Full Length Research Paper

Association study of single nucleotide polymorphism of human Toll like receptor 9 and susceptibility to pulmonary tuberculosis in Egyptian population

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Toll-like receptors (TLRs) are known to play important roles in human innate immune systems. Polymorphisms in and functions of TLRs have been investigated to identify associations with specific infectious diseases including tuberculosis (TB). This study was performed for 166 samples of unrelated individual’s diagnosis of pulmonary tuberculosis and 98 household healthy samples. Genomic DNA was extracted from EDTA-anticoagulated peripheral blood. The alleles of (rs352140) TLR9 gene polymorphisms were detected using polymerase chain reaction restriction fragment length polymorphism. The resulting fragments were separated in 3% agarose gel electrophoresis. The sequence results generated by the forward and reverse sequencing primers were analyzed with the software program sequencing analysis. Sequence comparisons of three genotypes AG, AA, and GG were performed using the multiple-alignment algorithm in Megalign. The direct counting was used to determine the allele and genotype frequencies of each polymorphism. Hardy-Weinberg equilibrium (HWE) tests were performed in controls by Fisher exact test. Significant deviations from the Hardy-Weinberg equilibrium in the distribution of the TLRs SNP genotypes in TB patients and controls were not detected for the SNP TLR9rs352140 for both patients and controls. These results do not indicate a major influence of these putative functional TLR SNP on the susceptibility to (or protection from) tuberculosis in Egyptian population.

Key words: Association, TLR9, tuberculosis, Egyptian population.

INTRODUCTION

Tuberculosis (TB), an old and destructive disease, is a considerable public health problem. TB is an infectious disease caused by Mycobacterium tuberculosis (Mtb) (Jahantigh et al., 2013; WHO, 2010b). World Health
Organization (WHO) evaluated one third of the population of the world is infected with *Mtb* (Dye et al., 1999). One billion people are infected in the 2000 to 2020, and about 200 million people will evolve active TB. Only 5 to 10% of the infected patients develop the active disease in their lifetime and 90% remain as latently *Mtb* infected individuals (Torres-Garcia et al., 2013). In spite the fact that Egypt is not found in the WHO list of 22 high TB infected countries, it is considered one of the high percentage in Eastern Mediterranean countries. In Egypt, TB is considered the third most important public health problem after schistosomiasis and hepatitis C (National Tuberculosis Control Program, 2006; WHO, 2010a). The National Tuberculosis Control Program of the Ministry of Health and Population (MOHP) in Egypt registers over 12,000 new TB patients every year. More than 50% of the cases are sputum smear-positive pulmonary TB (Helal et al., 2009). A previous study reported a co-infection with *Schistosoma mansoni* in every third hospitalized patient with tuberculosis in Tanzania. They suggested that schistosomiasis may reduce the host’s immune response to the bacille calmette-guérin vaccine, which is widely used in endemic areas for protection against *Mtb*, and hence may lower the protective efficacy of the vaccine (Knopp et al., 2013). Furthermore, the joint effects of smoking, TB and human immunodeficiency virus (HIV) greatly increase the risk of chronic obstructive pulmonary disease in the long term (Ajagbe et al., 2014). The progression to active TB is the result of the environmental, host genetic factors and pathogenic characteristics of the *Mtb* strain (Torres-Garcia et al., 2013). Multiple genes have been involved in the control of *Mtb* and progression to TB (Pan et al., 2005; Yim and Selvaraj, 2010). Since a twin study established the importance of a genetic component as a factor in TB susceptibility (Comstock, 1978), linkage studies (Mahasirimongkol et al., 2009), genome wide association studies (Thye et al., 2010), and many candidate gene studies have been performed, and the candidate gene studies often focused on genes involved in immune function, including human leukocyte antigen (HLA) (Lombard et al., 2006), IL12B1 (Remus et al., 2004), Toll-like receptors (TLRs) (Velez et al., 2010), SLC11A1 (Van Crevel et al., 2009), IFNG (Pacheco et al., 2008), and CD209 (Vannberg et al., 2008).

