

# Vitamin D receptor expression levels determine the severity and complexity of disease progression among leprosy reaction patients

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## Abstract

We studied the roles of vitamin D and its receptor, VDR, in the progression of leprosy. The majority of individuals with leprosy from Kolkata, India, with a type 1 or type 2 reaction have low levels of vitamin D<sub>3</sub> in serum samples. Interestingly, individuals with a type 2 reaction associated with neuritis/erythema nodosum leprosum had very low VDR mRNA expression levels, ranging from 5% to 10%, compared to that of healthy control subjects; these patients also had a high bacilli index, ranging from 3+ to 5+. This is the first report to indicate that VDR expression levels may determine the complexity and severity of the progression of leprosy.

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## Introduction

Leprosy reactions are immunological complications that occur before, during or after the completion of multidrug therapy. Two major types of complications may occur, type 1 and type 2 [1–3]. These distinct conditions occur separately and arise at different times in some patients. The type 1 complication is a delayed hypersensitivity reaction; the dermatopathologic features of acute type 1 reactions are oedema, increased number of lymphocytes in the dermis and loss of normal granuloma organization. These reactions appear to be mediated via Th1 lymphocytes, and cells from reaction lesions express the pro-inflammatory cytokines interferon (IFN) gamma, interleukin 12 and the free radical producer inducible nitric oxide synthase. The type 2 reaction, erythema nodosum leprosum (ENL), is

serious and difficult to manage, particularly the immunologic complications of borderline lepromatous and lepromatous leprosy. High levels of circulating tumour necrosis factor alpha (TNF- $\alpha$ ) have been demonstrated in the plasma of some individuals with type 2 reactions. *In vitro* peripheral blood mononuclear cells (PBMCs) from individuals with ENL secrete increased amounts of TNF- $\alpha$  after stimulation [4–6].

Vitamin D<sub>2</sub> (ergocalciferol) and vitamin D<sub>3</sub> (cholecalciferol) are the two major forms of vitamin D. Vitamin D<sub>3</sub>, the active form of vitamin D, not only regulates calcium and bone metabolism but also plays an immunomodulatory role mediated through binding of its receptor (VDR) in monocytes, macrophages and activated lymphocytes.

Previous studies have demonstrated a deficiency of the secosteroid vitamin D in mycobacterial diseases. This vitamin D deficiency has been found to have a strong negative correlation with the rise in pro-inflammatory cytokines such as TNF- $\alpha$ . As observed previously, a significant increase in TNF- $\alpha$  has been found the skin lesions of patients with leprosy having type 2 reactions, where it acts as an inflammation modulator. Moreover, high levels of TNF- $\alpha$  have been implicated in the direct damage of myelin sheath of the neurons, stimulation of bone reabsorption and inhibition of bone collagen synthesis. These

properties likely provide an explanation for clinical symptoms such as nerve impairment, disability and deformity that are often observed in leprosy patients. Although no evidence for a deficiency in serum vitamin D in leprosy cases has yet been reported, the indirect evidence of having bone deformities due to low calcium absorption suggests that vitamin D deficiency may play a role in leprosy [7]. However, it has been well established that host cell-mediated immunity decides the progression and severity of leprosy diseases, although the role of secosteroid cannot be neglected. Because vitamin D<sub>3</sub> exerts its immunomodulatory effects through binding to VDR, its cell expression is a crucial determinant for the pathogenesis of viral or bacterial diseases. A few studies have suggested that certain mutations of the VDR gene might be associated with leprosy disease progression [8,9], but no study has yet reported that an alteration in VDR expression can change the complexity of a leprosy reaction. In the present study, we conducted a systematic study on patients with leprosy reaction to assess VDR expression in PBMCs.

