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Original Article

Comparison of single nucleotide polymorphisms [SNP] at TNF-α promoter region with TNF receptor 2 (TNFR2) in susceptibility to pulmonary tuberculosis; using PCR-RFLP technique

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Abstract: Since apoptosis and survival of the immune cells are crucially important in prevention or predisposition of individual from/to infections, especially in intracellular ones, the current study was performed to assess the correlation of host genetic polymorphisms with susceptibility to TB. For this reason, we investigated the difference of the single nucleotide polymorphisms [SNPs] in tumor necrosis factors [TNF-α] genes at (-238, -308, -857 and -863 position) and tumor necrosis factors receptors two [TNFR2] at (T 587 G position) between patients [n=151] and control [n=83]. The genotyping was studied by using PCR-RFLP which had high sensitivity in detecting compared with other techniques. The results showed a strong correlation between two polymorphisms in different loci of TNF-α gene including TNF-α T-857 C and A 238 G. But no association were found in TNFR2 genes with susceptibility to TB. And we found no correlation between TNFR2 and TNF-α gene polymorphisms. Therefore, the TNF-α T 857 C and A 238 G SNPs could be promising marker for identifying risk populations.

Keywords: Pulmonary tuberculosis, susceptibility, TNF alpha, TNFR2, Iran, PCR-RFLP

Introduction

Mycobacterium tuberculosis is an oldest human pathogen, which has plagued countless human societies, despite the well plan strategies in the last century. Accordingly, to World Health Organization (WHO), the estimated number of patients that fallen ill with TB was recorded as much as 9.6 million people with 1.5 million deaths [1-3]. Despite several attempts to control the disease, TB is too hard to control mainly due to chronic character, long term treatment and difficult vaccine preparation as well as more coincidence with HIV infection [4-6]. The susceptibility to TB depends upon different factors and the risk of developing diseases after infection with M *tuberculosis* ranges from 5% to 10%. This suggests that besides the mycobacterial itself, the host genetic factors may determine the differences in host susceptibility to TB [7-9]. Among the important risk factor, cytokines and specially tumor necrosis factor alpha (TNF-α) genes, are thought to be responsible in regulating the protective immune responses [10-14]. Tumor necrosis factor alpha (TNF-α), gene that encodes the cytokines TNF-α is located within the class III region of the MHC [15]. TNF-α is expressed as a trans-membrane protein (memTNF) that can be processed into a soluble form (sTNF) [16] that exerts its functions via two receptors TNF receptor 1 (TNFR1) and TNFR2. The former exists in almost all cells aims to trigger apoptosis signaling pathways while the latter restricted expression and activation of cell survival. TNFR2 is one of the main receptors on CD4+ and CD8+ lymphocytes; cranial neural crest cells (CNCs) and endothelial cells. CD4+ and CD8+ seem to be important in immune cell replication and differentiation to prevent the growth of intracellular microorgan-

isms like *M tuberculosis* [15-17]. TNF-α gene and its receptors have significant suppressive effects on bacterial growth into macrophages [18]. Overall, TNFR1 promotes inflammatory signaling pathways, whereas TNFR2 mediates immune modulator functions and promotes tissue homeostasis and regeneration [19]. During recent years, allelic polymorphisms of TNF-α gene at the various positions (-238, -308, -857, -863) have been shown to be associated with risk of TB [8, 9, 20, 21]. However, limited investigation has discussed the importance of TNFR2 in susceptibility to TB. The *in-vivo* experiment set up has shown the correlation of serum level of TNFR2 with TNF-α activity, but the importance of allelic polymorphism in TNFR2 and susceptibility to TB is not very clearly discussed. *Mizoguchi* demonstrated that single nucleotide polymorphisms (SNP) in exon 6 of the TNFR2 gene leads to a non-conservative change at position 196 of the amino acid methionine into arginine (T 587 G or Met196Arg) [22]. This SNP, which causes the increase in cytokine production were shown to be associated with rheumatoid arthritis, systemic lupus erythematosus and Crohn's diseases (CD) [23, 24]. Therefore, the SNP at position 587 seems to be important in susceptibility to diseases development [22-24]. In this study, we aimed to genotyped for polymorphism of 5 selected SNPs. Four single-nucleotide polymorphisms in the TNF-α promoter region (-238, -308, -857 and -863) were selected as well as one SNP in TNF receptor II (T 587 G). In order to establish whether any polymorphism was positively associated with the risk of developing tuberculosis, healthy controls were also genotyped. To our knowledge this is the first report that investigates relation between TNF-α gene and the TNFR in susceptibility to pulmonary tuberculosis.

