ASSOCIATION BETWEEN IFN-γ +874A/T, TNF-α -308G/A AND IL-12Rβ2 -237C/T GENE POLYMORPHISMS AND SUSCEPTIBILITY TO PULMONARY TUBERCULOSIS IN A TURKISH POPULATION

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ABSTRACT

Introduction: Host genetic factors may be associated with an increased risk of developing tuberculosis (TB). The specific aim of this study was to investigate the associations between interferon-gamma (IFN-γ) +874A/T, tumor necrosis factor-alpha (TNF-α) -308G/A and interleukin-12 receptor beta2 (IL-12Rβ2) -237C/T gene polymorphisms and the development of pulmonary tuberculosis (PTB) in a Turkish population.

Materials and methods: The study was performed as a case-control study. The data was collected using prospective methods. Genotyping of the rs2430561 region of IFN-γ gene, rs11810249 region of IL-12Rβ2 gene and rs1800629 region of TNF-α gene were analyzed by Polymerase Chain Reaction (PCR)-based direct sequencing. Bidirectional sequence traces were analyzed with SeqScape® Software v3.0 (Applied Biosystems, USA) and manually reviewed.

Results: 92 patients with a smear-positive PTB and 42 healthy subjects were enrolled in the study. Sex and age were similar in both groups (p> 0.05). The genotype frequencies of the IFN-γ +874A/T, TNF-α -308G/A and IL-12Rβ2 -237C/T polymorphisms showed no statistically significant difference between the two groups (p>0.05). The distribution of IFN-γ +874A/T, TNF-α -308G/A and IL-12Rβ2 -237C/T polymorphisms in patients with PTB were not significantly different from those of controls in allele frequencies (p> 0.05).

Conclusion: Our findings suggest that IFN-γ +874A/T, TNF-α -308G/A and IL-12Rβ2 -237C/T gene polymorphisms are not risk factors for susceptibility to the development of PTB in a Turkish population.

Key words: Pulmonary tuberculosis, IFN-gamma, interleukin-12, TNF-alpha, genetic polymorphism.

Introduction

Tuberculosis (TB) remains a major global health problem⁴. It is the second leading cause of death from an infectious disease worldwide, after human immunodeficiency virus (HIV) infection. Although Mycobacterium tuberculosis infects approximately one-third of the world’s population, only 5-10% of these latent individuals will develop active TB disease during their lifetime⁵.

Host genetic factors may be associated with an increased risk of developing active TB. Genetic variations in an increasing number of genes have been associated with human susceptibility to TB disease⁴. T helper (Th) cells were classified as Th1 and Th2 by Mossman et al. on the basis of the cytokines they express⁶. Th1/Th2 cytokine balance is known to play a key role in controlling TB infection. The major immunologic host defense mechanism for TB
is based on a Th1-type cytokine response, including interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and interleukin-12 (IL-12). IFN-γ plays a critical role in the initial protective immune response against *Mycobacterium tuberculosis* and is involved in macrophage activation\(^5\). Mice defective in IFN-γ genes were unable to control a virulent *Mycobacterium tuberculosis* infection\(^6\). TNF-α is the cytokine that plays a key role in both granuloma formation and in macrophage activation\(^3\). In the absence of TNF-α, the immune system is unable to manage a latent TB infection\(^7\). The increased risk of developing active TB in patients receiving anti-TNF-α agents for chronic inflammatory diseases, also supports the role that TNF-α has in controlling latent TB in humans\(^8\). IL-12 is the main Th1-response inducing cytokine and is important in mediating a protective immune response to *Mycobacterium tuberculosis*\(^9\). IL-12 has a crucial role in the induction of IFN-γ secretion\(^9\). IL-12 binds to a membrane receptor complex that is composed of two subunits: IL-12 receptor (IL-12R) β1 and IL-12Rβ2. Mice that were deficient in IL-12Rβ2 were unable to produce IFN-γ and to generate a Th1 cytokine response, which shows that IL-12Rβ2 has an essential role in mediating the biological functions of IL-12 in mice\(^10\).

