Toll Like Receptor-4 Gene Polymorphism and Susceptibility to Pulmonary Tuberculosis

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Tuberculosis (TB) affects human life globally for a long time. The difference in clinical outcome of infection suggests that host genetic makeup is responsible for such variability. Toll like receptors (TLRs) are pattern recognition receptors and have a significant role in mycobacterial recognition by the innate immune system. TLR-4 is the key receptor in initiation of innate immunity against *M. tuberculosis.* This study investigated whether variants in TLR-4 896A/G (Asp299Gly) and TLR-4 1196C/T (Thr399Ile) genes are related with susceptibility or resistance to pulmonary tuberculosis (PTB) in Saudi population. Genotyping of TLR-4 896A/G, TLR-4 1196C/T gene was performed by polymerase chain reaction followed by restriction fragment length polymorphism (PCR –RFLP) in 60 PTB patients and 60 control subjects. The A allele at (896A/G) was more frequent in the control group while G allele was more common in PTB patients. The frequency of T allele of (1196C/T) polymorphism was significantly increased in PTB patients as compared to the control group (*P*<0.001; Odds ratio (OR) 2.79, 95% Confidence interval (CI) 1.65-4.72). A trend toward increased frequency of TT and CT genotypes of TLR4 at (1196C/T) were also observed in PTB patients as compared to control group (48.3% vs. 26, 7%, and 21.7% vs. 15%), respectively. This study suggests that that TLR4 polymorphism especially TLR4 rs4986791 may be associated with increase susceptibility to pulmonary tuberculosis, and C allele of rs4986791 is a promising protective factor for tuberculosis susceptibility in Saudi population.

Tuberculosis (TB) is a major global health problem, causing health problems among millions of people each year. Despite substantial progress toward the goal of its elimination, it is the second important cause of death from infectious disease globally [1]. In 2017, it was estimated that 10.0 million people developed TB disease worldwide: 5.8 million men, 3.2 million women and 1.0 million children. TB caused about 1.3 million deaths among HIV-negative people and supplementary 300,000 deaths from TB among HIV-positive people [2]. Among the Arab countries, Saudi Arabia is the third-largest country in the Middle East by land area with a moderate annual burden of tuberculosis. Saudi Arabia reported an annual TB incidence rate of 18/100,000 population [3]. Despite the government's extensive efforts, TB is among some communicable diseases that have not been carried under control Saudi Arabia faces the danger from increasing extents of extrapulmonary TB, non-tuberculous mycobacterial infections, and drug resistance [3].

The TB infection outcome depends on several factors, including age, sex, society, and the host genetic factors are assumed to play an important role in tuberculosis pathogenesis [4]. Innate and acquired immune responses are essential for host control of tuberculosis infection [5]. However, if the immunity becomes debilitated, active pulmonary or extra pulmonary tuberculosis developed [6]. So, the genetic variants of molecules involved in innate host-defense mechanisms are

associated with host susceptibility to tuberculosis [7].

Toll like receptor (TLR) play a significant role in stimulation of innate immunity against TB infection. These receptors are present on the cell surface or intracellular in cytoplasm or on endosomal membranes. TLRS recognize pathogen associated molecular patterns (PAMP) and trigger a host defense response. Polymorphisms in TLR genes are widely studied to investigate its association with tuberculosis susceptibility in different ethnicities, but results are conflicting [8]. Single nucleotide polymorphisms (SNPs) in TLR encoding genes might modify receptors, ligands interactions or regulate TLR expression and so the susceptibility of individual to infectious disease [9].

TLR4 was originally recognized as one of the mediators of lipopolysaccharide (LPS) inflammatory responses. It acts with both a heat-labile soluble mycobacterial factor and complete viable *M. tuberculosis* to activate innate responses. Two non-synonymous mutations have been described in the extracellular domain of TLR4 (Asp229Gly and Thr399Ile) and related to hypo-responsiveness to LPS in alveolar macrophages, epithelial cells, and peripheral blood mononuclear cells [10].

