Iranian Journal of Clinical Infectious Diseases
2009;4(4):207-213

ORIGINAL ARTICLE

The VDR and TNF-α gene polymorphisms in Iranian tuberculosis patients: The study on host susceptibility

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ABSTRACT

Background: Vitamin–D receptor (VDR) and tumor necrosis factor–alpha (TNF-α) genes are thought to be important in the intracellular killing of mycobacteria. This study aimed to determine the association of VDR and TNF-α variant with development of pulmonary tuberculosis (PTB) among Iranian patients.

Patients and methods: Selected regions of VDR and TNF-α were amplified, and then the PCR products were digested using restriction enzyme (RFLP). Digested products were run on 8% polyacrylamide gel, and were stained with silver–nitrate. Single nucleotide polymorphisms (SNPs) at restriction sites of B*smI*, and F*okI* of VDR gene and SNPs of TNF-α at -238,-308, -244,-857,-863 positions were analyzed by PCR-RFLP among 117 PTB cases and 60 healthy controls.

Results: No statistically significant difference was observed in allele frequencies of *FokI* of VDR and TNF-α at -238, -244,-863 and –857 position. Although, the frequency of b allele of B*smI* ($p=0.001$) and -308 A variant in TNF-α promoter region ($p=0.006$) were significantly more in PTB patients than healthy controls. The frequency of extended diplotypes were different in patients and control subjects ($p<0.05$).

Conclusion: This study confirmed the association of VDR *BsmI* and TNF-α -308A with susceptibility to tuberculosis in Iranian PTB patients. In addition, the results showed the importance of haplotypes and diplotypes analysis in determining the host susceptibility against TB.

Keywords: *VDR, TNF-α gene, Tuberculosis*.
(Iranian Journal of Clinical Infectious Diseases 2009;4(4):207-213).

INTRODUCTION

In humans, the development of tuberculosis is a two-stage process, through which a susceptible person first becomes infected and then after an interval of years or even decades, later on may develop the active diseases (1). For individuals who are infected with *Mycobacterium tuberculosis* (MTB), the risk of developing disease ranges from 5% to 10% (2). This suggest that besides the mycobacteria itself, the host genetic factors may determine the differences in host susceptibility to TB (3). Recently, a number of genes have been investigated in various case control studies, out of which the vitamin–D receptor (VDR) and tumor necrosis factor–alpha (TNF-α) genes are thought to be important in the intracellular killing of mycobacteria (4,5). Vitamin–D receptor (VDR) gene is located on chromosome 12q and has several

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common allelic variants (4). In overall, the VDR gene consists of nine exons and has several polymorphisms in intron 8 and exon 9, which are in linkage disequilibrium with each other (4). VDR exerts immuno-modulatory effects, which activates the monocytes and restrict the growth of MTB in macrophages (6). The TNF-α gene that encodes the cytokines TNF-α is located within the class III region of the MHC. TNF-α is an important mediator in the inflammatory response against TB and its production controlled both transcriptionally and post transcriptionally (7,8). Some reports have shown that production of TNF-α could be influenced by TNF promoter polymorphisms, TNF microsatellites and HLA-DR gene (7). Generally, serum levels of TNF-α are significantly elevated in patients with advanced tuberculosis compared with those with mild tuberculosis and controls (7). These studies suggested that individual differences in TNF-α production may be genetically determined (8). Polymorphisms in the VDR and TNF-α genes have been associated in susceptibility to tuberculosis in different ethnic groups (9-11), however the results have been inconclusive.

The aim of this study was to investigate the association of polymorphisms in VDR and TNF-α –genes in the Iranian PTB patients**.** Furthermore, the combined polymorphisms (haplotype/ diplotype) of these genes were assessed. To our knowledge this is the first report on the single nucleotide polymorphisms (SNPs) of TNF-α at -244,-857 and -863 positions in TB patients.

PATIENTS and METHODS

The study involved 117 newly smear positive TB patients referred to Iranian National Reference TB Laboratory from January 2007 to January 2008. All patients had positive acid-fast bacilli (AFB) smear microscopy results and their chest X-ray (CXR) had classical picture of TB, i.e., upper lobe infiltration with presence of cavities. Sixty healthy individuals were included in this study as controls.

The Institutional Review Board at NRITLD approved the study and all patients were requested to sign an informed consent. Patients and control subjects were matched for age, sex and nationality.

DNA isolation: Genomic DNA was extracted using the standard protocol with slight modifications (12). Briefly, peripheral blood leukocytes (PBLs) were separated from two milliliters of the whole blood using RBC lysis buffer (0.155M NH_4Cl, 0.01M $NaHCO_3$). Thereafter, PBLs re-suspended in 500µl of SE buffer (NaCl 3M, EDTA 0.5M, PH=8), containing 40µl of 10% SDS and 3µl of 20mg/ml of proteinase K. The suspension was incubated at 60°C for 30 minutes. After incubation, 200µl of equilibrated phenol (PH=8) was added to the mixture and centrifuged for 10 min at 12000g. The aqueous phase transferred to a new tube and the DNA was precipitated using cold propanol.

