1. Introduction

Tuberculosis (TB) caused by Mycobacterium tuberculosis (M. tuberculosis) is a major public health problem. Despite use of bacillus Calmette-Guérin vaccine, approximately a third of the world population is infected by this microorganism[1]. It is imaginable that many genetic factors play roles in the pathogenesis of this disease because only 10% of the patients infected by M. tuberculosis reveal clinical TB[2]. For example, tumor necrosis factor is implicated in the prevention of mycobacterial infections and progression from latent TB infection to active TB disease[3]. Also, Shahsavar et al. indicated that KIR3DS1 and its combination with HLA-B BW4 Ile80 are associated with the susceptibility to respiratory TB in the Lur population of Iran[4]. Furthermore, human and mouse studies on the M. tuberculosis infection have represented different loci in the susceptibility or resistance to TB including toll like receptors (TLRs)[5-9]. TLRs are the pattern recognition receptors expressed on the macrophages and the other leukocytes. They play a basic role in the phagocytosis and other host immune mechanisms including microbial pathogens recognition pattern. In humans, TLRs comprise 10 receptors that are important for innate immunity. These 10 receptors are classified as the members of the great receptor family interleukin-1 according to a common cytoplasmic region called toll/interleukin-1 receptor domain. TLR activation triggers a complicated cascade leading to induce wide spectrum of proinflammatory genes[10-12]. TLRs activate nuclear factor-kB which is the first line defense against pathogens[13]. Human and animal TB studies have demonstrated that TLRs contribute in the innate immunity response to the TB infection. Among TLRs, TLR1, TLR2, TLR4, TLR6, TLR8 and TLR9 were analyzed widely as the candidate genes in the susceptibility to TB infection in the different populations[14-20]. Because of ethnic and racial significant
differences in the distribution of TLR genes on one hand and TLRs association, especially TLR2 and TLR4 with respiratory TB on the other hand, our study was conducted on the innate immunity role in the TB to know whether common TLR2 and TLR4 polymorphisms have association in the susceptibility to pulmonary TB infection in the Lur population of Iran. Therefore, in this study, the susceptibility to pulmonary TB infection was ascertained by the studying of 677 C/T TLR2 (Arg677Trp), 753 G/A TLR2 (Arg753Gln), 299 A/G TLR4 (Asp299Gly) and 399 C/T TLR4 (Thr399Ile) polymorphisms in the TB group and was compared with that in the healthy control group.

2. Materials and methods

2.1. Patients and controls

This study was designed as a case-control study. The patient group was consisted of 100 unrelated Iranian TB patients referring to health center of Khorramabad City of Lorestan Province. Their TB was confirmed by using smear/culture of sputum. The control group was consisted of 100 unrelated Iranian people. The control individuals were from the same race and geographic region. Controls were asymptomatic with normal radiologic results, no history of TB and negative purified protein derivative test. The matching of the patient group with the control one was established. All patients and controls have parents with the same race. The study was approved by the Ethical Committee of Lorestan University of Medical Sciences and informed consent was provided according to the Declaration of Helsinki. All samples were collected with the written consent of the patients.

2.2. TLR polymorphisms genotyping

The DNA samples of patients and controls were extracted from peripheral leukocytes by using QIAmp kit (Qiagen, Germany) in accordance with the manufacture commands. We utilized PCR-restriction fragment length polymorphism presented before by Folwaczny et al.[21] and Selvaraj et al.[22] to identify 677 C/T TLR2 (Arg677Trp), 753 G/A TLR2 (Arg753Gln), 299 A/G TLR4 (Asp299Gly) and 399 C/T TLR4 (Thr399Ile) polymorphisms from patients’ and controls’ genomic DNA. The forward and reverse primer sequences (Qiagen, Germany), restriction enzymes (Biolabs, USA) and digestion patterns for variable alleles are listed in Table 1.