TLRs are a family of phylogenetically conserved genes, that are essential for recognition of pathogen associated molecular patterns (PAMPs) on dendritic cells and macrophages (Azad et al., 2012; Bafica et al., 2005; Carvalho et al., 2011; Chen et al., 2010; Chow et al., 1999). TLR9, an endosomal localized receptor on B cells, plasmacytoid dendritic cells (pDCs), and monocytes/macrophages, recognizes unmethylated nucleic acid motifs in bacterial and viral DNA (Hemmi et al., 2000). TLR9 is one of the most important receptors in the initiation of protective immunity against intracellular pathogens by activation signaling cascade of intracellular receptor signaling (Akira, 2006). TLR9 encoding gene is located on chromosome 3p21.3. It spans about 5 kb and contains two exons (Akira, 2006; Jahanthig et al., 2013). TLR-knockout mouse studies indicate that TLR2, TLR4, and TLR9 participate to host resistance to *Mtb* infection (Bafica et al., 2005). Genetic variations of TLR1, TLR2, TLR4, TLR6 and TLR9 have been associated with the susceptibility to TB in different ethnic groups (Ocejo-Vinyals et al., 2013; Thada et al., 2013; Zaki et al., 2012), but other studies have failed to demonstrate significant associations of TLRs polymorphisms with TB (Sanchez et al., 2012; Tian et al., 2013). Up to now, no previous studies have addressed the prevalence of TLRs polymorphisms in Egyptian patients with TB. Therefore, this study examined whether polymorphisms in TLR9 is associated with the susceptibility to pulmonary TB Egyptian individuals or not.

**MATERIALS AND METHODS**

**Subjects**

In this study, samples from 264 unrelated individuals were obtained. They were divided into 166 patient subjects with diagnosis of pulmonary TB collected from Abbassia Chest Hospital according to approval from health ministry in Egypt (Serial No. 09/2014) and 98 household healthy subjects as controls. This study included only subjects of 18 to 65 years old according to bioethics principles. The diagnosis of pulmonary TB was based on the WHO criteria with the presence of clinical symptoms, detection of acid-fast bacilli in sputum smear samples, *Mtb* positive cultures in Lwenstein-Jensen medium, X-ray evidence of cavitary lesions in lung. In the absence of clinical symptoms of active pulmonary tuberculosis, no medical history of TB, other infectious or autoimmune diseases, cancer and other diseases affecting host immunity were found in the control subjects. Informed consent was obtained from all subjects or their legal representatives before participation in the study. The protocol was IRB-approved at VACSERA-EGYPT Institutional Bioethics Review Board (BERD- VACSERA, EGYPT). A questionnaire including a full medical history regarding different variables as age, sex, residence, and medical history of TB was recorded. The study was explained to all participants using the consent form. TB outcome of adult subjects are to be tested by the current study, so subjects under 18 years old are not included. There is need to evaluate TB outcome in normal adult subjects whereas immunity of children, pregnant and immuno-compromised are out of our scope. About 3 to 5 ml EDTA-anticoagulated peripheral blood was drawn from each subject and stored at -20°C.

**DNA isolation and TLR9 genotyping**

Genomic DNA was extracted from EDTA-anticoagulated peripheral blood using TIAN amp genomic DNA kit (Tiangen, Korea, Cat #DP304-02) according to its manufacturer instructions. The extracted DNA was stored at -20°C until further analysis. The alleles of (rs352140) TLR9 gene polymorphisms were detected using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). The amplification of TLR 9 (rs352140) fragments using PCR was performed according to Sambrook and Russel (2001) with some modifications using forward and reverse primers 5'-AAAGCTGAGCCTCTACCAAGCA-3’ and 5'.
**Table 1.** Demographic and clinical characteristics of the recruited subjects (patients and controls).

