities. Associations between low serum vitamin D_3 levels and increased risk of tuberculosis have been reported.
Study Subjects and Methods Plasma 1,25 dihydroxy vitamin D_3 levels ($1,25(OH)_2$ D_3) and ex vivo levels of VDR protein from peripheral blood mononuclear cells were studied in 65 pulmonary tuberculosis (PTB) patients and 60 normal healthy subjects (NHS) using enzyme-linked immunosorbent assay-based methods. Using real-time polymerase chain reaction (PCR), induction of VDR, cathelicidin, and CYP27B1 mRNA were studied in live *Mycobacterium tuberculosis*-stimulated macrophage cultures treated with or without 1,25 dihydroxy vitamin D_3. VDR and CYP27B1 (-1077 A/T) gene polymorphisms were studied using PCR-based methods.
Results $1,25(OH)_2$ D_3 were significantly increased ($p=0.0004$), while ex vivo levels of VDR protein were significantly decreased in PTB patients ($p=0.017$) as compared to NHS. $1,25(OH)_2$ D_3 levels were not different between variant genotypes of CYP27B1. A trend towards decreased levels of VDR protein was observed among NHS with *Bsm*I BB and *Taq*I tt genotypes compared to NHS with other genotypes. Relative quantification of mRNA using real-time PCR revealed increased VDR mRNA expression in live *M. tuberculosis*-stimulated culture in PTB patients ($p<0.01$) than normal healthy subjects. Cathelicidin mRNA expression was significantly increased in vitamin D_3-treated cultures compared to unstimulated and *M. tuberculosis*-stimulated culture in both patients ($p<0.001$) and NHS ($p<0.05$).
Conclusions The present study suggests that PTB patients may have increased $1,25(OH)_2$ D_3 levels, and this might lead to downregulation of VDR expression. Decreased VDR levels could result in defective VDR signaling. Moreover, addition of $1,25(OH)_2$ D_3 might lead to increased expression of cathelicidin which could enhance the immunity against tuberculosis.

Keywords 1,25 Dihydroxy vitamin D_3 · vitamin D receptor · cathelicidin · tuberculosis

Introduction

Tuberculosis is a global emergency, and one third of the world population is estimated to be latently infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis. High rates of *M. tuberculosis* (MTB) infection and vitamin D deficiency have been reported in African immigrants in Australia [1], the USA [2], and Europe [3]. An association between 25 hydroxy vitamin D_3 levels and tuberculosis (TB) has been described in several case-control studies [4–6].

1,25 Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is synthesized from 7-dehydrocholesterol in the skin by exposure to ultraviolet rays (200–300 nm) from the sun. Biological activation of vitamin D involves firstly 25-hydroxylation, followed by 1α-hydroxylation to synthesize $1,25(OH)_2$ D_3. In humans, a hepatic mitochondrial hydroxylase enzyme named CYP27A is involved in the 25 hydroxylation. 25-Hydroxyvitamin D_3 is the major circulating vitamin D

P. Selvaraj (✉) · S. Prabhu Anand · M. Harishankar · K. Alagarasu
Department of Immunology, Tuberculosis Research Centre, Indian Council of Medical Research, Mayor V.R. Ramanathan Road, Chetput, Chennai 600 031, India
e-mail: p.selvaraj53@yahoo.com

metabolite and is modified by 1α-hydroxylation catalyzed by 1α hydroxylase coded by CYP27B1 gene to produce hormonal 1,25$(OH)_2$ D_3. While kidneys represent a major site of 1α-hydroxylation of 25-hydroxyvitamin D_3, activated macrophages and dendritic cells also express CYP27B1 [7]. Studies have shown that Toll-like receptor (TLR) stimulation of human macrophages induces expressions of CYP27B1, vitamin D receptor (VDR), and relevant downstream targets of VDR (including cathelicidin, antimicrobial peptide gene) [8]. Polymorphisms in the CYP27B1 gene have been shown to be associated with susceptibility to autoimmune diseases [9, 10] and might also influence endogenous levels of 1,25 $(OH)_2$ D_3.

1,25$(OH)_2$ D_3, the active metabolite of vitamin D, traditionally known for the maintenance of mineral homeostasis, is being increasingly recognized for its potent antiproliferative, prodifferentiative, and immunomodulatory activities [11]. Recent studies have shown that 1,25$(OH)_2$ D_3 induces the expression of antimicrobial peptide cathelicidin which restricts the growth of *M. tuberculosis* in monocytes under in vitro culture conditions [12]. Our studies on 1,25 $(OH)_2$ D_3 have shown that vitamin D_3 could enhance macrophage phagocytosis of live *M. tuberculosis* and spontaneous lymphocyte proliferation, while it might suppress *M. tuberculosis* culture filtrate antigen-induced lymphocyte proliferation in normal healthy subjects [13]. Moreover, our study also revealed that 1,25$(OH)_2$ D3 differentially modulates production of cytokines in response to *M. tuberculosis* antigens by predominantly suppressing IL-12p40 and IFN-γ production in a dose-dependent manner [14]. A more recent trial of vitamin D supplementation in Indonesian pulmonary TB patients has reported more rapid sputum clearance of acid-fast bacilli and radiological improvement [15].

Vitamin D_3 exerts its effect through vitamin D receptor. A polymorphism in the 5′ regulatory region (Cdx2) is known to influence the transcriptional activity of VDR gene while polymorphisms in the 3′ untranslated region (UTR; *Bsm*I, *Apa*I, and *Taq*I) have been shown to be associated with stability of VDR mRNA. A polymorphism located in the translation initiation codon (*Fok*I) gives rise to three amino acid differences in the VDR length and is known to affect the protein function [16]. Our studies have shown that variant genotypes of 5′ regulatory and 3′ UTR polymorphisms of VDR gene differentially regulate vitamin D_3-modulated immune functions in pulmonary tuberculosis [17, 18]. In the present study, we have investigated the levels of 1,25 $(OH)_2$ D_3, VDR protein from the peripheral blood mononuclear cell lysates of pulmonary tuberculosis patients and normal healthy subjects. Moreover, relative expression of VDR, cathelicidin, and CYP27B1 mRNA in *M. tuberculosis*-stimulated macrophage cultures treated with or without 1,25 $(OH)_2$ D_3 were also studied. Further analysis were also done to find out whether CYP27B1 promoter and VDR gene polymorphisms influence the plasma 1,25$(OH)_2$ D_3 levels and ex vivo levels of VDR protein.