The aim of this work was to investigate whether variants in TLR-4 896A/G (Asp299Gly) and TLR-4 1196C/T (Thr399Ile) genes are associated with susceptibility or resistance to pulmonary tuberculosis (PTB) in Saudi population.

Subjects and Methods

This case control study was conducted on 60 adult patients with pulmonary tuberculosis attending inpatients and outpatient clinic of Internal Medicine Departments and Chest Department, Armed force hospital, Southern area, Kingdom of Saudi Arabia (KSA), during the period from November 2017 to July 2018. Sixty clinically and radiologically TB free subjects with age and sex-matched to patients were used as a control group. Members of the control group were previously vaccinated with BCG with a positive tuberculin test.

The study was reviewed and approved by the ethics committee of the Armed Forces Hospital of Southern Region, KSA (SRCMP18012017), date of decision 29-1-2017. A written consent from patients and controls was obtained before including in the study.

The patients and controls were subjected to full history taking (age, sex, family and past history of tuberculosis and history of intake of antituberculous drugs). Complete physical examination, plain chest X-ray: posterior-anterior (P.A) and lateral view if needed. Sputum smears for acid fast bacilli (Ziehl–Neelsen staining). Sputum culture on Lowenstein-Jensen (LJ) or Mycobacteria growth indicator tube (MGIT) media.

TB diagnosis was based on sputum positive microscopy, confirmed by positive culture. Patients recognized to be immune-deficient (e.g., HIV infection, diabetes, cancer, immunosuppressive therapy, renal failure or liver cell failure,) were excluded from the study. Control subjects were excluded if they had a history of prior anti-tuberculous therapy, signs or symptoms suggestive of active TB, and/or notable infiltrates on chest X-ray.

Sample collection

- Sputum samples

Morning sputum samples were collected for 3 consecutive days by asking the patient to cough deeply into a sterile screw cap cup. The specimens were refrigerated when they could not be processed immediately.

Sputum liquefaction, decontamination and concentration procedures were done by the N-acetyl L-cysteine sodium hydroxide (NALC-NaOH) method (Sigma-Aldrich) . Then sputum samples were subjected to Z.N stain and culture on LJ (Hardy Diagnostics ,CA , USA) or MGIT media.(Becton Dickinson, New Jersey, U.S.A)

- Blood sample

Two mL venous blood sample was collected under complete aseptic technique from each patient and control subject into vacutainer tube containing EDTA and stored at -80°C for further processing.

Genotyping of the TLR4 by Polymerase chain reaction–restriction fragment length polymorphism PCR -RFLP

- DNA extraction

DNA was extracted from the whole blood using Gene JET Whole Blood Genomic DNA Purification kit (Thermo Scientific, EU, Lithuania) according to the manufacturer's instructions. The extracted DNA concentration was measured by Nano drop Spectrophotometer 2000 (Thermo-Fisher Scientific, Wilmington, USA). Readings were taken at wave lengths 260 and 280 nm. The ratio of readings of optical density at wave lengths; 260 nm and 280 nm provide an estimate of purity of DNA. Pure preparations of DNA have OD260/OD280 of 1.7 - 2.0. Purified DNA samples were stored at -20°c till used in the amplification step.

- DNA amplification

Two PCR runs were performed independently one for each mutation.

The primer sequences, for each SNP are given in Table (1) according to Davoodi & Seow [11]. The PCR mixture contained 25 μl Maxima Hot Start PCR Master Mix (2X) (Thermo Scientific, EU Lithuania), 10 μl of tested DNA, 0.5μM of each primer (Biosearch Technologies, USA) and water, nuclease-free was added to a PCR mixture to give a final volume of 50 μl. Water, nuclease-free was used as a negative control in each PCR run. Rapid cycler PCR (G- Storm Thermal cycler, England) was used in running PCR cycles with the following conditions: an initial denaturation step at 95°C for 3min, 35 repeated cycles at 94°C for 30s, annealing at 52°C

(Asp299Gly) and 60°C (Thr399Ile), for 30s, and extension at 72°C for 30s, and a final extension step at72°C for 10 min. Ten μL of each amplified DNA & 50 bp ladder (molecular weight marker) (Fermentas, Germany) were separated by electrophoresis using 2% agarose gel containing 0.3 μg/ml of ethidium bromide. The bands were visualized using a UV transilluminator (254nm).