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Patient</th>
<th>Controls</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>46</td>
<td>27.8</td>
<td>46</td>
</tr>
<tr>
<td>Male</td>
<td>120</td>
<td>72.2</td>
<td>52</td>
</tr>
<tr>
<td>Age</td>
<td>38.2 ± 13.44</td>
<td>35.36 ± 7.86</td>
<td>0.057</td>
</tr>
<tr>
<td>Sputum analysis</td>
<td>143</td>
<td>86.1</td>
<td>0</td>
</tr>
<tr>
<td>Sputum plantation</td>
<td>2</td>
<td>1.25</td>
<td>0</td>
</tr>
<tr>
<td>X-ray examination</td>
<td>166</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Diabetes</td>
<td>6</td>
<td>3.6</td>
<td>1</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Autoimmune disease</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

**Preparing sequencing cycle reaction**

The deoxy ribonucleoside chain termination was employed for sequencing the double-stranded recombinant DNA obtained during the PCR procedure. Amplified DNA fragments were sequenced directly using the ABI Prism Big Dye Terminator V.3.1 Cycle sequencing Kit on an ABI 310 DNA automated sequencer (Applied Biosystems). The reactions were done in 20 μl mixture reaction, according to the manufacturer’s instructions. Sequencing cycle for SNP at TLR9 rs352140 was performed with the following parameters: initial denaturation of 95°C for 5 min, 35 cycles of 95°C for 45 sec, 56°C for 1 min, and 72°C for 30 sec, followed by a final extension of 72°C for 7 min. But sequencing cycle was performed with the following parameters: preheating at 95°C for 1 min, 35 cycles of 95°C for 1 min, 47°C for 1 min, and 72°C for 1 min; a final 72°C for 10 min as a final extension step (Nguyen et al., 2009).

**Removal of unincorporated dye terminator**

DyeEx Kits for purification was used to remove all unincorporated dye terminator directly from sequencing reaction. Spin columns were gently vortexed to re-suspend the resin using centrifugation for 3 min at 750 ×g. The sequencing reaction 20 μl was slowly applied to the gel bed and centrifuged for 3 min at 750 ×g. The samples were dried in a vacuum centrifuge (Van Houdt et al., 2010).

**Sequencing**

Sequencing was carried out at Genetic Engineering Research Department (Vacsera, Egypt). After removing all the unincorporated dye terminator as mentioned earlier, all samples have been re-suspended in Hi Di-ionized formamide, denatured at 95°C for 3 min and 30 s, then applied on chilled ice for 5 min. Electrophoresis process was performed on ABI Prism 310 Genetic Analyzer, by using ABI Prism 310 collection data base. All samples were analyzed using sequencing analysis software. The sequence results generated by the same forward and reverse sequencing primers of PCR were analyzed with the software program sequencing analysis v5.3.1. For sequence comparisons of three genotypes AG, AA, and GG, sequence alignment was performed using the multiple-alignment algorithm in Megalign (DNASTAR, Window version 3.12e). Also, the resulted sequence was aligned with National Center for Biotechnology Information (NCBI).

**Statistical analysis**

The direct counting was used to determine the allele and genotype frequencies of each polymorphism. Hardy–Weinberg equilibrium (HWE) tests were performed in controls by Fisher exact test. A P-value of less than 0.01 was considered to indicate deviation from HWE. Association analysis between SNPs and TB was also performed by Fisher exact test. A P-value of less than 0.05 was considered to be statistically significant. Odds ratio (OR) with 95% confidence interval (CI) were calculated for the SNP for evaluating the relative risk using Statacalc program (Graphpad prizm version 5.0).

