Association between vitamin D receptor polymorphisms and haplotypes with pulmonary tuberculosis

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Abstract. The vitamin D receptor (VDR) is an important factor in activating immune response in different infectious diseases. The aim of the present study was to investigate the association between the *VDR* gene polymorphisms and pulmonary tuberculosis (PTB). The case control study was performed on 120 PTB patients and 131 healthy controls. Genetic analysis was performed by polymerase chain reaction and the restriction fragment length polymorphism method. The *VDR Fok1 Ff* genotype was associated with TB and the risk of PTB was two times higher in individuals with the *Ff* genotype. A higher frequency of *f* allele was observed in PTB patients and therefore, the *f* allele may be a risk factor for PTB susceptibility. There were no associations between the *Taq1* and *Bsm1* polymorphisms and PTB. In addition, haplotype analysis showed that the *f-T-B* and *f-t-b* haplotypes (*Fok1, Taq1* and *Bsm1*) may have the potential to increase PTB susceptibility. In conclusion, the *Ff* genotype and *f* allele of the *VDR Fok1* polymorphism were associated with PTB susceptibility. In addition, the *f-T-B* and *f-t-b* haplotypes may be the susceptible haplotypes for PTB.

Introduction

Tuberculosis (TB) is the result of infection with *Mycobacterium tuberculosis* (*M. tuberculosis*) and is a significant cause of morbidity and mortality worldwide. Each year >9 million people are infected by TB and >1.7 million succumb to TB annually (1). The incidence of TB in Iran has been reported as 13.7 per 100,000 in 2009; however, its incidence was higher in the Sistan-Balouchestan province, southeastern Iran. The higher incidence is due to bordering with Afghanistan and Pakistan; two countries with a high TB prevalence (2). Cell-mediated immunity is essential for suppression of *Mycobacterial* infection as it is an intracellular parasite (3). The fact that only 10% of those infected with *M. tuberculosis* progress to clinical disease revealed that genetic factors, as well as environmental factors are involved in the pathophysiology of TB (4).

In addition, the host genetic basis of TB has been confirmed by twin studies that indicated a two times higher risk of disease in identical twins compared to non-identical twins (5).

Several genes have been found to play a role in TB susceptibility and the relative significance of these genes in disease progression or various forms of disease is often modified by the ethnicity in different populations (6).

The active form of vitamin D, 1-25-dihydroxyvitamin D_3, is an important hormone that modulates the activity of different defense and immune cells, including lymphocytes, monocytes, macrophages and epithelial cells (7). Since vitamin D_3 increases phagocytosis via the activation of macrophages and affects immune response, it is potentially involved in the development of several diseases (8). Vitamin D_3 may limit the growth of *M. tuberculosis* in macrophages (7). Vitamin D_3 exerts its effects through the vitamin D receptor (VDR) and regulates numerous target genes by binding to its nuclear receptor. Active VDR binds to vitamin D response elements that are located in the promoter region of target genes and controls the transcription of these genes (9). The *VDR* gene is located in chromosome 12cen-q12, including at least five promoter regions, eight exons that code proteins and six untranslated exons, which are alternatively spliced. Since there are several polymorphisms in the *VDR* gene that may affect VDR activity, those polymorphisms have been known as potential candidates for genetic susceptibility to TB (10,11).

The *Fok1* polymorphism (rs2228570) of the *VDR* gene, which is located in the translation initiation start site, produces two versions of the VDR protein with different lengths (three amino acids). The short protein, which is encoded by the 'F' allele, is more active than the longer one. Additionally,

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other studies have presented several polymorphisms in strong linkage disequilibrium (LD) in the 3' untranslated region (3'UTR) of the *VDR* gene, including *Taq1* (rs731236), *Bsm1* (rs154410) and *Apa1* (rs7975232). Polymorphisms can be detected by restriction fragment length polymorphism (RFLP). This region of the *VDR* gene regulates gene expression. Therefore, the polymorphisms that are located in this region may influence VDR activity (12). Thus, the present study was designed to evaluate the possible role of the *VDR Fok1*, *Taq1* and *Bsm1* polymorphisms and haplotypes on pulmonary TB (PTB) susceptibility in a local population of southeastern Iran.

