TNF- \( \alpha \) -308 G/A POLYMORPHISM AND SUSCEPTIBILITY TO TUBERCULOSIS IN AZERI POPULATION OF IRAN

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Single nucleotide polymorphisms (SNPs) in cytokine genes may alter the level and function of secreted cytokine; therefore, SNPs can influence the immune response. The aim of the present study was to determine the association of TNF-\( \alpha \) -308G/A single nucleotide polymorphism in tuberculosis patients in the Azeri population of Iran. The TNF-308G/A single nucleotide polymorphism in the promoter region was genotyped by using the allele-specific PCR method in 200 healthy controls and 124 tuberculosis patients.

The distribution of allele frequencies for TNF-\( \alpha \) -308G/A polymorphism between control and tuberculosis patient groups was not significant \((P\text{-value} = 0.058, \text{OR} = 1.5)\). Furthermore, no statistically significant association was found between TNF-\( \alpha \) -308G/A genotype and resistance/susceptibility to TB \((P\text{-value} = 0.102)\). Our results suggest that TNF-\( \alpha \) -308G/A polymorphism has no measurable effect on the development of tuberculosis in Azeri population of Iran.

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**INTRODUCTION**

It is estimated that 9.0 million new cases of TB (13% co-infected with HIV), also 1.5 million deaths occurred due to TB in 2013 (World Health Organization, 2014). Only 10% of tuberculosis-infected individuals develop active TB (Khalilullah et al., 2014). In addition, twin studies have shown that monozygotic twins (monozygotic twins are genetically identical) have a higher concordance for tuberculosis when compared with dizygotic twins or other pairs of siblings (Cheepsattayakorn and Cheepsattayakorn, 2009). These findings suggest that host genetic factors play a key role in the development of TB disease.

TNF-α produced by macrophages, T cells, and dendritic cells in response to *Mycobacterium tuberculosis* infection (Yim and Selvaraj, 2010). The TNF-α have the important role in granuloma formation and maintenance the structure of formed granulomas. The granuloma makes a suitable local environment for the interaction of immune cells that leads to an effective immune response. Eventually cytokine production, macrophage activation, and T cytotoxic lymphocyte effector function resulted in the killing of the pathogen. Moreover, the granuloma inhibits the spread of the infection (Algood et al., 2003). In contrast to this protective role of TNF-α in the development of TB, It is reported that the serum level of TNF-α is significantly higher in patients with advanced tuberculosis when compared to the mild case of TB and control individuals (Fiorenza et al., 2005). Furthermore, the expression of TNF increases in pleural fluid of patients with pleural TB (Liang et al., 2011).

The TNF-α gene is located within major histocompatibility complex class III (MHC III) a highly polymorphic region on chromosome 6p21. There are many SNPs in the promoter of this gene (Hajeer and Hutchinson, 2001). One of the most important polymorphisms in the TNF-α gene is located at -308G/A position that relates to variable levels of TNF-α. Indeed, TNF-α -308 A allele compared with G allele has higher transcriptional activity (Wilson et al., 1997; González et al., 2003; Sallakci et al., 2005). On the other hand, some studies reported the TNF-α -308G/A polymorphism has no effect on the expression of TNF-α gene (Brinkman et al., 1995; Uglialoro et al., 1998).

In addition to the above conflicting studies, the association studies between -308G/A SNP and tuberculosis have shown different results, some studies reported a significant association (Scola et al., 2003; Merza et al., 2009), but the results of other studies were not significant (Oh et al., 2007; Ates et al., 2008; Velayati et al., 2013). Therefore, further study was needed to clarify these discrepancies. The aim of the present study was to determine the association of TNF-α -308G/A single nucleotide polymorphism in TB patients in the Azeri population of Iran.

