

Serum vitamin D and vitamin D receptor gene *FokI* polymorphisms in children with tuberculosis

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Abstract

Background Vitamin D deficiency and vitamin D receptor (VDR) gene polymorphisms are strongly associated with tuberculosis (TB) susceptibility in countries with four seasons. As a country with sufficient sunlight for vitamin D production in skin, the incidence of TB in Indonesia remains high.

Objective To assess for possible associations between the incidence of tuberculosis and serum vitamin D level, as well as VDR *FokI* polymorphisms in children.

Methods A case-control study was conducted at the Department of Child Health, Dr. Mohammad Hoesin Hospital, Palembang from November 2011 to April 2012. Subjects were children with TB (the case) and children without TB who had been exposed to TB in the home (the control). Serum vitamin D [1,25(OH)₂D₃ or calcitriol] level was measured by immunodiagnostic system (IDS) 1,25-dihydroxy vitamin D enzyme immunoassay (EIA) kit. The VDR *FokI* polymorphisms were identified by polymerase chain reaction (PCR) and restriction-fragment length polymorphism (RFLP) analysis.

Results Sixty subjects was divided equally into the case and control groups. The mean serum calcitriol level in the case group was significantly lower than that of the control group [105.5 (SD 66.9) pmol/L vs. 162.9 (SD 52.9) pmol/L, respectively; (P=0.001)]. We found 9 subjects with calcitriol deficiency, 8 in the TB group and 1 in the healthy contact group (OR 10.5; 95%CI 1.2 to 90.7) The VDR *FokI* polymorphism was seen in 28 subjects in the case group and 22 in the control group (OR 5.0; 95%CI 0.9 to 26.4).

Conclusion Vitamin D (calcitriol) deficiency and lower serum levels are associated with higher risk of TB in children. The VDR gene *FokI* polymorphism also contributes to susceptibility for TB. [Paediatr Indones. 2015;55:263-7].

Keywords: Vitamin D deficiency, VDR *FokI* polymorphism, tuberculosis, children

The role of vitamin D on host immunity defense against TB infection has long been known. The binding of vitamin D's active metabolite form (calcitriol) to the vitamin D receptor (VDR) in immune cells modulates macrophage activity against mycobacterium infection.¹ Therefore, low levels of calcitriol and/or abnormalities of vitamin D receptors may impair macrophage function, increasing the host's susceptibility to tuberculosis.^{2,3}

Host susceptibility to developing active TB is influenced by several factors such as mycobacterium virulence, host immunity, environment, genetics, or interactions among them.¹ As a country with sufficient sunlight exposure, which is known to be essential in the vitamin D production process, Indonesia has a high incidence of TB in children. A recent study showed that Indonesia ranks 5th in the list of countries with high TB incidence, 480,000/year of whom 10% are children.⁴ The contribution of low serum vitamin D levels and vitamin D receptor polymorphisms in TB development has been investigated in numerous studies in countries with four seasons on a variety

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of population groups.^{2,3} We performed this study to assess for possible associations between the incidence of tuberculosis and calcitriol levels as well as vitamin D receptor gene *FokI* polymorphisms in children at Palembang, South Sumatera.

Methods

This case-control study was conducted at the Department of Child Health, Dr. Mohammad Hoesin Hospital, Palembang, South Sumatera, from November 2011 to April 2012. Subjects were recruited by consecutive sampling and were children under 18 years of age. The case subjects had been diagnosed with active pulmonary or extrapulmonary TB, based on clinical manifestations, laboratory and radiological findings, and confirmed by a positive tuberculin test. The control group subjects were recruited from our Pediatric and Adult Respiriology Specialist Outpatient Clinic and consisted of children who had household contact with TB patients proven by a positive tuberculin purified protein derivative test, but were symptom free. Patients with malabsorption, liver abnormalities, renal dysfunction, immunosuppressed condition, severe malnutrition, or undergoing long-term anticonvulsant therapy were excluded. All participants' parents gave informed consent for inclusion, and permission to carry out this study was obtained from the Research Ethics Committee at Sriwijaya University Medical School.

Blood specimens were taken from all patients at or shortly after diagnosis. Calcitriol was assayed using the IDS® 1,25-dihydroxy vitamin D EIA kit. Calcitriol deficiency was defined to be a concentration of <67 pmol/L.⁵ Blood was collected in EDTA tubes for VDR gene polymorphism identification and stored at -20°C until DNA extraction. The DNA was purified by a Chelex-based DNA purification protocol.⁶ The DNA was amplified with a standard PCR technique. The VDR *FokI* polymorphisms were identified by polymerase chain reaction (PCR) and restriction-fragment length polymorphism (RFLP) analysis. The sense primer: 5'-AGC TGG CCC TGG CAC TGA CTCTGC TCT-3', and the reverse primer: 5'-ATG GAA ACA CCT TGC TTC TTC TCC CTC-3' were used. After initial denaturation for 10 minutes at 94°C, samples were subjected to 35 cycles of amplification, consisting of a 60-second denaturing