VDR genotyping: VDR gene polymorphisms were studied using PCR and RFLP. For FokI polymorphisms, the following primers were used to amplify a 265bp product from the region flanking exon 2 of VDR gene: 5' AGCTGGCCCTGGCAC TGACTCTGCTCT-3'; 5'AGGAAACACCTTGC TTCTTCTCCCTC-3' (11). For BsmI polymorphisms, the following primers were used to amplify a 825bp product: 5' CAACCAAGACTA CAAGTACCGCGTCAGTGA-3' ;5'AACCAG CGGGAAGTAAAGGG-3' (13).Cycling conditions for all reaction involved 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min (11).

PCR-RFLP of VDR: PCR products were digested in an excess of restriction enzyme for 3 h (at 65°C) with BsmI at 37°C and with FokI (13). Digested products were run on 8% polyacrylamide gel, and were stained with silver–nitrate. The presence of a restriction site was assigned a lowercase letter and its absence an uppercase letter, according to the convention (table 1).

TNF-α genotyping: TNF-α gene polymorphisms was studied using PCR and RFLP. For TNF –308

polymorphisms, the following primers were used to amplify a 107bp product: 5' AGCAATAGGTGG TTTTGACTCGGGCCCAT-3';5'TCCTCCCTG CTCCGATTCCG-3' (14). For -238 and -244 polymorphisms, the following primers were used to amplify a 230bp product: 5'CCTCAAGGACTC CAAAGCTTTCTG -3'; 5'ACACTCCCCATC CTCCCAGATC -3' (10). For -857 polymorphisms, the following primers were used to amplify a 127bp product: 5' GGCTCTGAGGAATGGGTT AC-3' ;5'CCTCTACATGGCCCTGTCTAC-3'(14). The amplification was accomplished by an initial denaturation at 94°C for 5 min, and 30 cycles at 94°C for 40s, at 56°C for 40s, at 72°C for 1 min, followed by an extension at 72°C for 6 min (14).

PCR-RFLP of TNF-α: PCR products of TNF -238, TNF-244, TNF-308 ,TNF-857 and TNF-863 digested with 2 U enzymes of BgI II, Bsaj I, NcoI, TaiI and TaiI, respectively (10,14). Digested products were run on 8% polyacrylamide gel, and were stained with silver–nitrate.

Finally, the frequency of the genotypes in patient and control groups were estimated by direct gene counting and then the data were analyzed using SPSS (version 11, SPSS Inc., Chicago, IL, USA). In order to test the Hardy-Weinberg equilibrium, all frequencies of various genotypes were compared using the chi-square test. The odds ratio and P-value were calculated for each allele in patient and control groups. All P value was two tailed. A P-value of <0.05 was considered significant with 95% confidence intervals (CI).

RESULTS

Allele frequencies of VDR gene polymorphisms: Each of F*okI* and B*smI* of VDR polymorphisms showed three types of patterns; frequent homozygote allele (wild type), infrequent heterozygote and infrequent homozygote alleles (mutant type). The frequency of F*okI* polymorphisms were FF, F*f*, and *ff*=57.3%, 39.3%, and 3.4% for patients and 58.3%, 41%, and 0% for controls. For *BsmI*, the total number of infrequent allele (B/b + b/b) was more in TB cases (94%) when compared with controls (78%) (OR: 0.24, 95%CI:0.07-0.67, p=0.001). The frequency of BB genotype was 21.7% in controls versus 6% in TB cases (table 1).

Allele frequencies of TNF-α gene polymorphisms: Table 1 represents five polymorphisms in TNF-α gene: a G to A substitution at position-308, a G to A substitution at position -238, a C to T substitution at position-857, a C to A substitution at position -863, and a G to A substitution at position-244. No statistical significant difference was observed in the allele frequencies of TNF-238 C/A and TNF-863C/A in control and TB cases. Although, the frequency of -308A allele were more in TB cases (23.1%) than control subjects (6.7%) (OR:0.26, 95%CI:0.07-0.77, p=0.006). No individual with mutant allele was found at TNF-*α* 244 positions. The TNF-*α*-857 T allele was more in TB cases (41%) than control subjects but the difference was not statistically significant (OR:0.162, 95%CI:0.3-0.77, p=0.181).

