The Relationship Between Toll-Like Receptor 2 Mutation And IL-1 Receptor Antagonist Polymorphism With Tuberculosis Disease

Levent Dalar¹, Seda Tural Onur¹, Mesut Bayraktaroğlu¹, Sinem Nedime Sükcü¹,

Pinar Özkan Epözütk¹, Sedat Altın

Summary

Background: Toll-like receptor 2 (TLR-2) plays role in the immune response of mycobacteria. Interleukin-1(–IL-1) is a cytokine that plays a role in T cell activation and effect with TNF. Interleukin-1 receptor antagonist(IL-1RA) is a natural inhibitor of IL-1. The aim of this study was to demonstrate the relationship between tuberculosis and Arg753Gln mutation in the TLR-2 gene and the effect of IL-1RA polymorphism, which is claimed to increase the susceptibility to tuberculosis in several studies.

Materials and Methods: We compared mutation analysis of 119 consecutive patients with active tuberculosis or tuberculous pleurisy and 83 ethnically matched healthy volunteers.

Results: GG frequency was 97.48 % and GA frequency was 2.52% in the tuberculosis group. We found no statistically significant risk for the GA genotype and IL-1RA alleles. Allele-2 polymorphism correlation with reduced delayed-type hypersensitivity response to PPD (purified protein derivative) was not statistically significant in our series (p=0.084).

Conclusion: The results of this study do not support the hypothesis that there is a relationship between Arg753Gln mutation and the risk of developing tuberculosis. The results of the present study do not support the presence of a relationship between IL-1RA polymorphism and the development and course of tuberculosis.

Key words: toll-like receptor 2, IL-1 receptor antagonist, tuberculosis

Introduction

Tuberculosis(TB) is a common infectious agent in underdeveloped and developing countries. It is believed that genetic factors may be associated with the occurrence of disseminated disease in some people(1,2). Toll-like receptors(TLR) are a set of immune receptors that recognize structures common to many different pathogens and to some endogenous molecules (3). TLRs are transmembrane proteins located on the surface of antigen presenting cells (APCs) as dendritic cells or macrophages (3–7). TLR mediated signalling
induces production pro-inflammatory and immune related cytokines by recognizing pathogen derived structures(8-10).

Recent studies describe TLRs as pattern recognition receptors (PRR) of the host against viruses, bacteria, and fungi. TLRs are transmembrane proteins characterized by an extracellular leucine-rich domain that participates in ligand recognition and an intracellular tail that contains a conserved region called the toll interleukin 1 receptor (IL-1R) homology domain(3-11). Stimulation of TLR initiates a signalling cascade that involves a number of proteins such as MyD 88 and IL-1 receptor associated kinase(12). TLR-2 has been reported to be the principle mediator of macrophage activation in response to mycobacteria(13).

Interleukin receptor antagonist (IL-1RA) is a competitive inhibitor of proinflammation induced by IL-1(14). The polymorphic IL-1RA gene can make quantitative changes in IL-1RA and IL-1β production(15). Individuals with homozygous allele 2 IL-1RA gene have more severe and prolonged proinflammatory immune response than the other genotypes(15).

The IL1-RA polymorphic gene has been investigated extensively and thoroughly(15). This gene is located on chromosome 2 like IL-1α and IL-1β coding gene with very close proximity. IL-1RA also binds to the same IL-1 receptor but does not start signal transduction. Therefore IL-1RA is a competitive inhibitor of IL-1 bioactivity(16). The levels of IL-1RA and IL-1 at the site of inflammation are the decisive whether the proinflammatory response is commencing, progressing or ceasing.

In the second intron sequence of the IL-1RA gene there is a tandem repeat length of 86 base pairs(17). The number of repeat sequence varies from 2 to 6 in humans. The incidence of each allele varies among different ethnic or geographic populations, but the 4 repeat allele 1(IL-1 RN * 1) is more common than the 2 repeat allele 2(IL-1 RN * 2). The remaining alleles with 3, 5 and 6 repeats are seen in less than 1% of the populations.

Individuals with IL-1 RN * 2 allele have a more prolonged and more severe proinflammatory immune response if that is the case homozygosity must make these people more powerful against microbial infection and colonization(15).

