

Association Between TLR8 and TLR9 Gene Polymorphisms and Pulmonary Tuberculosis

Seyed Mohammad Hashemi-Shahri¹; Mohsen Taheri^{2,3,*}; Ali Gadari¹; Mohammad Naderi¹; Gholamreza Bahari⁴; Mohammad Hashemi⁴

¹Infectious Diseases and Tropical Medicine Research Center, Zahedan University of Medical Sciences, Zahedan, IR Iran

²Genetics of Non-communicable Disease Research Center, Zahedan University of Medical Sciences, Zahedan, IR Iran

³Department of Genetics, School of Medicine, Zahedan University of Medical Sciences, Zahedan, IR Iran

⁴Department of Clinical Biochemistry, School of Medicine, Zahedan University of Medical Sciences, Zahedan, IR Iran

*Corresponding author: Mohsen Taheri, Department of Genetics, School of Medicine, Zahedan University of Medical Sciences, Zahedan, IR Iran. Tel: +98-5413425793, Fax: +98-5413425796, E-mail: amirt112@yahoo.com

Received: February 3, 2014; Revised: February 10, 2014; Accepted: February 16, 2014

Background: Tuberculosis is still a health problem throughout the world. Both genetic and environmental factors may contribute the susceptibility to tuberculosis. Toll-like receptors play a critical role in the recognition of mycobacterium tuberculosis (TB).

Objectives: The aim of this study was to evaluate the possible association between TLR8 rs3764880 and TLR9 rs148805533 polymorphisms and pulmonary tuberculosis (PTB) in a sample of Iranian population.

Patients and Methods: In this study, blood samples of 320 subjects including 160 PTB patients and 160 healthy subjects were collected. DNA was extracted and TLR8 rs3764880 polymorphism was analyzed by Tetra Amplification Refractory Mutation System-Polymerase Chain Reaction (TARMS-PCR) and TLR9 rs148805533 polymorphisms was analyzed by allele specific PCR.

Results: The allelic and genotypic frequencies of the TLR8 rs3764880 did not differ significantly between PTB and the controls. No significant difference was found between the groups regarding TLR9 rs148805533 polymorphism.

Conclusions: TLR8 rs3764880 and TLR9 rs148805533 polymorphisms may not be risk factors for susceptibility to tuberculosis in a sample of Iranian population. Larger studies are required to validate our findings.

Keywords: Tuberculosis; Toll-Like Receptor 8; Toll-Like Receptor 9; Polymorphism

1. Background

Tuberculosis (TB) caused by *Mycobacterium tuberculosis*, remains a major health problem. WHO predicted about 8.6 million cases of tuberculosis and 1.3 million death from the disease in 2012 (1). The incidence rate of TB in Zahedan (central of Sistan and Baluchistan province) is higher than other regions of the country (2). Tuberculosis predominately affects the lungs, but can affect almost all of the body organs, including the brain, the kidneys and the bones. Only 5% to 10% of the human beings who infected with mycobacterium develop the active disease in their life span and 90% remain as latent. Host genetic variation, environmental and characteristics of the mycobacterium strain play important roles in susceptibility to TB. Toll-like receptors (TLRs) are a transmembrane protein playing key role in the innate immune system. TLRs are the first line of protection against pathogens and have a critical role in inflammation and immune regulation (3). They are usually expressed on various immune cells such as macrophages and dendritic cells, which recognize certain molecules derived from bacteria (4). They belong to a receptor su-

perfamily with Interleukin-1 receptors recognized as Interleukin-1 Receptor / Toll-Like Receptor Superfamily. Indeed, they are a family of pattern-recognition receptors (PRR) and recognize pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), lipoproteins and peptidoglycans (5). Stimulation of TLRs by PAMPs leading to the activation of NF- κ B transcription factor which induces the secretion of several proinflammatory cytokines, production of interferon (IFNs) lead to activation of macrophage in response to mycobacterium (6). Thirteen TLRs have been characterized in humans, each one recognizes different PAMPs from various microbial pathogens, such as viruses and bacteria. Some of these TLRs are localized on the cell surface such as TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11, and the rest are located on endosomal/lysosomal compartment such as TLR3, TLR7, TLR8, and TLR9 (7). It seems that TLR1, TLR2, TLR4, TLR6, TLR8 and TLR9 are involved in the recognition of mycobacterium (8).