Study Subjects and Methods

Study Subjects

The study subjects consist of 65 pulmonary tuberculosis patients (mean age±SE, 36.0±8.06) attending Institute of Thoracic Medicine and Government Thiruvotteeswarar hospital of Thoracic Medicine, Chennai and 60 normal healthy subjects (mean age±SE, 29.9±7.74). Patients included were clinically and radiologically diagnosed for pulmonary tuberculosis (PTB) and confirmed by sputum positivity for *M. tuberculosis* by smear and culture. All the PTB patients were HIV negative and recruited for the study before the commencement of antituberculosis treatment. The normal healthy subjects (NHS) comprising staffs and trainees of Tuberculosis Research Centre, Chennai who were clinically normal and volunteered to give blood for the study. Among the PTB patients, 52 were men, and 13 were women. Similarly, in NHS, 40 were men and 20 were women. An informed consent was obtained from all the patients before blood collection, and this study was approved by Ethical Committee of the Institute.

Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood of healthy donors and pulmonary tuberculosis patients by standard Ficoll-Hypaque density gradient centrifugation. The separated cells were resuspended in Roswell Park Memorial Institute (RPMI) 1640 tissue culture medium (Sigma Chemical Co., St. Louis, USA) supplemented with 2 mM glutamine. Plasma samples were stored at −80°C.

Estimation of Plasma 1,25 Dihydroxy Vitamin D_3 Levels

Plasma 1,25 dihydroxy vitamin D_3 levels were estimated using a competitive enzyme-immuno assay technique with a selected monoclonal antibody recognizing 1,25 dihydroxy vitamin D_3. Enzyme-linked immunosorbent assay (ELISA) was performed using a kit (Immuno diagnostic, Bensheim, Germany) according to the manufacturer's instruction. The detection limit was 4.8 pg/ml.

Estimation of Ex Vivo Levels of Vitamin D Receptor Protein from Peripheral Blood Mononuclear Cells

Ex vivo levels of VDR protein from peripheral blood mononuclear cells were estimated using an ELISA kit

(Diagnostic System Laboratories Inc, Webster, TX, USA) according to the manufacturer's instruction. PBMCs (5×10^6) were used for extraction of total protein. Total protein levels were estimated using Bicinchonic acid protein assay kit (Sigma Chemical). VDR protein levels were expressed as femtomoles per milligram of total protein.

Monocyte-Derived Macrophage Culture

About 100 μl of the cell suspension containing 3×10^6 PBMCs was layered in a 24-well culture plate (Costar, Cambridge, MA, USA). The plate was incubated at 37°C and 5% CO_2 in an incubator (Heraeus, Kendro Laboratories, Germany) for 40 min. After incubation, the non-adherent cells were aspirated gently and washed thrice in warm RPMI. The remaining adherent cells, i.e., monocytes, were cultured in RPMI containing 10% autologous serum at 37°C and 5% CO_2. After 48 h, the monocyte-derived macrophages were infected with 3×10^6 live *M. tuberculosis* H37Rv. The infecting dose of *M. tuberculosis* H37Rv was normalized to average monocyte count (10% of total PBMC) so that the multiplicity of infection was one macrophage to ten bacilli). The cultures were incubated for 3 h at 37°C. After incubation, the uningested bacilli were removed by gentle washing three times with warm medium. Then, the monocytes were cultured in RPMI containing 10% autologous serum with or without 1,25$(OH)_2$ D_3 (10^{-7} M) at 37°C and 5% CO_2 for 3 days. 1,25 Dihydroxyvitamin D_3 (Sigma Chemical) was dissolved in 95% ethanol, and the final concentration of the ethanol did not exceed 0.5% in the cultures.

Total RNA Extraction and complementary DNA Synthesis

Total RNA was extracted from *M. tuberculosis* infected macrophages as per the manufacturer's protocol using TRI Reagent (Ambion, Austin, TX, USA). Briefly, 200 μl of TRI Reagent was added to each well and incubated for 10 min. Then, the contents of the well were transferred to a 1.5-ml vial and centrifuged at 12,000×g for 10 min at 4°C. To the supernatant, 40 μl of chloroform was added and incubated at room temperature for 15 min and centrifuged at 12,000×*g* for 15 min at 4°C. The aqueous phase was separated, and equal volume of isopropanol was added, vortexed, and incubated at room temperature for 15 min. Centrifugation was done at 12,000×*g* for 10 min at 4°C, and the supernatant was discarded. Two hundred microliters of 70% ethanol was added to the pellet and centrifuged at 7,500×*g* for 10 min at 4°C. The above step was repeated once again. The pellet was air dried and suspended in 10 μl of nuclease-free water and used for complementary DNA (cDNA) synthesis.

cDNA was synthesized from 10 μl of total RNA using high-capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA). Twenty-microliter reaction mixture was prepared using 10× reverse transcription buffer, 25× dNTPs, 10× random primers, RNaseInhibitor (20 U/μl), and MultiScribe™ Reverse Transcriptase (50 U/ μl). The cycling condition used for cDNA conversion was 25°C for 10 min followed by 37°C for 2 h.

Relative Quantification of mRNA by Real-Time Polymerase Chain Reaction

The relative quantification for the target genes was done using 2 μl cDNA template per reaction in a total volume of 20 μl. Validated TaqMan assay primers and probes (Applied Biosystems) and TaqMan Universal polymerase chain reaction (PCR) master mix were used for the analyses of the expression of target genes—VDR (Hs01045840_A1), cathelicidin (Hs00189038_m1), and CYP27B1 (Hs00168017_A1) and house keeping gene, glyceraldehyde-3 phosphate dehydrogenase (GAPDH). All analyses were performed in triplicates in an ABI Prism® 7500 Sequence detection system. Data were normalized to GAPDH content, and fold induction relative to the unstimulated cultures was calculated using the $2^{(-\Delta\Delta Ct)}$ method, where ΔCt is $\Delta Ct_{(stimulated)}-\Delta Ct_{(unstimulated)}$, ΔCt is $Ct_{(target)}-Ct_{(GAPDH)}$, and Ct is the cycle at which an arbitrary detection threshold is crossed.