**RESULTS**

Demographic and clinical characteristics of TB patients and controls are summarized in Table 1. One hundred and sixty six patients with TB and 98 controls were investigated for the presence of the TLR9 polymorphisms. The demographic characteristics of the 166 patients with TB participating in this study show that they had a mean age (± standard deviation) of 38.2 ± 13.44. The result shows 86.1% of the TB patients were sputum positive and 6.3% had diabetes mellitus. TB infection was confirmed using X-ray examination for both patients and controls as shown in Table 1. Notably, none of the subjects reported any history of other clinical symptoms.
Table 2. Contribution of the TLR9 C2848T (rs352140) polymorphisms in Egyptian patients with pulmonary tuberculosis.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients (frequency)</th>
<th>Control (frequency)</th>
<th>χ²</th>
<th>P value</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>43 (25.9)</td>
<td>19 (19.4)</td>
<td>1.46</td>
<td>0.23</td>
<td>1.45</td>
</tr>
<tr>
<td>GA</td>
<td>81 (48.8)</td>
<td>53 (54.1)</td>
<td>0.74</td>
<td>0.39</td>
<td>1.17</td>
</tr>
<tr>
<td>AA</td>
<td>42 (25.3)</td>
<td>26 (26.5)</td>
<td>0.05</td>
<td>0.83</td>
<td>0.94</td>
</tr>
</tbody>
</table>

Table 3. Assessment of gender in relation to TLR9 C2848T (rs352140) polymorphisms in study group.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Genotype</th>
<th>Male (Frequency, %)</th>
<th>Female (Frequency, %)</th>
<th>χ²</th>
<th>P value</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>GG</td>
<td>34 (28.3)</td>
<td>9 (19.6)</td>
<td>1.33</td>
<td>0.25</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>56 (46.7)</td>
<td>25 (54.3)</td>
<td>0.64</td>
<td>0.42</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>30 (25.0)</td>
<td>12 (26.2)</td>
<td>0.02</td>
<td>0.89</td>
<td>1.06</td>
</tr>
<tr>
<td>Controls</td>
<td>GG</td>
<td>8 (15.4)</td>
<td>11 (23.9)</td>
<td>1.14</td>
<td>0.29</td>
<td>1.73</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>27 (51.9)</td>
<td>26 (56.5)</td>
<td>2.16</td>
<td>0.14</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>17 (32.7)</td>
<td>9 (19.6)</td>
<td>2.31</td>
<td>0.12</td>
<td>1.55</td>
</tr>
</tbody>
</table>

at the time of sampling. Mean age, gender and the prevalence of other comorbidities such as systemic hypertension, immunodeficiency and autoimmunity were similar among groups.

Using PCR-RFLP, the observed genotype and allele frequency distribution of TLR9 C2848T (rs352140) gene polymorphisms between Egyptian patients with pulmonary tuberculosis and controls are illustrated in Table 2. Significant deviations from the Hardy-Weinberg equilibrium in the distribution of the TLRs SNP genotypes in TB patients as well as controls were not detected for the SNP TLR9rs352140 in both TB patients and controls. The TLR9 C2848T (rs352140) genotypic and allelic frequency distributions between Egyptian patients with pulmonary tuberculosis and healthy controls were similar and no significant association was observed for pulmonary tuberculosis development (p > 0.05). When univariate analysis was performed by age, gender and clinical data, no statistical difference was observed (p > 0.05) between healthy and patient subjects in all criteria except for gender which is in accordance with published data indicating that in most low-income countries, the ratio of male to female cases of tuberculosis is approximately 2:1 (WHO, 2004) attributable to biological characteristics and socioeconomic and cultural barriers to access healthcare (Borgdorff et al., 2000). Table 3 illustrates genotype and allele frequency distribution of TLR9 C2848T (rs352140) gene polymorphisms between male to female in the study group and healthy control subjects indicating no significant differences based on gender difference.

Figure 1 shows the digested PCR products with BstUI digestion for TLR9 rs-352140. DNA bands at 135 and 42 bp corresponded to the homozygous TLR9 GG genotype, while bands of 177, 135 and 42 bp were designated as heterozygous GA; a band of 177 bp corresponded to the homozygous AA genotype. Digested samples were separated by electrophoresis on 3% agarose gel and visualized by ethidium bromide staining.