- Restriction endonuclease digestion

PCR products were subjected to restriction enzyme digestion, as indicated in table (1); according to according to Davoodi & Seow [11]. A 10 μL aliquot of the amplified product was digested by fast digest restriction enzyme (Thermo Scientific, EU Lithuania) using *Hinf*I and *Nco*I restriction enzymes according to the manufacturer's instructions. The tubes were incubated at 37°C for 5 min (*Hinf*I) and 10 min (*Nco*I). Then thermal inactivation was done at 65 ℃ for 20 min ((*Hinf*I) and 15min ((*Nco*I). The digested PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide.

Statistical Analysis

The data were tabulated, coded then analyzed using the computer program SPSS (Statistical package for social science) version 20. Continuous variables were expressed as mean and SD. Comparisons between the proportions of different genotypes and allele distributions in the studied groups were carried out using the Chi-squared test (χ2) and the Fisher's exact test as appropriate and the corresponding Odd ratio and 95% CI (OR; 95%CI) were calculated. A *P*-value for the calculated tests statistics was obtained. A *P*-value < 0.05 was considered statistically significant.

Table 1. Primers and restriction enzymes used for genotyping of TLRs-4

TLR4 polymorphisms	Sequences of the primers	Restriction Enzymes	Size of the Restriction fragments
TLR4 (896A/G)	F: 5' AGCATACTTAGACTACTACCTCCATG 3' R: 5' GAGAGATTTGAGTTTCAATGTGGG 3'	*Nco* I	Allele A: 188 bp(wild) Allele G: 168 bp + 20 bp (mutant)
TLR4 (1196C/T)	F: 5' GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA 3' R: 5' GGAAATCCAGATGTTCTAGTTGTTCTAAGCC 3'	*Hinf* I	Allele C: 124 bp(wild) Allele T: 98 bp + 26 bp (mutant)

Results

The results of this study revealed that the mean age of the patients was (41±6.6 years), they were 75% males and 25% females. The mean age of the control group was (39.6±9.7 years), they were 66.7% males and 33.3% females. No significant difference was detected between patients and control groups as regard to age and sex.

Allelic and genotypic frequencies of TLR4 Gene Polymorphisms at (896A/G) and (1196C/T) among controls and PTB Patients are shown in Table (2), (Figures: 1a, 1b, 2a, & 2b). For TLR 4 at (896A/G), G allele frequency was more common in PTB patients, but with no significance difference between A and G allelic frequency in controls and PTB patients (P=0.88). Regarding to genotype frequency, AA genotypes were the most common genotypes in controls and PTB patient . The 3 genotypes did not show any statistically significant differences (P1= 0.5, P2=0.7 and P3=0.42) between PTB patient and controls

For TLR4 at (1196C/T), the frequency of both C and T alleles showed significant difference between control and PTB patients

(OR=2.79, 95%CI 1.65-4.72 & P<0.001). PTB patients were more often carriers of T allele than control group (60% vs. 34.1%), respectively. Patients were more likely to have TT genotype CT genotype as compared to controls (48.3% vs. 26, 7%, and 21.7% vs. 15%), respectively. However, genotype TT was associated with increased risk of being a case of pulmonary tuberculosis. There was a significant difference in frequency between CC and CT genotypes (OR= 2.81, 95%CI=1.01-7.81, P=0.04) and CC and TT genotypes (OR=3.5, 95%CI=1.53-8.12, P=0.003). However there was no significant difference in frequency between CT and TT genotypes (OR=1.25, 95%CI=0.44-3.57, P=0.67) between the control and patient groups.