Genotyping of VDR and CYP27B1 Gene Polymorphisms

Genomic DNA was isolated from peripheral blood white cells obtained from the blood of patients and controls using salting out procedure. Cdx2 polymorphism of VDR gene was studied by polymerase chain reaction with allele specific primers [19]. *Fok*I, *Bsm*I, *Apa*I, and *Taq*I polymorphisms of VDR gene and CYP27B1 gene promoter region polymorphism (−1077) were studied using PCR-based restriction fragment length polymorphism methods as described earlier [20, 10].

Statistical Analysis

Plasma 1,25 dihydroxy vitamin D_3, ex vivo VDR protein levels, and fold change of VDR, cathelicidin, and CYP27B1 mRNA were represented as mean±standard error. One-way analysis of variance with Bonferroni correction and Student's *t* test were used to compare the 1,25 dihydroxy vitamin D_3 and ex vivo VDR protein levels between the different genotypes. For single comparisons between PTB patients and NHS, Student's *t* test was used. All computations were done using Graph pad prism software (version 4). *p* value less than 0.05 was considered statistically significant.

Results

Plasma 1,25 Dihydroxy Vitamin D_3 Levels

Plasma vitamin D_3 levels ($1,25(OH)_2$ D_3) were estimated in 60 PTB patients and 65 NHS. Plasma $1,25(OH)_2$ D_3 levels were found to be significantly increased among PTB patients compared to NHS (p=0.0004). When the PTB patients and NHS were stratified as men and women, male patients had significantly increased $1,25(OH)_2$ D_3 levels as compared to male NHS (p=0.0043), while a trend towards increased $1,25(OH)_2$ D_3 levels were noticed among female PTB patients than in female NHS (p=0.0642; Fig. 1). However, no difference in the levels of $1,25(OH)_2$ D_3 was observed between men and women within PTB patient group and NHS.

Seasonal Pattern of Plasma $1,25(OH)_2$ D_3 Levels

Both PTB patients and NHS were recruited throughout the year, and hence, we analyzed whether the study subjects displayed a predictable seasonal pattern of plasma $1,25(OH)_2$ D_3 levels depending on the month the subjects were recruited. When both PTB patients and NHS were separated into four groups based on seasons, temperature and rainfall, among NHS, $1,25(OH)_2$ D_3 levels were significantly lower in the months of September, October, and November compared to June, July, and August months (p<0.001) and December, January, and February months (p<0.001). Although $1,25(OH)_2$ D_3 levels were lower in the months of September, October, and November compared to March, April, and May months, it was not statistically significant. Among the PTB patients, no such seasonal pattern of $1,25(OH)_2$ D_3 levels were observed. Except for December, January, and February months, $1,25(OH)_2$ D_3 levels differed significantly between PTB patients and NHS (p<0.05) for all the other three seasons (Fig. 2).

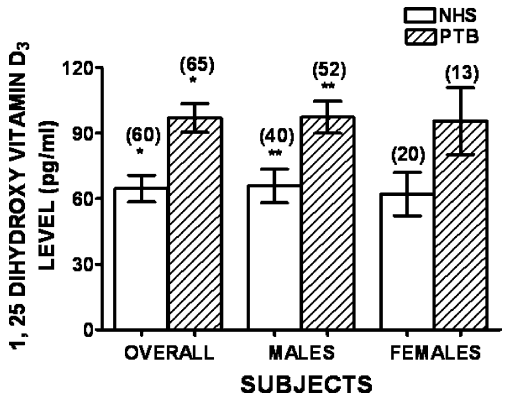

Fig. 1 Plasma 1,25 dihydroxy vitamin D_3 levels in pulmonary tuberculosis (*PTB*) patients and normal healthy subjects (*NHS*). *Numbers in parentheses* represent the subjects studied in each group. Results are expressed as mean±standard error. PTB vs. NHS *p=0.0004; PTB men vs. NHS men **p=0.0043

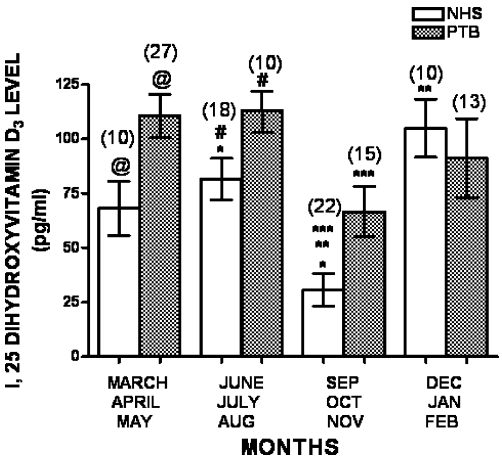

Fig. 2 Plasma 1,25 dihydroxy vitamin D_3 levels in pulmonary tuberculosis (*PTB*) patients and normal healthy subjects (*NHS*) based on seasons. *Numbers in the parentheses* represent the subjects studied in each group. June, July, and August vs. Sep, Oct, and Nov * p<0.001; Dec, Jan, and Feb vs. Sep, Oct, and Nov **p<0.001; PTB vs. NHS @, #, *** p<0.05

CYP27B1 Gene Polymorphism and Plasma $1,25(OH)_2$ D_3 Levels

1α Hydroxylase coded by CYP27B1 gene is the enzyme involved in converting 25-hydroxyvitamin D_3 to $1,25(OH)_2$ D_3. We studied a promoter polymorphism (−1077 A/T) in CYP27B1 gene and analyzed whether variant genotypes of −1077 A/T polymorphism are associated with differential levels of $1,25(OH)_2$ D_3. Allele and genotype frequencies of −1077 A/T polymorphism were not different between patients and NHS (Table 1). Moreover, $1,25(OH)_2$ D_3 did not differ among individuals with variant genotypes of −1,077 A/T polymorphism in both PTB patients and NHS (Fig. 3)

Ex Vivo Levels of Vitamin D Receptor Protein from Peripheral Blood Mononuclear Cells

Ex vivo levels of VDR protein from PBMCs were studied in 17 PTB patients and 36 NHS. PTB patients had significantly low levels of VDR protein compared to NHS (p=0.0172). Stratification of PTB patients and NHS into men and women revealed a trend towards decreased levels of VDR protein in men and women of PTB patients as compared to men (p=0.0598) and women (0.0834) of NHS, respectively. However, no difference in the levels of VDR protein was observed between men and women of both PTB patients and NHS (Fig. 4).