Toll-like receptor 8 is encoded by the TLR8 gene also known as CD288. It is located on the Xp22.2 chromosome

Implication for health policy/practice/research/medical education:

Due to the importance of Tuberculosis in the world, this study focuses on the possible association between TLR8 rs3764880 and TLR9 rs148805533 polymorphisms and pulmonary tuberculosis (PTB).

Copyright © 2014, Zahedan University of Medical Sciences. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

close to another family member, TLR7 (9). TLR8 is expressed in monocytes, macrophages and myeloid dendritic cells (DCs) and is able to recognize single-stranded RNA and short double-stranded RNA of pathogens (10). Toll-like receptor 9 is encoded by the TLR9 gene, which is also known as CD289. It is located on 3p21.3 chromosome (11). TLR9 is expressed in different cells of the immune system such as dendritic cells, B lymphocytes, monocytes and natural killer (NK) cells, and is able to recognize unmethylated CpG islands in DNA molecules leading to production of cytokines such as type-I interferon and IL-12 (12). TLRs activation by its ligands cause a lot of biological processes such as cytokine secretion, apoptosis and antimicrobial activity (8). Several studies investigated the role of TLRs polymorphism in patients with pulmonary tuberculosis (PTB), but the results of genetic association studies are controversial.

2. Objectives

Therefore, the aim of the present study was to investigate the association between TLR 8, 9 polymorphism and PTB patients and healthy individuals.

3. Patients and Methods

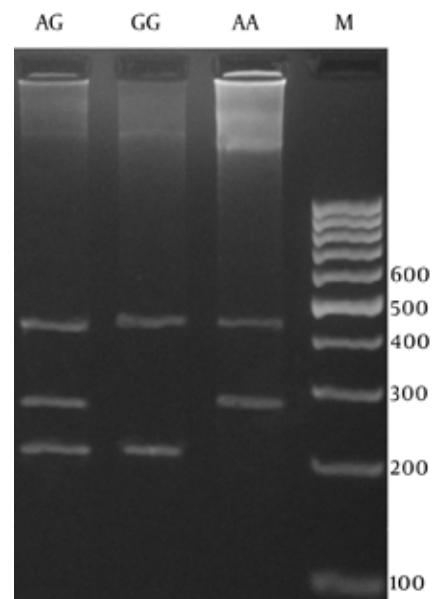
This case control study was performed in the Research Center for Infectious Diseases and Tropical Medicine, Bou-Ali Hospital, Zahedan, Iran. A total of 160 patients with pulmonary tuberculosis (newly diagnosed PTB cases and underwent treatment subjects for PTB), and 160 unrelated healthy subjects with no clinical symptoms or family histories of TB belonged to same ethnicity as patients and living in the same region as the patients with PTB (Southeast Iran) were enrolled in the study. The Ethics Committee of Zahedan University of Medical Sciences approved the project, and informed consent was obtained from both PTB patients and healthy control subjects. Tuberculosis was diagnosed by clinical symptoms, radiological evidence, sputum Acid Fast Bacillus (AFB) smear positivity, culture and response to antituberculosis chemotherapy as described in our previous study (13). Two milliliters of venous blood was collected from each patient and healthy subject using EDTA as anticoagulant and stored at -20 °C until further use. Genomic DNA was isolated from peripheral blood samples using salting out method as described previously (14). We searched the TLR8, 9 genes in the SNP database of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/SNP>) and selected two SNPs: rs3764880 A/G among the TLR8 and rs148805533 among the TLR 9 SNPs.