Table 2. Allelic and Genotypic Frequencies of TLR4 Gene Polymorphisms at (896A/G) and (1196C/T) among controls and PTB Patients.

Alleles and Genotypes		Control (N=60) N (%)	PTB (N=60) N (%)	OR	95% CI	*P value
TLR4 (896A/G) (rs4986790)	A allele	91 (75.8)	90 (75)	1.05	0.58-1.88	NS
	G allele	29 (24.2)	30 (25)			
	AA	40 (66.7)	41(68.3)	0.7	0.26-1.95	P1=0.5
	AG	11(18.3)	8 (13.3)	1.19	0.44-3.19	P2=0.7
	GG	9 (15)	11(18.3)	1.68	0.47-5.97	P3=0.42
TLR4(1196C/T) (rs496791)	C allele	79(65.9)	49 (40)	2.79	1.65-4.72	<0.001
	T allele	41(34.1)	71(60)			
	CC	35 (58.3)	18 (30)	2.81	1.01-7.81	0.04*
	CT	9 (15)	13 (21.7)	3.5	1.53-8.12	0.003+
	TT	16 (26.7)	29 (48.3)	1.25	0.44-3.57	NS€

Obtained by X2 test, P<0.05 is significant.

P1: between AA ad AG genotypes   *: between CC and CT genotypes

P2: between AA and GG genotypes   +: between CC and TT genotypes

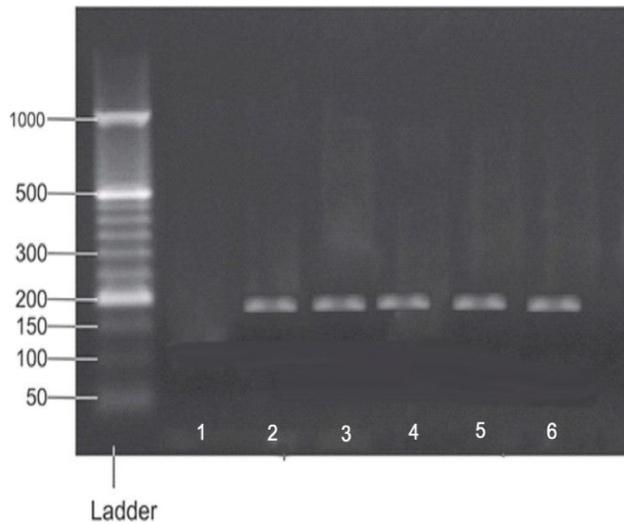P3: between AG and GG genotypes   € : between CT and TT genotypes