Three random samples from each genotype were chosen for sequencing to confirm TLR9 rs352140 SNP detection using RFLP-PCR assay. Figure 2 shows the sequencing results within the TLR9-TIR. All the sequencing results matched with the RFLP-PCR assay results. Direct sequencing of the 177-bp region containing the single nucleotide polymorphism site of interest confirmed the genotyping results at nucleotide 135. The resulting sequence was: AGCTGAGGTCCAGGGCTCCAGTCG[C/T]GGTAGCTCCGTGAATGAGTGCTCG.

To confirm these results, the sequence alignment was done in NCBI blast. The Identities was 50/50 (100%) (Figure, 3).

**DISCUSSION**

TB caused by Mtb, is a main health problem worldwide, with about 10 million new patients diagnosed every year. Innate immunity plays an important role in the host defense against Mtb. The recognition of Mtb by cells of the innate immune system is the first step in this process. Some human epidemiological studies detected that genetic variation in genes encoding for recognition receptors (PPRs) and downstream signaling products influence disease susceptibility, severity, and outcome. TLRs are a family of PPRs consisting of 12 members in
human and other mammals. TLRs expression is carried out on the surface of the cell membrane or on the membrane macrophages and dendritic cells. In spite of the interaction of \textit{Mtb} with TLRs leads to phagocyte activation, the interaction itself does not lead to immediate ingestion of the mycobacteria (Kleinnijenhuis et al., 2011). Host genetic may involve multiple genes and their polymorphisms to develop TB (Leandro et al., 2013). TLR association may differ from population to another. For example, TLR2 polymorphisms are not responsible for the increased prevalence of TB in the Indian population. While significant TLR2 gene has been reported exclusively in the Caucasian population, a Korean population and in a Tunisian population. Other authors have failed to detect these polymorphisms in the Korean population (Biswas et al., 2009).

Previous studies revealed that TLR9 has critical role in the incidence of TB. The gene of TLR9 is located on chromosome 3p21.3. The total length of TLR9 gene is approximate 5 kb. Its coding gene has two exons, and the main coding region is in the second exon (Chen et al., 2015). So, the objective of the current study was to detect the presence of association between TLR9 C2848T (rs352140) polymorphism and tuberculosis infection among patients with tuberculosis in Egyptian population. This study found that there were no significant differences between neither male and female pulmonary
TB nor control groups for genotype frequencies regarding TLR9 C2848T (rs352140) polymorphism. In agreement with these findings, Salimi (2015) revealed that TLR8 rs3764880 and TLR9 rs148805533 polymorphisms may not be risk factors for susceptibility to pulmonary tuberculosis in a sample of Iranian population. In their study, they found no association between polymorphism and pulmonary tuberculosis neither in their female nor in their male patients. In addition, they did not observe the Del allele of 14 bp Ins/Del polymorphism of TLR9 gene in the studied population and reported no association between this polymorphism and pulmonary tuberculosis. In another study, Iranian population was performed in 124 newly diagnosed TB cases and 149 healthy controls in a TB-endemic region of Iran. They found that no significant relation between TLR4 and TLR9 polymorphisms alone and TB (Jahantigh et al., 2013). Also, similar to the current results, there was no association between TLR9 rs148805533 (14 bp Ins/Del) polymorphism and TB in South India (Selvaraj et al., 2010). Other candidate gene studies have examined the relationship between TLR9 SNPs and pulmonary TB. In previous study, a meta-analysis was performed to assess the association between seven extensively studied TLR9 polymorphisms (rs187084, rs352165, rs5743836, rs5743842, rs352139, rs352140 and rs352167) and TB risk. The analysis revealed an association between certain TLR9 polymorphism and TB risk. In addition, 5 different genetic models (Allele, Heterozygote, Homozygote, Dominant and Recessive model) were analyzed in all polymorphisms. A subgroup analysis by race was also performed for rs187084, rs352139 and rs5743836 polymorphisms, the studies included Indians, Iranian and West African, Indonesians, Vietnamese, Chinese and Mexicans. The results showed that rs187084 and rs5743836 polymorphisms were not associated with TB risk, while the association between rs352139 polymorphism and TB risk may vary by race (Chen et al., 2015). Also, expression of TLR7, TLR8 and TLR9 was determined in monocytes from HIV-infected patients and control subjects, which were activated with specific ligands. The expression of MyD88 and NF-kBp65 were determined by flow cytometry. No statistical difference was found in the expression of TLR7, 8 and 9 in monocytes from patients compared to controls, but they observed the non-significant increased expression of TLR9 in patients (Valencia et al., 2013). In an animal trial, the relation between TLR1 and TLR9 with TB susceptibility in Chinese Holstein cattle was examined. They suggested that variants in the TLR1 gene are associated with susceptibility to TB, whereas no significant association can be inferred from the polymorphisms in the TLR9 gene (Sun et al., 2012). In another study to investigate the role of TLR9 in innate immunity to *Mycobacterium avium*, TLR9, TLR2, and MyD88 knockout (KO) mice were infected with this bacterium. They proved that TLR2 and MyD88, but not TLR9, played a major role in interleukin-12 and TNF-α production by *M. avium*-infected macrophages and dendritic cells (DCs). They also found that major histocompatibility complex class II molecule expression on DCs is regulated by TLR2 and MyD88 signaling, and not by TLR9 (Carvalho et al., 2011).