Vitamin D Receptor Gene Polymorphisms and Ex Vivo Levels of VDR Protein

Vitamin D receptor gene polymorphisms in the 5′ regulatory (Cdx2), coding (*Fok*I), and 3′ untranslated regions

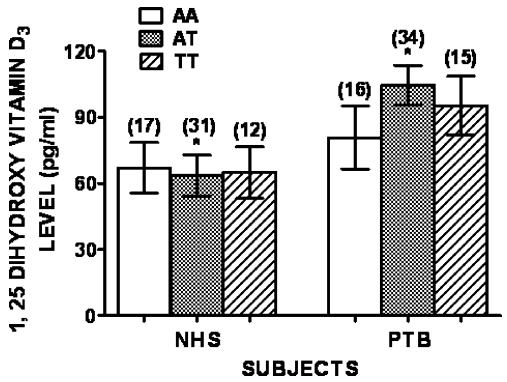

Fig. 3 Influence of −1077 A/T promoter region polymorphism of CYP27B1 gene on plasma 1,25 dihydroxy vitamin D_3 levels. *Numbers in the parentheses* represent the subjects studied in pulmonary tuberculosis (*PTB*) patients and normal healthy subjects (*NHS*) groups

(*Bsm*I, *Apa*I, and *Taq*I) were genotyped in PTB patients and NHS, and the polymorphisms were analyzed for their influence on ex vivo levels of VDR protein. With regard to the 5′ regulatory region polymorphism Cdx2, patients with GG genotype had significantly increased levels of VDR protein compared to patients with AA genotype (p=0.0221). However, no such difference was observed among NHS. The levels of VDR protein were not different among the variant genotypes of coding region polymorphism *Fok*I in both PTB patients and NHS (data not shown).

Among the 3′ UTR polymorphisms, a trend towards decreased levels of VDR protein was observed in NHS with BB genotype compared to bb genotype of *Bsm*I polymorphism (p=0.135). NHS with variant genotypes of *Apa*I polymorphism did not differ in the levels of VDR protein. A trend towards higher levels of VDR protein was seen among NHS with TT genotype compared to NHS with tt genotype of *Taq*I polymorphism (p=0.1106). Among PTB patients, levels of VDR protein were not different between the variant genotypes of 3′ UTR polymorphisms (Fig. 5).

Since haplotypes of VDR gene polymorphisms might collectively influence VDR levels, influence of 3′ UTR extended genotypes (combination of haplotypes) on VDR protein levels were analyzed collectively. A trend towards increased levels of VDR protein was observed among NHS with bbaaTT extended genotype compared to NHS with BBAAtt extended genotype. However, no such difference was observed among PTB patients (data not shown).

Relative Quantification of VDR mRNA Expression in Macrophage Cultures Using Real-Time PCR

Relative quantification of VDR mRNA expression was studied in macrophage cultures stimulated with *M. tuberculosis*, treated with or without 1,25$(OH)_2$ D_3 using real-time PCR. Among PTB patients, expression of VDR mRNA was significantly higher in *M. tuberculosis*-stimulated macrophage cultures compared to unstimulated cultures (p<0.01). Moreover, expression of VDR mRNA was also significantly higher in *M. tuberculosis*-stimulated macrophage cultures compared to cultures with 1,25$(OH)_2$ D_3 (p<0.05) alone or with 1,25$(OH)_2$ D_3 and *M. tuberculosis* (p<0.01). In NHS, VDR mRNA expression was not significantly different in the presence of 1,25$(OH)_2$ D_3 or *M. tuberculosis* compared to unstimulated cultures. When the fold induction levels were compared between PTB patients and NHS, PTB patients had relatively increased expression of VDR mRNA in 1,25$(OH)_2$ D_3-treated cultures (p<0.05) or in cultures stimulated with *M. tuberculosis* (p<0.05; Fig. 6).

Table 1 Percent Genotype Frequencies of Vitamin D Receptor and CYP27B1 Gene Polymorphisms in NHS and PTB Patients

Genotypes	NHS n=60	PTB patients n=65
VDR gene polymorphisms		
Cdx-2		
GG	40.0 (24)	30.8 (20)
GA	48.3 (29)	52.3 (34)
AA	11.7 (7)	16.9 (11)
*Fok*I		
FF	55.0 (33)	50.8 (33)
Ff	43.3 (26)	44.6 (29)
ff	1.7 (1)	4.6 (3)
*Bsm*I		
BB	26.7 (16)	41.5 (27)
Bb	38.3 (23)	33.9 (22)
bb	35.0 (21)	24.6 (16)
*Apa*I		
AA	38.3 (23)	38.5 (25)
Aa	41.7 (25)	44.6 (29)
aa	20.0 (12)	16.9 (11)
*Taq*I		
TT	45.0 (27)	36.9 (24)
Tt	35.0 (21)	50.8 (33)
tt	20.0 (12)	12.3 (8)
CYP27B1 gene polymorphism		
−1077		
AA	28.3 (17)	24.6 (16)
AT	51.7 (31)	52.3 (34)
TT	20.0 (12)	23.1 (15)

Genotype frequencies are given in percentage; numbers in parentheses represent individuals positive for the particular genotype.

