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Is There Any Impact of VDR Gene Polymorphism in Bataks Ethnic to Have Tuberculosis?

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Abstract: The active metabolite of vitamin D activates macrophages and results in the restriction of growth of Tuberculosis (TB) pathogen *M. tuberculosis*. The effect of vitamin D is achieved through binding to the Vitamin D Receptor (VDR), which ultimately produces the antimicrobial peptide Cathelicidin. Matched case-control study with 76 pulmonary tuberculosis Indonesian Batak ethnic patients and 76 healthy normal control were completed. Genetic polymorphisms of VDR gene were analysed using PCR and RFLP. The genotype frequencies of *Apa*I were AA 10.6%, Aa 44.7%, aa 44.7% for the control group and AA 10.6%, Aa 43.4%, aa 46% for the PTB patients. For the *Fok*I genotype the frequencies were FF 39.5%, Ff 44.7%, ff 15.8% for the control group and FF 35.5%, Ff 55.3%, ff 9.2% for the PTB patients. In these two genotypes there were no significant differences found between the control group and the PTB patients ($P > 0.05$). Besides, there genotypic frequencies were in agreement with the Hardy-Weinberg Equilibrum ($P > 0.05$). However, for the *Bsm*I genotype frequencies, BB 2.6%, Bb 23.7% and bb 73.7% for the control group and BB 0%, Bb 68.4%, bb 31.6% for the PTB patients, there was a significant association found with PTB; the bb genotype is correlated with a decreased risk for PTB (OR 0.22, 95% CI: 0.11-0.45). *Apa*I and *Fok*I polymorphisms of VDR gene do not appear to be responsible for host susceptibility to pulmonary tuberculosis in the Indonesian Batak ethnic population but *Bsm*I polymorphism had association with host resistant to PTB.

Key words: Pulmonary tuberculosis, VDR polymorphism, Batak, Indonesia.

1. Introduction

Tuberculosis (TB) is a contagious disease that remains one of the world's deadliest health problems to date. The World Health Organization (WHO) reported in 2013 that is was estimated that 9 million people worldwide suffer from TB, of which 56% of the cases occur in South East Asia and West Pacific, and that 1.5 million deaths yearly are caused by TB [1]. Among several other transmission diseases, Pulmonary tuberculosis (PTB) is considered a high burden in Indonesia. After infection of the pathogen which is responsible for TB, *Mycobacterium tuberculosis*, it is known that environmental and host genetic factors can influence the susceptibility to develop active TB [2, 3]. Many studies were able to produce convincingly prove that the genetic factor plays an important role in the disease enhancement [3, 4]. Recently, researches are focusing on the Vitamin D Receptor (VDR), which caught the interest as it was shown that the growth of *M. tuberculosis* is restrained after vitamin D administration [4, 5]. This effect is achieved through the binding of vitamin D to VDR located in macrophages, which activates the antimicrobial peptide cathelicidin synthesis [4], and consequently eliminates *Mycobacterium tuberculosis* in the phagolysosome [5]. This process and therefore the effect of vitamin D might be influenced by polymorphisms in the VDR gene.

One of these polymorphism is *Fok*I, with a transition of C to T (ACG-ATG) at the first of the two potential translation initiation sites in exon 2, and can be distinguished by RFLP using endonuclease *Fok*I. If translation start at the primary ATG site, individuals with the T allele designed as f, the VDR protein is

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synthesized in the full-length of 427 amino acids. In contrast, if the translation start at the second ATG site, individuals with the C allele designed as F, the VDR protein lacks three NH2-terminal amino acids [6]. Several studies showed that the transcription of the F allele occurs 1.7 times more than the f allele and that the F allele interacts with the transcription factor IIB more efficiently, resulting in a more potent VDR protein transcription [7, 8]. The second polymorphism in this study is *Bsm*I, which is located at the intron between exon VIII and IX. The *Bsm*I polymorphism control transcription level, transcription stability and post modification transcription of VDR gene [9]. The final investigated polymorphism is *Apa*I located at intron 8 with a transtition of T to G. The term A indicates the T allele and a the G allele [4]. *Apa*I variation could generate splicing errors henced affect to the protein synthesis.