phase at 95°C, a 60-second annealing phase, and a 60-second extension phase at 72°C. A 7-minute, 72°C hold was the final step of the program. Following amplification, 0.2 µl of *FokI* restriction enzyme, 1.2 µl of buffer, and 2.6 µl of ddH₂O were added to 8 µl of the PCR product and digested at 37 °C. Genotypes were assigned as follows: F/F 265 bp only; F/f 265 bp, 196 bp, and 96 bp; f/f, 196 bp and 96 bp. The *FokI* alleles were designated 'F' as the infrequent allele (mutant), and 'f' as the common allele (wild type).

We analyzed the difference between calcitriol level using independent t-test. Differences in proportions between the groups were analyzed by Fisher's exact and Chi-square tests. Odds ratios (OR) with 95% confidence interval were calculated, with P values of < 0.05 considered to be significant. Logistic regression analysis with Backward-Wald method was used to assess for significant associations between the incidence of tuberculosis and vitamin D deficiency, VDR *FokI* polymorphism and also other confounding factors such as age, nutrition status, and economical status.

Results

Thirty patients with active TB and 30 healthy contacts as controls were enrolled in this study. The mean age of all subjects was 4.7 (SD 3.1) years, ranging from 5 months to 11.4 years. The characteristics of subjects are shown in **Table 1**.

The mean calcitriol level was significantly lower in the active TB group [105.5 (SD 66.9) pmol/L] than in the healthy contact group [162.9 (SD 52.9) pmol/L]; (P=0.001). We also found 8 children with calcitriol deficiency in the active TB group and 1 child in the healthy contact group. Statistical analysis revealed a significant association between calcitriol deficiency and active TB in children (OR 10.5; 95%CI 1.2 to 90.7; P=0.026).

The results of the PCR-RFLP genotyping of the VDR *FokI* polymorphisms are presented in **Figure 1**. **Table 2** shows the distribution of genotypes and alleles for the VDR gene *FokI* polymorphisms in our subjects. Vitamin D receptor gene *FokI* polymorphisms (FF and Ff) were found in 28/30 subjects in the TB group and in 22/30 subjects in the healthy contact group. We found an association between tuberculosis disease and VDR *FokI* polymorphisms (OR=5; 95%CI 0.9 to 26.4;

Table 1. Characteristics of subjects

Characteristics	TB group (N = 30)	Healthy contact group (N = 30)
Age, n		
≤ 5 years	17	17
> 5 years	13	13
Gender, n		
Male	16	18
Female	14	12
Nutritional status, n		
Undernourished	12	11
Well-nourished	18	19
Family income, n		
Above regional minimum wage	25	22
Below regional minimum wage	5	8
Paternal education, n		
High school or below	10	7
Above high school	20	23
Maternal education, n		
High school or below	8	9
Above high school	22	21

Table 2. Distribution of genotypes and alleles for the VDR gene *FokI* polymorphisms

	TB group	Healthy contact group
Genotype, n (N=30)		
FF (mutant)	15	5
Ff (heterozygote)	13	17
ff (wild type)	2	8
Allele, n (N=60)		
F	43	27
f	17	33

P=0.038). The F allele frequency was significantly higher in the TB group compared to the healthy contact group (OR=3.1; 95%CI 1.4 to 6.5; P=0.003).

Logistic regression analysis indicated that the incidence of tuberculosis disease was influenced by vitamin D deficiency (RR=8.9; 95% CI 0.97 to 81.76; P=0.032) and VDR *FokI* polymorphisms (RR=4.45; 95% CI 1.27 to 15.47; P=0.019).

Discussion

Our study found that lower serum calcitriol level, calcitriol deficiency, and VDR *FokI* polymorphisms have associations with active TB disease in children. Children with vitamin D deficiency had a greater risk in developing tuberculosis (10-fold higher), while those with VDR gene *FokI* polymorphism had a 5-fold higher risk of having active tuberculosis compared to healthy contacts. Similarly, a previous study showed that calcitriol together with vitamin D receptor, may have an important role in modulating the immune response to TB infection. Lower serum calcitriol level and genetic variation, in this case the VDR *FokI* polymorphism, increased the risk of developing active tuberculosis.³

In a region that is considered to have sufficient sunlight exposure essential to vitamin D production, the finding of vitamin D deficiency in this population should alert medical staff. Low vitamin D levels in children could be caused by their higher requirements, due to growth, lifestyles or cultural practices that

