

Original Article

Pulmonary tuberculosis susceptibility and association with Toll-Like receptor 2 Arg753Gln polymorphism

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Abstract

Introduction: Tuberculosis has been a concern of healthcare professionals due to the serious threats it poses on public health safety. However, regardless all the efforts, no appropriate goals for immunological diagnosis or tuberculosis treatment were established. Toll-like receptor 2 is one of the toll-like receptors, which plays a fundamental role in recognizing and hosting defense against *Mycobacterium tuberculosis* infection. Toll-like receptor 2's genetic polymorphism (arginine-to-glutamine substitution at residue 753 (Arg753Gln)) was linked to negative effects on the function of Toll-like receptor 2 which, in turn, impacts the body's resistance or susceptibility to tuberculosis. The current study aimed at investigating the single Arg753Gln nucleotide polymorphism of the Toll-like receptor 2 gene in patients with tuberculosis infection versus a sample of healthy subjects as controls.

Methodology: A comparative study was conducted to investigate Toll-like receptor 2 polymorphism of the single nucleotide gene Arg753Gln in 30 patients with pulmonary tuberculosis and compare their results with other 20 healthy controls matched by age and sex.

Results: TLR-2-Arg polymorphism allele A occurred in 36.7% of the patient group. Homozygous carriers of allele A/A polymorphism occurred in 13.4% compared to 5% among controls, while GA genotype was found in 23.3% among the study group and 10% among controls. The association between GA genotype and pulmonary tuberculosis was found statistically significant ($p = 0.002$) than other genotypes. Allele frequency for both G and A were ($p = 0.002$) in patient groups and ($p = 0.000$) among the control group.

Conclusions: TLR-2 Arg753Gln polymorphisms may have a crucial role in pulmonary tuberculosis susceptibility among Egyptian patients.

Key words: Polymorphism; *Mycobacterium*; TB; TLR2.

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Introduction

Tuberculosis (TB) is bacterial infection that has been recognized long ago and has remained a significant concern of global health to-date. The organism causing this infection is called *Mycobacterium Tuberculosis* [1]. Among the reported 10 million new global TB cases in 2018, nearly half a million have been multidrug-resistant (MDR-TB) [2]. About one third of the population around the globe are affected by *Mycobacterium tuberculosis* infection. However, only 5–15% of infected people show clinical manifestations of TB during their lives [3].

People's response to infection varies; some of them develop uncontrolled disease while others eradicate the infection. The exact reason behind this huge variation is still unknown [4]. Genes are also thought to be an

essential factor in determining disease susceptibility [5-10].

The disease's immune pathogenesis involves several aspects of the immune system. The most prominent ones are tumor necrosis factor- α (TNF- α), macrophages, T-lymphocytes, and interferon- γ . These components are essential in the immune response and defense against this infection [11-15].

The macrophages are stimulated by different components of the mycobacterium, which leads to an increased TNF production which stimulates the macrophages further, contributing to the formation of granulomas. Multiple mycobacterial components also result in activation of macrophages, this mainly occurs through toll-like receptor 2 (TLR-2) which are types of proteins expressed on either surface of macrophages and dendritic cells (TLR-1, 2, 4, 5, 6) or on the cytosol

or endosomal membranes (TLR3, 7, 8, 9) [16]. These are a conserved gene family that codes ten functional proteins in humans. TLRs are essential to the innate immune system's response to infectious organisms through discriminatory molecular patterns (PAMPs) that occurs in conjunction with self-pathogen like lipopolysaccharide (LPS), surface lipoproteins and teichoic acid and the initiation of the signal transduction pathway NF- μ B. The Nuclear translocation of NF-Cytokine B leads to transcription of pro-inflammatory cytokine genes which are necessary for establishing a defensive immune response. various TLRs can identify different groups of pathogens synthesized products and regulating the host's protective process against pathogen invasions [17]. When it comes to TB, light has been shed on TLR-2 because it is thought to sense the PAMPs of *Mycobacterium tuberculosis* and thus recruits the adaptor molecules of MyD88 and TIRAP into the receptors' complex. Serine/threonine kinase is linked to receptor IL-1 (IRAK)-1 and IRAK-4 are consequently recruited and activated. This allows for the receptors to interact with a new adaptor protein, (i.e., downstream molecule TRAF-6). TRAF-6 activates TAK-1 and MKK6. This activation leads to further activation of NF-kB, ERK, Jun N-terminal kinase (JNK), and p38 kinase transcription factor. The TIR domain that contains adaptors MyD88 and TIRAP is linked to the TIR domain of TLR2 and are fundamental for activating PGN-induced NFkB and MAPK. [18,19].