Materials and methods

Setting and study population

Through a cross-sectional study, 151 TB cases and 83 healthy people as controls were recruited in the investigation. Patients and control subjects were matched for age, sex and nationality [The Institutional Review Board at the NRITLD approved the study and all the patients have signed informed consent]. Inclusion criteria for patients with tuberculosis (TB) were two positive sputum smear test results, and a positive sputum culture results, as well as chest radiograph findings and clinical symptoms that were compatible with pulmonary TB. Exclusion criteria were history of any other diseases e.g., malignancy, autoimmune diseases or infection with other mycobacterium than tuberculosis.

Mycobacterial isolation

Collected sputum samples from each patient were digested and decontaminated by Petroff's method. Lowenstein-Jensen media were used for bacterial growth. The extracted DNA from culture positive samples was used for identification [11].

Mycobacterial identification

The isolates were characterized using hsp65 genes spacer PCR-RFLP. For hsp65 gene, a PCR reaction was amplified in 50 ml mixtures containing 4 pmol of specific primers (TB15; 59-CGT AYG ACG AAG AGG CCC GT-39 and TB17; 59-WAS GGR TCC TCS AGG ACS GC-39), 1 ml deoxynucleotide triphosphates, 1.5 ml MgCl, 0.25 ml (1 U) Taq polymerase, 2.5 ml (1%) DMSO, 5 ml PCR Buffer, and 5 ml (20 ng) of extracted DNA. The reaction mixture was subjected to 30 amplification cycles (20 sec at 95°C, 1 min at 60°C, 40 sec at 72°C) followed by a 5 min extension at 95°C [26]. The PCR product of the first step (470 bp) was used for the second amplification using primers TB11 (59-ACC AAC GAT GGT GTG TCC AT-39) and TB12 (59-CTT GTC GAA CCG CAT ACC CT), which produced a segment of 439 bp [25]. PCR products were digested by 5 U of restriction enzyme HaeIII and BstEII for 24 hours at 37°C. The pattern of digested products was analyzed using 8% polyacrylamide gel.

TNF-genotyping

Polymorphisms in the TNF promoter region, namely TNF single nucleotide polymorphisms [SNP], -238, -308, -857 and -863 were studied using PCR-RFLP [9, 11, 27, 28]. For TNF-238 polymorphisms, the following primers were used to amplify a 230 bp product: 5'CCT CAA GGA CTC CAA AGC TTT CTG-3'; 5'ACA CTC CCC ATC CTC CCA GATC-3. For TNF-308 polymorphisms, the following primers were used to amplify a 107 bp product: 5'AGC AAT AGG TGG TTT TGA CTCGGGC CCAT-3'; 5'TCC TCC CTG CTC CGA TTC CG-3'. For -857 polymorphisms, the following primers were used to amplify a 127 bp product: 5'GGC TCT GAG GAA TGG GTT AC-3';

