The Correlation between TLR9 (rs148805533 Del/Ins) Gene Polymorphisms and Susceptibility to Pulmonary Tuberculosis

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Abstract

Background: One of the main infectious causes of morbidity and mortality worldwide is *Mycobacterium tuberculosis*. The Toll-like receptor (TLR) genes' polymorphisms and mutations have been linked to an increased risk of infection in general. **Objectives:** This study aimed to investigate the association among TLR9 (rs148805533 Del/Ins), gene polymorphism, and pulmonary tuberculosis (PTB). **Materials and Methods:** Blood and sputum samples were collected from 70 patients with PTB and 30 healthy as a control group. Patients were diagnosed clinically by the specialized physician, in addition to use the acid-fast smear and culture on Lowenstein–Jensen. In addition, the molecular diagnostics technique was used by Gene Xpert device. The genotyping was carried out by using allele specific-polymerase chain reaction technique, and the TLR9 concentration was estimated by enzyme-linked immunosorbent assay (ELISA) technique. **Results:** The results of TLR9 polymorphism showed that the Ins/Ins allele, Del/Ins allele, and Del/Del allele genotypes frequencies at the site of (rs148805533) polymorphisms were significantly higher in PTB patients than the control groups ($P = 0.05$). The Ins/Ins allele genotype was the most frequent in PTB patients. In addition, the insertion allele was the most frequent in PTB patients and control groups. **Conclusion:** Our results indicated that in a sample of the local Iraqi population, the TLR9 (rs148805533) gene polymorphism may be a significant genetic determinant for PTB susceptibility.

Keywords: Pulmonary tuberculosis (PTB), single-nucleotide polymorphisms (SNPs), TLR9 (rs148805533 Del/Ins)

Introduction

Tuberculosis (TB) has been asymptomatically (latently) infecting one-third of the global population and remains a major concern to global public health.[1] However, due to some factors between host and pathogen interaction, including the pathogenic characteristics of the *Mycobacterium tuberculosis* (*M. tuberculosis*), environmental factors, individual lifestyle and diet, and host genetic and immunological factors, only 10% of latently infected subjects progress to develop the active form of the disease.[2-4]

The incidence of TB differs among races, ethnicities, and families, which suggests a genetic factor affecting TB susceptibility.[5] Host genetic factors such as Toll-like receptors (TLRs) that are associated with the susceptibility to TB will serve as genetic markers to predispose or predetermine the development of the disease. TLRs are a family of phylogenetically conserved genes, which are essential for the recognition of a broad repertoire of pathogen-associated molecular patterns on macrophages and dendritic cells and play an important role in the innate responses against *M. tuberculosis*.[5-7] To date, among the 11 mammalian TLRs that have been identified, TLR1, TLR2, TLR4, TLR6, TLR8, and TLR9 are functional in humans and are involved in the recognition of mycobacteria-associated products.[2] Mutations and polymorphisms within the genes involved in the TLR pathway are associated with susceptibility to infection in general[8] as well as to TB specifically.[8,9]

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TLR9 is encoded by the TLR9 gene. It is situated on chromosome 3p21.3.[1] It spans approximately 5 kb in size and contains two exons, the second of which is the major coding region.[10,11] TLR9 can detect unmethylated nucleic acid and is expressed in a variety of immune system cells, including dendritic cells, B lymphocytes, macrophages, monocytes, and natural killer cells, specifically cytosine-phosphate-guanine (CpG) motifs, cytokines like type-I interferon and interleukin 12 are produced as a result of bacterial and viral DNA.[12]

TLR9 plays an elementary role in pathogen recognition and activation of innate immunity. In humans, TLR9 genetic variants have been associated with HIV progression as well as more broadly with asthma, systemic lupus erythematosus, and atherosclerosis.[8,13] To our knowledge, the present study is the first study about the association of genetic polymorphism in TLRs (TLR9) with TB susceptibility in Iraqi population. Therefore, the identification of host genes responsible for susceptibility and resistance to TB should provide a significant contribution for understanding the pathogenesis of the disease and may lead to the development of new prophylaxis and treatment strategies.[14]

Materials and Methods

Patients groups

Seventy Iraqi pulmonary TB (PTB) patients were enrolled in this study and thirty health control, at consultant clinic for respiratory diseases in Babylon and Karbala provinces, Iraq. All cases in this study were pathologically confirmed by acid-fast stain, X-ray techniques, and Gene Xpert technique, which reveals the bacterium *M. tuberculosis* and DNA and resistance to rifampicin, which indicates to occur mutation.