Figure 1a. Gel electrophoresis of the PCR products ofTLR4 (896A/G) polymorphisms. Lanes 1: negative control.
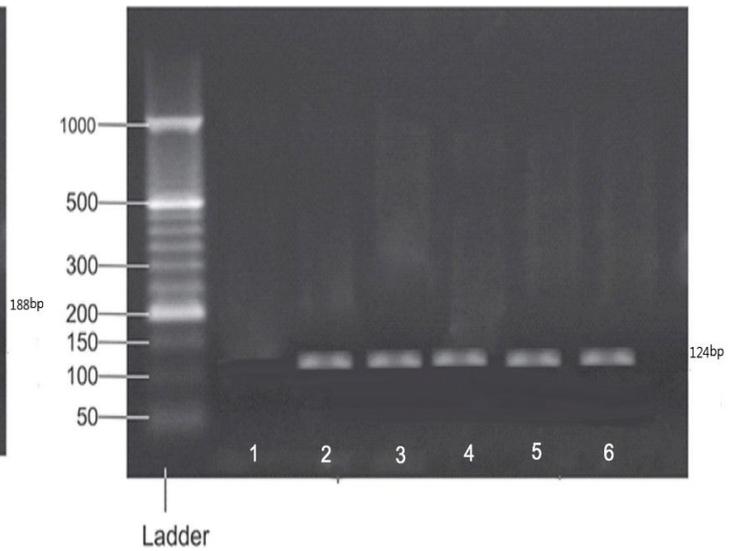
Figure 1b. Gel electrophoresis of the PCR products of TLR4 (1196C/T) polymorphisms. Lanes 1: negative control.
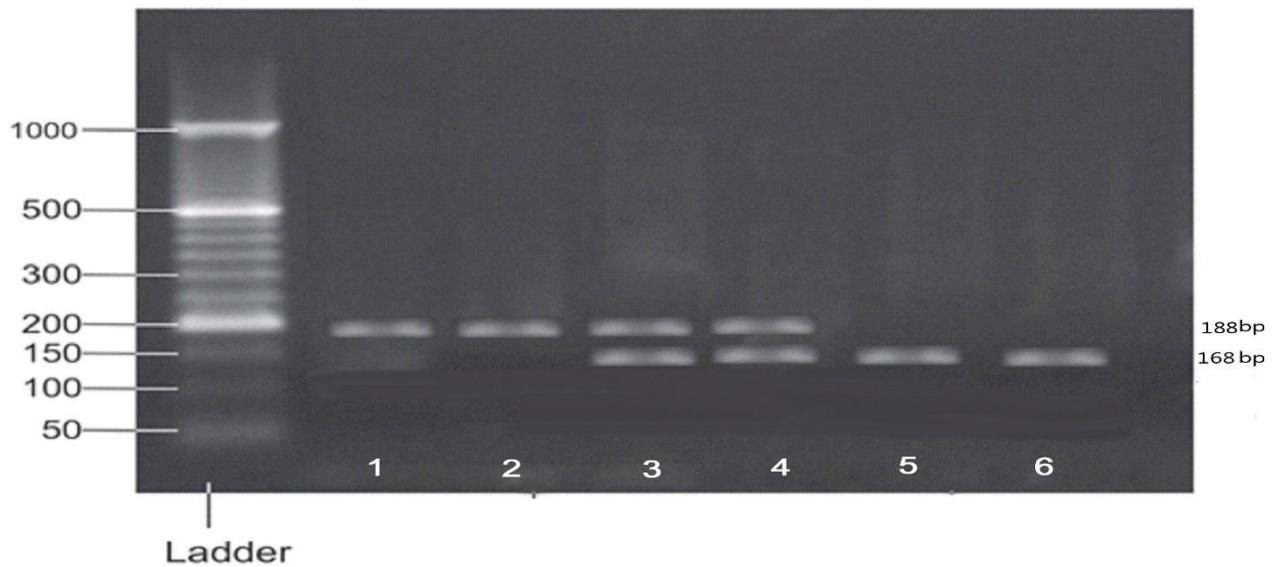