Materials and methods

Patient selection. The case-control study was conducted prospectively at a university-affiliated hospital (Boo-Ali Hospital, Zahedan, southeastern Iran). The hospital is a referral center for TB. The study was conducted between March 2010 and May 2011 and a total of 120 patients were selected. Diagnosis of pulmonary TB was made by clinical findings; positive sputum smear for acid-fast bacilli and the results of chest X-ray, but only patients who were confirmed by culture were included in the study. Patients affected with other diseases or conditions, such as myocardial infarction, cirrhosis, acute pancreatitis and septic shock, were excluded from the study. A total of 131 normal healthy subjects who underwent the physical examination at Boo-Ali Hospital were recruited during the study period and were matched for age, gender, ethnicity and geographical origin to patients. The inclusion criteria for normal healthy subjects were absence of clinical symptoms and signs suggestive of active PTB and had a normal chest X-ray. No medical history of TB or other infectious diseases, autoimmune diseases, cancer or other diseases that affect host immunity were observed in the control group. C reactive protein (CRP) was measured for the control group and only negative CRP results were used in the final analysis. The Dean for research affairs of the University Ethics Committee approved the protocol prior to commencing the study.

DNA extraction. Genomic DNA was extracted from 200 μl of peripheral blood in EDTA using the DNA extraction kit (Roche Diagnostics, Mannheim, Germany).

Genotyping of VDR Fok1, Taq1 and Bsm1 polymorphisms. Genotypes were detected using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The primer sequences, annealing temperature, restriction enzymes and fragments sizes are shown in Table I. PCR was performed in a 25 μl final volume containing 25 pmol of each primer, 0.1 mmol/l dNTP (Fermentas, Lithuania), 0.3 μg genomic DNA, 1.5 mmol/l MgCl$_2$, 2.5 μl 10X PCR buffer and 1.5 units Taq DNA polymerase (Fermentas), according to the following protocol: Initial denaturation at 94°C for 4 min; 30 cycles of denaturation at 94°C for 45 sec, annealing for 30 sec and extension at 72°C for 45 sec; and final extension at 72°C for 5 min. The PCR products were digested overnight with *Fok1*, *Taq1* and *Bsm1* restriction endonucleases (Fermentas) and visualized in 2.5% agarose gel electrophoresis.

The presence and absence of a restriction site were assigned a lowercase and uppercase letter, respectively (*a* and *A* for *Apa1*, *t* and *T* for *Taq1*, *f* and *F* for *Fok1*, *b* and *B* for *Bsm1*).

Statistical analysis. The statistical analysis of the data was performed using SPSS software for Windows, version 20 (SPSS, Inc., Chicago, IL, USA). The differences between the groups were analyzed by independent sample t-test, χ^2 test or Fisher's exact test, as appropriate. The χ^2 test was used for deviation of genotype distribution from the Hardy-Weinberg equilibrium. Allele frequencies were calculated by the gene counting method. The odds ratio (OR) and 95% confidence interval (CI) for each variable were also estimated. The frequency of haplotypes was calculated using PHASE software, version 2.1 (13). Logistic regression analysis was used to assess the independent effect of each risk polymorphism and haplotypes on TB. Bonferroni's post hoc correction was applied to confirm the association of haplotypes with the disease. A two-sided significance level of 0.05 was considered to indicate a statistically significant difference. The computation of LD between single-nucleotide polymorphisms (SNPs) was estimated using the normalized measure of allelic association *D'* and the characterization of these patterns was determined using Haploview software, version 4.2 (http://www.broad.mit.edu/mpg/haploview).

Results

Patient characteristics. The demographic and clinical characteristics of PTB patients and controls are shown in Table II. There was no statistically significant difference in gender, age and ethnic characteristics in the patients compared to the control subjects. The frequency of smokers was significantly higher in the PTB patients compared to controls (47 vs. 35; P=0.0001).

Genotype frequencies. The genotype and allele frequencies of *VDR* polymorphisms in PTB patients and healthy controls are shown in Table III. All loci conformed to the Hardy-Weinberg equilibrium in the patient and control groups (P>0.05).