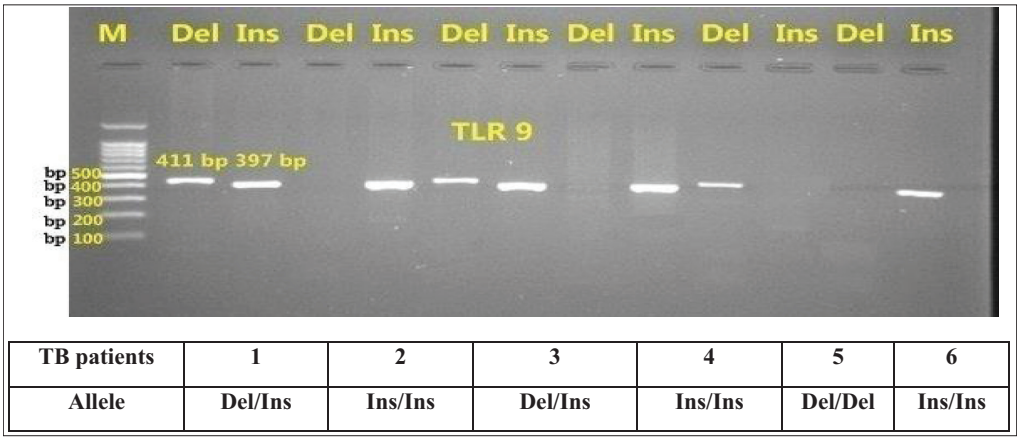
DNA extraction

Using a genomic DNA extraction kit (Promega, Madison, Wisconsin), genomic DNA was isolated from venous blood samples of both the patients and the healthy control subjects, according to the manufacturer's protocol. The quantity of DNA was confirmed by Bio Photometer (Eppendorf AG, Germany), and DNA was stored at −20°C. All genotypes of TLR9 at position (rs148805533 Del/Ins) have been mapped to chromosome (3p21.3) for all individuals by using allele specific-polymerase chain reaction (PCR) technique to detect deletion/insertion TLR9 polymorphism (rs148805533). The primers used in the study for the TLR9 rs148805533 polymorphism and their amplicon size are as follows: forward (Del allele): 5′-CAGGGCCATGTCACTGTTGC-3′ with 411 bp, forward (Ins Allele): 5′-CAGGGCC ATGTGGGTGACAA-3′ with 397 bp, and reverse primer: 5′-GTAG-GCTGTCTGAGAGGGG-3′.[15]

PCR was carried out as follows: 95°C for 5 min; 30 cycles of 95°C for 30 s, 64°C for 30 s; 72 °C for 30 s, and final extension was performed at 72°C for 5 min using reagents from Promega company. Two percent of agarose gel electrophoresis was used to evaluate PCR products, and the results were seen under an ultraviolet light source. The sizes of the PCR products for the insertion and deletion alleles (rs148805533) were 411 bp and 397 bp, respectively [Figure 1].

Statistical analysis

The statistical analysis to the association study was performed by using a commercially available software program Statistical package for the social science (Statistical Package for the Social Sciences [SPSS]), Version 18.0, SPSS software, Inc., Chicago, Illinois). The allele and genotype frequencies in TB patients and healthy controls were compared using Pearson's Chi-square test to look into any potential associations between the chosen polymorphisms and the disease. Associations between the disease and genotypes were assessed by calculating odds ratios (ORs) and 95% confidence intervals (CIs) of



TB patients	1	2	3	4	5	6
Allele	Del/Ins	Ins/Ins	Del/Ins	Ins/Ins	Del/Del	Ins/Ins

Figure 1: Allele specific-PCR representative agarose gel electrophoresis finding of TLR9 (rs148805533 Del/Ins) Gene Polymorphism (2% agarose gel, 1X TBE buffer (pH 8) at 100V for 45 min) showing the product of PCR for detection of TLR9 (rs148805533) gene variation visualized under UV light after staining with 1 μL of ethidium bromide at the concentration of 0.5 mg/mL; band with 411 base pair for deletion allele and band 397 base pair for insertion allele. Lane M 100 bp ladder

PTB genetics. A *P* value of 0.05 or less was regarded as statistically significant.

Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients' verbal and analytical approval before the sample was taken. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee according to the document number dated December 1, 2015, to get this approval.

Results

We recruited 70 PTB patients and 30 unrelated healthy subjects in this study. The patient's age was ranging from 20 to 75 years old. A total of 40 were males and 30 were females, and among the healthy controls, 20 were males and 10 were females. The infection percentage increased in the age group of 50–59 to reach 21.4% of the total number of TB patients, whereas the number of cases decreased in the age groups of 30–39 (17.1%) and 40–49 (15.7%). High percentage of infection (25.7%) was found in the age group of >60 years. The results that were expressed in Table 1 revealed the wide age range of TB patients. This suggests that all age groups were susceptible to TB infection, but the rate of infection varies depending on the age. The current results revealed that the most predominant age groups (>60) years of TB patients. There was no significant difference between the PTB patients and healthy controls regarding sex and age ($P > 0.05$), as shown in Table 1.

Among these 70 patients, 38 (54.28%) give positive acid-fast bacillus (AFB) smear and culture on Lowenstein-Jensen (LJ) medium or both, while 32 (45.71%) were diagnosed by Gene Xpert technique as shown in Table 2.

For the detection of the TLR9 (rs148805533) gene polymorphism, allele specific-PCR was utilized. The results showed the genotypes and alleles frequencies of TLR9 (rs148805533 Del/Ins) gene polymorphisms in PTB patients and control groups, which are presented in Table 2 and Figure 1.

In Table 2, the results showed that the Ins/Ins allele, Del/Ins allele, and Del/Del allele genotypes frequencies at the site of rs148805533 polymorphisms were significantly higher in PTB patients than in control groups ($P = 0.05$), the Ins/Ins allele homozygous genotype frequency was overrepresented among the PTB patients, 40 (57.15%) which is more than the other two genotypes Del/Ins allele 17(24.28%) and Del/Del allele 13 (18.57%), respectively. Therefore, the Ins/Ins allele genotype was the most frequent in PTB patients.

The TLR9 (Ins/Ins allele) homozygous genotype was significantly associated with increased susceptibility to TB.

The individuals with Ins/Ins allele homozygous genotype were significantly overrepresented among TB patients, 40 (57.15%), compared with healthy control subjects, 8 (26.66%) had a 1.244-folds increased risk of developing TB than the other two genotypes Del/Ins allele and Del/Del allele ($P = 0.05$; OR = 1.244; 95% CI, 0.872–1.775, for Ins/Ins allele genotype). In contrast, the individuals

Table 1: Characteristics of age and gender in TB patients in this study

Age groups	Gender		Total	Chi-square	*P* value
	Male	Female			
20–29 years	8 (11.4%)	6 (8.6%)	14 (20%)	7.04	0.13
30–39 years	10 (14.2%)	2 (2.9%)	12 (17.1%)		
40–49 years	7 (10.0%)	4 (5.7%)	11 (15.7%)		
50–59 years	5 (7.1%)	10 (14.2%)	15 (21.4%)		
>60 years	10 (14.2%)	8 (11.4%)	18 (25.7%)		

Table 2: Genotypes and alleles frequencies of TLR9 (rs148805533 Del/Ins) gene polymorphism in PTB patients and control

TLR9 (rs148805533) genotype	Genotype frequency (%)			
	PTB patients ($n = 70$)	Control ($n = 30$)	*P* value of F-test	OR (95% CI)
Del/Ins allele	17 (24.28%)	20 (66.67%)	0.05	0.145 (0.015–1.423)
Ins/Ins allele	40 (57.15%)	8 (26.66%)		1.244 (0.872–1.775)
Del/Del allele	13 (18.57%)	2 (6.67%)		0.115 (0.011–0.852)
TLR9 (rs148805533) alleles		**Allele frequency (%)**		
Insertion allele	97 (69.29%)	36 (60%)	0.05	
Deletion allele	43 (30.71%)	24 (40%)		

CI: confidence intervals, OR: odds ratio, PTB: pulmonary tuberculosis

with Del/Ins allele heterozygous genotype were obviously more presented among control individuals, 20 (66.67%), when compared with TB patients, 17 (24.28%) (P = 0.05; OR = 0.145; 95% CI, 0.015–1.423, for Del allele/Ins allele genotype). While the individuals with Del/Del allele homozygous genotype were obviously more presented among TB patients, 13(18.57%), when compared with control individuals, 2(6.67%) with significant difference (P = 0.05; OR = 0.115; 95% CI, 0.011–0.852, for Del allele/Del allele genotype), as shown in Table 2.