Figure 2a. Gel electrophoresis of the PCR products of TLR 4 (896A/G) polymorphisms after digestion by *Nco* I lanes 1&2: indicate AA (homozygous), Lanes 3 & 4: indicate AG (heterozygous) and Lanes: 5 & 6: indicate GG (homozygous).
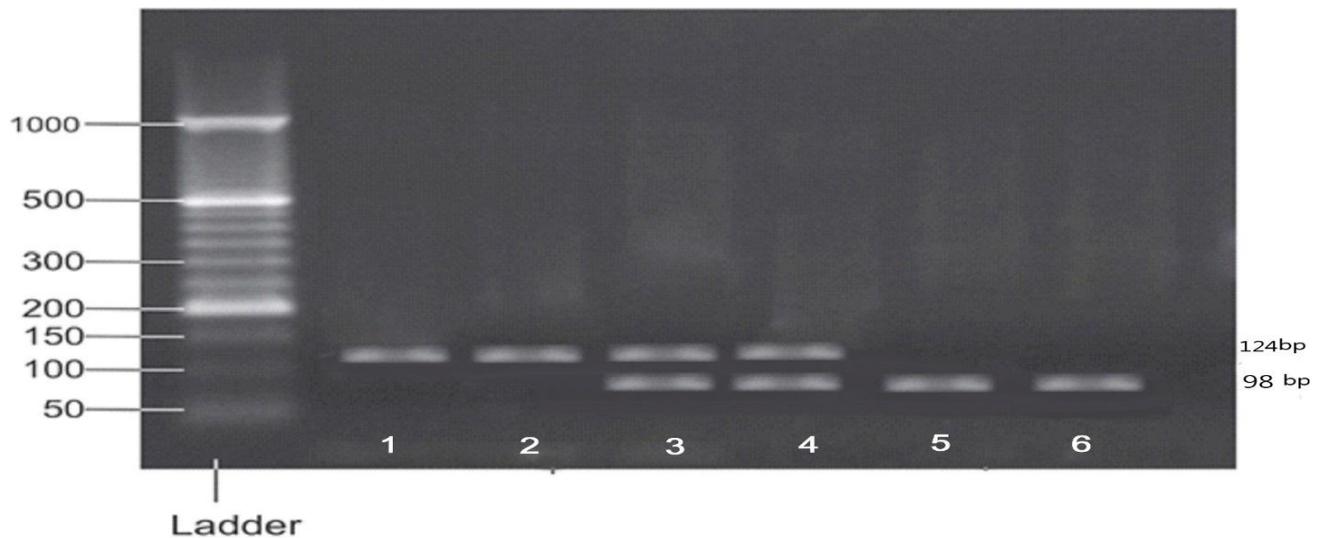
Figure 2b. Gel electrophoresis of the PCR products of TLR 4(1196C/T) polymorphisms after digestion by *Hinf*I lanes 1&2: indicate CC (homozygous), Lanes 3 & 4: indicate CT (heterozygous) and Lanes: 5 & 6: indicate TT (homozygous).

Discussion

Innate immunity acting a key role in host protection against *Mycobacterium tuberculosis* (MTB) infection. The first step is recognition of MTB by molecules such as TLRs of macrophages and other leukocytes. TLR4 binds to different components of MTB such as 3- and 4-acylated lipomannan, 60- and 65-heat shock proteins, and 50S ribosomal protein, resulting in the stimulation of the innate immune system and the production of specific cytokines to complete or eliminate the microbial infection [12]. So it is probable that the genetic variants of TLRs involved in innate host-defense mechanisms are connected with the resistance or susceptibility to tuberculosis

Our study aimed to evaluate whether the variation in TLR4 896A/G and TLR-4 1196C/T are associated with susceptibility or resistance to pulmonary tuberculosis in Saudi population. The present study revealed that regarding TLR 4 at (896A/G), there was

no significant difference between A and G allelic frequency and between 3 genotypes in controls and PTB patients. These results are in harmony with a study of associations between the polymorphisms in TLR2, TLR4 and toll interacting protein and susceptibility to latent tuberculosis infection (LTBI) or subsequent PTB in a Chinese Han population [13]. This study observed no significant association between polymorphisms of TLR2 and TLR4 with PTB or LTBI [13].

Our results are in agreement with those of a study carried out on the Chinese population, which reported that 28 of the 29 patients with tuberculosis had normal polymorphism in The TLR-4 nucleotide Asp299Gly; the lasting patient had a heterozygote-type change. Three control subjects had a heterozygote change, and one revealed a mutation and there was a higher ratio of TLR-4 polymorphisms in the patients with tuberculosis than in the controls, no statistical significant differences

were observed, this finding was explained by the limited number of patients with tuberculosis [14]. Also, Selvaraj *et al.* [15] reported that there was no association between mis-sense mutations in the extracellular domain of TLR-4 (896A/G) and PTB patients in South India.