## Materials and methods

### Patient selection

Patients with leprosy from Kolkata, India, were selected according to World Health Organization guidelines. Reaction status was diagnosed as redness, swelling with tenderness of existing lesion or ulceration (Fig. 1(A)). The corresponding nerves also exhibited swelling, pain and tenderness, often accompanied by loss of function, which was taken to be a type 1 reaction, and recurrent crops of red, pink or dusky brown, painful, small dome-shaped papules or large nodules with ill-defined margins that were tender upon touch, along with tender and enlarged nerves (Fig. 1(A)). Type 2 reactions are a systematic disorder affecting many organ systems. The lesions may be superficial or deep, causing panniculitis (Fig. 1(A)). ENL may present as a systemic illness; high fever, systemic upset and prostration were taken to indicate type 2 reactions. Equal numbers of subjects having no symptoms of infection, having no



(B)

Parameters	Healthy control (n=15)	Leprosy patient (n=15)	Reaction patient (n=15)
Vitamin D	33.40±3.41	27.47± 4.17	19.83± 3.6
TNF-α	10.02 ± 2.42	12.35 ± 1.81	23.80 ± 2.42
IFN-γ	10.66 ± 0.88	8.31 ± 1.076	4.44 ± 1.19
IL-10	3.46 ± 1.06	5.179 ± 1.193	8.525 ± 1.143
IFN-α	1.679 ± 0.709	2.619 ± 1.384	8.898 ± 1.79
IFN-β	8.963 ± 1.205	5.199 ± 1.776	0.908 ± 1.450

**FIG. 1.** (A) Skin lesions in typical type 1 and type 2 reactions among leprosy patients from Kolkata, India. (B) Tabular presentation of serum levels vitamin D<sub>3</sub> (ng/ml), pro- and anti-inflammatory cytokines (pg/ml) among leprosy reaction patients and healthy controls.

other inflammatory diseases such as diabetes or tuberculosis and having normal levels of serum 25-hydroxy vitamin D (25(OH)D; 30–40 ng/mL) were assigned as healthy controls.

### Bacillary index

Bacillary index (BI) was determined by Ziehl-Neelsen staining. In brief, slit skin was stained with carbol fuchsin and counter-stained with methylene blue. Stained slides were observed under a 100× oil immersion lens, and BI was clustered according to World Health Organization guidelines.

### TNF- $\alpha$ estimation

Blood samples were collected from each of the patients having type 1 and type 2 reactions before treatment with steroids, which are commonly used to suppress inflammation. The serum was separated and stored in aliquots at  $-65^{\circ}\text{C}$  to  $-70^{\circ}\text{C}$ . The assay was performed as per the protocol supplied using the commercially available sandwich micro-ELISA assay (Ray Biotech, UK).

### 25(OH)D estimation

Blood collected for serum analysis was allowed to clot for 30 minutes at room temperature before centrifugation at 3000 rpm for 10 minutes. Serum 25(OH)D concentrations were determined from serum samples via immunochemiluminometric assay by a commercial laboratory. The normal level was taken to be 30 to 40 ng/mL.

### RNA isolation and real-time quantitation for VDR expression

PBMCs from leprosy reaction patients were isolated by Ficoll-Hypaque (Histopaque; Sigma, USA) gradient. Total RNAs were isolated by Trizol reagent (Ambion, USA) following the manufacturer's protocol. Total RNA (500 ng) was used to prepare cDNA using a reverse transcription kit (Fermentus, UK) as per the manufacturer's instructions. Quantitative real-time PCR for VDR expression was performed using a VDR-specific primer pair and SYBR Green dye (Power SYBR Green; Applied Biosystems, USA). GAPDH (glyceraldehyde phosphate dehydrogenase) mRNAs were also quantitated and used as internal control for real-time PCR. Cycle threshold (Ct) values for VDR were determined and VDR expressions normalized to GAPDH compared. All experiments were performed in triplicate; average values are presented.

### Western blot analysis

VDR proteins from healthy and leprosy reaction patients were extracted using radioimmunoprecipitation assay lysis buffer in presence of protease inhibitor cocktail. Proteins were separated in 10% polyacrylamide gels and transferred to

nitrocellulose membrane. Proteins were then blotted using rabbit anti-VDR antibody (Santa Cruz Biotechnology, USA). Rabbit anti-GAPDH antibody was used as internal control. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was used for detection in the presence luminol substrate (Pierce, USA).