TLR and IL-1RA signal collaboration cascades are worthy of further investigation. The aim of current study was to investigate the relationship between the TLR2 Arg753Gln mutation and IL-1RA genotype polymorphism with tuberculosis disease.

Materials And Methods

1. Patient population: The study population was divided into tuberculosis and control groups. A total of 202 subjects, composed of 119 consecutive patients with active pulmonary tuberculosis or tuberculous pleurisy, who were admitted to Yedikule Chest Diseases Centre in Istanbul and 83 healthy controls were included in this study.

Tuberculosis bacilli were required to be seen in sputum or culture, and a clinical, radiologic and histopathologic diagnosis of tuberculous was a requisite to achieve a definite diagnosis of tuberculosis in lung parenchyma. For a definite diagnosis of tuberculous pleurisy, tuberculosis bacilli had to be seen in pleural fluid or otherwise typical caseous granulomas were required to be shown in the pleural membrane. The control group were required to have no problems on their chest X-ray and sputum, no history of tuberculous, and Tuberculin skin test(TST) <15 mm (It is mandatory to BCG(Bacillus Calmette Guérin) vaccine in Turkey both whom has BCG under <15 mm of TST and at first month of the life).

This study was approved by the local hospital ethics committee on human research (Yedikule Chest Diseases and Thoracic Surgery Training and Research Hospital Local Ethics Committee, Approval number: 0028-170904-061004-0028). Written informed consent was obtained from all participants.

2. Collection of blood samples and Genetic Analysis: Blood samples were taken from patients on the day of definitive diagnosis. Samples were centrifuged (Shimadzu UV160A, S. No: 28006648, Japan) at 3000 rpm for 10 min and the serum was stored at –80°C.

i.PCR method: The TLR-2 gene polymorphism (Arg753Gln) was analyzed using polymerase chain reaction-restriction fragment length polymorphism. Genomic DNA was isolated from the peripheral blood samples according to a standard protocol using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA)(18).

PCR reaction was performed in a total volume of 25 µl, which contained approximately 100 ng DNA, 2.5 µl of 10X polymerase buffer, 2.0 mmol/l MgCl2, 0.2 mmol/L dNTPs, 0.4 µmol/l of each primer, and 1 U of Taq polymerase (MBI Fermentas). The PCR program on PTC-150 Minicycler(MJ Research) thermal cycler
protocol was as follows: an initial denaturation step at 94°C for 4 min, followed by 33 cycles of 30 sec at 94°C, 30 sec at 56°C, 30 sec at 72°C, and a final extension step of 8 min at 72°C(19).

A 176-bp product from the TLR-2 gene was amplified using primers TLR2-F:5’- tggatcgtcactcctgcc-3’ and TLR2-R: 5’- tgggaacctgacctttaccg-3’. A total of 5 µl from the PCR product was run on 2% agarose to check for any nonspecific bands. A 10 µl aliquot of the PCR product was digested with 3U of the Pst I enzyme and 2 µl of its 10X reaction buffer in a 20 µl reaction volume (MBI Fermentas). The mixture was incubated at 37°C for 3 hours or overnight. The digestion of the 176 bp product resulted in 72 and 104 bp fragments for 753Gln allele whereas 176 bp product remained undigested with the 753Arg allele. The digested products were electrophoresed on 3% agarose gels at 100 Volt for 30 min. The gel and running buffers were 1X TBE (0.89 M Tris Base, 0.89 M Boric Acid, 20 mM Na2EDTA adjusted to pH 8). The fragments were visualized by ethidium bromide under a UV transilluminator.

The IL-IRN exon 2 polymorphism was analyzed using PCR with primers IL-IRN-F:5’-ctcagcaacactccatctcgtcctgggtcctgccattctcattc-3’ and IL-IRN-R:5’-ctcagcaacactccatctcgtcctgccattctcattc-3’. The PCR reaction was performed in a total volume of 25 µl as above. The conditions used were as follows: 94°C for 5 min, then 35 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 30 s, and, finally, 72°C for 10 min. The PCR product was analyzed using electrophoresis on a 2% agarose gel stained with ethidium bromide. Allele 1 (4 repeats) was 410 bp, allele 2 (2 repeats) was 240 bp, and allele 3 (5 repeats) was 500 bp, allele 4 (3 repeats) was 325 bp, and allele 5 (6 repeats) was 595 bp in length.