TLR-2 helps recognizing various types of bacterial lipoproteins. For instance, peptides that originate from Mycoplasma, Borrelia, Treponema, as well as Mycobacterium Tuberculosis [20,15]. When these receptors are blocked, the macrophages' ability to sense the meaning of mycobacterial peptides is reduced. Later, they detect presence of mycobacteria by activating TLR-2 (the first line of defense), resulting in activating a series of immune responses (including TNF-a, antigen processing, interleukin-1 and interferon-gamma development, T-lymphocyte activation, etc.). research have shown that activating TLR-2 directly results in the intracellular killing of *Mycobacterium tuberculosis* by means of alveolar macrophages. When an individual has a specific TLR-2 genotype, he can be affine with *Mycobacterium tuberculosis* ligands resulting in variations in signal transduction [21].

Single nucleotide polymorphisms (SNPs) are deoxyribonucleic acid (DNA) sequence variants that arise when the genome sequence alters a single nucleotide (adenine (A), thymine (T), cytosine (C) or

guanine (G)) [21]. Several SNPs do not influence cell function. However, they can increase an individual's risk for disease or affect diseases' response to pharmacological treatment [19]. Lorenz *et al.* [22] reported a new TLR-2 gene polymorphism (arginine substitution to glutamine at residue 753 (Arg753Gln)) which results in a suppressed macrophagic response to bacterial peptides. Subsequently leading to suppression in the host's immune response to bacteria [22].

The goal of the current study was identifying the TLR-2 polymorphisms–Arg753Gln (753G/A) and demonstrating the role it plays in susceptibility to pulmonary TB among Egyptian population (TB patients versus healthy controls).

Methodology

This case-control analysis was carried out from October 2019 to March 2020 and the study included two groups

First group included 30 patients newly diagnosed with pulmonary TB.

Morning sputum samples were collected from 30 patients attending to Menoufia university hospital in the chest outpatient clinic. The selected patients were suspected to have TB based on their clinical signs and symptoms of the disease. TB was suggested based on the chest x-ray findings and the clinical presentation. Diagnosis was then confirmed by a sputum smear/culture [23].

The diagnostic criteria for TB disease had been identified as containing at least one of the following:

- Clinical and radiological findings consistent with TB disease and positive sputum smears for acid-fast bacilli on at least two separate occasions
- A positive result of the sputum culture, bronchial lavage, and pleural fluid, along with presence of *Mycobacterium tuberculosis* in an organ other than the lung (urine, semen, cerebrospinal fluid, etc.)
- Pathological confirmation in biopsy specimens of TB disease (lymph node, lung, etc.)

Patients with no convincing evidence for pulmonary TB diagnosis, patients with diabetes mellitus, autoimmune diseases, malnutrition were excluded from this study

Pulmonary TB infection was categorized into three grades [24,25].

- Minimally advanced: These lesions are characterized by having mild to moderate densities with cavitation not well-administrable, and a unilateral gross lung

volume. They are usually located superior to the 2nd chondro-sternal joint, and the spinous process of the 4th dorsal vertebra.

- Moderately advanced: These lesions are characterized by being disseminated and their severity range from mild to moderate. They can occur bilaterally within the total lung volume of one lung or the equivalent. When the lesions are small or concomitant, they are limited to one third of one lung’s total volume. Cavitation is occasionally present with a diameter < 4 cm.
- Far advanced: Lesions are characterized by increased severity in comparison with the mildly advanced lesions.

Second group included 20 healthy control subjects from chest department, outpatient clinic and blood donors from blood transfusion bank, Menoufia university hospital. These control groups were investigated for TB and found to be healthy. Patients with no history of acute or chronic respiratory infections as well as those with no history of TB disease were included as control groups in our study.