The frequency of the *VDR Ff* genotype was significantly higher in PTB patients compared to controls and the PTB risk was two times higher in individuals with *Ff* genotype prior and subsequent to adjustment for age, gender, smoking and ethnicity. However, the frequency of the *ff* genotype was not different between the two groups prior and subsequent to adjustment for age, gender, smoking and ethnicity. A higher frequency of the *f* allele was observed in TB patients and the *f* allele may be a risk factor for PTB predisposition (OR, 1.8; 95% CR, 1.2-2.8; P=0.006). These findings showed that there were no significant difference regarding *VDR Bsm1* and *Taq1* polymorphisms among the PTB patients and control group.

The LD patterns of the three *VDR* SNPs are shown in Fig. 1. The frequency of seven common haplotypes of the three *VDR* SNPs [*Fok1*(*C/T*), *Taq1*(*T/C*) and *Bsm1*(*A/G*)] are shown in Table IV. The frequency of *f-T-B* and *f-t-b* haplotypes was significantly higher in PTB patients compared to controls and haplotype-based association analysis revealed that the *f-T-B* and *f-t-b* haplotypes may have the potential to increase PTB susceptibility (OR, 1.3; 95% CR, 1.1-1.5; P=0.014 and OR, 1.1;

Table I. Primer sequences, annealing temperature, restriction enzymes and fragment sizes of the vitamin D receptor gene polymorphisms.

Target sequence	Primer sequence	Annealing temperature, ˚C	PCR product	RFLP fragments	Refs
Fok1	F: 5'-AGCTGGCCCTGGCACTGACTCTGGCT-3'	57	267	*F*(C): 305	
	R: 5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3'			*f*(T): 115, 190	11
Taq1	F: 5'-GGGACGATGAGGGATGGACAGAGC-3'	61	716	*T*(T): 512, 204	
	R: 5'-GGAAAGGGGTTAGGTTGGACAGGA-3'			*t*(C): 311, 201, 204	11
Bsm1	F: 5'-AACTTGCATGAGGAGGAGCATGTC-3'	61	813	*B*(A): 813	
	R: 5'-GGAGAGGAGCCTGTGTCCCATTTG-3'			*b*(G): 335, 478	11

PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

Table II. Demographic characteristics of pulmonary tuberculosis (PTB) patients and controls.

Variables	PTB n=120	Controls n=131	P-value	OR (95% CI)
Age, years	51.5±19.7	48.1±12.2	0.1	
Gender (M/F)	45/75	38/93	0.09	
Smoking, n (%)	47 (39)	35 (27)	0.0001	3.2 (1.8-5.8)
Race, n (%)			0.1	
Persian	46 (38)	34 (26)		
Balouch	70 (59)	90 (69)		
Afghan	4 (3)	7 (5)		

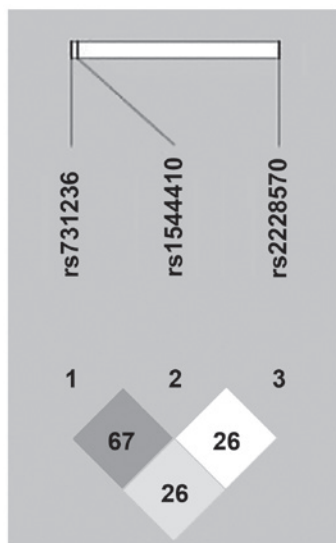OR, odds ratio; CI, confidence interval; M, male; F, female.

Figure 1. Linkage disequilibrium pattern of three single nucleotide polymorphisms in the vitamin D receptor gene.

95% CR, 1-1.2; P=0.012 respectively). The association was also statistically significant following post hoc Bonferroni's correction.

Discussion

TB is a global health problem and its incidence is not the same in different countries, ethnic groups and populations. Much evidence supports an important role for host genetic variations in the predisposition to TB, therefore, the combination effect of genetic and environmental factors may influence the development of TB (14). In addition, there is other evidence that emphasizes the variations in ethnicity for the susceptibility to TB (15). Different candidate genes have been examined in associated studies to evaluate the identity of the TB 'susceptibility factors,' including human leukocyte antigen (16,17), natural-resistance-associated macrophage protein 1 (16,17), *VDR* (11,16), cluster of differentiation 14 (18), interleukins (19) and Toll-like receptors (20).