3. Statistical evaluation: The results were determined statistically as averages ± standard deviation or median. Mann-Whitney U test was used to compare median values and also t test was used to compare averages ± standard deviation values. For nonparametric values, Chi-square test was used to compare groups. ROC curve analysis was used to determine area under the curve for the Toll-like receptor 2 Arg753Gln mutation and interleukin-1 receptor antagonist polymorphism with descriptive characteristics and biochemical parameters of various boundary value levels.

Results

The mean ages were found to be 35.49±13 years (range, 30-75 years) and 46.47±16.9 years (range, 20-65 years) in patients and healthy controls, respectively. Summarized data of the patients and control groups are given in Table 1.

The tuberculosis group comprised 87 patients(75%) with newly diagnosed disease, 22 patients(18.96%) with relapse or treatment after failure tuberculosis, and 7(6.04%) patients with multidrug resistance. Nineteen(15.96%) cases of pleurisy and 97(84.04%) with parenchymal involvement were found among the patients under radiologic assessment. Twenty-seven(23%) patients had unilateral parenchymal pathology located in the apical lesion and 70(60%) had disseminated parenchymal infiltration. The relationship between Arg753gln genotype and IL-1 R with varieties of tuberculosis was not detected (respectively p=0.096 and p= 0.106). Similarly there was also no association between radiological extent or appearance with TLR and IL1RA allele (p=0.278 ve p=0.096). Previous studies have determined in individuals with IL1RA allele-2 polymorphism’s size reduction of PPD did not show statistical significance in our series(p=0.084).

GG frequency was 97.48% and GA frequency was 2.52% in the tuberculosis group and these values were 97.76% and 2.24%, respectively, in the control group. TLR-2 genotypes on agarose gel shown in Figure 1. AA allele was not detected in the study and control groups.

The allele frequencies of IL- RA gene with a penta-allelic 86-bp tandem repeat in intron 2 were as below: allele-1: 57.2% (111 cases); allele-2: 36.1% (70 cases); allele-3: 5.2% (10 cases) and allele-4: 1.5% (3 cases)(Table 1). IL- RA Allels; Allele 1(4 repeats) was 410 bp, allele 2(2 repeats) was 240 bp, and allele 3(5 repeats) was 500 bp, allele 4 (3 repeats) was 325 bp, and allele 5(6 repeats) was 595 bp in length shown on agarase gel in Figure 2.

Chi-square and correlation analyses demonstrated that there was no statistically significant relationship between the development of tuberculosis and possession of IL-IRA alleles. Similarly, there was no relation between the dissemination of the disease and the size of purified protein derivative(PPD) induration. Allele-2 polymorphism correlation with reduced delayed-type hypersensitivity response to PPD demonstrated in previous studies was not statistically significant in our series(p=0.084). Graphics of genomic distribution shown in Figure 3.
Table 1: Data and Genetic Analysis of the patients and control groups. PPD: Purified protein derivative; BCG: Bacillus-Calmette-Guerin; a: Toll-like b: IL-RA alleles’ repeats; Ns: not significant

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>35.49</td>
<td>84.03</td>
<td>28.21</td>
<td>present</td>
<td>97.48%</td>
<td>3/31.0%</td>
<td>68 (57.1%)</td>
<td>66.5%</td>
<td>31.2%</td>
<td>46 (55.4%)</td>
<td>2/2.4%</td>
<td>0/0%</td>
<td>5/6.0%</td>
</tr>
<tr>
<td>Control group (n: 83)</td>
<td>46.47</td>
<td>14</td>
<td>10.03</td>
<td>present</td>
<td>97.26%</td>
<td>2.24%</td>
<td>29 (34.9%)</td>
<td>2/2.4%</td>
<td>0/0%</td>
<td>5/6.0%</td>
<td>0/0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>46.47</td>
<td>14</td>
<td>10.03</td>
<td>present</td>
<td>97.26%</td>
<td>2.24%</td>
<td>29 (34.9%)</td>
<td>2/2.4%</td>
<td>0/0%</td>
<td>5/6.0%</td>
<td>0/0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.0065</td>
<td>0.001</td>
<td>0.001</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 1:** A 3% (w/v) agarose gel showing Arg753Gln genotypes of TLR-2 gene. Lane 1: homozygote 753Gln alleles; lane 4: 753Gln/753Arg alleles; lane 2, 3, 5, 6 and 7: homozygote 753Arg alleles; and lane 8: 100 bp ladder.