Haplotypes and diplotypes analysis of VDR & TNF-α gene polymorphisms: For two VDR polymorphisms, four haplotypes (fB,fb,FB,Fb) were identified. Their frequencies were similar in both groups and no statistical significant differences were noted. The frequencies *of FFBb* (31.6%) and *FfBb* (24%) diplotypes were more in patients (31.6% and 24.8%) than controls (11.3% and 23.3%) (p<0.05). For five TNF-α gene polymorphisms, 9 haplotypes and 19 diplotypes were identified. The ACGGG, *CCGGG* and CTGGG were the most frequent haplotypes in both groups. The overall distribution of diplotypes of TNF-α gene was different between TB and control subjects. The following diplotypes were found in TB cases only; CAAAGAGGGG (6%), CCTTGG GGGG (3.4%), and CCCCGAGGAG (1.7%) (table 2,3).

Table 1. Allele frequencies of VDR & TNF-α genes in Iranian patients with pulmonary tuberculosis (PTB) and controls

Genotypes	Group		x2 (p value)		Odd' ratio (95%CI)
	PTB (n=117)	Control (n=60)	Overall genotypic frequency	Individual genotypic frequency	
VDR-fokI	4(3.4)	0 (0.0)	2.11(P=0.348)		
f/f(homozygote	46(39.3)	25(41.7)			
F/f (heterozygote)	67(57.3)	35(58.3)			
F/F(frequent type)					
VDR bsmI	43(36.8)	26(43.3)	13.03(p=0.001)	-0.157(p=0.001)	B/b+b/b=0.24(0
b/b(homozygote	67(57.3)	21(35.0)			.07-0.67)
B/b(heterozygote)	7(6.0)	13(21.7)			
B/B(frequent type)					
TNF-238	9(7.7)	3(5.0)	0.0455(p=0.552)		
G/A(heterozygote)	108(92.3)	57(95.0)			
G/G(homozygote	0(0.0)	0(0.0)			
A/A(frequent type)					
TNF-308			7.6(p=0.02)	-0.164(p=0.006)	A/G+G/G=0.26
A/A(frequent type)	3(2.6)	0(0.0)			(0.07-0.77)
G/A(heterozygote)	24(20.5)	4(6.7)			
G/G(homozygote	90(76.9)	56(93.3)			
TNF-244			2.25(p=0.325)		
G/G(frequent type)	117(100)	60(100)			
TNF-863					
A/A(homozygote	0(0.0)	1(1.7)			
C/A(heterozygote)	35(29.9)	20(33.3)			
C/C (frequent type)	82(70.1)	39(65.0)			
TNF-857			4.2(p=0.119)	-0.11(p=0.181)	C/T+T/T=0.162
C/T(heterozygote)	42(35.9)	18(30.0)			(0.3-1.25)
C/C(frequent type)	69(59.0)	42(70.0)			
T/T(homozygote	6(5.1)	0(0.0)			

Table 2. Haplotypes frequencies in Iranian patients with pulmonary tuberculosis (PTB) and controls

Haplotypes	Extended haplotype frequency (%)			
	TB cases	Control	P-value	Total population
VDR				
fb	49(20.9)	22(18.3)	NS	71(20.1)
fB	5(2.1)	3(2.5)	NS	8(2.3)
Fb	104(44.4)	51(42.5)	NS	155(43.8)
FB	76(32.5)	44(36.7)	NS	120(33.9)
TNF				
ACAGG	7(3.0)	0 (0)	NS	7(2.0)
ACGGG	22(9.4)	19(15.8)	NS	41(11.6)
ATGGG	6(2.6)	3 (2.5)	NS	9(2.5)
CCAGG	13(5.6)	2 (1.7)	NS	15(4.2)
CCGGA	8(3.4)	3 (2.5)	NS	11(3.1)
CCGGG	130(55.6)	78(65)	NS	208(58.8)
CTAGG	10(4.3)	2 (1.7)	NS	12(3.4)
CTGGA	1(0.4)	0 (0)	NS	1(0.3)
CTGGG	37(15.8)	13(10.8)	NS	50(14.1)

Table 3. Genotypes frequencies in Iranian patients with pulmonary tuberculosis (PTB) and controls