Several studies have reported a higher frequency of vitamin D deficiency among TB patients and high doses of vitamin D were extensively used for TB treatment (11,21). *In vivo* studies showed that vitamin D suppressed intracellular growth of *M. tuberculosis* (22). Cathelicidin expression, which is the first line of defense in patients, is induced by vitamin D (23).

The active form of vitamin D can lead to macrophage activation and subsequently limit the intracellular growth of *M. tuberculosis*. This vitamin exerts its effect via binding to VDR in the monocytes, therefore the *VDR* gene polymorphisms are suggested to be involved in genetic susceptibility to TB (24). The association between the VDR polymorphisms and TB susceptibility has been studied in different populations and the results were contradictory (10,25-27).

In the present study, a higher frequency of the *VDR Ff* genotype of the *Fok1* polymorphism was observed in the patients compared to the controls. Therefore, this genotype may be considered as a genetic risk factor for the PTB susceptibility. Additionally, the presence of the *Fok1* mutated allele (*f* allele), either in the heterozygous or homozygous state, increased the disease risk.

There were no associations between the *VDR Taq1* and *Bsm1* polymorphisms and PTB. The frequency of the *f-T-B* and *f-t-b* haplotypes of the *VDR Fok1*(*C/T*), *Taq1*(*T/C*) and *Bsm1*(*A/G*) polymorphisms were significantly higher in PTB patients.

An association between 25-hydroxycholecalciferol deficiency and occurrence of TB among the Gujarati Asian

Table III. Genotype and allele frequencies of vitamin D receptor (*VDR*) gene polymorphisms in pulmonary tuberculosis (PTB) patients and healthy controls.

VDR polymorphisms	PTB, n=120 (%)	Controls, n=131 (%)	P-value	OR (95% CI)	P-value	OR[a] (95% CI)
Fok1						
FF	65 (54)	93 (71)		1		
Ff	44 (37)	31 (24)	0.013	2.0 (1.2-3.6)	0.015	2.0 (1.2-3.6)
ff	11 (9)	7 (5)	0.1	1.5 (0.9-2.5)	0.08	1.6 (1-2.6)
Ff + ff	55 (46)	38 (29)	0.006	2.1 (1.2-3.5)	0.006	2.1 (1.2-3.6)
F	174 (73)	217 (83)		1		-
f	66 (27)	45 (17)	0.006	1.8 (1.2-2.8)		
Taq1						
TT	52 (43)	67 (51)		1		
Tt	54 (45)	50 (38)	0.2	1.4 (0.8-2.4)	0.17	1.5 (0.9-2.5)
tt	14 (12)	14 (11)	0.6	1.1 (0.8-1.7)	0.5	1.2 (0.8-1.8)
Tt + tt	68 (57)	64 (49)	0.2	1.4 (0.8-2.3)	0.2	1.4 (0.9-2.3)
T	158 (66)	184 (70)		1		-
t	82 (34)	78 (30)	0.3	1.2 (0.8-1.8)		-
Bsm1						
BB	31 (26)	39 (30)		1		
Bb	66 (55)	70 (53)	0.6	1.2 (0.7-2.1)	0.7	1.1 (0.6-2)
bb	23 (19)	22 (17)	0.5	1.2 (0.8-1.7)	0.4	1.2 (0.8-1.7)
Bb + bb	89 (74)	92 (70)	0.5	1.2 (0.7-2.1)	0.6	1.2 (0.7-2.1)
B	128 (53)	148 (57)		1		
b	112 (47)	114 (43)	0.5	1.1 (0.8-1.6)		

[a]OR, adjusted OR for age, gender, smoking and ethnicity. OR, odds ratio; CI, confidence interval.

Table IV. Haplotypes frequency of vitamin D receptor gene polymorphisms in pulmonary tuberculosis (PTB) patients and controls.