However, the results for TLR9 (rs148805533 Del/Ins) polymorphism showed that insertion allele frequency was 97 (69.29%) in the TB patients and 36 (60%) in the control individuals, whereas deletion allele frequency was 43 (30.71%) in the TB patients and 24 (40%) in the control individuals with significant difference (P = 0.05), as shown in Table 2.

Our findings revealed that there were statistically significant variations in genotype and allele frequencies were revealed between PTB patients and healthy groups related rs148805533 gene polymorphism of TLR9 in our population (P = 0.05).

Figure 1 shows the genotypes of six patients: patients 1 and 3 with the heterozygous genotype Del/Ins allele and patients 2, 4, and 6 with the homozygous genotype Ins/Ins allele, whereas patient 5 with the homozygous genotype Del/Del allele of TLR9 (rs148805533) gene polymorphism.

TLR9 concentration in PTB patients and control

The mean serum level of TLR9 in all patients with active TB was higher (6.19 ± 0.79 pg/mL) than the control individuals (5.92 ± 0.33 pg/mL). This study reveals a significant difference between the serum level of TLR9 for TB patients and control subjects (P < 0.05). Figure 2, shows the result of TLR9 concentration in PTB patients' group and control group. There was a significant increase in TB patients' group than the control (P = 0.026). Serum level of TLR9 in all patients with active TB was higher (6.19 ± 0.79 pg/mL) than the serum level of TLR8 (2.84 ± 0.99 pg/mL).

The mean of serum concentration of TLRs (TLR9) among patients with TB was significantly higher in the PTB group than in the control group, suggesting a relationship of high serum levels of these receptors with active TB and the progression to more serious forms of the disease.

In conclusion, our results in the present study revealed might be that the level of TLRs concentration in the serum of PTB patients was affecting the site and severity of TB infection.

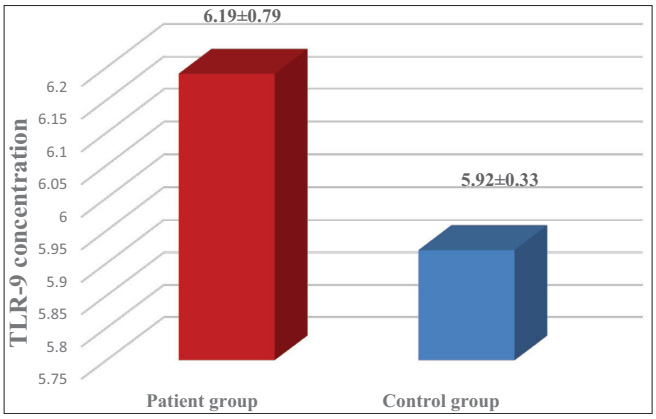


Figure 2: TLR9 concentration in PTB patients and control

Discussion

In the present study, the TB infection percentage increased in the age group of 50–59 to reach 21.4% of the total number of TB patients, whereas the number of cases decreased in the age groups of 30–39 (17.1%) and 40–49 (15.7%). This finding was matched with WHO,[16] which reported that TB is mainly a disease of older people or of the immunocompromised. Zaman *et al.*[17] reported that the age groups' studies were also different between studies and covered all ages.