In this study, we designed a Tetra amplification refractory mutation system polymerase chain reaction (T-ARMS PCR) for detection of TLR8 polymorphism, and allele specific PCR for detection of TLR9 polymorphism. We used four primers in T-ARMS PCR for detection of TLR8 SNP rs3764880 A/G, two external primers (forward outer: 5'- AAATCACAAGTTCCTTCTTTTCATGTA -3', reverse outer: 5'- CATCACTGCATTGATTTTCAAATTTA -3') and

two internal primers (forward inner [A allele]: 5'- GGAATGAAAATTAGAACAAACAGAACCA 3' reverse inner [A allele]: 5'- TTTGCTAAAGAAATAGAAGTGGCTTACAAC -3'). Primers for the TLR9 rs148805533 polymorphism were as follows: Forward (Del allele): CAGGGCCATGTCACTGTTGC, Forward (Ins Allele): CAGGGCCATGTGGGTGACAA, Reverse: GTAGGCTGTCTGAGAGGGGA. The PCR mixture included 0.4 μM of each primer, 250 μM of each dNTP, 1 U Taq DNA polymerase with 2 mM MgCl₂, and 50 ng genomic DNA. Polymerase chain reactions (PCRs) were performed as follows: 5 min at 95°C followed by 30 cycles of 30 s at 95°C, 30 s at 50°C for rs3764880, 30 s at 64°C for rs148805533, and 30 s at 72°C, and final extension was performed at 72°C for 5 min. PCR products were analyzed by electrophoresis on 2% agarose gel and observed under ultraviolet light.

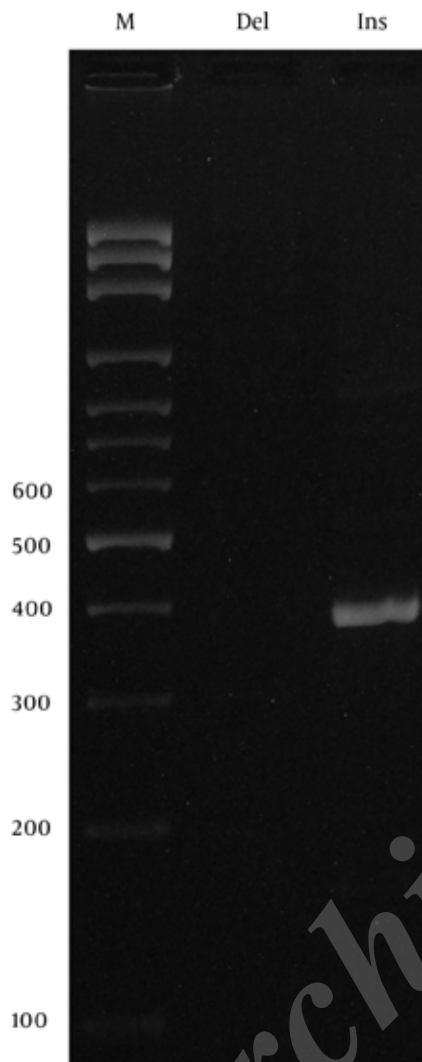
The product sizes of rs3764880 polymorphism were 272bp for A allele, 209bp for G allele and 423bp for the two outer primers (Figure 1). The PCR products for insertion and deletion alleles (rs148805533) comprised 411bp and 397 bp, respectively (Figure 2). Random samples were re-genotyped to verify the genotyping accuracy. Re-genotyping confirmed the previous results and there were no genotyping errors. Statistical analysis was performed by using SPSS for Windows, V18.0, SPSS, Inc., IL, USA. To investigate potential association of the selected polymorphisms and tuberculosis, the allele and genotype frequencies in patients and healthy controls were compared using Pearson's chi-squared test. Logistic regression analysis was applied to estimate odds ratio (OR) and 95% confidence intervals (CI) of genetic risk in PTB. A P value less than 0.05 was considered as statistically significant.

Figure 1. Photograph of the PCR Products of TLR8 rs3764880 Gene Polymorphism



In T-ARMS-PCR method two external primers (control band) and two inner primers (allele specific primers) were used. The product sizes for detection of TLR8 rs3764880 polymorphism were; 272 bp for A allele, 209 bp for G allele and 423 bp for control band. M: DNA Marker.

Figure 2. Photograph of the PCR Products of TLR9 rs148805533 Gene Polymorphism



The product sizes for detection of TLR9 rs148805533 polymorphism were 411 bp for insertion and 397 bp for control band. M: DNA Marker.