On the contrary, many researchers proved that TLR genes have been variably associated with tuberculosis infection and there is strong evidence indicating that host genetic factors play critical roles in tuberculosis susceptibility, severity and development (Azad et al., 2012; Davila et al., 2008; Khalilullah et al., 2014). It was suggested that the allele A of the intronic polymorphism rs352139 on TLR9 gene might contribute to the risk of developing TB in Mexican Amerindiands. Polymorphisms and functions of TLRs have been investigated to identify associations with specific infectious diseases, including TB. A statistically significant association was observed between TB susceptibility in a classified Indonesian female group and rs352139, an SNP located in the intron of TLR9 (Torres-Garcia et al., 2013). Meta-analysis of the Indonesian and Vietnamese populations showed that rs352139 was significantly associated with TB in the recessive model. This finding indicated that a TLR9 polymorphism might have an important role in the susceptibility to *Mtb* in Asian populations (Kobayashi et al., 2012). In Vietnam, using a case population design,
they evaluated whether SNPs in the TLR9 gene region were associated with susceptibility to pulmonary or meningeal TB as well as neurologic presentation and mortality in the meningeal TB group (Graustein et al., 2015). It was found to be essential for cellular responses to mycobacterial CpG DNA. In vitro studies showed that DCs release IL-12 in response to TLR9 (Thada et al., 2013). A report demonstrates that TLR9-deficient mice are susceptible to Mtb infection, and mice lacking both TLR2 and TLR9 are more susceptible to TB (Bafica et al., 2005). In a study in three independent population samples indicate that variations in TLR2 and TLR9 might play important roles in determining susceptibility to TB in African-Americans population when TLR1, TLR2, TLR4, TLR6, and TLR9 were examined (Velez et al., 2010). In another study in India, it is found, a significantly lower minor allele frequency (MAF) of T-1486C in the Baiga tribe, wherein fewer PTB cases were reported, than that in the Gond and Korku tribes. These data suggest that the minor “C” allele at rs187084 locus may be associated with susceptibility to PTB (Bharti et al., 2014).

Conclusions

The results of this study indicate that the single nucleotide polymorphisms in TLR9 (SNP TLR9 rs352140) gene might not be associated with TB risk in Egyptian population.

Conflict of Interests

The authors have not declared any conflict of interest.

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