Gene polymorphisms, related to cytokine production, which may negatively influence the secretion of these cytokines, may be associated with the development of TB. The identification of genetic host factors may provide new prophylaxis and treatment strategies for TB prevention and control. The specific aim of this study was to investigate the associations between IFN-γ +874A/T, TNF-α -308G/A and IL-12Rβ2-237C/T gene polymorphisms and susceptibility to pulmonary tuberculosis (PTB) in a Turkish population.

**Materials and methods**

A case-control, multi-center study was performed prospectively from December 2013 to December 2014 at the Gulhane Military Medical Academy (GMMA) Haydarpasa Training Hospital, Yedikule Chest Diseases Hospital and Sureyyapasa Chest Diseases Hospital. The Institutional Ethics Committee of GMMA Haydarpasa Training Hospital approved the study. All subjects signed written informed consent.

**Study population**

Patients with newly diagnosed smear-positive PTB undergoing anti-tuberculosis treatment were enrolled in the study. The diagnosis of smear-positive PTB was based on clinical symptoms and radiological findings, along with at least one sputum smear positive for acid fast bacillus (AFB) as described by WHO\(^5\). Healthy subjects who did not have recent signs, symptoms or history of TB were enrolled in a control group. Table

Patients with immune-mediated inflammatory diseases, acquired immune deficiency syndromes, cancer and patients receiving immunosuppressive treatment were excluded. These patients were excluded given the complex nature of their primary disease and treatment, which may result in an increased risk of TB.

Blood samples were used for genetic analysis. Blood was drawn into disodium ethylene diamine tetra acetic acid (Na-EDTA) tubes and stored at -20°C until DNA extraction.

**Genetic Analysis:** Genotyping of the rs2430561 region of IFN-γ gene, rs11810249 region of 12Rβ2 gene and rs1800629 region of TNF-α gene were done by Polymerase Chain Reaction (PCR)-based direct sequencing. Genomic DNAs were isolated from 1 mL of EDTA anticoagulated whole blood by using QIAamp DNA Blood Mini Kit (250) (catalog #: 51106) (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. DNA concentrations of samples were assessed spectrophotometrically by using Nanodrop 1000 spectrophotometer (Thermo Scientific, USA). PCR amplifications of related regions of IFN-γ, IL-12Rβ2 and TNF-α genes were performed in a Thermal Cycler (ABI, Applied Biosystems, USA) by using HotStarTaq DNA Polymerase kit (catalog #: 203205) (QIAGEN, Hilden, Germany) and the appropriate primers that are listed in table 1.

**Table 1:** Primer sequences of IFN-γ, IL-12Rβ2 and TNF-α cytokine genes and PCR product sizes are listed.

bp: base pair; IFN-γ: interferon-γ, TNF-α: tumor necrosis factor-alpha, IL-12Rβ2: interleukin-12 receptor beta2. PCR: Polymerase Chain Reaction.
PCR reactions were run as total volume of 50 µl reaction mixtures consisting of nuclease-free water, 5 µl 10x PCR Buffer, 25mM MgCl2 (none for rs2430561, 2 µl for rs11810249 and 1 µl for 1800629), 2 µl 10 mM dNTP mix (ABI, Applied Biosystems, USA), 5 µl of each primer (4pmol/µl), 0.25 µl of HotStarTaq DNA polymerase and 100 ng of genomic DNA. After an initial denaturation at 95°C for 15 minutes, 36 cycles were performed of 30 seconds denaturation at 95°C, 30 seconds annealing at 59°C for rs2430561, 60°C for rs11810249, 55°C for rs1800629 and 30 seconds extension at 72°C, followed by a final extension of 10 minutes at 72°C. The intensity of PCR products of 389 bp (rs2430561), 455 bp (rs11810249) and 328 bp (rs1800629) were checked by running 5 µl on each PCR reaction with 2 µl of loading dye on a 2% agarose gel (Figure 1).