Moumita *et al.*[18] explained that GeneXpert's cartridge-based nucleic acid amplification test was discovered to be an effective molecular assay technique for the quick diagnosis of TB as well as RR-TB. Another study concluded that GeneXpert assay is a suitable technique for the early diagnosis of *M. tuberculosis* that is rifampicin resistant.[19]

Mammalian TLRs are a key family of innate immune proteins and a major class of PRR, serving principal recognition and signaling functions critical to host defense mechanisms.[20] TLRs are expressed in innate immune cells such as dendritic cells (DCs), macrophages, monocytes, neutrophils, T and B cells, as well as nonimmune cells such as fibroblast cells and epithelial cells, endothelial cells, and even neural cells serving as critical mediators of the immune response to a variety of pathogens, including *M. tuberculosis*.[21]

Our study is in disagreement with previous studies by Selvaraj *et al.*,[12] which indicated that TLR9 genotype and allele frequencies were identical in healthy control persons and patients with PTB, and that variation in TLR9 is not linked to PTB susceptibility in the south Indian population.

The current study was incompatible with many other studies,[15,22] which demonstrated that TLR9 polymorphism is not related to susceptibility to PTB in the Iranian population and that allele and genotype frequencies

of TLR9 were identical in healthy control people and patients with PTB.[15]

Another study by Jahantigh *et al*.[11] on TB-endemic region of Iran (Zahedan) indicated that there was no significant difference in the genotype and allele frequency of −1486 T/C TLR9 between PTB patients and controls.

Our results are in agreement with the results obtained by Velez in a study on Caucasians and African-Americans, determined that TLR9 may be crucial in determining TB susceptibility after research result indicated five SNP in TLR9 in African-Americans and one SNP in TLR9 Caucasians had statistically significant relationships with PTB.

Numerous earlier investigations indicated that TLR9 mutations and TB susceptibility are related.[3,23] The importance of TLR9 in the antimycobacterial responses has been supported in experimental models using the TLR9-deficient mice.[6,7]

The immune response to TB may be impaired by polymorphisms in TLR9, which are considered to impact their expression, function, and RNA splicing.[2,3]

In the present study, we found a possible association between TLR9 (rs148805533 Del/Ins) gene polymorphism and PTB in Babylon and Karbala population of Iraq. We observe the Del allele of 14 bp Del/Ins polymorphism of TLR9 gene in the studied population and reported association between this polymorphism and PTB in our population. Our study is in disagreement with previous studies by Hashemi *et al*.[15] and Salimi,[22] in Iranian population and in Egyptian population[24] also in south India,[12] 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 PTB.

Previous studies indicated that the SNPs in TLR9 gene might be associated with TB risk.[25] Therefore, the objective of the current study was to detect the presence of association between TLR9 (rs148805533 Del/Ins) gene polymorphism and TB infection among patients with TB and control groups in Babylon and Karbala population, Iraq. This study found that there were significant associations regarding TLR9 (rs148805533 Del/Ins) gene polymorphism with TB, but other studies have failed to demonstrate significant associations of TLRs polymorphisms with TB.[26,27] 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 no significant relation between TLR9 polymorphisms alone and TB.[11] This study is in disagreement with our results, we found a possible association between TLR9 polymorphism and TB in the local population of Babylon and Karbala provinces, Iraq.

A large number of studies showed that polymorphisms in TLR1, TLR2, TLR4, TLR6, TLR8, and TLR9 have been implicated in TB susceptibility.[28] But other studies have failed to demonstrate significant associations of TLRs polymorphisms with TB.[26,27]

Our study was incompatible with several authors[15,22,24] who revealed that TLR9 (rs148805533) polymorphisms may not be risk factors for susceptibility to PTB in a sample of Iranian and Egyptian populations, respectively. In their study, they found no association between these polymorphisms and PTB neither in their female nor in their male patients.

Evaluation the TLR9 concentration by ELISA

By using enzyme-linked immunosorbent assay (ELISA) technique to determine the concentration of TLR9 in PTB patients' serum and control groups, the level of TLR9 concentration in serum was measured in all 70 active TB patients (40 were attended as new TB patients, and 30 patients were attended to follow-up evaluation of the Directly Observed Treatment, Short-course [DOTS]) as well as in the 30 control individuals. The expressions of serum TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR9 were detected by ELISA techniques.[29] Therefore, the serum TLRs may be a biomarker in early diagnosis of TB disease.

Conclusion

Our results indicated that in a sample of the local Iraqi population, the TLR9 (rs148805533) gene polymorphism may be a significant genetic determinant for PTB susceptibility.

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Conflicts of interest

There are no conflicts of interest.

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