Other works studied the role of SNPs in TLRs in predisposition to TB and/or bacillary load. One study [16] reported a minor allele frequency (MAF) of 896A/G (3.8% and 2.4%) and 1196C/T (2.7% and 0.2%) among patients and the control group, respectively. The same finding was reported by another case control study that investigated patients suffered from malaria in Amazonas [17]. Whereas other two studies detected higher MAF among Brazilian population ranging from 4.6% to 7.0% [18,19].

Several studies highlighted ethnicity as an important factor. In Moroccan patients there was significant interaction between TLR2 (rs3804099) and TLR4 (rs4986790) SNPs to protect against PTB (corrected p (PC) = 0.036), suggesting that TLR4 (rs4986790) SNP may act synergistically with TLR2 (rs3804099) SNPs [20]. Another two studies in China and Iran supported that TLR4 polymorphism modify latent TB infection and PTB susceptibility [21, 22]. Note that the TLR4 896A/G and1196C/T SNPs are co-segregated and the frequency of haplotype of the mutant alleles ranges from 0 to 5.0% among different peoples universal. But, this frequency is 10–20 times higher among African populations and it is suggested that may be a result of selective pressure due to malaria infection [23].

The current study revealed significant difference between the control and patient groups in the TLR4 (1198) C and T allele frequencies and in the frequency between CC and CT genotypes and CC and TT

genotypes. In contrast to our results, a study on south Indian population showed no relationship between TLR-4 (1196C/T) polymorphisms and tuberculosis infection [15], this was due to small sample size. Also, two smaller meta-analysis studies on TLR4 rs4986791SNP found no cross population association with TB, but subgroup analysis could not be performed as a limited number of studies were available [27-28]. Another study investigated the association between TLR polymorphism and infection progress in Chinese Han population, revealed a higher ratio of TLR-4 polymorphisms in the patients with tuberculosis than in controls. However, this did not reach statistical significance because of the limited number of patients with tuberculosis [14]. It is worth noting that such discrepancy in the result of genetic polymorphisms in TLR4 and resistance or susceptibility to tuberculosis might be due to a dynamic host-pathogen interplay between genetic and pathogen phenotypes [29].

A published study had shown that TLR4 rs4986791 was associated with both tuberculin skin test conversion and development of active TB disease. Thus, highlighting the significance of innate immunity, especially TLRs, in the pathogenesis of MTB infection in human and TB disease [24]. The TLR4 ectodomain shows an important role in recognition of pathogen-associated molecular patterns. Especially, TLR4 rs4986791 has been associated with hypo-responsiveness to ligand interaction due its location near the central ectodomain region. And this polymorphism leads to increase the severity pulmonary TB as measured by sputum bacillary loads and chest X-ray. In response to the interaction between MTB ligands and TLR4, stimulated myeloid cells produce interleukin 12 and other inflammatory

substances, which are essential to stimulate T helper 1 responses. So, TLR4 can be important to stimulate the protecting response of Th1 due to MTB infection and hypo-responsiveness may increase susceptibility to TB [16].

A meta-analysis study included nine studies on TLR4 rs4986791 SNPs reported that there was no worldwide association with TB susceptibility and TLR4 rs4986791, however, subgroup analysis (included 4 studies) reported that T allele, TC and TT genotypes were connected with increased susceptibility to TB in the allelic, heterozygous and dominant models [25]. In addition, the findings of a study in Asian populations by Najmi *et al* (26), agree with our finding that the TT and TC genotypes of TLR4 rs4986791 have been related with increased TB risk.

One limitation of this study is the small sample size of the study population. Thus further studies are needed on a larger scale to clarify the association of different TLRs polymorphism with MTB and their potential role in pathogenesis of TB diseases. , Findings of the suggested study may support in the policy of designing new vaccines to TB or in the combination of chemotherapy with immunotherapy.

In conclusion, TLR4 polymorphism, especially TLR4 rs4986791 may be a risk factor for increased susceptibility to PTB. The C allele of rs4986791 may be a promising protective factor against tuberculosis in Saudi population. These results are reliable with process of TB pathogenesis, in which TLRs are important for host immunity against MTB.

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