Reagent contamination controls were performed by examining lane for “No DNA” blank tubes. Then, all successful PCR products were purified using PureLink® PCR Purification Kit (catalog #: K3100-01) (Invitrogen Life Technologies, USA) according to the manufacturer’s instructions. The purified amplicons were submitted to direct sequencing in both directions (forward and reverse) using reagents from the BigDye Terminator v3.1 Cycle Sequencing kit (ABI, Applied Biosystems, USA) according to the manufacturer’s protocol (Figure 2 and Figure 3).

After ethanol precipitation subsequent products were run on the ABI-3730 (48 capillary) automatic sequencer (Applied Biosystems, USA). Bidirectional sequence traces were analyzed with SeqScape® Software v3.0 (Applied Biosystems, USA) and manually reviewed.

**Figure 1**: Agarose gel electrophoresis of Polymerase Chain Reaction (PCR) products with 100 base pair (bp) deoxyribo nucleic acid (DNA) ladder. Every lane shows expected bands and no contamination is observed.

**Figure 2**: Samples of forward sequencing electropherograms of rs2430561 (AA, AT, TT) of the IFN-γ gene and rs1800629 (GG, GA, AA) of the TNF-α gene. Black peaks represent guanine (G), blue peaks represent cytosine (C), green peaks represent adenine (A) and red peaks represent thymine (T). IFN-γ: interferon-γ, TNF-α: tumor necrosis factor-alpha.

**Figure 3**: Sample of forward sequencing electropherogram of rs11810249 (CC) of the IL-12Rβ2 gene. Black peaks represent guanine (G), blue peaks represent cytosine (C), green peaks represent adenine (A) and red peaks represent thymine (T). IL-12Rβ2: interleukin-12 receptor beta2.

**Statistical Analysis**

Statistical Package for the Social Sciences (SPSS) software version 18.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The data was expressed as the means ± standard deviation (SD) for continuous data and the frequency and percentage for categorical data. The t-test was used to
compare differences between the groups for the continuous data. Chi-square test was used to compare the distribution of the categorical data between the groups and for deviation from the Hardy Weinberg equilibrium. The association between genotype and PTB was estimated by computing the odds ratio (OR) and 95% confidence interval (CI) from logistic regression analyses. A p value of less than 0.05 was considered statistically significant.

Results

92 patients with smear-positive PTB and 42 healthy subjects were enrolled in the study. Of the 92 study patients, 22 (23.9%) were female and 70 (76.1%) were male. In the control group, nine (21.4%) were female and 33 (78.6%) were male. The mean ages for the study and control groups were 29.75 ± 13.87 and 31.42 ± 13.10 years, respectively. Age and sex were similar in both groups (p=0.51 and 0.75, respectively). Both groups were from the same geographical area and were Caucasian.

The frequency of IFN-γ +874A/T gene polymorphism in the study and control groups is shown in table 2. The genotype distribution of IFN-γ +874A/T was in the Hardy-Weinberg equilibrium for both the study and control groups (p=0.69 and 0.06, respectively). Genotype frequency analysis in the study group revealed that A/T heterozygote (47.8%) was the largest group followed by A/A homozygous (28.3%), and the least frequent was the T/T homozygous (23.9%) group. The genotype frequencies of the IFN-γ +874A/T polymorphism showed no statistically significant difference between the two groups. The distribution of IFN-γ +874A/T polymorphism in patients with PTB did not show a significant difference from the controls in allele frequencies (p=0.83).