**MATERIALS AND METHODS**

**Patients and controls**

One hundred twenty-four patients (71 males and 53 females) with active TB and 200 (183 males and 17 females) healthy subjects participated in the study. Blood samples of patients were collected from Sina Hospital, Tuberculosis and Lung Disease Research Center and Imam Reza Hospital from 2011 to 2014. Patients and healthy individuals had the same ethnic background and were from East Azerbaijan province of Iran. Tuberculosis in the patient group was proven by conventional bacteriological methods (smear/ culture positive). The inclusion
criteria for the patient group were the absence of human immunodeficiency virus (HIV) infection and immunosuppressive conditions, and for the control group was the absence of autoimmune diseases, family history of tuberculosis disease and acute or chronic pulmonary disease. Informed consents were obtained from all subjects. This study was approved by the Ethical Committee of Tabriz University of Medical Sciences, Iran (project number 9253).

**DNA extraction and purification**

Extraction of DNA was performed by using a standard method with slight modifications (ASGHARZADEH *et al.*, 2007). About 300 μl of buffy-coat obtained from blood samples was suspended in 150 μl of TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0) by vortexing. After 60 μl of 10% SDS and 10 μl of 20 mg/mL proteinase K were added, mixed vigorously and incubated overnight at 60°C. Next, 100 μl of 5 M NaCl was added and shaken and about 80 μl of pre-warmed (65°C) CTAB NaCl solution (10%CTAB + 0.7M NaCl) was added, mixed vigorously and incubated for 10 min at 65°C. After the addition of 700 μl of chloroform/isoamyl alcohol (24:1), the suspension was mixed for 20 s and centrifuged for 8 min at 11,000 × g. The upper phase was transferred to a new microtube and 0.6 volumes of 2-propanol were added and incubated for 30 min at -20°C. The solution was centrifuged at 12,000 × g for 15 min. The DNA pellet was washed with cold 70% ethanol and centrifuged at 12,000 × g for 5 min. The supernatant was discarded, and the DNA pellet was dried at room temperature. The DNA pellet was resuspended in 50 μl of distilled water and stored at -20°C.

**TNF-α -308G/A genotyping**

The TNF-α -308G/A SNP in the promoter region was genotyped by using the allele-specific PCR method as previously described (OH *et al.* 2007). The PCR primers used for genotyping were as follows: (generic primer, antisense) 5'-tcgctgggagccc-3', (Primer G, allele1, sense) 5'-ataggttttgagggga-3', (Primer A, allele2, sense) 5'-aataggtttgaggggcatg-3'.

PCR was performed in a total volume of 20μl containing 100 ng of genomic DNA, 100μM of each dNTP, 0.5μM of each primer, 50mM KCl, 20 mM Tris-HCl(pH = 8.4), 1.5 mM MgCl2, and 1.25 units of recombinant Taq DNA polymerase (CinnaGen, Iran). Cycling was performed in a Mastercycler gradient (Eppendorf-Germany) as follows: a pre-PCR step of 7 min denaturation at 94°C, followed by 35 cycles at 94°C for 40s, at 59°C for 45s, at 72°C for 45s, with final extension was performed at 72°C for 7 min. The amplified products were separated by agarose gel (1.2%) electrophoresis, stained with 0.5μg/ml ethidium bromide and visualized under UV light (PCR product size = 185 bp). The PCR product size was determined by comparison with the 100bp DNA ladder plus size marker (Fermentas, Lithuania).

**Statistical analysis**

The genotype and allele frequencies were compared between patient and healthy control groups. Moreover, the odds ratios (ORs) with 95% confidence intervals (CIs) were calculated for TNF-α -308G/A SNP for evaluating the relative risk to TB. *P*-values were calculated using the Pearson chi-square probability; *P*-values were corrected for multiple testing (Bonferroni correction; Pc). Pc (P corrected = Number of variables × *P*-value) less than 0.05 was considered to be statistically significant. Observed and expected genotypes in both control and patient groups were tested for Hardy–Weinberg equilibrium, *P*-value< 0.05 was considered a deviation
from Hardy–Weinberg equilibrium. All statistical analyses were performed by chi-square test, SPSS software (version 22).

