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**Title:** **TLR2 Arg677Trp Polymorphism increases Susceptibility to Tuberculosis in Homozygous Wild Type Individuals**

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# TLR2 Arg677Trp Polymorphism Increases Susceptibility to Tuberculosis in Homozygous Wild Type Individuals

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## ABSTRACT

Single nucleotide polymorphisms (SNPs) of Toll-like Receptors TLR2 and TLR4 have been associated with the susceptibility and severity of tuberculosis in different populations, worldwide. However, no such report is available about the Pakistani population. Genetic polymorphisms can be considered as markers for the likelihood of developing pulmonary tuberculosis. TLR family has a significant role in enhancing innate immunity which mediates the inflammatory reaction against a broad array of pathogens. TLRs are critical in determining immune response to mycobacteria by interacting with various bacterial molecular patterns. In this case-control study, genomic DNA from 100 patients and 100 controls were extracted. SNP genotyping of 6 SNPs, namely TLR2 NC_000004.12:g.153703800C>T, NP_001305716.1:p.Ser298Phe, NC_000004.12:g.153703946A>G, NP_001305716.1:p.Lys347Glu, NC_000004.12:g.153704936C>T NP_001305716.1:p.Arg677Trp, and NC_000004.12:g.153705165G>A, NP_001305716.1:p.Arg753Gln and TLR4, NC_000009.12:g.117713024A>G NP_612564.1:p.Asp299Gly, and NC_000009.12:g.117713324C>T, NP_612564.1:pThr399Ile were performed. with amplification refractory mutation system (ARMS) PCR. The results showed that out of the 6 above-mentioned SNPs, only NC_000004.12:g.153704936C>T (NP_001305716.1:p.Arg677Trp), C>C wild-type genotype has a statistically significant association with tuberculosis ($p < 0.05$). Although this genotype showed no significant association with bacillary load, which shows disease severity. Furthermore, no significant association was found between bacillary load and duration of symptoms. It was concluded that in the study population of newly diagnosed cases of TB, as well as the positive association of NC_000004.12:g.153704936C>T (NP_001305716.1:p.Arg677Trp) wild-

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type genotype with TB susceptibility, needs to be validated on a larger population.

**Keywords:** genotype, mycobacterium tuberculosis, single nucleotide polymorphism, tuberculosis, toll-like receptor

## 1. INTRODUCTION

Tuberculosis (TB) is a granulomatous disease caused by *Mycobacterium tuberculosis* (MTB). It creates a considerable global health burden, specifically in Southeast Asia [1]. However, from the estimated one third of the world's population infected with MTB, only 5-10% develop clinical tuberculosis, resulting in >8 million TB cases and 2 million deaths each year [2]. The occurrence of TB at different rates among particular races, ethnicities, and families indicates a genetic predisposition to TB susceptibility. MTB interacts with the environmental and host genetic factors in complex ways, which play a critical role in TB development [2].

During the last few decades, many studies have been carried out regarding the mechanisms and responses against MTB. The presence of pathogen is detected by the innate immune system which provides the first line of host defense by employing its several components, such as macrophages, T-lymphocytes, tumor necrosis factor-α (TNF-α), and interferon-γ [3]. Human toll-like receptors (TLR), expressed on macrophages as pattern recognition receptors (PRRs), play a crucial role in innate immunity by recognizing pathogen-associated molecular patterns (PAMP), which further direct adaptive immune responses [4]. TLRs are conserved mediators of innate immunity and essential for microbial recognition by macrophages [5]. They comprise a class of 11 transmembrane proteins with extracellular Leucine-rich repeat (LRR) domain involved in ligand recognition and an intracellular tail with toll interleukin 1 receptor (TIR) homology domain. Among the TLR family, TLR2 and TLR4 remain the principal mediators of macrophage activation against MTB. After MTB inhalation, TLR2 (in association with TLR1) activates an innate immune response by recognizing various bacterial lipoproteins and lipopeptides [6]. Whereas TLR4 (along with its coreceptor MD-2) binds to lipopolysaccharide (LPS), which is a part of the outer membrane of gram-negative bacteria [7]. This MTB molecular pattern recognition triggers an intracellular signaling cascade which involves the activation of NFκB. This results in the stimulation of pro-inflammatory

cytokines gene transcription [8]. Cytokines production, in turn, activates macrophages, leading to granuloma formation [9, 10].