The potential roles of VDR *Fok*I, *Apa*I and *Bsm*I polymorphisms in the occurence of PTB have been investigated in many ethnic groups. Different outcome of results produced by this studies are probably due to the diversities of the ethnic groups. A meta analysis study performed by Gao *et al*. in 2010, found that in Asians, the subjects with ff genotype were more susceptible to PTB and the subjects with bb genotype have a decrease risk for PTB. However, none of the polymorphisms was significantly related to PTB among Africans or South Americans [10]. For the *Bsm*I polymorphism in the European population, the variant homozygote and heterozygote genotypes were associated with a significantly decreased risk of tuberculosis when compared to the wild type homozygote [11]. *Apa*I polymorphism has no influence in the levels of VDR protein among 65 pulmonary tuberculosis (PTB) patients and 60 normal healthy subjects in Chennai, India [12]. Because the genetic effect may be different in various ethnic groups, this study wanted to investigate the effect of VDR polymorphism on PTB susceptibility in the Indonesian Batak-ethnic population.

2. Methods

2.1 Cases and Controls

Pulmonary tuberculosis patients were recuited from several TB services in Medan city, Indonesia, from November 2012 to April 2013. The cases were newly diagnosed pulmonary tuberculosis patients, age 16-55 years old, Batak ethnic, have symptoms of pulmonary TB, positive sputum smear and chest radiography consistent with active disease. Cases who were HIV positive and known to present diabetes mellitus and other severe disease, imunnosupressive drug consumption were excluded from the study.

A control group composed of sex, age and ethnically matched were healthy subjects with normal chest X ray and no history of previous tuberculosis. A control group were healthy workers like doctors, nurses and medical students. All participants were from the Batak ethnic for 3 generations. This study was approved by the Ethics Committee of the Faculty of Medicine, University of Sumatera Utara, Medan, Indonesia.

2.2 VDR Genotyping

All subjects were interviewed and an informed consent was obtained. An anticoagulated peripheral blood specimen was collected and DNA was extracted (Promega, USA) and stored at minus 20 ^{o}C. Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) was used to identify *Apa*I, *Fok*I and *Bsm*I polymorphism of the Vitamin D Receptor gene. The primer sequences used in this study were as follows: *Forward primer*: AGA GCA TGG ACA GGG AGC AAG; *Reverse Primer*: GCA ACT CCT CAT GGC TGA GGT CTC A for *Apa*I, *Forward Primer*: 5'-AGC TGG CCC TGG CAC TGA CTC TGC TCT-3' and *Reverse Primer*: 5'-ATG GAA ACA CCT TGC TTC TTC TCC CTC-3' for *Fok*I [13]. *Forward Primer*: 5'-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA-3' and *Reverse Primer*: 5'-AAC CAG CGG GAA GAG GTC AAG GG-3' for *Bsm*I [13].

PCR conditions were as follow; for *Fok*I and *Bsm*I was denaturation at 94 ^{o}C for 5 min, followed by 35 cycles of PCR at 94 ^{o}C (30 s), annealing at 61 ^{o}C (30 s), and 72 ^{o}C (1 min) [13]. *Apa*I proceed through 40 cycles of PCR at 94 ^{o}C (30 s), annealing at 60 ^{o}C (30 s) and extension at 72 ^{o}C (30 s). Final extension was continued at 72 ^{o}C for 7 min. Following PCR, the amplified PCR products were digested with *Apa*I (NEB) restriction enzyme at 25 ^{o}C for 1 h, *Fok*I (Takara, Bio-Inc, Japan) restriction enzyme at 37 ^{o}C for 3 h and *Bsm*I (Thermo Scientific, Lithuania) restriction enzyme at 37 ^{o}C for 4 h [13]. Digested products were analyzed using electrophoresis in 2% agarose gel and ethidium bromide stained. The bands were visualyzed by Gel Documentation System.

Depend on the digestion pattern of *Fok*I polymorphism, individuals were scored as "ff" by homozygous for the presence of the *Fok*I site (169 bp and 96 bp), "FF" homozygous for the absence of the *Fok*I site (265 bp), or "Ff" in presence and absence of *Fok*I site (265 bp, 169 bp and 96 bp) [13]. Digestion of the amplified 745 bp *Apa*I PCR product gives two fragments, 531 bp and 214 bp respectively, if the product was excessable. Depending on the digestion pattern, individuals were scored as "aa" homozygous for the presence of the *Apa*I site, "AA" homozygous for the absence of the *Apa*I site, or "Aa" in presence and absence of the *Apa*I site (745 bp, 531 bp, and 214 bp). The digestion pattern of *Bsm*I polymorphism were "bb" for the presence of *Bsm*I site (175 bp and 650 bp), "BB" homozygous for the absence of the *Bsm*I site (825 bp), and "Bb" in presence and absence of the *Bsm*I site (825 bp, 175 bp and 650 bp) [13].