Ethics statement

Ethical approval was attained from Jouf University’s local bioethical committee – Approval No (05-02-42) in addition to an approval of the Research Ethical Committee at the Faculty of Medicine - Menoufia University. Moreover, participants consented verbally to participation in this research.

Sample collection

Sputum samples were taken from individuals with suspected pulmonary tuberculosis. Ziehl-Neelsen stain used for microbiological diagnosis of acid-fast bacilli. TB culture was done on Lowenstein – Jensen media at a temperature of 37°C for a duration of eight weeks [26,27].

5 mL of blood were withdrawn and labeled as blood samples from both groups. Samples were collected in EDTA tubes and were stored at a temperature of -80 °C until used for genomic extraction.

DNA extraction and Arg753Gln polymorphism of TLR-2 was studied for all participants in the Central

Laboratory, Faculty of Medicine - Menoufia University.

Genomic DNA extraction

The Genomic DNA was extracted from blood using G-spin TM, Total DNA Extraction kits (iNtRON BiotechnologQMS-GT1704) as per manufacturer’s instructions. Following extraction, DNA was kept at a temperature of -20 °C until used later.

Genotyping of arginine-to-glutamine substitution at residue-753 polymorphism of TLR-2

The TLR-2 coding sequence (GenBank accession No. 88878) was used to construct the primer. This process was done by using TLR-2 polymorphism detection with amplification refractory mutation system polymerase chain reaction (PCR) at position 2258 of the open reading frame as per the description of Newton *et al.* [28]. Primer sequence [21] used in the study were listed in Table 1.

This polymorphism leads to replacing arginine (CGG) with glutamine (CAG), and the end-result genotypes are thereby: arginine/glutamine (AG), glutamine/glutamine (AA), as well as arginine/arginine (GG).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed using the technique demonstrated by Oges *et al* (2004) as the following: 120 ng of DNA and 1 U Taq DNA polymerase (MBI Fermentas) DNA amplification, eight mM of deoxyribonucleoside triphosphates (MBI Fermentas) and 20 pmol of each primer.

The reactions of the common allele (G), with TLR-2-R, TLR-2-F, TLR-2-G, and then the reactions of the rare allele (A), with TLR-2-F, TLR2-A, and TLR-2-R (TIBMOLBIOL, Berlin, Germany), were carried out in an end volume of 50 mL as follow: Initial denaturation was performed for 4 minutes at 94 µC. After that 35 denaturation cycles were performed for a duration of 1 minute at 94 µC, annealing was performed at 62 µC and extension was performed at 72 µC, with variations in the annealing temperatures. The final extension was performed for a duration of 4 minutes at 72 µC. Applied Biosystems 7500 real time PCR (Germany) was utilized to perform the amplification.

Table 1. Primers used for polymorphism detection in Toll-like Receptor 2 (TLR2) gene location 2258 [21].

Primer	Nucleotide sequence
TLR2-F	TATGGTCCAGGAGCTGGAGA
TLR2-R	TGACATAAAGATCCCAACTAGACAA
TLR2-G	GGTCTTGGTGTTTCATTATCTTCC
TLR2-A	GGTCTTGGTGTTTCATTATCTTCT

T: thymine; A: adenine; G: guanine; C: cytosine.

Table 2. Demographic data of the studied groups.

Demographic Variables	Pulmonary TB Patients (n = 30)		Control Group (n = 20)	
	No of cases	Percentage	No of cases	Percentage
Age (in years)				
< 20	3	10.0	2	10.0
21 - 30	5	16.7	4	20.0
31 - 40	8	26.7	8	40.0
41 - 50	8	26.7	5	25.0
51 - 60	4	13.3	1	5.0
> 60	2	6.7	-	0.0
Mean ± SD	34.90 ± 9.711		39.23± 13.866	
Gender				
Male	19	63.7	12	60.0
Female	11	36.7	8	40.0
Malignancy				
Yes	6	20.0	-	0.0
No	24	80.0	20	100.0
Smoking				
Yes	8	26.7	4	20.0
No	22	73.3	16	80.0
Chronic Illness				
No	18	60.0	18	90.0
Diabetes	7	23.3	1	5.0
COPD	2	6.7	-	0.0
Hypertension	3	10.0	1	5.0
Family History of Pulmonary Tuberculosis				
Yes	3	10.0	3	15.0
No	27	90.0	17	85.0
Family history of Autoimmune Diseases				
Yes	7	23.3	0	0.0
No	23	76.7	20	100.0

Table 3. Hardy–Weinberg equilibrium for TB patients and controls by asymptotic Pearson’s chi-square test.