There is substantial evidence that single nucleotide polymorphisms (SNPs) in TLR encoding genes modulate receptor/ligand interactions and consequently, the individual's susceptibility to TB [11]. Defect in TLR2 and TLR4 genes has been linked with the suppression of response by macrophages [12]. Many SNPs have been identified in exon 3 of TLR2 and TLR4 genes. Although, only a few of them have been linked to reduced immune response activation and an increased risk of infection [13]. The NC_000004.12:g.153704936 SNP in TLR2 remains biologically relevant because it alters the receptor's intracellular signaling domain, impairing NF-κB activation and cytokine release. This functional defect weakens pathogen recognition and has been linked to increased susceptibility to infectious and inflammatory diseases, including sepsis. Recent studies (2022-2023) confirmed that TLR2 polymorphisms, including NC_000004.12:g.153704936, can destabilize protein structure and compromise innate immune responses [13, 14]. In order to find the association of SNPs with TLR2 and TLR4 genes in the Pakistani population, a case control study on NC_000004.12:g.153704936 (rs121917864) and NC_000004.12:g.153705165 (rs5743708) substitution in intracellular TIR, reportedly associated with TB [15], NC_000004.12:g.153703800 (rs377645847) and NC_000004.12:g.153703946 (rs779986684*)* substitutions in the extracellular domain of TLR2, was executed. Whereas, for TLR4, NC_000009.12:g.117713024 (rs4986790) and NC_000009.12:g.117713324 (rs4986791) substitutions in the extracellular domain was examined in the same patients of pulmonary tuberculosis (PTB).

## 2. MATERIAL AND METHODS

This case control study was performed at the Molecular Biology Laboratory of Gulab Devi Educational Complex, Lahore. Blood samples from 100 smear positive patients of tuberculosis and 100 healthy subjects selected as control were collected. All subjects gave their informed consent prior to their inclusion in the study. The diagnosis of pulmonary tuberculosis was confirmed by sputum smear positivity. This system involves the reporting of the number of acid-fast bacilli seen at 1000x magnification. According to the number of acid-fast bacilli seen, the smears

were classified as 4+, 3+, 2+, or 1+. According to the laboratory grading of sputum smear, 1+ accounts for 1-9AFB/100 field, 2+ for 1-10AFB/50 fields, 3+ for 1-10/field, while 4+ accounts for AFB >9/field. Control samples were collected randomly from healthy subjects having no disease. The inclusion criteria involved both male and female controls and patients of age between 18 to 60 years. The screening of controls for HIV, HCV, and HBsAg was performed by immunochromatographic strip test. Patients with diabetes mellitus, HIV, HCV, HBsAg, extra pulmonary tuberculosis, and other secondary complications such as fibrosis, malignant diseases, or any disease other than pulmonary tuberculosis were excluded. Genomic DNA was extracted from the peripheral leukocytes according to the protocol, while amendments in the protocol were made according to laboratory requirements [16]. RBCs lysis was performed by adding STE (25% sucrose, 50mM EDTA, and 50mM Tris) to buffy coat layer containing white blood cells, followed by the addition of 10% SDS. The aqueous phase containing DNA was further processed by the addition of isoamyl alcohol. Isopropanol and sodium acetate were used for DNA precipitation. The DNA pallet was washed with cold 75% ethanol and resuspended in TE buffer (10mM Tris, 1mM EDTA). The tube containing the purified DNA was stored at -20°C for SNP amplification.

To detect TLR2 and TLR4 polymorphisms, the following set of primers (Table 1) were designed for ARMS-PCR using primer3 software (http://bioinfo.ut.ee/primer3-0.4.0) and optimized for similar melting temperatures. Two parallel reactions of each SNP were carried out, one for the wild-type allele using TLR-F, TLR-RW and one for mutant allele using TLR-F, TLR-RM. PCR was performed with 1µl of extracted genomic DNA in a 0.2ml tube. Amplification reaction involved 0.2µl of Taq DNA polymerase, 2.5µl of buffer (1X), 2.5µl of dNTPs (2.5 µM), 2.5µl of $MgCl_2$ (2.5mM), and 1µl of each primer. The reactions were performed in a final volume of 25µl under the following conditions: 5 minutes of initial denaturation at 95°C, followed by 30 cycles of denaturation for 45 seconds at 95°C, annealing for 45 seconds at 55°C, and extension for 1 minute at 72°C. The final extension was carried out for 10 minutes at 72°C. PCR amplified products were then subjected to electrophoresis in 2% agarose gel containing 0.5 µg/ml ethidium bromide. The amplified fragments were assessed by comparing them with the corresponding size marker.