<table>
<thead>
<tr>
<th>Patient, no. (%)</th>
<th>Control, no. (%)</th>
<th>OR (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ +874A/T polymorphism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>26 (23.9%)</td>
<td>9 (21.4%)</td>
<td>0.78 (0.34-1.76)</td>
</tr>
<tr>
<td>AT</td>
<td>44 (47.8%)</td>
<td>27 (64.3%)</td>
<td>0.44 (1.01-1.25)</td>
</tr>
<tr>
<td>TT</td>
<td>22 (23.9%)</td>
<td>14 (32.6%)</td>
<td>Reference</td>
</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>96 (52.17)</td>
<td>45 (53.57)</td>
<td>0.992 (0.75-1.35)</td>
</tr>
<tr>
<td>T</td>
<td>88 (47.83)</td>
<td>53 (46.43)</td>
<td>Reference</td>
</tr>
</tbody>
</table>

Table 2: Comparison of the genotype and allele frequencies of +874A/T polymorphism in the IFN-γ gene between pulmonary tuberculosis patients and controls. Data presented as no. [%], No: number, IFN-γ: interferon-γ, OR: odds ratio, CI: confidence interval, A: adenine, T: thymine.

The frequencies of TNF-α -308G/A gene polymorphism in the study and control groups are presented in table 3. The genotype distribution of TNF-α -308G/A was in the Hardy-Weinberg equilibrium for the control group but not for the study group (p=0.44 and <0.001, respectively). Genotype frequency analysis in patients showed that the largest group comprised GG homozygous (84.8% of 92 genotypes). The G/A homozygous group followed (10.9%), and the least frequent was the A/A group (4.3%). The genotype frequencies of the TNF-α -308G/A polymorphism showed no statistically significant difference between the two groups. The comparison of genotype frequencies between GG (reference) and AA was not performed; instead, GG and GA+AA genotype frequencies were compared. The distribution of TNF-α -308G/A gene polymorphism in patients with PTB did not show a significant difference from the controls in allele frequencies (p=0.814).

The frequencies of IL-12Rβ2 -237C/T gene polymorphism in the study and control groups are
presented in table 4. The C/C homozygous genotype frequency was detected in 100% of the patients and controls. There was no gene polymorphism associated with IL-12Rβ2 -237C/T in the study.

Discussion

In this study, we investigated the IFN-γ +874A/T, TNF-α -308G/A and IL-12Rβ2 -237C/T gene polymorphisms and their association with developing PTB. IFN-γ +874 A/T heterozygote (47.82%), TNF-α -308 GG homozygous (84.8%) and IL-12Rβ2 -237 C/C homozygous (100%) were the most frequent genotypes. There were no statistically significant differences in genotype frequencies and there was no association between these gene polymorphisms and PTB development.

The development of TB is the result of a complex interaction between the host and pathogen bacteria. The immunopathogenesis of TB has not yet been fully elucidated. Several genes coding for different cytokines, which have important roles in the immunopathogenesis of TB, may affect a host’s susceptibility to TB.

In a Spanish population, the relationship between IFN-γ +874T/A polymorphism and susceptibility to TB was investigated using cytokine production. The genetic defect in the production of IFN-γ in individuals homozygous for the A allele was shown to be a contributor for an increased risk of developing TB(13). The +874T allele of IFN-γ was considered to be protective for TB and IFN-γ +874T/A was suggested as a genetic marker for TB resistance and could be used to better design a TB vaccine(13). IFN-γ also has a role in limiting PTB. IFN-γ +874 TT genotype and T allele were detected more in minimal or moderate PTB, and IFN-γ AA genotype was detected, especially in advanced PTB and disseminated TB(15). An association of IFN-γ +874T/A gene polymorphism with disease severity rather than susceptibility to TB was also reported in a Croatian Caucasian population(16). A positive association between IFN-γ +874A/T polymorphism and TB was observed in Sicilians, South Africans, Hong Kong Chinese and Spanish populations, but this association was not observed in Malawians, Texans, West Africans, South Indians and Chinese populations(17).