2.3 Statistical Analysis

The genotype frequencies of each of the SNPs were compared by the Chi-square test. The strength of the association between VDR *Apa*I, *Fok*I and *Bsm*I polymorphisms and TB risk was evaluated by calculating odds ratio (OR) with 95% confidence interval (95% CI). The 2-sided exact $P < 0.05$ was considered statistically significant. Conditional logistic regression was performed to calculate the odds ratio. Data were managed and analysed using Epi info. Hardy-Weinberg equilibrium test was done in case and control groups for *Apa*I, *Fok*I and *Bsm*I polymorphisms using the web tool HWE Testing calculator, available on line; $P < 0.05$ was considered as a significant disequilibrium [14].

3. Findings

The characteristics of PTB patients and normal control are summarized in Table 1. Sex, age and ethnic characteristics between PTB patients and controls were matched.

The results of VDR genotyping for PTB patients and healthy controls are summarized in Table 2.

The characteristics of PTB patients and normal control are summarized in Table 1. There was no significant difference in sex, age and ethnic between PTB patients and controls ($P = 1.000$).

Table 1 Characteristic of PTB cases and controls group.

Characteristic	Cases n (%)	Controls n (%)	*P* value
Sex			
Male	53 (69.7)	53 (69.7)	1.000
Female	23 (30.3)	23 (30.3)	
Age groups			
16-25	22 (28.9)	22 (28.9)	1.000
26-35	32 (42.1)	32 (42.1)	
36-45	14 (18.4)	14 (18.4)	
46-55	8 (10.5)	8 (10.5)	
Ethnicity (Batak)	76 (100)	76 (100)	1.000

Table 2 Allele frequencies and genotype of VDR gene *Apa*I, *Fok*I and *Bsm*I polymorphism in PTB cases and controls.

Polymorphism	Cases n (%)	Controls n (%)	*P*	HWE in cases χ^2 (*P*)	HWE in controls χ^2 (*P*)
*Apa*I					
Genotype AA	8 (10.6)	8 (10.6)	0.987	0 (> 0.05)	0.01 (> 0.05)
Aa	33 (43.4)	34 (44.7)			
aa	35 (46.0)	34 (44.7)			
Total	76 (100)	76 (100)			
Allele frequencies					
A	51 (33.6)	50 (32.9)	1.000		
a	101 (66.4)	102 (67.1)			
Total	152 (100)	152 (100)			
*Fok*I[13]					
Genotype FF	27 (35.5)	30 (39.5)	0.314	2.67 (> 0.05)	0.21 (> 0.05)
Ff	42 (55.3)	34 (44.7)			
ff	7 (9.2)	12 (15.8)			
Total	76 (100)	76 (100)			
Allele frequencies					
F	96 (63.2)	94 (61.8)	0.813		
f	56 (36.8)	58 (38.2)			
Total	152 (100)	152 (100)			
*Bsm*I[13]					
Genotype BB	0	2 (2.6)	< 0.001	20.55 (< 0.05)	0.14 (> 0.05)
Bb	52 (68.4)	18 (23.7)			
bb	24 (31.6)	56 (73.7)			
Total	76 (100)	76 (100)			
Allele frequencies					
B	52 (34.2)	22 (14.5)	< 0.001		
b	100 (65.8)	130 (85.5)			
Total	152 (100)	152 (100)			

HWE, Hardy- Weinberg Equilibrium; $P < 0.05$ was considered as significant disequilibrium.