**Table 1.** Primers Required for PCR

| Primer Name | Size (bp) | Product Size | Sequence |
|---|---|---|---|
| TLR2-F | 20 | 264 | TGTGCTCTGTTCCTGCTGAT |
| 677RW | 21 | 264 | CTTGCCAGGAATGAAGTTCCG |
| 677RM | 21 | 264 | CTTGCCAGGAATGAAGTGCCA |
| 753RW | 24 | 495 | AGGTCTTGGTGTTCATTATATTCC |
| 753RM | 23 | 495 | GGTCTTGGTGTTCATTATCGTCT |
| TLR2-F | 23 | 410 | TTTTGCTGGACTTACCTTCCTTG |
| 298RW | 24 | 410 | GATCTATAACTCTGTCATTGTCAG |
| 298RM | 25 | 410 | GGATCTATAACTCTGTCATTGTCAA |
| 347RW | 26 | 558 | AGTAAACAAGGAACCAGAAAAAGTTT |
| 347RM | 26 | 558 | AGTAAACAAGGAACCAGAAAAATTTC |
| TLR4-F | 20 | 165 | AGTCCATCGTTTGGTTCTGG |
| 299RW | 31 | 165 | TTTGTCAAACAATTAAATAAGTCAATAGTAT |
| 299RM | 29 | 165 | TGTCAAACAATTAAATAAGTCAATAGTAC |
| 399RW | 25 | 459 | CTCAGATCTAAATACTTTAGGATGG |
| 399RM | 25 | 459 | CTCAGATCTAAATACTTTAGGGTGA |

**Note.** (Forward F, Reverse R, Wild W and Mutant M) 677: NP_001305716.1:p.Arg677Trp, 753: NP_001305716.1:p.Arg753Gln, 298: NP_001305716.1:p.Ser298Phe, 347: NP_001305716.1:p.Lys347Glu, 299: NP_612564.1:p.Asp299Gly, 399: NP_612564.1:p. Thr399Ile.

### 2.1. Statistical Analysis

Data analysis for genotype and allele frequencies of TLR2 and TLR4 polymorphisms among healthy controls and newly diagnosed patients were carried out (with chi-square test or Fisher exact test using SPSS (version 21). Odd ratios at 95% confidence interval were calculated to evaluate the relative risk with a particular allele or genotype.

## 3. RESULTS

In this case control study, 100 subjects were included in each group. All of the subjects included in the study had the same ethnic origin. The control group consisted of 62 (62%) males and 38 (38%) females with a mean ± *SD* age of 34.7±10.8. Out of 100 patients, 60 (60%) were males and 40 (40%) were females with a mean± *SD* age of 31.4± 13.8. Only 33 patients were presented with a family history of tuberculosis. Among the 100 subjects, the number of smokers and non-smokers was 24 and 76 in patients and 14 and 86 in controls, respectively. The subjects included in case group were presented with symptoms having a minimum duration of 1 week and a

maximum duration of 144 weeks.

### 3.1. Association of TLR2 Polymorphisms with Tuberculosis in Control and Case Groups

The genotype distribution of TLR2 polymorphisms in healthy controls and PTB patients is shown below in Table 2. In NC_000004.12:g.153703800 polymorphism, allele frequencies for C and T were found to be 0.98 and 0.02 in control group and 0.985 and 0.015 in patient group, respectively. In NC_000004.12:g.153703946 polymorphism, allele frequencies for A and G were found to be 0.925 and 0.075 in control group and 0.97 and 0.03 in patient group, respectively. In NC_000004.12:g.153704936 polymorphism, allele frequencies for C and T were found to be 0.61 and 0.39 in control group and 0.97 and 0.03 in patient group, respectively. In NC_000004.12:g.153705165 polymorphism, allele frequencies for G and A were found to be 0.87 and 0.13 in control group and 0.915 and 0.085 in patient group, respectively. Among the four studied SNPs in TLR2 gene, none showed significant association ($p$>0.05) with tuberculosis, except NC_000004.12:g.153704936 polymorphism. The chi square analysis showed that the CC genotype has an odd ratio of 2.201 (CI 1.198-4.041) and a $p$-value of 0.03.