The amplification was accomplished by Master cycler set (BioRad, USA) in 50 μL reaction mixture consisting of 200 μmol/L deoxyribonucleoside triphosphates, 20 pmol each of forward (F) and reverse (R) primers, 1 unit of Hot Start Taq DNA polymerase (Qiagen, Germany), and 1× PCR buffer (Qiagen, Germany) and 100 ng genomic DNA. The PCR was carried out according to the following temperature conditions: PCR cycle including an initial denaturation step at 95 °C for 5 min followed by 35 cycles at 95 °C for 30 s, annealing performed specifically at 72 °C for 30 s and a final spreading step at 72 °C for 2 min for each polymorphism. The annealing temperatures were 65 °C, 65 °C, 62 °C and 60 °C, respectively, for polymorphisms expressed above. Then, PCR products were incubated at 37 °C for 2 h to digest by respective restriction enzymes. The PCR products were electrophoresed on a 3% agarose gel including 0.5 mg/mL ethidium bromide and were visualized under an ultraviolet light. The results validation obtained from the designed method approved randomly by the sequencing of the chosen samples.

2.3. Statistical analysis

Genotypes and allele frequencies of the TLR2 and TLR4 polymorphisms were determined by direct calculation in the TB population and healthy controls. The whole polymorphisms were consistent with the Hardy-Weinberg equilibrium in both patient and control groups. The differences in the genotypes and allele frequencies of TLR2 and TLR4 genes polymorphisms were determined by the Chi-squared test and Fisher’s exact test between TB population and healthy control population.

Because of multiple comparisons, we utilized Yate’s correction test to correct the significant differences. Overall, P < 0.05 was statistically significant after correction. The odds ratio (OR) was calculated by the cross-product ratio and the exact confidence intervals (CI) of 95% were obtained.

3. Results

Out of the 100 study patients, 50 were female and 50 were male with the mean age of (48.54 ± 7.35) years; while out of the 100 study controls, 50 were female and 50 were male with the mean age of (48.54 ± 7.35) years. The amplification was accomplished by Master cycler set (BioRad, USA) in 50 μL reaction mixture consisting of 200 μmol/L deoxyribonucleoside triphosphates, 20 pmol each of forward (F) and reverse (R) primers, 1 unit of Hot Start Taq DNA polymerase (Qiagen, Germany), and 1× PCR buffer (Qiagen, Germany) and 100 ng genomic DNA. The PCR was carried out according to the following temperature conditions: PCR cycle including an initial denaturation step at 95 °C for 5 min followed by 35 cycles at 95 °C for 30 s, annealing performed specifically at 72 °C for 30 s and a final spreading step at 72 °C for 2 min for each polymorphism. The annealing temperatures were 65 °C, 65 °C, 62 °C and 60 °C, respectively, for polymorphisms expressed above. Then, PCR products were incubated at 37 °C for 2 h to digest by respective restriction enzymes. The PCR products were electrophoresed on a 3% agarose gel including 0.5 mg/mL ethidium bromide and were visualized under an ultraviolet light. The results validation obtained from the designed method approved randomly by the sequencing of the chosen samples.

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Table 1

<table>
<thead>
<tr>
<th>TLR spolymorphisms</th>
<th>Sequences of the primers</th>
<th>PCR product size (bp)</th>
<th>Restriction enzymes</th>
<th>Length of the restriction fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2 (677 C/T)</td>
<td>F: 5’ CCCCTCTCAAGTTGTTGCTTCAAG 3’</td>
<td>152</td>
<td>MwoI</td>
<td>C-130 bp + 22 bp</td>
</tr>
<tr>
<td></td>
<td>R: 5’ AGTCCAGTCTACATGCTCAACCAC 3’</td>
<td></td>
<td></td>
<td>T-152 bp</td>
</tr>
<tr>
<td>TLR2 (753 G/A)</td>
<td>F: 5’ CATTCACAGGCCTTCTGGCAAGCTCC 3’</td>
<td>129</td>
<td>MspI</td>
<td>G-104 bp + 25 bp</td>
</tr>
<tr>
<td></td>
<td>R: 5’ GGAACCTAGAGACTTTATCGCAAGCTC 3’</td>
<td></td>
<td></td>
<td>A-129 bp</td>
</tr>
<tr>
<td>TLR4 (299 A/G)</td>
<td>F: 5’ AGCATCTTAGACTTACACTCCCATGG 3’</td>
<td>188</td>
<td>NcoI</td>
<td>A-188 bp</td>
</tr>
<tr>
<td></td>
<td>R: 5’ GAGAGATTTGAGTTTCAATGTTGGG 3’</td>
<td></td>
<td></td>
<td>G-168 bp + 20 bp</td>
</tr>
<tr>
<td>TLR4 (399 C/T)</td>
<td>F: 5’ GGTTGCTGTTCTCAAAAGTGATTTTGGAGAAG 3’</td>
<td>124</td>
<td>Hinfl</td>
<td>C-124 bp</td>
</tr>
<tr>
<td></td>
<td>R: 5’ GGAATCCAGATGTTCTAGTTGTTCTAGGCC 3’</td>
<td></td>
<td></td>
<td>T-98 bp + 26 bp</td>
</tr>
</tbody>
</table>