RESULTS

The distribution of allele frequencies for TNF-α -308G/A polymorphism between control and TB patient groups was not significant (P-value = 0.058, Pc = 0.116, OR = 1.5) (Table 1). Furthermore, no statistically significant association was found between TNF-α -308G/A genotype and resistance/susceptibility to TB (P-value = 0.102). The GG genotype (patients = 62.9%, controls = 74%) and G allele (patients = 80.65%, controls = 86.25) were more frequent in both patient and control groups, although were less frequent in the patient group than in the controls (Table 2). The genotype frequencies for TNF-α -308G/A polymorphism was in Hardy–Weinberg equilibrium in both patient and control groups (P-value = 0.44, P-value = 0.91, respectively).

Table 1. Allele frequency of the TNF-α -308G/A polymorphism in TB patients and controls.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Controls N (%)</th>
<th>TB patients N (%)</th>
<th>χ²</th>
<th>OR</th>
<th>95% CI</th>
<th>P-value</th>
<th>Pc</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>55 (13.75%)</td>
<td>48 (19.34%)</td>
<td></td>
<td>3.59</td>
<td>1.5</td>
<td>0.98-2.30</td>
<td>0.058</td>
</tr>
<tr>
<td>G</td>
<td>345 (86.25%)</td>
<td>200 (80.65%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>400</td>
<td>248</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N: number of subjects; Pc: corrected P-values (P-value × number of Genotypes); OR: odds ratio; CI: confidence intervals

TNF-α alleles in TB patients versus controls. Chi-square test (2×2 comparison).

Table 2. Genotype frequency of the TNF-α -308G/A polymorphism in TB patients and controls.

<table>
<thead>
<tr>
<th>*Genotype</th>
<th>Controls N (%)</th>
<th>TB Patients N (%)</th>
<th>χ²</th>
<th>OR</th>
<th>95% CI</th>
<th>P-value</th>
<th>Pc</th>
</tr>
</thead>
<tbody>
<tr>
<td>*AA(referent)</td>
<td>3 (1.5%)</td>
<td>2 (1.61%)</td>
<td>1</td>
<td>referent</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>49 (24.5%)</td>
<td>44 (35.49%)</td>
<td>0.74</td>
<td>0.12-4.65</td>
<td>0.75</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>148 (74%)</td>
<td>78 (62.9%)</td>
<td>1.26</td>
<td>0.21-7.75</td>
<td>0.799</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>**AA</td>
<td>3 (1.5%)</td>
<td>2 (1.61%)</td>
<td>4.57</td>
<td>-</td>
<td>-</td>
<td>0.102</td>
<td>NS</td>
</tr>
<tr>
<td>AG</td>
<td>49 (24.5%)</td>
<td>44 (35.49%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>148 (74%)</td>
<td>78 (62.9%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
<td>124</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N: number of subjects; OR: odds ratio; CI: confidence intervals; NS: Not significant; Pc: corrected P-values (P-value × number of Genotypes/alleles)

*TNF-α genotypes frequencies in TB patients versus controls, AA genotype was used as reference, Chi-square test (2×2 comparison).