The results of VDR genotyping for PTB patients and healthy controls are summarized in Table 2. PTB patients and healthy controls has a similar distribution for *Apa*I genotype and allele frequencies ($P > 0.05$) and as well for *Fok*I. On the *Bsm*I genotype and allele frequencies, there was a significant difference between PTB patients and healthy controls ($P < 0.001$) [13]. The genotypes of *Apa*I, *Fok*I polymorphism in cases and controls group were in Hardy-Weinberg equilibrium ($P > 0.05$) and *Bsm*I polymorphism in the control group as well. The *Bsm*I polymorphism case group was not in the Hardy-Weinberg equilibrium ($P < 0.05$) [13]. The relation between *Apa*I, *Fok*I and *Bsm*I polymorphisms and pulmonary tuberculosis are summarized in Table 3.

There was no significant association found between the *Apa*I polymorphism and PTB (OR 0.98, 95% CI: 0.33-2.91) for the Aa genotype and the aa genotype (OR 1.03, 95% CI: 0.34-3.06). The relation of *Fok*I polymorphism and PTB (OR 1.37, 95% CI: 0.69-2.73) was also found not to be associated for Ff genotype and (OR 0.65, 95% CI: 0.22-1.89) for ff genotype [13]. For the *Bsm*I polymorphism, however the bb genotype did showed to be associated with a decreased risk to PTB (OR 0.22, 95% CI: 0.11-0.45) [13].

4. Discussion

The development of active B after infection is depend on the multiply interaction between host, bacteria (agent) and environment. One of the host factors that

Table 3 Analysis of VDR gene *Apa*I, *Fok*I and *Bsm*I polymorphisms in PTB cases and controls.

Polymorphism	Cases n (%)	Controls n (%)	OR (95% CI)	*P*
*Apa*I				
Genotype AA	8 (10.6)	8 (10.6)	1	
Aa	33 (43.4)	34 (44.7)	0.98 (0.33-2.91)*	0.964
aa	35 (46.0)	34 (44.7)	1.03 (0.34-3.06)*	0.964
*Fok*I[13]				
Genotype FF	27 (35.5)	30 (39.5)	1	
Ff	42 (55.3)	34 (44.7)	1.39 (0.69-2.77)*	0.352
ff	7 (9.2)	12 (15.8)	0.64 (0.22-1.86)*	0.418
*Bsm*I[13]				
Genotype BB+Bb	52 (68.4)	20 (26.3)	1	< 0.001
bb	24 (31.6)	56 (73.7)	0.22 (0.11-0.45)*	

*Odds ratio was calculated using conditional logistic regression analysis.

affect the susceptibility to PTB are genetics. Evaluation to some research showed that population or various ethnic gives different results. No significant relationship was found in this study between VDR *Apa*I and *Fok*I polymorphism and PTB, which is the same result as in the population of Asia including Korea [15], India [16], China [10], and Iran [17], Africa [18-21] and South American population [22]. A meta analysis among the Asian population demonstrates that genotype ff was associated with susceptibility to TB [10]. Besides; association was found between the *Fok*I polymorphism and TB in the Han population of China [23] and the males in India [24]. Another meta-analysis in China on the Han ethnic illustrates an association between the ff genotype of *Fok*I polymorphism of VDR gene with PTB [25]. Also, a meta-analysis from 13 studies also confirmed the association between the ff genotype of *Fok*I polymorphism with TB [26].

There is no correlation found between *Bsm*I polymorphism of the VDR gene and TB in studies from Africa [19, 30] and Asia including India [27] and Korea [25]. Other studies from Asia, including Iran [28] and Turki [29], and from Africa [30] showed that the bb genotype was a protective factor towards TB, while in another study, the bb genotype correlated to the susceptibility to TB in Iran [17, 31]. Among the Asian population using meta-analysis from 15 studies demonstrates that the b allele and bb genotype are associated with a decreased risk to TB [32]. For the BsmI polymorphism in the European population, the variant homozygote and heterozygote genotypes were associated with a significantly decreased risk of tuberculosis when compared to the wild type homozygote (bb+Bb vs BB) [11]. In the current related study, we found that bb genotype was associated with decreased risk to PTB [13].