TLR2 polymorphism	Genotype	Pulmonary TB patients (n = 30)		Control Group (n = 20)		χ^2	p-value
		No of cases	Percentage	No of cases	Percentage		
Arg753Gln	GA	7	23.3	2	10.0	12.600	0.002*
	GG	19	63.3	17	85.0	2.820	0.030*
	AA	4	13.4	1	5.0	-	-
	Total	30	100.0	20	100.0		
χ^2		12.600		24.100			
p-value		0.002*		0.000*			

GA: Heterozygous carrier of polymorphism; AA: homozygous carrier; GG: normal genotype; * significant.

Table 4. Relation between genotypes and clinical severity of pulmonary TB infection.

Pulmonary TB Grading	Genotype					
	GA		GG		AA	
	No of cases	Percentage	No of cases	Percentage	No of cases	Percentage
Minimally advanced	6	85.7	11	57.9	3	75.0
Moderately advanced	1	14.3	6	31.6	1	25.0
Far advanced	0	0	2	10.5	0	0.0
χ^2			2.378			
p-value			0.667*			

* statistically non- significant.

The end-result PCR products underwent electrophoresis in a 2% agarose gel and ethidium bromide was used to stain them. Amplification of the 470-base pair (bp) PCR product was performed using TLR-2-F and TLR-2-R, while the 328-bp product was performed using TLR-2-A and TLR-2-F, or TLR-2-G and TLR-2-F.

A *P*-value was considered significant at *P*=0.05. Chi-squared test was conducted to analyze the distribution of allele and genotypes in both groups, Hardy-Weinberg equilibrium was tested using the same test [29].

Results

Total number our study was performed on 30 patients attending Menoufia university hospital in the chest outpatient clinic suspected to have pulmonary TB.

Pulmonary TB was diagnosed by sputum smear positivity. This study included two groups of pulmonary TB patients diagnosed clinically and laboratory by sputum and bronchial lavage smear (n = 30). Their mean age ± SD was 34.90 ± 9.711 years.

The control group consists of 20 subjects 12(60%) males and 8 (40%) females with their mean age ± SD equal to 39.23± 13.866 yrs.

Among the patient group, 8 (26.7%) were smokers. Among the control group, 4 (20%) were smokers. Seven (23.3%) of diseased patients were diabetic, 2 (6.7%) were COPD, and 3 (10%) were hypertensive. Three (10%) of patients with pulmonary TB had a family history of pulmonary TB and 7 (23.3%) had a history of autoimmune diseases (Table 2).

Distribution of TLRs into two polymorphism genotypes among the studied groups (pulmonary TB and control) were shown in Table 2. TLR-2-Arg polymorphism allele (A) was found in 11 out of 30 patients in the study group (i.e., 36.7%). Homozygous carriers of allele A/A polymorphism occurred in 4

(13.4%) compared to 1 (5%) among controls. Meanwhile, GA genotype was found in 7 (23.3%) among the patient group and 2(10%) among the control group.

Ratios between these genotypes among both groups were calculated and the differences between them were analyzed using the Chi-square test. The statistical analysis revealed a significant correlation between GA genotype and pulmonary TB (*p* = 0.002) compared to other genotypes (Table 3). Allele frequencies for both A and G were 0.002 in patient groups and 0.000 among the control group as shown in Table 3

Based on Hardy-Weinberg exact test, the two groups included in this study were not in equilibrium. *P*-values were calculated for both groups. The results were *p* = 0.002 for the study group and *p* = 0.000 for the control.

The correlation between TLR-2 polymorphism and staging of pulmonary TB was found non-significant (*p* = 0.667). Six (85.7%) of patients with minimally advanced pulmonary TB had GA allele genotype, and 3 of them (75%) had AA allele genotype (Table 4).