n Number of individuals studied

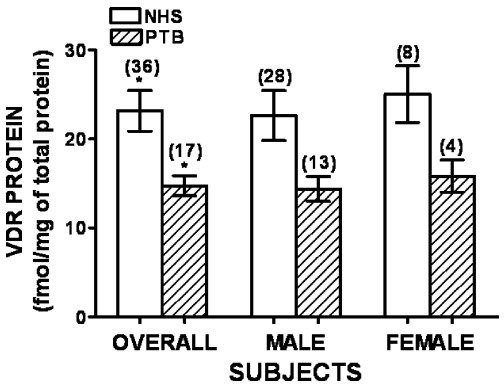

Fig. 4 Ex vivo levels of VDR protein from peripheral blood mononuclear cells of pulmonary tuberculosis (*PTB*) patients and normal healthy subjects (*NHS*). *Numbers in the parentheses* represent the subjects studied for that particular group. PTB vs. NHS *p=0.0172

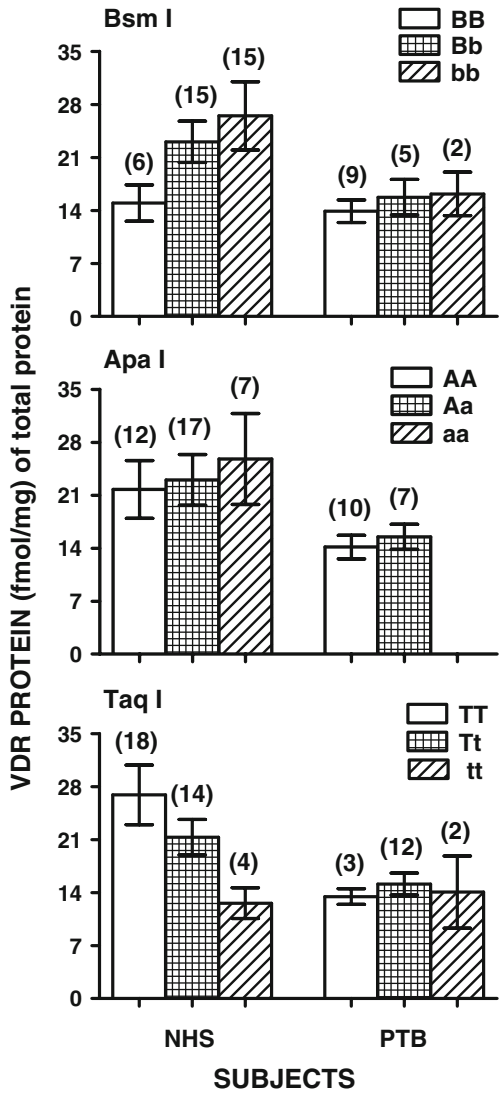

Fig. 5 Influence of *Bsm*I, *Apa*I, and *Taq*I polymorphisms of 3′ untranslated region of vitamin D receptor gene on ex vivo levels of VDR protein. *Numbers in the parentheses* represent the subjects studied in each group

Relative Quantification of Cathelicidin and CY27B1 mRNA Expression in Macrophage Cultures

Relative quantification of cathelicidin and CYP27B1 mRNA expression was studied in macrophage cultures stimulated with *M. tuberculosis*, treated with or without 1,25$(OH)_2$ D_3 using real-time PCR. In both patients and NHS, stimulation with *M. tuberculosis* has no effect on the expression of cathelicidin mRNA. Cultures treated with 1,25$(OH)_2$ D_3 showed increased expression of cathelicidin mRNA compared to unstimulated as well as *M. tuberculosis*-stimulated cultures of both PTB patients (p<0.001) and NHS (p<0.05). In PTB patients, cathelicidin mRNA expression was higher in cultures with1,25$(OH)_2$ D_3 alone compared to cultures with 1,25$(OH)_2$ D_3 and stimulated with *M. tuberculosis* (p<0.05). However, in NHS, no such difference was observed between cultures treated with 1,25 $(OH)_2$ D_3 and stimulated with or without *M. tuberculosis* (Fig. 7). When cathelicidin mRNA expression was compared between PTB patients and NHS, cathelicidin mRNA expression was significantly higher in patients in cultures with 1,25$(OH)_2$ D_3 alone (p=0.034). Although CYP27B1 mRNA expression was not significantly different under different culture conditions compared to unstimulated cultures, a tendency towards increased expression of CYP27B1 was observed in cultures stimulated with *M. tuberculosis* in PTB patients. Similarly, a tendency towards increased expression of CYP27B1 was observed in cultures

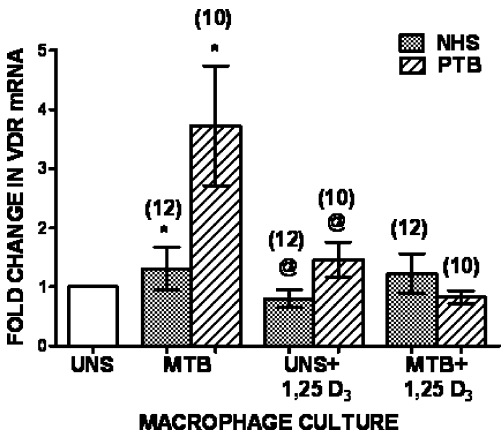

Fig. 6 Relative expression of vitamin D receptor mRNA in macrophage cultures of pulmonary tuberculosis (*PTB*) patients and normal healthy subjects (*NHS*). VDR mRNA expression was quantified (relative quantification) by real-time PCR and normalized to house-keeping gene, Glyceraldehyde-3 phosphate dehydrogenase and fold induction over unstimulated cultures was calculated by ΔΔCT method. *Numbers in the parentheses* represent the number of subjects studied in each group. *UNS* unstimulated macrophage cultures, *MTB* macrophage cultures stimulated with *M. tuberculosis*, *UNS+1,25 D_3* unstimulated cultures with vitamin D_3, *MTB+1,25 D_3* cultures stimulated with *M. tuberculosis* and treated with vitamin D_3; PTB vs. NHS * and @p<0.05