**Figure 2:** A 2% (w/v) agarose gel showing different patterns for IL-RN genotypes. Lane 1:100 bp ladder; lane 2: alleles 1-1; lane 3: alleles 2-2; lane 4: alleles 1-2; lane 5: alleles 2-3; and lane 6: alleles 1-4.

**Figure 3:** Graphs of genomic distribution of IL1RA alleles

**Discussion**

Molecules that are homologous to interleukin I receptors in humans are called “toll-like receptors”. TLRs act as primary sensors for microbial products, activate the immune and inflammatory mechanisms to initiate the synthesis of genes, and affect adaptive responses(4,20). TLR ligands become activated and attach to the intracellular portions of nuclear factor-kappa beta(NF-KB) and stimulate the mitogen-activated protein kinase family(4,21). NF-KB activates cytokines such as TNF-α, IL-1, IL-6 and IL-8 and the genes of proinflammatory products. In current study, the inflammatory process involved in the same pathway both in TLR-2 and IL-1RA has been used to investigate the pathogenesis of tuberculosis.

Shey et al. studied the innate response of the BCG vaccination in the first 9 months of life, they determined different cytokine levels in older and newborn infants in their interesting study. Also these functional and phenotypic differences were possibly due to differential activation of key pro-inflammatory signal transduction pathways downstream of mycobacterial recognition receptor triggering; specifically, lesser TLR1/2-mediated coactivation of monocyte NF-xB and MAPK pathways was observed in newborns than in older infants. This study highlights the importance of the TLR-2 pathway, such differences as may be established(22).

Jensen and colleagues suggest that these effects are also found in infants; BCG vaccination seemed to induce both a Th1-polarizing(IFN-γ) innate response and an increased pro-inflammatory cytokine response (IL-1β, IL-6, and TNF-α) in Guinean 4-week-old infants, including an increased baseline cytokine production in their study. Emphasizing researchers of this pathway was ruled by TLR-4 have shown the importance of the TLR pathway in tuberculous disease(23). Two polymorphisms of the TLR-2 gene have recently been described: Arg753Gln correlated with the incidence of sepsis in a white population and Arg677Trp correlated with the incidence of lepromatous leprosy in an Asian population(24). Other data shows that the polymorphisms of the TLR-2 gene may lead to sensitivity for infections induced by mycobacterial structures(25). Schröder et al. showed that 30 of 319 patients(9.4%) were heterozygotes for Arg753Gln. This rate varied between 2.73% and 14.56% in previous studies. In our study, this rate was 2.52% in the tuberculosis group(3 of 119 cases) and 2.24% in the...
healthy control group (2 of 83 cases) (26). In the Turkish population study of Öğüş et al., AA and GA frequencies were 9.3% and 8.6% in patients with tuberculosis, and 8.5% and 9.3% in those with pulmonary tuberculosis, respectively (27). Naderi et al. studied on 174 patients with Iranian. There was no significant difference in the polymorphism of Arg677Gln of the TLR2 gene among PTB and control groups (p>0.05). The results showed that there was a significant difference between case and control groups regarding 597T/C polymorphism (28).

Hashemi et al. includes series of 265 patients with pulmonary tuberculosis in their study. Our data suggest that VNTR IL1RN polymorphism may not be associated with the risk of PTB in a sample of Iranian population (29).

In contrast to Wu and colleagues compared 205 latent tuberculosis and 109 active tuberculosis cases to 405 healthy volunteers in their study. TLR2, the frequencies of the CC genotype and C allele (SNP) rs3804100 were significantly higher in the latent tuberculosis group than in the healthy control group (30).