Different results of this research compared to others, came from some reasoning. One of them is ethnic factor. Indonesia has more than 300 ethnics and this study restricted to Batak ethnic Indonesian population only, to prevent genetic bias from ethnic influence. Ethnicity is found to be very important factor on genetic function in PTB. *Apa*I polymorphism for PTB patients in Chennai, India was for 60.76% for the A allele and 39.23% for the a allele [12] and this was similar for TB in Bandung, Indonesia that was frequency of A allele 59.5% and for a allele was 40.5% [33] while frequency of A- and a- allele in Batak ethnic in Medan was differ, i. e 33.6% and 66.4% respectively. The distribution of *Fok*I allele in the world diverge between populations or ethnics. Frequency of F allele in North Indian population was 71.5% and f allele was 28.5% [34], comparable to Chennai, India the frequency of F allele was 76.6% and f allele was 23.3% [12], whereas in one study (Batak ethnic in Medan,

North Sumatera) the frequency of F allele was 61.8% and f allelle was 38.2% [13] and this is vary from population in Bandung, Indonesia which is 22.6% for F allele and 77.4% for f allele [33]. Frequency of f allele is lower among African race (24%) if compared to the Caucasians (34%) and Asians (51%) [35]. For *Bsm*I polymorphism, the frequency of B allele in PTB patients in Chennai, India was 58.46% and for b allele was 41.53% [12] but in one linked to this study, the frequency of B allele and b allele were 34.2% and 65.8% respectively [13]. This results also vary form TB patients in Bandung, Indonesia, i.e. frequency of B allele was 14.3% and frequency of b allele was 85.7% [33]. The frequency of B allele is 7% among Asians, 36% among Africans and 42% among Caucasians [35]. Another studies found the *Bsm*I bb genotype frequency was 2% among Asians, 5% among Africa Americans and 17% among Caucasians [6].

The difference in classification of case and control in each study could also have altered the results. Some studies assume the case group as negative acid fast bacilli bacteria sputum or extra pulmonary TB, whereas for the control group, some studies took blood samples from blood banks where history of exposure to TB is not known. Correspondingly, not all studies have conducted HIV tests to determine the HIV status of the subjects [36]. A variety of results could also be caused by gene-environment interaction, gene-gene interaction and gene-agent interaction. In the Indian population, the levels of VDR protein were not fluctuate in both PTB patients and Normal Healthy Subjects (NHS) on polymorphism *Fok*I but a trend towards decreased levels of VDR protein was observed in NHS with BB genotype compared to bb genotype of BsmI polymorphism [12]. In this study and one connected study [13], among those polymorphism *Apa*I, *Fok*I and *Bsm*I, only bb genotype was associated to decreased risk for PTB. A study on Indian Gujarat ethnic resides in London showed no association between *Fok*I polymorphism with TB, but together with vitamin D deficiency, ff genotype is associated with susceptibility to TB [37]. Another study in Bandung, Indonesia proved that FF and Ff genotype 2.94 more frequently found on Child TB than the healthy one [33]. Gene-gene interaction has been shown in some studies. A single gene that is not associated with susceptibility to TB will show an association if combined with other genes [38, 39]. Besides, gene-agent interaction showed association of a certain host gene with a certain strain of *Mycobacterium tuberculosis* [40, 41].

These results might also be affected by other factors. As a ligand of vitamin D, VDR could be activated if Vitamin D Receptor form heterodimer with Retinoid X Receptor (RXR) hence binding to Vitamin D Response Elements (VDRE) which are controlled by Vitamin D Response Elements—Binding Protein (VDRE-BP) on the gene target promoter of cathelicidin [5]. Cathelicidin promotes the elimination of Mtb in phagolysosome. It is known that before vitamin D enters the macrophage, inactive vitamin D in the blood serum could be bound to vitamin D binding protein (DBP) or stay in a free state. After inactive vitamin D bind to Toll-Like Receptor on macrophage, the CYP27b1 enzyme on mitochondria forms the active state of vitamin D. Consequently, along with heat shock protein 70 (hsc70) and bcl-2 associated athanogene (BAG-1), active vitamin D and its receptor enter the nucleus and forms heterodimer with Retinoid X Receptor (RXR) [5, 42]. Therefore, all that occur in transcription of cathelicidin can influence the results of the study.

5. Conclusions

The development of PTB is a complicate interaction between host, pathogen and environmental factors. From this study, which was performed in the Indonesian Batak ethnic population, revealed that *Apa*I gene of VDR polymorphism is not responsible for host susceptibility to PTB and in one related study *Fok*I polymorphism as well [13]. Concerning the *Bsm*I polymorphism [13], the bb genotype was associated

with decreased risk to PTB. This outcome may give further clarification why some people are more resistant against TB than the others.

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