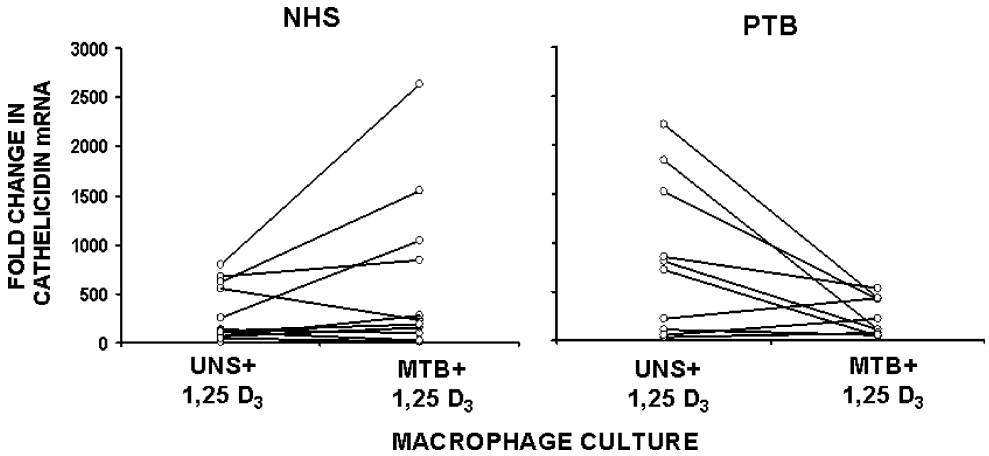

Fig. 7 Relative expression of cathelicidin mRNA in unstimulated macrophage cultures with vitamin D_3 and cultures stimulated with *M. tuberculosis* and treated with vitamin D_3 of pulmonary tuberculosis (*PTB*) patients and normal healthy subjects (*NHS*). Relative quantification of cathelicidin mRNA expression was carried out by real-time PCR. Results are expressed for each Individual subjects under both conditions. In PTB, UNS+1,25 D_3 vs. MTB+1,25 D_3, $p<0.05$

stimulated with *M. tuberculosis* in PTB patients as compared to NHS (data not shown).

Discussion

Studies on people migrating to Europe from countries with high incidence of latent TB infection reported that the immigrants experienced rates of TB that exceeded rates in their country of origin. This increased rates of TB coincided with development of vitamin D deficiency arising probably due to decreased sunlight exposure. A meta-analysis on association of low serum vitamin D levels and active tuberculosis reported that low serum vitamin D levels are associated with increased risk of active tuberculosis [21]. Previous studies associating increased risk of TB and vitamin D deficiency have investigated 25 hydroxy vitamin D_3 levels only [4–6, 21]. In the present study, we investigated plasma levels of 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) in active PTB patients and NHS. Significantly increased levels of $1,25(OH)_2D_3$ were observed in PTB patients than in NHS. Increased serum levels of 1,25 $(OH)_2D_3$ have also been observed in Taiwanese PTB patients compared to NHS [22].

Increased levels of $1,25(OH)_2D_3$ observed in the present study might be due to the upregulation of CYP27B1 expression involved in the conversion of 25 hydroxy vitamin D_3 to $1,25(OH)_2D_3$. Specific upregulation of CYP27B1 by dendritic cells and monocytes stimulated by a synthetic 19-kDa lipopeptide of *M. tuberculosis* has been shown earlier [8]. Increased $1,25(OH)_2D_3$ levels might lead to hypercalcemia in tuberculosis patients [23]. Increased synthesis of $1,25(OH)_2D_3$ might lead to the utilization of available 25 hydroxy vitamin D_3. Since $1,25(OH)_2D_3$ has a lower half-life and is utilized for various metabolic process, PTB patients might suffer from deficiency of substrate for synthesis of active metabolite. However, whether the increase in $1,25(OH)_2D_3$ is associated with 25 hydroxy vitamin D_3 deficiency needs investigation. Increased 1,25 $(OH)_2D_3$ levels might also have a role in reducing the disease severity through suppression of inflammatory cytokines. Further studies with $1,25(OH)_2D_3$ levels and disease severity might throw more light on this aspect.

We also analyzed whether the study subjects displayed a predictable seasonal pattern of plasma $1,25(OH)_2$ D_3 levels depending on the month the subjects were recruited. Among PTB patients, $1,25(OH)_2$ D_3 did not display any seasonal pattern. However, a trend towards decreased levels of plasma $1,25(OH)_2$ D_3 was observed in the months of September, October, and November. NHS displayed a predictable seasonal pattern of $1,25(OH)_2$ D_3 levels depending on the months with $1,25(OH)_2$ D_3 being lower at months of September, October, and November. Since the study area receives most rainfall during the months of September, October, and November (Northeast Monsoon), exposure of subjects to sunlight might be low resulting in reduced $1,25(OH)_2$ D_3 levels. Exposure to UV-B rays which trigger the synthesis of vitamin D might also be low in these months compared to summer and other months.

Since 1α hydroxylase is the enzyme involved in synthesis of $1,25(OH)_2$ D_3, we hypothesized that polymorphisms in the gene CYP27B1 coding for 1α hydroxylase might be associated with $1,25(OH)_2$ D_3 levels and investigated a polymorphism in the promoter region of CYP27B1. Variant genotypes of −1077 A/T polymorphism of CYP27B1 gene were not associated with the levels of $1,25(OH)_2$ D_3. Additional functional polymorphisms in CYP27B1 gene need to be studied.

Ex vivo levels of VDR protein was studied in PBMCs of PTB patients and NHS. Significantly decreased levels of VDR protein were observed in PTB patients compared to NHS. Observed decrease of VDR protein in patients might be due to downregulation of VDR expression by increased synthesis of $1,25(OH)_2$ D_3 [24]. Moreover, the decrease might also lead to defective VDR signaling due to the unavailability of receptors for $1,25(OH)_2$ D_3. Since human cathelicidin antimicrobial peptide gene is a direct target of VDR [25], defective VDR signaling could lead to impaired

immunity against *M. tuberculosis* mediated through VDR. Defective VDR signaling might also result in increased inflammation due to the increased expression of inflammatory cytokines.