	Extended genotype frequency (%)			
Diplotypes	**TB cases**	**Control**	**P-value /ORs**	**Total population**
VDR				
ffbb	2(1.7)	0(0)	NS	2(1.1)
ffBb	1(0.9)	0(0)	NS	1(0.6)
ffBB	1(0.9)	0(0)	NS	1(0.6)
Ffbb	15(12.0)	15(25)	0.4(0.2-1)	30(16.9)
FfBb	29(24.8)	7(11.7)	NS	36(20.3)
FfBB	2(1.7)	3(5.0)	NS	5(20.8)
FFbb	26(22.2)	11(18.3)	NS	37(20.9)
FFBb	37(31.6)	14(23.3)	NS	51(28.8)
FFBB	4(3.4)	10(16.7)	0.2(0.6-0.6)	14(7.9)
TNF				
AACCGGGGGG	0 (0.0)	1(1.7)	NS	1(0.6)
CACCGAGGGG	7(6.0)	0(0.0)	NS	7(4.0)
CACCGGGGAG	1(0.9)	1(1.7)	NS	2(1.1)
CACCGGGGGG	21(17.9)	16(26.7)	NS	37(20.9)
CACTGGGGAG	1(0.9)	0(0.0)	NS	1(0.6)
CACTGGGGGG	5(4.3)	3(5.0)	NS	8(4.5)
CCCCAAGGGG	1(0.9)	0(0.0)	NS	1(0.6)
CCCCGAGGAG	2(1.7)	0(0.0)	NS	2(1.1)
CCCCGAGGGG	8(6.8)	29(3.3)	NS	10(5.6)
CCCCGGGGAG	3(2.6)	0(0.0)	NS	3(1.7)
CCCCGGGGGG	26(22.2)	22(36.7)	0.5(0.3-0.7)	48(27.1)
CCCTAAGGGG	1(0.9)	0(0.0)	NS	1(0.6)
CCCTGAGGAG	1(0.9)	0(0.0)	NS	1(0.6)
CCCTGAGGGG	6(5.1)	2(3.3)	NS	8(4.5)
CCCTGGGGAG	0(0.0)	2(3.3)	NS	2(1.1)
CCCTGGGGGG	28(23.9)	11(18.3)	NS	39(22.0)
CCTTAAGGGG	1(0.9)	0(0.0)	NS	1(0.6)
CCTTGGGGAG	1(0.9)	0(0.0)	NS	1(0.6)
CCTTGGGGGG	4(3.4)	0(0.0)	NS	4(2.3)

DISCUSSION

In a study carried out in PTB patients of south Indian, the *Bb* genotype of VDR *BsmI* was associated with susceptibility to TB, whereas AA genotype of A*paI* and BB genotype of B*smI* were associated with resistance to PTB ((11,13,15). In another study, the frequency of B*b* genotype was higher in spinal TB than healthy controls (16). In fact, various diallelic polymorphisms have been identified in the vitamin D receptor gene and these polymorphic variants have been shown to be associated with susceptibility or resistance to tuberculosis (13,15,16). Our data reconfirmed the association of B*smI* polymorphisms (B*b*+*bb*) with tuberculosis. Although, the frequency of FF genotype of *VDR FokI* was more than its corresponding mutant one, in both studied groups (table 1). Totally, 58.3% of PTB cases and 57.3% of control subjects had FF alleles. Generally, the VDR gene with FF genotype demonstrated an increased transcription rate, and this has provided the main explanation for association between *Fok*I genotype and the development of diseases. Previous studies showed 65% frequency of FF genotype among Indian PTB patients and 62% among west-Africans (9,13). Although in Chinese and Peruvian the frequency was lowered to 24 and 9%, respectively (15,17). In the present study, the frequency of FF genotype was close to Indian communities.

Recently, two SNPs on TNF-α promoter gene (-238 and -308) have been described, and the presence of A at -308 has been associated with the high TNF-α production (7,10,14). Today, several polymorphisms have been found in TNF gene at position -1031, -863, -857, -575, -376, -308, -244, -238, and +70 (14). Although, the polymorphisms in position, -238, and -308 have been extensively studied in tuberculosis (18,19), previous studies demonstrated no association of particular allele or genotype of TNF-α with TB in different Asians .i.e., Indian, Turkish and Thai population (7,20,21).

In this study, only TNF-α-308A allele was associated with PTB (table 1). Similar to our finding, Bikmaeva et al suggested an association of -308A allele with risk of pulmonary TB (21). Basically, the study of single nucleotide polymorphisms is a traditional approach and today investigators determine the frequency of haplotypes or diplotypes polymorphisms for identifying the candidate genes in molecular epidemiology (9,16,21). In our study, no significant difference was observed in the frequency of haplotypes in the studied groups. Although, their diplotypes were more specific (table 2,3). Therefore, the haplotype or diplotype variables can be considered as an alternative way to study the SNPs of VDR and TNF-α genes in susceptibility to tuberculosis.

In conclusions, the molecular studies would contribute to better understanding the pathogenic

processes that underlie major infectious diseases by allowing a more systematic study of the genetic influences. In the present study, we showed the association of b allele of B*smI* and TNF-α-308A variants in susceptibility to TB.

ACKNOWLEDGEMENT

We thank all the TB patients and their families who have patiently helped us to complete the required information. The project was founded by MRC/NRITLD/WHO Grant No, 0112-23-2007.

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