The family history and the genotype were found positively correlated, and statistical significance was found at a 1% level of significance. However, there was no statistical significance between them at a 5% level of significance. This implies that family history is positively associated with the presence of Genotype among the control group (Table 5).

Discussion

Recently, MDR-TB has shown a high prevalence and a rapid rate of spreading resulting in a steep rise in the regional burden of TB infection and thus forming a threat to public health.

Genetic factors are considered important determinants in individual susceptibility for TB infection. Several research in literature have reported

Table 5. Relationship between family history of Pulmonary TB, autoimmune disease & the presence of genotype among patients with pulmonary TB.

	Family History	Autoimmune	Arg753Gln polymorphism
Family History			
Pearson Correlation	1	-0.079	-0.130
Sig. (2-tailed)		0.679	0.493
N	30	30	30
Autoimmune Diseases			
Pearson Correlation	-0.079	1	0.172
Sig. (2-tailed)	0.679		0.365
N	30	30	30
Arg753Gln polymorphism			
Pearson Correlation	-0.130	0.172	1
Sig. (2-tailed)	0.493	0.365	
N	30	30	30

the correlation between genes and risk for TB. These studies have also pointed out the important role that TLR-2 gene plays for the innate immunity in modulation of host susceptibility to TB. TLRs are important in immune responses because they contribute to detecting pathogens and thus aid in establishing an adaptive defensive response. Many studies investigated the presence of TLRs polymorphisms in different healthy and diseased persons [30]. Despite all these research efforts, no conclusive result was reached regarding TB susceptibility in people with different ethnic backgrounds. [31-34].

This study aims at investigating the relation between 753G/A TLR-2 (Arg753Gln) polymorphism and the susceptibility to TB. It was hypothesized that this gene polymorphism leads to an increased risk for pulmonary tuberculosis in infected individuals.

Several experimental studies have investigated the effects of TLR-2 Arg753Gln gene polymorphism on risk of TB. Yet, there is still a contradiction in the results of these research. Most studies have demonstrated an essential correlation between TB susceptibility and TLR-2 Arg753Gln gene polymorphism. Agus *et al.* [21] reported a relation between this polymorphism and pulmonary TB infection in Turkish population. Similar results were also reported in other study [35]. On the contrary, Ma *et al.* reported a lower risk for TB in association with the presence of TLR-2 Arg753Gln gene polymorphism [36]. Moreover, a study conducted by Xue *et al.* revealed that TLR-2 Arg753Gln gene polymorphism had no correlation with the risk of TB [37].

Interestingly, these are the consequences of gene polymorphism on TLR-2's action. Arg753Gln polymorphism's involvement seemed to cause defective agonist-induced tyrosine phosphorylation, TLR-6 hetero-dimerization, and Mal and MyD88 recruitment. There was a correlation between these deficits in proximal signaling and TLR-2's decreased capacity to mediate p38 phosphorylation, NF- κ B activation and induction of IL-8 mRNA in cells MTB infection, without changes in TLR-2 expression. These alterations in TLR-2 features mean that it is involved in TB susceptibility [38-41].

The current study compared pulmonary TB patients with healthy controls and revealed a significant difference in their TLR-2 polymorphism genotypes and allele distribution. Regarding allele frequency of TLR-2 Arg753Gln polymorphism, homozygous and heterozygous allele A accounted for 23.3% and 13.4% respectively in patients with TB. While in healthy controls, homozygous accounted for 10% and

heterozygous accounted for 5%. These findings suggest a higher susceptibility to develop TB in carriers of GA and AA genotypes of TLR2 Arg753Gln polymorphism compared to those who carry GG genotype. Similar findings were previously reported by Ogus *et al.* whose study revealed a significant difference between TB patients and healthy control [21]. In addition, a research by Wang *et al.*, revealed a significant difference between study and controls after comparing their homozygous variant genotypes [42]. Many other studies revealed a significant correlativity between TLR-2 Arg753Gln polymorphism and the higher risk to develop TB in allele genetic module, dominant and heterozygote genetic modules. AA, AG genotypes and A allele, in the heterozygote, dominant, and allelic model were linked to a higher risk for TB [43,44].

In previous study, TLR-2 polymorphism was found to compromise the macrophages' capability to respond to different types of bacterial polypeptides [22].