## Results

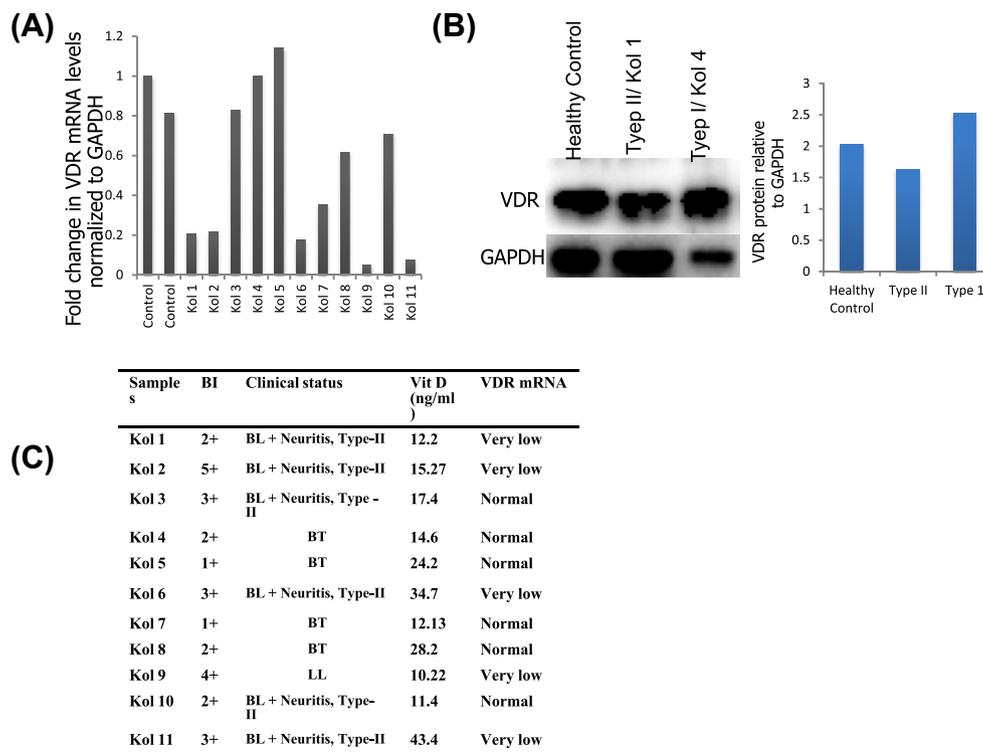
### Pro- and anti-inflammatory cytokine and vitamin D<sub>3</sub> profile

Leprosy infection was determined in tissue samples of reaction patients by PCR using primers specific for *Mycobacterium leprae*, as described elsewhere [10] (data not shown). The BI was also measured from tissue samples of leprosy reaction patients by Ziehl-Neelsen staining, as described in Materials and Methods. BI was found to range from 1+ to 6+. Higher BIs were observed among individuals with type 2 reactions and ENL or neuritis.

Blood samples were collected from 15 individuals with leprosy but no reaction and 15 individuals with leprosy reaction and compared to healthy controls ( $n = 15$ ). Serum samples were examined for vitamin D<sub>3</sub> levels and for pro- and anti-inflammatory cytokines by CLIA and ELISA to better understand infection status. The levels of vitamin D<sub>3</sub> in healthy controls were  $33 \pm 3.76$  ng/ml, whereas it was  $19 \pm 3.6$  ng/ml in reaction patients (Fig. 1(B)). Increased TNF- $\alpha$ , interleukin 10 and IFN- $\alpha$  levels were observed among reaction individuals, whereas decreased levels of IFN- $\gamma$  and IFN- $\beta$  were determined compared to healthy individuals (Fig. 1(B)). These results are typical of leprosy disease progression.