4. Results

The study subjects included 160 PTB patients (mean age \pm SD: 51.27 ± 20.52) and 160 healthy controls (mean age \pm SD: 47.68 ± 15.86 years). Among the PTB patients, 77 were males and 83 were females, and among the healthy controls, 62 were males and 98 were females. There was no significant difference among the PTB patients and healthy controls regarding sex and age ($P > 0.05$). Table 1 summarized the genotype and allele frequencies of rs3764880 A/G polymorphisms in females. There were no significant differences between female patients with pulmonary TB and female control ones for genotype frequencies regarding the TLR8 rs3764880 polymorphism.

The wild-type genotype AA was observed in 43 (43.9%) of the female patients, whereas 34 (34.7%) were heterozygote (AG) and 21 (21.4) were homozygote for the mutant genotype (GG). In the female control group, the frequencies of genotypes were 40 (48.2%) for AA and 16 (19.3%) for AG and 27 (32.5%) for GG. No significant differences were found in allelic frequencies between PTB and control subjects females regarding rs3764880 polymorphism of TLR8 ($P = 0.51$). As shown in Table 2 similarly, there were no significant differences between the male pulmonary TB and male control cases for genotype distribution frequencies at rs3764880 ($p > 0.05$). The analysis of the PTB patients and healthy control revealed no statistically significant difference between the groups regarding 14-bp insertion/deletion polymorphism of the TLR9 gene. All of the samples had Ins/Ins genotypes. Our finding demonstrated that the 14-bp deletion polymorphism of TLR9 is not a risk factor for PTB.

5. Discussion

The objective of the current study was to detect the presence of rs3764880 polymorphism in TLR8 and rs148805533 polymorphism in TLR9 genes and to assess its distribution among patients with tuberculosis, and healthy subjects in a sample of Iranian population. Our findings revealed that there were no significant differences between female pulmonary TB and female control groups for genotype frequencies regarding TLR8 rs3764880 polymorphism; similar to females, there were not significant differences between the male pulmonary TB and male control cases. In agreement with our findings, Dalgic et al. reported that genotype distributions and allele frequencies at rs3764880 between the PTB and control groups did not show significant differences. They did not find any significant differences between the female pulmonary TB and female control groups for genotypes GG, AG and AA, or between alleles G and A frequencies at rs3764880. However they found a significant association between genotype A/(-) and susceptibility to PTB in males (15). In contrast to our findings, Davila et al. in a study on PTB patients from Indonesia and Russia found that four polymorphisms in the TLR8 gene were associated with TB susceptibility in males, that one of them was polymorphism rs3764880, In addition they found a strong allelic association with susceptibility to PTB in males (9). They demonstrated that the mechanism through TLR8 recognizes mycobacterium and intracellular signaling is unknown, however they mentioned that expression level of TLR8 were up-regulated significantly in patients during the acute phase of disease (9). TLRs have a critical role in pathogen recognition and activation of innate immunity and act in multiple cellular processes such as cytokine secretion (6), modulation of the adaptive immune response and apoptosis (8). TLR8 is located on X chromosome and is activated by bacterial nucleic acids (10). TLR8 has a role in immunity against mycobacterium through IRF-7 and

induced production of IFN(10).

No significant difference was found between PTB patients and healthy control regarding rs148805533 polymorphism in TLR9 genes in our population. In agreement with our study Selvaraj et al. in south India showed that allele and genotype frequencies of TLR9 were similar in healthy control subjects and patients with pulmonary tuberculosis, and polymorphism in TLR9 is not associated with susceptibility to pulmonary TB in south Indian population (12). Jahantigh et al. in a study on TB-endemic region of Iran (Zahedan) showed that genotype and allele frequency of -1486 T/C TLR9 were not significantly different between pulmonary TB patients and controls (16). Contrary with our results Velez et al. showed that five SNP in TLR9 had statistically significant associations with TB and concluded that TLR9 might play important roles in determining susceptibility to TB (11). Several prior studies also found that variants of TLR9 are associated with TB susceptibility (17, 18), furthermore the role of TLR9 polymorphisms in other infectious diseases, inflammation and cancers have verified by many researchers (19-22). TLR9 is expressed intracellularly by different cells of the immune system and has a key role in activation of the innate immune system against mycobacterium infection (17). TLR9 alerts the immune system by binding to unmethylated CpG DNA motifs and induction of IL-12

production through DCs (8).