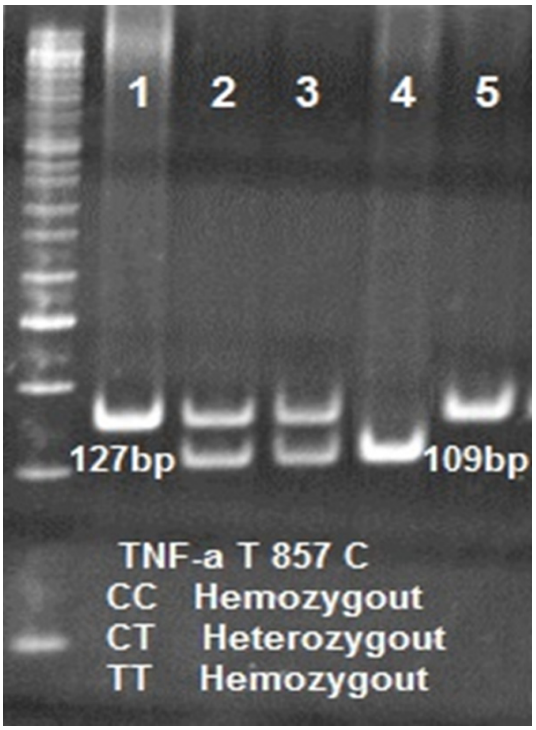

Figure 1. Electrophoresis pattern of polymerase chain reaction (PCR) and PCR-restriction fragments length polymorphisms (PCR-RFLP) of TNF-α at -857 position. The PCR product is 127 bp (lane 1 from left) and the digested pattern by Tai I is CT (line 2&3) 127-109-18 bp; TT (line 4) 109-18 bp and CC (line 5) 127 bp.

5'CCT CTA CAT GGC CCT GTC TAC-3' (**Figure 1**). For -863 the following primers were used to amplify a 126 bp product: A: 5'-GGC TCT GAG GAA TGG GTT AC-3'; B: 5'-CTA CAT GGC CCT GTC TTC GTT ACG-3'. The amplification was accomplished by an initial denaturation at 94°C for 5 min, and 30 cycles at 94°C for 40 s, at 56°C for 40 s, at 72°C for 1 min, followed by an extension at 72°C for 6 min [9, 11, 27, 28]. PCR products of, TNF-238, TNF-308, TNF-857 and TNF-863 digested with 2 U enzymes of Bgl II, Bsaj I, NcoI, TaiI and TaiI, respectively [9, 11]. Digested products were run on 8% polyacrylamide gel and were stained with Silver-Nitrate.

TNFR2-genotyping

For TNFR2 polymorphisms at 587 position, the following primers were used to amplify a 242 bp product: 5'-TTCTGGAGTTGGCTGCGTGT-3' and -ACTCTCCTATCCTGCCTGCT-3'. PCR products of TNFR2 digested with 2 U enzymes of NLA III [21, 23]. Digested products were run on 8% polyacrylamide gel and were stained with Silver-Nitrate (**Figure 2**).

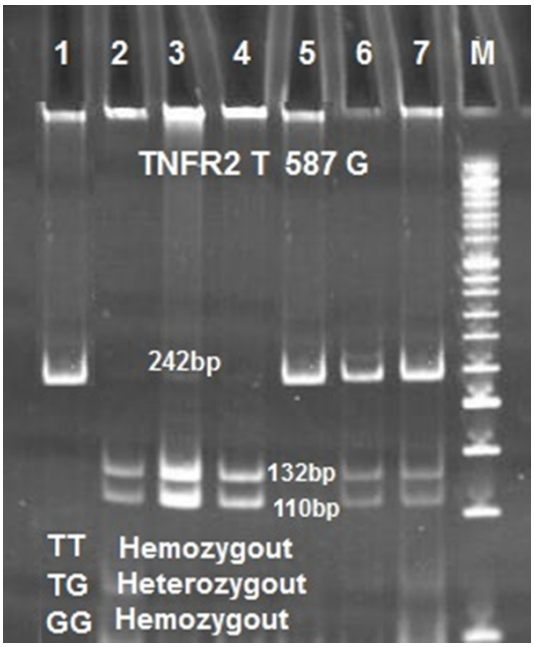

Figure 2. Electrophoresis pattern of polymerase chain reaction (PCR) and PCR-restriction fragments length polymorphisms (PCR-RFLP) of TNFR2. The PCR product is 242 bp (lane 1 from right) and the digested pattern by NIa III. The G allele is 132+110 bp (GG 2, 3, 4 lanes) & TG (7, 6 lanes).

Statistical analyses

Statistical analysis was performed using chi-square test to determine statistical associations between cases and control. *P*-value less than 0.05 were considered statistically significant. Data were analyzed using SPSS version 18 Software.

Ethics

All the patients with positive TB smear and culture were explained for the aim and process of the research project before giving their informed consent and the private and personal information were secured by the investigators. There was no limitation to quit the study and patients and controls paid no cost for genetic tests.