Sallakçı et al. found that IFN-γ +874T/A polymorphism was associated with TB and affected the magnitude of the IFN-γ response in a Turkish population, and the magnitude of the response decreased during transition from TT to TA and to AA genotypes(19). In contrast, Oral et al. reported that IFN-γ +874T/A and TNF-α -308G/A gene polymorphisms were not associated with susceptibility to TB and did not increase risk for TB development in a Turkish population(16). Ulger et al. also showed that there was not a statistically significant association between IFN-γ +874T/A gene polymorphism and susceptibility to TB in another Turkish population(17).

Consistent with most of the previous studies of the Turkish population, we also did not observe a significant association between IFN-γ +874A/T polymorphism and susceptibility to PTB.

TNF-α is a central mediator of granuloma formation and control of bacilli spread(19). Many studies have shown that polymorphisms in the TNF-α gene are implicated in susceptibility to TB, but the results are inconsistent. The TNF-α -308G/A polymorphism was found to be protective against TB in Sicily, and the -308A,-238G haplotype was protective in Colombia (3). Studies on TNF-α polymorphisms (-238 G/A, -308G/A and -376G/A) in Chinese, Cambodian and Indian TB patients revealed no association with either susceptibility or resistance to TB(3). A meta-analysis involving 18 studies, indicated that the TNF-α -308G/A polymorphism was not associated with the risk of TB in the general population; however, there was a significant risk for TNF-α -308 A allele among Asians but not Caucasians(18).

In contrast, another meta-analysis showed an increased association between TNF-α -857C/T polymorphism and TB risk among Asians but no association was found between TNF-α -308G/A and -863C/A with TB risk(19). In a study from Turkey, no statistically significant association was found between TNF-α 308G/A, -238G/A, -376G/A polymorphisms and TB(20). We also did not find any association between TNF-α -308G/A polymorphism and susceptibility to PTB.

IL-12 plays a critical role in Th1 cell development. It drives the development and differentiation of naive T cells into Th1 cells, leading to IFN-γ production(5). IL-12Rβ2 is bound to Janus kinase-2 (Jak2) and mediates signal transduction via transcription–4 (STAT4) that induces IFN-γ production(21). Mice deficient in either of the IL-12 components (p35 and p40) or in its receptor components (IL-12Rβ1 and β2) have deficient Th1 responses(22). Using antibodies to the IL-12Rβ2, IL-12Rβ2 expression on polarized Th cell populations generated in vitro was analyzed and IL-12Rβ2 expression...
was detected selectively in differentiated Th1 and T cytotoxic1 cells, but not Th2 or T cytotoxic2 cells. Controlling the expression of the IL-12Rβ2 subunit could be an important therapeutic target for the redirection of ongoing Th cell responses. It was shown that single nucleotide polymorphisms (SNPs) within the 5′ flanking region of IL-12Rβ2 affect the degree of expression of this gene and may be implicated in individual differences in cell-mediated immunity responsiveness to mycobacterial antigens. Polymorphisms in both the β1 and β2 subunits of IL-12R and its role in PTB were examined in the Japanese population and SPNs in IL-12Rβ1 but not in IL-12Rβ2 were reported to be a risk factor for PTB. Verma et al. examined the -237C/T polymorphism in the IL-12Rβ2 promoter (SNP ID: rs11810249) in Indian TB patients, as in our study. According to their study, the C allele predominated among patients (93.4%, 43/46), whilst the T allele was exclusively limited to a subset of patients (6.5%, 3/46) (27). There was no association between IL-12Rβ2 - 237C/T gene polymorphism and susceptibility to PTB development in our Turkish study population.

The main limitation of this study was restricted statistical power given the relatively small size of the study population.

In conclusion, our findings suggest that IFN-γ +874T/A, TNF-α -308G/A and IL-12Rβ2 - 237C/T gene polymorphisms are not risk factors for susceptibility to PTB in a Turkish population. Further studies with larger sample sizes are needed to interpret the role of these gene polymorphisms in PTB.

References

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