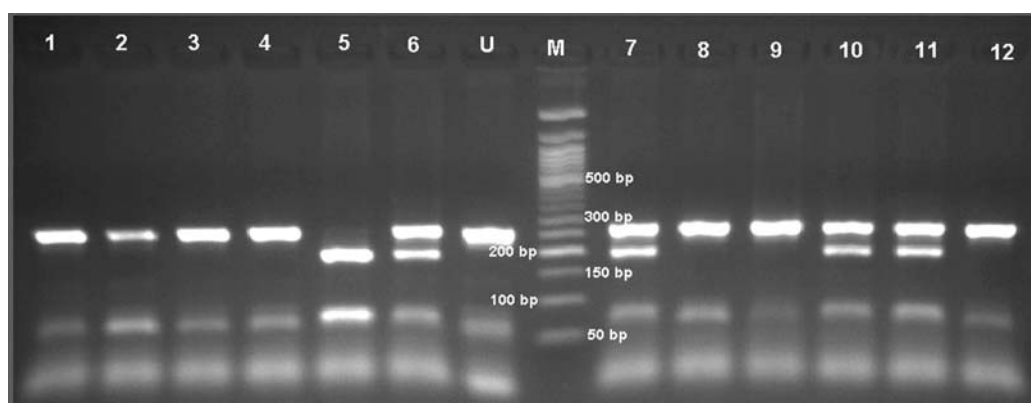


Figure 1. Genotyping of the VDR *FokI* polymorphism by PCR-RFLP [Notes: Lanes 1, 2, 3, 4, 8, 9, 12: FF genotype; lane 5: ff genotype; lanes 6, 7, 10, 11: Ff genotype].

decrease the time spent outdoors, or increase the amount of body surface area covered by clothing, darker skin pigmentation, use of sunscreens, and even prolonged breastfeeding without supplementation or adequate sunlight exposure.^{7,8}

Polymorphism is defined as a variation in genetic sequence that occurs in certain populations that might affect host susceptibility to certain diseases or response to therapy. Polymorphisms start as a mutation and result in differences in allele frequency among ethnic groups as a result of evolutionary processes and population genetics. There are several known VDR gene polymorphisms, such as *FokI*, *BsmI*, *ApaI* and *TaqI*. The VDR *FokI* polymorphism has two protein variants that correspond to two available start sites: a long version (427 aa) and a short version (424 aa). This variation leads to differences in transactivation capacity as a transcription factor. As such, some promoter regions of vitamin D target genes might be more sensitive to this genotype-dependent difference in activity compared to others.⁹

The mechanism of interaction between calcitriol and the vitamin D receptor that modulates the immune response against tuberculosis infection is still under study. However, two possible mechanisms have been hypothesized. Calcitriol enhances the fusion of phagosomes and lysosomes in infected macrophages and reduces the viability of *M. tuberculosis*. Calcitriol also enhances the production of LL-37, which is known to be an antimicrobial peptide from the cathelicidin family that appears to be involved as a first-line defense in tuberculosis prevention by direct bactericidal activity and by attracting monocytes, T cells, and neutrophils to the site of infection.¹

An association between low serum vitamin D or vitamin D deficiency and TB has been demonstrated in several other studies. A Pakistani study found that 79% of TB patients are in a state of vitamin D deficiency.¹⁰ A study from India reported significantly lower vitamin D levels in TB patients compared to controls.¹¹ Others such as Gibney *et al.* in Australia,¹² Ustianowski *et al.*¹³, and Wilkinson *et al.*¹⁴ in London, as well as Syafii *et al.*¹⁵ in Cimahi, West Java, Indonesia have also made similar findings. However, in a 1985 study in Indonesia, Grange *et al.* failed to demonstrate an association.¹⁶ This difference might be related to time difference or age of subjects, who may have had differences in lifestyle or host immune system maturation.

Previous studies have also suggested a higher risk of TB in people with VDR *FokI* polymorphisms. In West India, Selvaraj *et al.* found that 47% of spinal tuberculosis patients had VDR *FokI* polymorphisms.¹⁷ And Liu *et al.* in China found that 23% of TB patients had *FokI* polymorphisms.¹⁸ Similarly, Setiabudiawan *et al.* in West Java, Indonesia found mutant alleles in 66.7% of children with TB.¹⁹ In contrast, Wilbur *et al.* in Paraguay reported that VDR *FokI* polymorphisms had a protective effect in hosts against TB infection.²⁰ This result supports the idea that genetic variation may lead to different responses in different ethnic populations.

There were several limitations in this study. We did not measure calcidiol, which is the most common parameter for determining vitamin D status. As such, we could not evaluate for a relationship between serum calcidiol and calcitriol levels, with regards to a role in immunity. We also did not identify other VDR polymorphisms such as *BsmI*, *ApaI* and *TaqI* that might also have contributed to host susceptibility to tuberculosis infection.

In conclusion, calcitriol deficiency or low serum level may account for a proportion of the acquired susceptibility of children to active tuberculosis, and *FokI* polymorphisms in the VDR gene also contribute to higher risk of tuberculosis in children.

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Conflict of interest

None declared.

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