Polymorphisms within TLR9 may impair the immune response against TB, and it is thought that polymorphism in TLR9 may influence their expression, function and affecting RNA splicing (8, 17). In conclusion, our results indicated that TLR8 rs3764880 polymorphism and TLR9 rs148805533 polymorphism may not be major genetic factors for susceptibility to pulmonary tuberculosis in a sample of Iranian population. Additional studies in a large number of TB patients and functional studies are needed to validate our findings.

Acknowledgements

The authors would like to appreciate patients and healthy subjects who willingly participated in the study.

Authors' contributions

Mohsen Taheri designed the study concepts, analyzed the data and prepared the manuscript. Ali Gadari and Gholamreza Bahari collected the data and performed the laboratory studies. Seyed Mohammad Hashemi-Shahri and Mohammad Naderi were involved in sample and data collection, and final approval of the manuscript. Mohammad Hashemi was involved in data analysis, drafting the manuscript and final approval.

Table 1. Analysis of the Association of the TLR8 rs3764880 Polymorphism and PTB in Females^{a, b}

Polymorphism	PTB	Normal	OR (95% CI)	P Value
Codominant				
AA	43 (43.9)	40 (48.2)	1.00	-
AG	34 (34.7)	16 (19.3)	1.97 (0.0949-4.11)	0.06
GG	21 (21.4)	27 (32.5)	0.72 (0.35-1.47)	0.37
Dominant				
AA	43 (43.9)	40 (48.2)	1.00	-
AG + GG	55 (46.1)	43 (51.8)	1.2 (0.66-2.10)	0.56
Recessive				
AA + AG	77 (88.6)	56 (67.5)	1.00	-
GG	21 (21.4)	27 (32.5)	0.56 (0.29-1.10)	0.09
Alleles				
A	120 (61.2)	96 (57.8)	1.00	-
G	76 (38.8)	70 (42.2)	1.15 (0.75-1.75)	0.51

^a Abbreviation: PTB, pulmonary tuberculosis

^b Data are presented as No. (%)

Table 2. Analysis of the Association of the TLR8 rs3764880 Polymorphism and PTB in Males^a

Alleles	PTB	Normal	OR (95% CI)	P Value
A	48 (62.3)	42 (67.7)	1.00	-
G	29 (37.7)	20 (32.3)	1.15 (0.84-1.59)	0.80

^a Abbreviation: PTB, pulmonary tuberculosis

Financial Disclosure

The authors did not have a conflict of interest.

Funding/Support

This work was supported by a dissertation grant from Zahedan University of Medical Sciences.