**Table 2.** Distribution of Toll-like Receptor 2 Polymorphisms in Control and Case Groups

| NC_000004.12:g.153703800 | | | | Allele Frequency | |
|---|---|---|---|---|---|
| | C>C | C>T | T>T | C (f) | T (f) |
| Controls | 96 | 4 | 0 | 196 (0.98) | 4 (0.02) |
| Cases | 97 | 3 | 0 | 197 (0.985) | 3 (0.015) |
| Total (%) | 193 (96.5) | 7 (3.5) | --- | | |
| Odd Ratio (CI 95%) | 1.347 (0294-6.180) | 0.742 (0.162-3.405) | ---- | | |
| $\chi^2$=0.148, $p$=0.7, $F$=0.5 | | | | | |
| NC_000004.12:g.153703946 | | | | Allele Frequency | |
| | A>A | A>G | G>G | A (f) | G (f) |
| Controls | 86 | 13 | 1 | 185 (0.925) | 15 (0.075) |
| Cases | 95 | 4 | 1 | 194 (0.97) | 6 (0.03) |
| Total (%) | 181 (90.5) | 17 (8.5) | 2 (1) | | |
| Odd Ratio (CI 95%) | 3.093 (1.069-8.945) | 0.279 (0.088-0.887) | 1.00 (0.062-16.212) | | |
| $\chi^2$=5.212, $p$=0.07 | | | | | |

| | NC_000004.12:g.153704936 | | | Allele Frequency | |
|---|---|---|---|---|---|
| | C>C | C>T | T>T | C (f) | T (f) |
| Controls | 24 | 74 | 2 | 122 (0.61) | 78 (0.39) |
| Cases | 41 | 57 | 2 | 139 (0.695) | 61 (0.305) |
| Total (%) | 65 (32.5) | 131 (65.5) | 4 (2) | | |
| Odd Ratio (CI 95%) | 2.201 (1.198-4.041) | 0.466 (0.256-0.846) | 1.00 (0.138-7.242) | | |
| $\chi^2$=6.652, $p$=0.03 | | | | | |
| | NC_000004.12:g.153705165 | | | Allele Frequency | |
| | G>G | G>A | A>A | G (f) | A (f) |
| Controls | 74 | 26 | 0 | 174 (87) | 26 (13) |
| Cases | 83 | 17 | 0 | 183 (91.5) | 17 (8.5) |
| Total (%) | 157 (78.5) | 43 (21.5) | --- | | |
| Odd Ratio (CI 95%) | 1.715 (0.863-3.410) | 0.583 (0.293-14.159) | --- | | |
| $\chi^2$=2.4, $p$=0.121, $F$=0.084 | | | | | |

### 3.2. Association of TLR4 Polymorphisms with Tuberculosis in Control and Case Groups

The genotype distribution of TLR4 polymorphisms (NC_000009.12:g.117713024 and NC_000009.12:g.117713324) in healthy controls and PTB patients is shown below in Table 3. Neither of the two SNPs, namely NC_000009.12:g.117713024 and NC_000009.12:g.117713324, showed a statistically significant association (p>0.05) with tuberculosis. In NC_000009.12:g.117713024 polymorphism, allele frequencies for A and G were found to be 0.61 and 0.39 in control group and 0.62 and 0.38 in patient group, respectively. In NC_000009.12:g.117713324 polymorphism, allele frequencies for C and T were found to be 0.785 and 0.215 in control group and 0.84 and 0.16 in patient group, respectively.