**TNF-α genotypes frequencies in TB patients versus controls, Chi-square test (3×2 comparison).
DISCUSSION

Single nucleotide polymorphisms in cytokine genes may alter the level and function of secreted cytokine; therefore, SNPs can influence the immune response (BIDWELL et al., 1999). In the present study, there was no statistically significant association between the –308G/A polymorphism and tuberculosis infection. The allele frequencies of TNF-α -308G/A polymorphism between the healthy individuals and TB patients was not significant (P-value = 0.058, OR = 1.5), no significant difference in the distribution of TNF-α genotype was also observed between controls and TB patients (P-value = 0.102). Banerjee et al indicated that the presence of −308 A allele and GA/AA genotype significantly associated with high-level of sera TNF-α (BANERJEE et al., 2011), in addition, high-level production of TNF-α is considered as a risk factor for progression of active extrapulmonary TB (BEN-SELMa et al., 2011). On the other hand, Brinkman et al. reported that the TNF-α -308G/A polymorphism has no measurable effect on the expression of TNF-α gene, thus this polymorphism has no impact on TB development (BRINKMAN et al. 1995), indeed our results may be due to the lack of effect of this polymorphism on the transcription as demonstrated by Brinkman et al (BRINKMAN et al. 1995).

Our result is consistent with previous studies that demonstrated no association between this polymorphism and development of TB (OH et al. 2007; VEJBAESYA et al., 2007; ATES et al. 2008; SHARMA et al., 2010; VEILAYATI et al. 2013). In contrast to our results, several other studies have shown a significant association between this polymorphism and tuberculosis (SCOLA et al. 2003; MERZA et al. 2009). Therefore, WANG et al. (2012) in a meta-analysis study showed that TNF-α -308G/A polymorphism was significantly associated with TB in Asian populations but was not associated in Caucasians populations. In addition, BEN-SELMa et al. reported that the 308 A allele was associated with the development of extrapulmonary TB, but no significant association was observed with pulmonary TB. These contradictory results may be due to study sample size, patients and controls selection criteria, distal promoter elements, and ethnic-specific genetic variations. Ethnic-specific genetic variations can influence the manner of immune responses to microbial pathogens. On the other hand, TNF-α -308G/A polymorphism may be in linkage disequilibrium with other functional polymorphism; several studies have shown that there is strong linkage disequilibrium between TNF-α polymorphisms and MHC (PRICE et al., 1999; LIO et al., 2001). TNF-α polymorphism association studies in Caucasian populations encounter with difficulties because there is a disequilibrium between TNF-α and MHC (ATES et al. 2008). As a result, the linkage disequilibrium in specific ethnic populations can affect the association studies.

The frequency of the high expression AA + AG genotype (BANERJEE et al. 2011), in our healthy population (control group) (26%) was similar to that of the Tunisian population (26%) (BEN-SELMa et al. 2011); however, was significantly different with Thais (10.2%) (VEJBAESYA et al. 2007), Indian (16.13%) (SHARMA et al. 2010), and other ethnic groups of Iran (MERZA et al. 2009; VEILAYATI et al. 2013).

In conclusion, our results suggest that TNF-α -308G/A polymorphism has no measurable effect on the development of TB in Azeri population of Iran. Further research with large sample size on TNF-α -308G/A polymorphism can lead to more reliable results regarding the relationship between TNF-α -308G/A polymorphism and tuberculosis.
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Polimorfizam pojedinačnih nukleotida (Single nucleotide polymorphisms-SNPs) u genima za citokinine može da izmeni nivo i funkciju sekrecije citokinina, na taj način SNPs mogu da utiču na imuni odgovor. Cilj ovih ispitivanja je bio određivanje asocijacije TNF-α -308G/A SNP kod pacijenata sa tuberkulozom Azeri populacije u Iranu. Izvršeno je genotipovanje polimorfizma regiona promotora korišćenjem atel- specifičneg PCR metoda kod kontrolnih, zdravih i 124 tuberkuloznih pacijenata. Distribucija frekvencije TNF-α -308G/A polimorfizma između grupa zdravih i tuberkuloznih pacijenata nije bila značajna ($P\text{-value} = 0.058$, OR = 1.5). Pored toga, nije uvrđena statistički značajna asocijacija između TNF-α -308G/A genotipa i rezistentnosti/osetljivosti na tuberkulozu ($P\text{-value} = 0.102$). Dobijeni rezultati ukazuju da TNF-α -308G/A polimorfizam nema merljiv efekat na razvoj tuberkuloze u Azeri populaciji Irana.

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