Fok1	*Taq1*	*Bsm1*	PTB %	Control %	P-value	OR (95% CI)
F	T	B	39.2	47.7		1
F	T	b	15.8	14.9	0.3	1.3 (0.8-2.2)
F	t	B	4.2	4.2	0.7	1.1 (0.7-1.7)
F	t	b	13.3	16	0.96	1.0 (0.8-1.2)
f	T	B	9.6	4.6	0.014	1.3 (1.1-1.5)
f	T	b	1.2	3.1	0.3	0.9 (0.7-1.1)
f	t	b	16.2	9.5	0.012	1.1 (1-1.2)

OR, odds ratio; CI, confidence interval.

population in west London has been reported previously. In addition, a significant interaction between the vitamin D status and *Fok1* and *Taq1* polymorphisms and TB was observed (11).

There was an association between ff genotype of *Fok1* but not *Taq1* polymorphism and susceptibility to PTB in Chinese Han population (25).

Although there has not been any reported association between the *VDR Taq1* and *Fok1* polymorphisms and PTB susceptibility in Peru, an association between the *VDR* gene polymorphism and response to treatment of PTB has been observed (26). In a case control study in West Africa, no association between TB and the *VDR Fok1*, *Bsm1*, *Apa1* and *Taq1* variants was reported; however, the *FA* haplotype of the *Fok1* and *Apa1* polymorphisms was correlated with TB susceptibility (10). Although the study by Lombard *et al* (28) did not report any correlation between the *VDR Fok1*, *Bsm1*, *Apa1* and *Taq1* polymorphisms and TB, the *F-b-A-T* haplotype was observed as a protective factor for TB in South Africa.

Similar to the results of the present study, the association between the *Fok1* polymorphism, but not the *Taq1* polymorphism, of the *VDR* gene with PTB has been observed in the Chinese Tibetan population (29).

The results of Alagarasu *et al* (30) indicated that the *b-A-T* haplotype of the 3'UTR *VDR* gene played a protective role against human immunodeficiency virus (HIV) infection, whereas the *B-A-t* haplotype may be associated with susceptibility to the development of TB in HIV-1-infected patients.

In contrast to the results of the present study, Banoei *et al* (31) revealed that the *tt* and *bb* genotypes of the *VDR Taq1* and *Bsm1* polymorphisms are associated with the predisposition to PTB in an Iranian population. In another study, Merza *et al* (32) also confirmed the association of the *VDR Bsm1* (*Bb + bb*) polymorphism and PTB in a local Iranian population.

In a meta-analysis that was performed on 23 studies in 2010, an association between the *Fok1 ff* genotype and TB has been observed among the Asian population (OR, 2.0; 95% CR, 1.3-3.2). Additionally a significant inverse association was observed for the *Bsm1 bb* genotype (OR, 0.5; 95% CR, 0.4-0.8). There were no associations between these polymorphisms and TB among the African or South American populations (27). The association between the *VDR Fok1* polymorphism and extra-PTB and spinal TB has been reported in American and Chinese Han populations, respectively (33,34).

In another study, no correlation between the *Taq1*, *Bsm1* and *Fok1* polymorphisms were found for host susceptibility to human TB in the Korean population (35).

Consistent with the findings of the present study, a higher frequency of the *Fok1 Ff* and *ff* genotypes in TB patients has been reported in the Chinese Kazak population. There were no significant differences of the *Taq1-Tt* and *tt* genotype frequencies between TB patients and healthy controls (16).

Although the reason for this discrepancy remains unclear, these different results in the association studies are common and may be due to the different genetic background of various populations, different selection criteria adopted for patients and controls in particular clinical presentation and environmental risk factors.

There were certain limitations in the present study, such as a small sample size and different ethnic groups (Fars and Balouch) existing in southeast Iran. Therefore, further investigations using a larger sample size and different ethnic groups are necessary to confirm the present results.

In conclusion, the results showed that the *VDR Ff* genotype and *f* allele of the *Fok1* polymorphism was associated with PTB susceptibility. There were no associations between the *VDR Taq1* and *Bsm1* polymorphisms and PTB. In addition, the frequency of the *f-T-B* and *f-t-b* haplotypes was significantly higher in the PTB patients.

Acknowledgements

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