Results

Participants and demographics

Totally 151 cases and 83 controls enrolled in this study. The mean age of patient and control

Table 1. It shows the allele and genotyping frequencies of TNF-α gene and TNFR2

Genotypes	Control (83)	TB cases (151)	OR [95% CI]	P
TNF863				
Allele				
C	122 [73.4%]	255 [84.4%]	0.6 [0.3-1.2]	NS*
A	25 [15.0%]	47 [15.5%]		
Genotype				
CC	61 [73.4%]	106 [70.1%]	0.6 [0.3-1.2]	NS
CA	19 [22.8%]	43 [28.4%]	1.3 [0.5-1.8]	NS
AA	3 [3.6%]	2 [1.3%]	1.5 [1.4-1.7]	NS
TNF857				
Allele				
T	56 [33.7]	72 [23.8]	0.6 [0.4-0.9]	0.02
C	110 [66.3]	230 [76.21]		
Genotype				
TT	8 [9.6]	6 [3.9]	0.3 [0.4-1.1]	0.08
TC	40 [48.1]	60 [39.7]	0.7 [0.4-1.2]	NS
CC	35 [42.1]	85 [56.21]	0.5 [0.3-0.9]	0.03
TNF308				
Allele				
G	154 [92.7]	272 [90.0]	0.7 [0.3-1.4]	NS
A	12 [7.3]	30 [10.0]		
Genotype				
GG	71 [85.5]	122 [80.7]	0.7 [0.3-1.4]	NS
GA	12 [14.5]	28 [18.5]	1.3 [0.6-2.8]	NS
AA	0 [0.0]	1 [0.6]	1.5 [1.4-1.7]	NS
TNF238				
Allele				
A	100 [60.2]	272 [89.4]	5.5 [3.4-9.0]	0.00
G	66 [39.8]	32 [10.6]		
Genotype				
AA	49 [59.0]	127 [84.1]	3.6 [1.9-6.8]	0.00
AG	2 [2.4]	16 [10.5]	4.8 [1.0-2.4]	0.02
GG	32 [38.5]	8 [5.2]	0.1 [0.03-0.2]	0.00
TNFR2 (587)				
Allele				
T	77 [46.3%]	144 [47.6%]	0.5 [0.2-1.4]	NS
G	89 [53.6%]	158 [52.3%]		
Genotype				
TT	8 [9.6%]	10 [6.6%]	1.3 [0.6-2.8]	NS
TG	61 [73.4%]	124 [82.1%]	0.7 [0.3-1.4]	NS
GG	14 [16.8%]	17 [11.2%]	1.3 [0.6-2.8]	NS

NS*: Nonsignificant.

was 49.2±21.2 and 42.5±20.16 years, respectively. The majority of patients [87; 57.6%] had over 50 years of old, and 12 had more than 65 years of old [12; 7.9%]. Seventy eight [51.6%] TB cases were male and seventy three [48.3%] were female. 46 patients [30.4%] had previous history of TB and the remaining were new smear positive TB cases [69.5%]. Fisher's exact test showed a correlation between old age and susceptibility to pulmonary TB (*P* value <0.001). Concerning past medical history, a *P* value <0.001 was enough to blame previous pulmonary TB involvement as a main factor for current disease among our patients.

TNF-α SNPs at -238, -308, -857 and -863 position

In overall, four types of polymorphisms were observed in TNF-α gene: an A to G substitution at position -238, a G to A substitution at position -308, a C to T substitution at position -857 and C to A substitution at position -863. Among these polymorphisms, C allele of TNF857 and A allele of TNF238 were more frequent in TB cases as compared to control group (**Table 1**). The SNPs at -308, -863 were not statistically significant in studied cases [Statistical analysis was performed using chi-square test]. But the genotypes of TNF at 857 C/C [85; 56.2%] and TNF238 A/A 127 [84.1%] genotypes were associated with increased risk of acquiring TB.

TNFR2 SNPs

No significance differences were observed in allele T and G between patients and control cases. The frequency of TT genotypes was 9.6% (8/83) in control and 6.6% (10/151) in TB patients. The most frequent genotypes in TNFR2 at 587 position was TG, which observed in 73.4% of controls and 82.1% of patients.