### Quantitation and analysis of VDR among leprosy reaction individuals

To determine whether vitamin D receptor protein or VDR play any role in immunity and control of leprosy disease progression, we measured VDR mRNA expression in PBMCs of infected individuals by quantitative real-time PCR and compared it to that of healthy controls (Fig. 2(A)). Comparison of VDR expression was made by determining the Ct values and was then normalized with GAPDH. Analysis revealed that six samples had significantly lower amounts of VDR mRNAs compared to healthy controls. VDR mRNAs in two of the samples were only 4% and 5%, respectively, compared to that of the control samples, whereas the other four had 10–20% VDR expression (Fig. 2(A)). Interestingly, all the individuals with low VDR expression manifested type 2 reactions, with neuritis and/or ENL. Four individuals had low vitamin D levels, and two had normal serum levels of vitamin D.



**FIG. 2.** (A) Real-time quantitative PCR for determining vitamin D receptor (VDR) mRNA expression levels among leprosy reaction patients and healthy controls. SYBR Green-based quantitative PCR was done using primers specific for VDR. The *GAPDH* (glyceraldehyde phosphate dehydrogenase) gene was quantified as an internal control. Experiments were performed in triplicate, and average values are presented. (B) VDR expression levels in leprosy reaction patients and healthy controls, as determined by Western blot analysis. Peripheral blood mononuclear cells (PBMCs) were isolated and total proteins were analyzed on a 10% PAGE gel. VDR and GAPDH proteins were detected with rabbit anti-VDR and rabbit anti-GAPDH antibody, respectively. Chemiluminescence was measured, and the ratio of VDR to GAPDH was calculated and graphically presented. (C) Tabular representation of bacillary index, reaction types and VDR expression levels among patients with leprosy.

To confirm that low VDR mRNA expression resulted in low VDR protein levels, we analyzed VDR protein expressions from individuals with low VDR mRNA and compared it to that of healthy control and type I samples. Our result from a representative type 2 sample clearly showed that low VDR mRNA-expressing samples had low VDR protein compared to controls with normal VDR mRNA levels, whereas control GAPDH protein levels remained similar (Fig. 2(B)).

## Discussion

Vitamin D<sub>3</sub> acts through its receptor, VDR, which, upon binding to vitamin D<sub>3</sub>, gets activated and regulates transcription of different classes of genes, including those involved in immune reaction. Thus, VDR expression levels in these cell types are important in controlling disease progression. VDR binds to VDR response element and regulates gene expression [11]. ChIP-seq analysis showed that upon vitamin D<sub>3</sub> stimulation,

significant changes occurred in the number of genes and was coupled with enriched VDR binding [12]. VDR binding sites are significantly enriched near autoimmune diseases and cancer-associated genes identified from genome-wide associated studies. Notable genes with VDR binding included *IRF8*, associated with multiple sclerosis, and *PTPN2*, associated with Crohn disease and type I diabetes [13]. VDR is suggested to play an important role in miRNA regulation and thus might be indirectly involved in the key regulation of other genes. VDR also regulates the *MCM7* gene, which encodes *MIR 106b* and is known to regulate the expression of *MIR181a* and *MIR-22* [14,15]. MicroRNA regulation is linked to several diseases, and their presence is sometimes used as a marker for them.

Previous studies have shown that certain mutations in the VDR gene are associated with leprosy complexity [8,9]. Here we have shown that individuals with complex leprosy reactions have very low VDR expression in association with low vitamin D<sub>3</sub>. These individuals had very high BI, and all had ENL. Interestingly, two individuals with normal vitamin D<sub>3</sub> levels but with

very low VDR levels (Fig. 2(C)) had high BI and manifested type 2 reaction with neuritis and/or ENL. This suggests not only that a certain level of vitamin D<sub>3</sub>-VDR interaction is crucial but also that the level of VDR expression is the determining factor in controlling leprosy reaction. We do not yet know how VDR expression affects the outcome of leprosy reaction. Understanding the exact mechanism is of interest and remains to be elucidated. ChIP-seq analysis of leprosy reaction patients' genes may provide detailed information.

This is a significant finding which may explain not only leprosy but also other diseases linked to vitamin D levels, such as diabetes. It will be interesting to discover the specific genes regulated by VDR expression that may affect disease outcome. Moreover, VDR therapy may be helpful in treating individuals with leprosy.

### Conflict of interest

None declared.

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