VDR gene polymorphisms are known to influence the expression of VDR. The 3′ UTR of the genes is known to be involved in regulation of gene expression, especially through regulation of mRNA stability [26]. Earlier studies have provided evidence of differential luciferase activity for the two 3′ UTR variants that are linked to the most frequent haplotypes, i.e., "baT" and "BAt" [27]. Other studies have analyzed differences in the expression levels according to the 3′ UTR polymorphisms, and they have revealed a tendency for the BAt haplotype to display higher levels of mRNA expression than the baT haplotype. However, the results are not consistent [28–31]. In the present study, analysis on the influence of VDR gene polymorphisms on ex vivo levels of VDR protein revealed a trend towards decreased levels of VDR in BB genotype of *Bsm*I polymorphism and tt genotype of *Taq*I polymorphism and BAt haplotype among NHS, suggesting that these polymorphisms might regulate the expression of VDR protein. However, no such trend was observed among PTB patients, and this might be due to downregulation of VDR protein irrespective of genotypes.

Relative expression of VDR, CYP27B1, and cathelicidin mRNA was studied in macrophage cultures under different culture conditions using real-time PCR. The results revealed that, in PTB patients, stimulation with *M. tuberculosis* resulted in increased VDR mRNA expression, while addition of $1,25(OH)_2$ D_3 has no effect on VDR expression compared to cultures without $1,25(OH)_2$ D_3. Despite the increased expression of VDR, stimulation with *M. tuberculosis* has no effect on cathelicidin expression. This might be due to the deficiency of 25 hydroxy vitamin D_3 for $1,25(OH)_2$ D_3 synthesis and utilization as well as loss of available $1,25(OH)_2$ D_3 due to its shorter half-life. Further, addition of $1,25(OH)_2$ D_3 resulted in increased expression of cathelicidin. This suggests that VDR becomes functional upon exogenous addition of $1,25(OH)_2$ D_3 and induces cathelicidin expression resulting in the enhancement of immunity against tubercle bacilli. Among NHS, no difference in the VDR mRNA expression was observed between unstimulated and live *M. tuberculosis*-stimulated culture conditions. This might be due to the downregulation of VDR expression by increased synthesis of $1,25(OH)_2$ D_3 from 25 hydroxy vitamin D_3 reserves due to stimulation with *M. tuberculosis*. Earlier studies have reported that stimulation through TLR could result in increased expression of CYP27B1 mRNA by 24 h [8]. In the present study also, stimulation with *M. tuberculosis* resulted in nonsignificant increase of CYP27B1 expression in PTB patients.

The present study reports that PTB patients have increased plasma $1,25(OH)_2$ D_3 levels, while their *ex vivo* levels of VDR protein are decreased. Increased $1,25(OH)_2$ D_3 levels might lead to downregulation of VDR and could cause defective VDR signaling. Addition of $1,25(OH)_2$ D_3 or 25 hydroxy vitamin D_3 might lead to increased expression of cathelicidin antimicrobial peptide which could supplement the immunity in pulmonary tuberculosis.

Acknowledgments We acknowledge the Council of Scientific and Industrial Research, New Delhi for providing senior research fellowship to Mr. S. Prabhu Anand and Indian Council of Medical Research, New Delhi for providing senior research fellowship to Mr. K. Alagarasu. The help rendered by clinician Dr. N. Meenakshi and staffs, namely Mrs. Kasturi Sasikumar and Mrs. Thilagavathi of Government Thiruvotteeswarar hospital of Thoracic medicine (GTHTM) and staffs of Institute of Thoracic medicine (ITM), Chennai for their kind assistance in obtaining the blood samples, is acknowledged.

References

1. Skull SA, Ngeow JY, Biggs BA, Street A, Ebeling PR. Vitamin D deficiency is common and unrecognized among recently arrived adult immigrants from The Horn of Africa. Intern Med J 2003;33:47–51. doi:10.1046/j.1445-5994.2003.00344.x.
2. Lifson AR, Thai D, O'Fallon A, Mills WA, Hang K. Prevalence of tuberculosis, hepatitis B virus, and intestinal parasitic infections among refugees to Minnesota. Public Health Rep 2002;117(1): 69–77.
3. Lopez-Velez R, Huerga H, Turrientes MC. Infectious diseases in immigrants from the perspective of a tropical medicine referral unit. Am J Trop Med Hyg 2003;69:115–21.
4. Sita-Lumsden A, Lapthorn G, Swaminathan R, Milburn HJ. Reactivation of tuberculosis and vitamin D deficiency: the contribution of diet and exposure to sunlight. Thorax 2007;62:1003–7. doi:10.1136/thx.2006.070060.
5. Grange JM, Davies PD, Brown RC, Woodhead JS, Kardjito T. A study of vitamin D levels in Indonesian patients with untreated pulmonary tuberculosis. Tubercle 1985;66:187–91. doi:10.1016/0041-3879(85)90035-2.
6. Wilkinson RJ, Llewelyn M, Toossi Z, Patel P, Pasvol G, Lalvani A, et al. Influence of vitamin D deficiency and vitamin D receptor polymorphisms on tuberculosis among Gujarati Asians in west London: a case-control study. Lancet 2000;355:618–21. doi:10.1016/S0140-6736(99)02301-6.
7. Anderson P, May B, Morris H. Vitamin d metabolism: new concepts and clinical implications. Clin Biochem Rev 2003;24:13–26.
8. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. Science 2006;311:1770–3. doi:10.1126/science.1123933.
9. Malecki MT, Klupa T, Wolkow P, Bochenski J, Wanic K, Sieradzki J. Association study of the vitamin D: 1alpha-hydroxylase (CYP1alpha) gene and type 2 diabetes mellitus in a Polish population. Diabetes Metab 2003;29:119–24. doi:10.1016/S1262-3636(07)70017-4.
10. Jennings CE, Owen CJ, Wilson V, Pearce SH. A haplotype of the CYP27B1 promoter is associated with autoimmune Addison's disease but not with Graves' disease in a UK population. J Mol Endocrinol 2005;34:859–63. doi:10.1677/jme.1.01760.
11. White JH. Vitamin D signaling, infectious diseases and regulation of innate immunity. Infect Immun 2008;76:3837–43. doi:10.1128/IAI.00353-08.