Kang and Chae [45] investigated another arginine to tryptophan residue replacement at 677, TLR-2 polymorphism in leprosy patients. This research revealed a close correlativity between polymorphism and lepromatous. However, the study was not specific to tuberculoid leprosy. These findings indicate that TLR-2 gene and its polymorphisms play a role in disease predisposition; perhaps by interrupting the pathway of the immune system's first-line defense mechanisms. Bochud *et al.*, conducted a study and reported that TLR-2 polymorphism led to severe impairments in the macrophagic response to *Mycobacterium tuberculosis* and *M. leprae* [46]. Another study by Ma *et al.*, investigated the exposure to airborne Mtb infection in TLR-2, TLR-4 and CD-14 knockout and control rodent classes. This study revealed that when TLR-2 knockout rodents were less tolerable to TB upon exposure to high doses. However, when both groups were subjected to conditions that resemble the natural low doses of airborne infection, no significant difference was found between both groups. Thus, these findings suggest that there may be an increased risk to develop progressive TB in case of defective TLR-2 gene [37].

These contradicting findings regarding the correlation between the polymorphisms and TB susceptibility may be contributed to the variation in sample size, methodology, studied population, age of participants, locations, and several environmental factors. Also, genetic factor may be an essential factor interfering with individuals' susceptibility to diseases.

However, there is lack of evidence supporting the hypothesis that these polymorphisms result in

compromised immune responses in general, and the response to Mycobacterium tuberculosis in particular. Despite the significant differences between patients and controls, TLR-2 polymorphism was observed in (36.6%) of individuals with pulmonary TB. So, it is considered an essential factor which affects these individuals' susceptibility to infection. This also indicates that there may be other impairments in different steps throughout the immune response. In other words, defects may also be present in other TLR-2 polymorphisms as well as in other TLRs resulting in a possible inability to stop the progression of TB infection. Literature has reported that individuals with dysfunction in interferon-gamma receptor, IL-12 or IL-12 receptors or those with signal transducer and transcription one mutation activator might hold a higher risk for getting infected with mycobacterium. [47–49] Some studies revealed a correlation between macrophage protein-1 SNPs and natural-resistance, while other studies reported a correlation between macrophage protein-1 SNPs and natural-resistance (solute carrier family 11, member 1) and vitamin D receptor genes that may be another contributing factor in susceptibility to mycobacterium TB [50,51].

A test of Hardy–Weinberg equilibrium was conducted for both groups to assess the deviation between them as this could be due to a true genetic correlation. Samples were taken from the same source group, and it was suggested that the SNP interaction A observed in TB can be caused by the direct functional effects of this polymorphism or may be caused by the interaction of the disequilibrium with another functional form.

In our study, there was non-significant correlation between 753G/A TLR2 (Arg753Gln) polymorphism (homozygous and heterozygous mutant) and the different grades of pulmonary TB characterized by the clinical severity of the infection. ($P=0.667$) six (85.7%) of patients with pulmonary TB grade 1 had GA allele genotype, and 3 (75%) had AA allele genotype. Agus *et al.* [21] noted the effect of heterozygosis on TB infection's severity. On the other hand, SALEH *et al.* in an Egyptian study reported no link between the polymorphism (heterozygous mutant) and the clinical severity of TB infection 753G / A TLR2 (Arg753Gln) (pulmonary and peritoneal).

Family history and Arg753Gln polymorphism of TLR2 were found positively correlated, and statistical significance is found at a 1% level of significance. However, there was no statistical significance between them at a 5 % level of significance. This implies that

family history is positively associated with the presence of Arg753Gln polymorphism among the patient group.

Our study limitations are small sample size, so more samples are needed for further studies. also, other gene polymorphisms should be investigated to research its effect on the increased risk for TB infection and the disease severity.

Conclusions

Polymorphisms of TLR-2 Arg753Gln may be a contributor to patients' susceptibility to developing pulmonary TB. For validating our findings and defining potential pathways for this interaction, more research, including broad sample size and ethnically diverse cohorts, are required.

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Authors Contributions

All authors contributed equally in conceptualization, planning, data collection and analysis, literatures acquisition, drafting and finalizing the article

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