References

- World Health Organization.. *Global tuberculosis report 2013*. Geneva, Switzerland: WHO; 2013. Contract No.: 9241564652.
- Ministry of Health and Medical Education.. [*Incidence of Tuberculosis*]. IR Iran: Division of TB and Leprosy Elimination. Center for Communicable Diseases Management;. Available from: http://cdc.hbi.ir/TB_Situation_in_Iran.aspx.
- Barton GM, Kagan JC. A cell biological view of Toll-like receptor function: regulation through compartmentalization. *Nat Rev Immunol*. 2009;9(8):535-42.
- Zhang Y, Jiang T, Yang X, Xue Y, Wang C, Liu J, et al. Toll-like receptor -1, -2, and -6 polymorphisms and pulmonary tuberculosis susceptibility: a systematic review and meta-analysis. *PLoS One*. 2013;8(5).
- Naderi M, Hashemi M, Hazire-Yazdi L, Taheri M, Moazeni-Roodi A, Eskandari-Nasab E, et al. Association between toll-like receptor2 Arg677Trp and 597T/C gene polymorphisms and pulmonary tuberculosis in Zahedan, Southeast Iran. *Braz J Infect Dis*. 2013;17(5):516-20.
- Kawai T, Akira S. Signaling to NF-kappaB by Toll-like receptors. *Trends Mol Med*. 2007;13(11):460-9.
- Areal H, Abrantes J, Esteves PJ. Signatures of positive selection in Toll-like receptor (TLR) genes in mammals. *BMC Evol Biol*. 2011;11:368.
- Thada S, Valluri VL, Gaddam SL. Influence of Toll-like receptor gene polymorphisms to tuberculosis susceptibility in humans. *Scand J Immunol*. 2013;78(3):221-9.
- Davila S, Hibberd ML, Hari Dass R, Wong HE, Sahiratmadja E, Bonnard C, et al. Genetic association and expression studies indicate a role of toll-like receptor 8 in pulmonary tuberculosis. *PLoS Genet*. 2008;4(10).
- Cervantes JL, Weinerman B, Basole C, Salazar JC. TLR8: the forgotten relative revindicated. *Cell Mol Immunol*. 2012;9(6):434-8.
- Velez DR, Wejse C, Stryjewski ME, Abbate E, Hulme WF, Myers JL, et al. Variants in toll-like receptors 2 and 9 influence susceptibility to pulmonary tuberculosis in Caucasians, African-Americans, and West Africans. *Hum Genet*. 2010;127(1):65-73.
- Selvaraj P, Harishankar M, Singh B, Jawahar MS, Banurekha VV. Toll-like receptor and TIRAP gene polymorphisms in pulmonary tuberculosis patients of South India. *Tuberculosis (Edinb)*. 2010;90(5):306-10.
- Kouhpayeh HR, Hashemi M, Hashemi SA, Moazeni-Roodi A, Naderi M, Sharifi-Mood B, et al. R620W functional polymorphism of protein tyrosine phosphatase non-receptor type 22 is not associated with pulmonary tuberculosis in Zahedan, southeast Iran. *Genet Mol Res*. 2012;11(2):1075-81.
- Hashemi M, Hanafi Bojd H, Eskandari Nasab E, Bahari A, Hashemzahi NA, Shafieipour S, et al. Association of Adiponectin rs1501299 and rs266729 Gene Polymorphisms With Nonalcoholic Fatty Liver Disease. *Hepat Mon*. 2013;13(5).
- Dalgic N, Tekin D, Kayaalti Z, Kadir E, Soylemezoglu T, Sancar M. Relationship between toll-like receptor 8 gene polymorphisms and pediatric pulmonary tuberculosis. *Dis Markers*. 2011;31(1):33-8.
- Jahantigh D, Salimi S, Alavi-Naini R, Emamdadi A, Owaysee Osque H, Farajian Mashhadi F. Association between TLR4 and TLR9 Gene Polymorphisms with Development of Pulmonary Tuberculosis in Zahedan, Southeastern Iran. *ScientificWorldJournal*. 2013;2013:534053.
- Torres-Garcia D, Cruz-Lagunas A, Garcia-Sancho Figueroa MC, Fernandez-Plata R, Baez-Saldana R, Mendoza-Milla C, et al. Variants in toll-like receptor 9 gene influence susceptibility to tuberculosis in a Mexican population. *J Transl Med*. 2013;11:220.
- Kobayashi K, Yuliwulandari R, Yanai H, Naka I, Lien LT, Hang NT, et al. Association of TLR polymorphisms with development of tuberculosis in Indonesian females. *Tissue Antigens*. 2012;79(3):190-7.
- Oliveira LB, Louvanto K, Ramanakumar AV, Franco EL, Villa LL, Ludwig-McGill Cohort S. Polymorphism in the promoter region of the Toll-like receptor 9 gene and cervical human papillomavirus infection. *J Gen Virol*. 2013;94(Pt 8):1858-64.
- Piotrowski P, Lianeri M, Wudarski M, Olesinska M, Jagodzinski PP. Contribution of toll-like receptor 9 gene single-nucleotide polymorphism to systemic lupus erythematosus. *Rheumatol Int*. 2013;33(5):1121-5.
- Omar AH, Yasunami M, Yamazaki A, Shibata H, Ofori MF, Akanmori BD, et al. Toll-like receptor 9 (TLR9) polymorphism associated with symptomatic malaria: a cohort study. *Malar J*. 2012;11:168.
- Huang CM, Huang PH, Chen CL, Lin YJ, Tsai CH, Huang WL, et al. Association of toll-like receptor 9 gene polymorphism in Chinese patients with systemic lupus erythematosus in Taiwan. *Rheumatol Int*. 2012;32(7):2105-9.