12. Martineau AR, Wilkinson KA, Newton SM, Floto RA, Norman AW, Skolimowska K, et al. IFN-gamma- and TNF-independent vitamin D-inducible human suppression of mycobacteria: the role of cathelicidin LL-37. J Immunol 2007;178:7190–8.
13. Chandra G, Selvaraj P, Jawahar MS, Banurekha VV, Narayanan PR. Effect of vitamin D3 on phagocytic potential of macrophages with live *Mycobacterium tuberculosis* and lymphoproliferative response in pulmonary tuberculosis. J Clin Immunol 2004;24:249–57. doi:10.1023/B:JOCI.0000025446.44146.52.
14. Vidyarani M, Selvaraj P, Jawahar MS, Narayanan PR. 1,25 Dihydroxyvitamin D3 modulated cytokine response in pulmonary tuberculosis. Cytokine 2007;40:128–34. doi:10.1016/j.cyto.2007.08.005.
15. Nursyam EW, Amin Z, Rumende CM. The effect of vitamin D as supplementary treatment in patients with moderately advanced pulmonary tuberculous lesion. Acta Med Indones 2006;38:3–5.
16. Uitterlinden AG, Fang Y, Van Meurs JB, Pols HA, Van Leeuwen JP. Genetics and biology of vitamin D receptor polymorphisms. Gene 2004;338:143–56. doi:10.1016/j.gene.2004.05.014.
17. Selvaraj P, Chandra G, Jawahar MS, Rani MV, Rajeshwari DN, Narayanan PR. Regulatory role of vitamin D receptor gene variants of Bsm I, Apa I, Taq I, and Fok I polymorphisms on macrophage phagocytosis and lymphoproliferative response to *Mycobacterium tuberculosis* antigen in pulmonary tuberculosis. J Clin Immunol 2004;24:523–32. doi:10.1023/B:JOCI.0000040923.07879.31.
18. Selvaraj P, Vidyarani M, Alagarasu K, Prabhu Anand S, Narayanan PR. Regulatory role of promoter and 3′ UTR variants of vitamin D receptor gene on cytokine response in pulmonary tuberculosis. J Clin Immunol 2008;28:306–13. doi:10.1007/s10875-007-9152-5.
19. Fang Y, van Meurs JB, Bergink AP, Hofman A, van Duijn CM, van Leeuwen JP, et al. Cdx-2 polymorphism in the promoter region of the human vitamin D receptor gene determines susceptibility to fracture in the elderly. J Bone Miner Res 2003;18:1632–41. doi:10.1359/jbmr.2003.18.9.1632.
20. Pani MA, Knapp M, Donner H, Braun J, Baur MP, Usadel KH, et al. Vitamin D receptor allele combinations influence genetic susceptibility to type 1 diabetes in Germans. Diabetes 2000;49(3):504–7. doi:10.2337/diabetes.49.3.504.
21. Nnoaham KE, Clarke A. Low serum vitamin D levels and tuberculosis: a systematic review and meta-analysis. Int J Epidemiol 2008;37:113–9. doi:10.1093/ije/dym247.
22. Chang JM, Kuo MC, Kuo HT, Hwang SJ, Tsai JC, Chen HC, et al. 1-Alpha,25-Dihydroxyvitamin D3 regulates inducible nitric oxide synthase messenger RNA expression and nitric oxide release in macrophage-like RAW 264.7 cells. J Lab Clin Med 2004;143:14–22. doi:10.1016/j.lab.2003.08.002.
23. Sharma OP. Hypercalcemia in granulomatous disorders: a clinical review. Curr Opin Pulm Med 2000;6:442–7. doi:10.1097/00063198-200009000-00010.
24. Lechner D, Kallay E, Cross HS. 1Alpha,25-dihydroxyvitamin D3 downregulates CYP27B1 and induces CYP24A1 in colon cells. Mol Cell Endocrinol 2007;263(1–2):55–64. doi:10.1016/j.mce.2006.08.009.
25. Gombart FA, Borregaard N, Koeffler P. Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly upregulated in myeloid cells by 1,25-dihydroxyvitamin D_3. FASEB J 2005;19:1067–77. doi:10.1096/fj.04-3284com.
26. Decker CJ, Parker R. Diversity of cytoplasmic functions for the 3′ untranslated region of eukaryotic transcripts. Curr Opin Cell Biol 1995;7:386–92. doi:10.1016/0955-0674(95)80094-8.
27. Morrison NA, Qi JC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, et al. Prediction of bone density from vitamin D receptor alleles. Nature 1994;367:284–7. doi:10.1038/367284a0.
28. Carling T, Rastad J, Akerstrom G, Westin G. Vitamin D receptor (VDR) and parathyroid hormone messenger ribonucleic acid levels correspond to polymorphic VDR alleles in human parathyroid tumors. J Clin Endocrinol Metab 1998;83:2255–9. doi:10.1210/jc.83.7.2255.
29. Ogunkolade BW, Boucher BJ, Prahl JM, Bustin SA, Burrin JM, Noonan K, et al. Vitamin D receptor (VDR) mRNA and VDR protein levels in relation to vitamin D status, insulin secretory capacity, and VDR genotype in Bangladeshi Asians. Diabetes 2002;51:2294–300. doi:10.2337/diabetes.51.7.2294.
30. Yamagata M, Nakajima S, Tokita A, Sakai N, Yanagihara I, Yabuta K, et al. Analysis of the stable levels of messenger RNA derived from different polymorphic alleles in the vitamin D receptor gene. J Bone Miner Metab 1999;17:164–70. doi:10.1007/s007740050080.
31. Fang Y, van Meurs JB, d'Alesio A, Jhamai M, Zhao H, Rivadeneira F, et al. Promoter and 3′-untranslated-region haplotypes in the vitamin d receptor gene predispose to osteoporotic fracture: the Rotterdam study. Am J Hum Genet 2005;77:807–23. doi:10.1086/497438.