Zhang et al. emphasized that TLR2 G2258A is associated with increased TB risk, especially in Asians and Europeans in a meta-analysis. TLR1 G1805T is associated with increased TB in Africans and American Hispanics. They have concluded that TLR6 C745T is associated with decreased TB risk (31).

In present study, the polymorphism frequency in the patients with pulmonary or pleural tuberculosis were below the values reported in previous studies and obtained no data to support that the Arg753Gln gene polymorphism increases the predisposition for tuberculosis. Similarly Schurz and colleagues conclude that most TLR variants showed no significant association of TB in their meta-analysis that they examined 32 study containing the 18907 patient (32).

Interleukin-1 (IL-1) is a cytokine which effects synergistically with TNF released by dendritic cells, macrophages and keratinocytes, plays a role in T cell activation among other activities. Glucocorticoids are known to be potent inhibitors of IL-1. Interleukin receptor antagonist (IL-1RA) which is also a natural inhibitor of IL-1 (20,33). The role and the effect of IL-1RA polymorphism in host susceptibility for tuberculosis have not yet been adequately explored. In this study the effect of IL-1RA polymorphism to the host susceptibility was investigated in pulmonary tuberculosis occurrence.

Bellamy et al. investigated different gene mutations in host susceptibility to tuberculosis in Gambia and suggested that IL-1 gene family and tuberculosis has an association and this association is focused at the IL-1RA gene polymorphism. They also demonstrated an increased susceptibility of tuberculosis in IL-1RA allele 2 (IL-1 RN *2) carriers among Gambians (34). Wilkinson et al. showed in an in vitro study that individuals with homozygous IL-1RA gene allele 2 (IL-1RN*2) have a more prolonged and severe proinflammatory immune response (35). Most populations studied before are have either IL-1 RN *1 homozygous or IL-1 RN *1/ IL-1 RN *2 heterozygotes. The prevalence of IL-1 RN *2 homozygotes is generally <10%. The IL-1 RN *2 homozygote frequency is low in black Africans and African-Americans compared with the white population (36). IL-1RA polymorphism has also recently been shown to influence mycobacterium tuberculosis infection (35). IL-1 RN *2 presence in response to M. tuberculosis was associated with increased IL-1RA messenger RNA and protein production and a higher IL-1RA/IL-1β ratio. The IL-1 RN *2 genotype also correlated with a reduced delayed-type hypersensitivity response to purified protein derivative and inversely related to the development of pleural tuberculosis. Therefore, a particular IL-1RA genotype presence may influence the course of tuberculosis (14). In our study, we obtained no data to support these claims. In our series, the most common allele was allele 1, and allele 2 was the second most frequent allele; however, there was no statistically significant difference between the study and control groups.

Strength of the study is the first clinical trial combined TLR-2 gene mutation and IL-1RA gene polymorphism in Turkish population and to the best of the knowledge in general.

The limitations of the study are relatively small number of patients, and could have not combined with cytokine levels and genotyping analysis. We speculate that the future studies should be combined with inflammatory cytokine levels in bronchoalveolar lavage fluid and genotypic analysis to reach more accurate results.

Conclusion

The results of this study do not support the hypothesis that claims that there is a relationship between Arg753Gln mutation and the risk of developing tuberculosis. The results of the present study do not
support the presence of a relationship between IL-1RA polymorphism and the development and the course of tuberculosis disease in the Turkish population. However, this potential relationship should be studied in other nationalities and large-scaled case-control studies, relating to the extent of parenchyma involvement in cases of pulmonary tuberculosis and Tuberculin Skin Test reaction. A lot of conflicting studies increases the importance of the work related to the presence of this pathway. Further and larger studies are needed to clarify those issues.

Conflict of Interest
The authors report no conflict of interest. Makale tüm yazarlar tarafından okunup kabul edildi, önceden belirtilden şekilde yazarlık ölçütlüğünün karşılanmamakta, her yazının makalenin dördüncü bir çıplaklayı yansıttığına inanmaktadır.

Acknowledgements
We thank all participating patients and volunteers. We thank David Chapman for English-language editing. We thank Ümrân Çetinçelik for laboratory support.

REFERANSLAR


doi:10.1371/journal.pone.0063357.


doi:10.1371/journal.pone.0139711.