F: Forward; R: Reverse; bp: Base pairs.
age of \((45.66 \pm 6.74)\) years. The genotypes and allele frequencies of 677 C/T TLR2 (Arg677Trp), 753 G/A TLR2 (Arg753Gln), 299 A/G TLR4 (Asp299Gly) and 399 C/T TLR4 (Thr399Ile) polymorphisms were exhibited in Tables 2 and 3.

### Table 2

Distribution of TLRs genotypes in pulmonary TB patients and healthy controls.

<table>
<thead>
<tr>
<th>TLRs polymorphisms</th>
<th>Genotypes</th>
<th>Tuberculosis patients (%)</th>
<th>Healthy controls (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2 (677 C/T)</td>
<td>CC</td>
<td>88</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TLR2 (753 G/A)</td>
<td>GG</td>
<td>65(^{\ast})</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>TLR4 (299 A/G)</td>
<td>AA</td>
<td>71</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>TLR4 (399 C/T)</td>
<td>CC</td>
<td>85</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

\(^{\ast}\): Significant difference after correction \((P < 0.05)\). \(n = 100\).

The genotypes and allele frequencies of 677 C/T TLR2 (Arg677Trp), 299 A/G TLR4 (Asp299Gly) and 399 C/T TLR4 (Thr399Ile) polymorphisms had no significant difference between the patients with pulmonary TB and the healthy controls. Also, TT genotype of 677 C/T TLR2 (Arg677Trp) polymorphism was not observed in the patient and control groups. G allele frequency of 753 G/A TLR2 (Arg753Gln) polymorphism decreased significantly in the patient group as compared with the control group (70% in the patient group vs. 90% in the control group, \(P = 0.0019\), OR = 0.328, \(CI = 0.263–0.412\)) (Table 2).

The different TLRs studies illustrate inconsistent results in the susceptibility to pulmonary TB in the variable ethnics[14-20]. In this study, we investigated the effect of 677 C/T TLR2 (Arg677Trp), 753 G/A TLR2 (Arg753Gln), 299 A/G TLR4 (Asp299Gly) and 399 C/T TLR4 (Thr399Ile) polymorphisms in the susceptibility to the pulmonary TB in the Lur population of Iran. The genotypes and allele frequencies of 753 G/A TLR2 (Arg753Gln) had significant difference between the patients with pulmonary TB and the healthy controls in this study.

In contrast, Xue et al.[24], in the south of China, and Selvaraj et al.[22], in the south of India, did not find any association between these polymorphisms and pulmonary TB. Due to the lack of genetic similarity between Caucasians, Huns and Indians (racial differences), this difference is not unexpected. Our study had similarity with former studies carried out by Ogus et al.[25] in 2004 and Dalgic et al.[26] in 2011 demonstrating the association of 753 G/A TLR2 (Arg753Gln) polymorphism and TB. Also, this outcome is expectable because of the genetic similarity in the Caucasians. In 2004, Ben-Ali et al.[27] also demonstrated the association between 677 C/T TLR2 (Arg677Trp) and TB in Tunisia. Furthermore, Naderi et al.[28] exhibited the association between 597 T/C TLR2 polymorphism and pulmonary TB in the south of Iran in 2013.

Therefore, it seems that host immune response to \(M.\) \textit{tuberculosis} is accomplished principally by the TLR2 signaling in the macrophages that leads to a direct bactericidal effect on \(M.\) \textit{tuberculosis} or apoptosis inducing in the infected macrophages[29]. It is also expressed before that TLR2 polymorphism leads to the decrease of the macrophages response to some bacterial peptides[30]. Finally, our findings suggest that 753 G/A TLR2 (Arg753Gln) polymorphism may have an association with the susceptibility to pulmonary TB in the Lur population of Iran. However, we demonstrate that more studies with large sample sizes are required to confirm the role of TLRs in the susceptibility to pulmonary TB.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Acknowledgments

We appreciate all the patients and healthy individuals participating in this study. This study was supported by the Lorestan University of Medical Sciences (Grant No. 1284).

### References