Correlation of TNF-α and TNFR2 SNPs with TB susceptibility

Combined correlation analysis was then conducted in terms of coincident SNPs in TNF-α

(-238, -308, -857 and -863) and TNFR2 (587) loci. No significant correlation was found.

Discussion

The current study found a strong correlation between two polymorphisms in different loci of TNF-α gene, including 857 C/C [85; 56.2%] and TNF 238 A/A 127 [84.1%]. Although, no association between polymorphisms of TNF-α gene and its receptor. i.e., TNFR2 (**Table 1**) was detected. For decades, several reports illustrated different association between genetic polymorphisms and susceptibility to a wide range of infectious diseases such as tuberculosis. Recently Yi performed a meta-analysis on TNF-α polymorphism and its association with pulmonary TB susceptibility [21]. This study evaluated and analyzed 18 previous studies which compromised a total of 2735 cases and 3177 controls. Among the various positions in the promoter region of TNF-α gene they could outline the protective roles of TNF-α SNPs at position -857 C>T, -308 G>A, 238 G>A in different ethnical populations, e.g., TNF-α SNPs at -308 G>A position is more in Africans origin than Asian [21]. In contrast, the -238 G>A was associated with PTB among Asians. In this regard, Varahram showed the importance of -308 G>A polymorphisms in Iranian PTB patients [11]. But in the present study, the frequency of G to A study was 92.7%, 90% and 7.3%, 10.0% in control and patients, respectively. As the number of studied cases in the present study was higher, we thereby conclude TNF-α SNPs at -308 G>A position may not be associated with Iranian PTB. More recently, Jafari showed an association between 14 gene polymorphisms and PTB [27]. They found that TLR4 (D 299 G and T 399 I) as well as TNF-α (-308 G>A) may influence the risk of PTB [27]. In this regards, Joshi studied the synergistic or prognostic role for cytokines like TNF-α t in three groups of individual including pulmonary TB patients, their household contacting people and healthy individuals [28]. They found significantly higher TNF-α serum levels among patients and their caregivers than healthy people. The significant ratio of TNF-α /IL-10 determined Th1 predominance in PTB patients and caregivers as well. Lee released a research report on the role of TNF-α polymorphisms in PTB susceptibility among European, Asian and Middle Eastern population [29]. They introduced TNF-α -857 T as a protective factor for PTB in Asians (OR=0.682, 95% CI=0.550-0.846) but no relationship was reported for TNF-α -308 A/G and -238 A/G. Our result showed a higher frequency of C allele in TNF-α -857 (76.21%) in patients than control group (66.3%), which is consistent with some early reports [9, 11, 21]. During recent years, it become clear that TNF-α exerts its pleiotropic function by activating signaling cascades via binding to two types of receptors i.e., TNFR1 and TNFR2 [12-16, 19]. TNFR2, encoded by the tumor necrosis factor receptor superfamily member IB gene (TNFRSFIB), can influence the biological activity of TNF-α both in a membrane-bound and a soluble form [17, 22]. In terms of TNFR2 polymorphisms and the risk of PTB, MÖller studied a South African population to find a protective impact of T allele of rs3397 alone and/or the 3'untranslated region haplotype GTT of TNF receptor 2 and concluded a role for TNF and TNF receptor mediated immune responses in the pathogenesis of the named disease [30]. In overall, GTT haplotype was found in an intermediate frequency (26%) among different studied population. We found no association between SNPs in TNFR2 and PTB patients. Thereby, further studies are needed to elucidate the functionally importance of TNFR2 SNPs.

Despite of the big dilemma and controversy for specific human genome diversity to shift individuals toward getting diseases, hereon, pulmonary TB, there are obvious evidence for their impact. Therefore, multidisciplinary studies with multiracial samples are highly advised to confirm the associations which have been raised during recent decades about the risk of involvement, prognosis and response to treatment regarding different demographic and genetic aspects. Concerning our current study, screening assessments for TNF-α -857 and -308 SNPs in Iran would be important in order to make future decisions for preventive treatments, they even could be promising marker to help design effective vaccine, identify risk populations and new treatment strategies.

Disclosure of conflict of interest

None.

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