**Table 3.** Distribution of Toll-like Receptor 4 Polymorphisms in Control and Case Groups

| | NC_000009.12:g.117713024 | | | Allele Frequency | |
|---|---|---|---|---|---|
| | A>A | A>G | G>G | A (f) | G (f) |
| Controls | 24 | 74 | 2 | 122 (0.61) | 78 (0.39) |
| Cases | 26 | 72 | 2 | 124 (0.62) | 76 (0.38) |
| Total (%) | 50 (25) | 146 (73) | 4 (2) | | |
| Odd Ratio (CI 95%) | 1.128 (0.594-2.14) | 0.891 (0.477-1.665) | 1.010 (0.139-7.317) | | |
| $\chi^2$=0.107, $p$=0.9 | | | | | |

| | NC_000009.12:g.117713324 | | | Allele Frequency | |
|---|---|---|---|---|---|
| | C>C | C>T | T>T | C (f) | T (f) |
| Controls | 61 | 35 | 4 | 157 (0.785) | 43 (0.215) |
| Cases | 71 | 26 | 3 | 168 (0.84) | 32 (0.16) |
| Total (%) | 132 (66) | 61 (30.5) | 7 (3.5) | | |
| Odd Ratio (CI 95%) | 1.565 (0.868-2.824) | 0.653 (0.356-1.97) | 0.742 (0.162-3.405) | | |
| $\chi^2$=2.228, $p$=0.3 | | | | | |

### 3.3. Association of TLR2 and TLR4 Polymorphisms with Bacillary Load and Symptoms Duration in PTB Patients

Further analysis was performed only in pulmonary TB patients by categorizing them into three classes (1+, 2+ and 3+) on the basis of sputum bacillary load and four classes on the basis of symptoms duration (0-2, 2-4, 4-6, and above 6 months). The genotypic distribution of TLR2 and TLR4 polymorphisms was observed among patients with different bacillary loads (Table 4). Neither TLR2 nor TLR4 showed a statistically significant association with disease severity. ANOVA test was performed to establish the cumulative relationship of patients falling in the four different categories of symptoms duration with sputum smear bacillary load (1+, 2+, 3). The results showed a statistically non-significant association ($F$=0.86, $p$=0.424) (Table 4). NC_000004.12:g.153703946-AA genotype was the most prevalent genotype observed in patients among all the categories of symptoms duration (Table 4). Tables 5 and 6 show the association of TLR2 and TLR4 polymorphisms with symptoms duration using the chi-square test. None of the studied polymorphisms showed a statistically significant association.

**Table 4.** Distribution of Different Grades of Bacillary Load and Symptoms Duration in Patient Group

| Duration of Symptoms | Sputum Smear Bacillary Load | | | Most Prevalent Genotype |
|---|---|---|---|---|
| | 1+ | 2+ | 3+ | |
| 0-2 Months | 33 | 21 | 2 | NC_000004.12:g.153703946_AA |
| 2-4 Months | 12 | 8 | 1 | NC_000004.12:g.153703946_AA |
| 4-6 Months | 4 | 5 | 2 | NC_000004.12:g.153703946_AA, NC_000004.12:g.153705165_GG, NC_000009.12:g.117713024_AG |
| Above Months | 8 | 3 | 1 | NC_000004.12:g.153703946_AA |
| Total | 57 | 37 | 6 | 100 |

| Duration of Symptoms | Sputum Smear Bacillary Load | | | Most Prevalent Genotype |
|---|---|---|---|---|
| | 1+ | 2+ | 3+ | |
| Mean± S | 15.8± 22.0 | 11.0± 9.5 | 17.0± 10.0 | 14.1±17.8 |
| F= 0.867, Sig= 0.424 | | | | |

**Table 5.** Association between Symptoms Duration and TLR2 Polymorphisms

| Duration of Symptoms | NC_000004.12:g.153703800 Polymorphism | | |
|---|---|---|---|
| | C>C | C>T | T>T |
| 0-2 Months | 55 | 1 | --- |
| 2-4 Months | 20 | 1 | --- |
| 4-6 Months | 11 | 0 | --- |
| Above 6 Months | 11 | 1 | --- |
| Total | 97 | 3 | --- |
| $\chi^2$=2.021, *p*=0.56 | | | |
| Duration of Symptoms | NC_000004.12:g.153703946 Polymorphism | | |
| | A>A | A>G | G>G |
| 0-2 Months | 53 | 2 | 1 |
| 2-4 Months | 21 | 0 | 0 |
| 4-6 Months | 9 | 2 | 0 |
| Above 6 Months | 12 | 0 | 0 |
| Total | 95 | 4 | 1 |
| $\chi^2$=7.95, *p*=0.24 | | | |
| Duration of Symptoms | NC_000004.12:g.153704936 Polymorphism | | |
| | C>C | C>T | T>T |
| 0-2 Months | 27 | 28 | 1 |
| 2-4 Months | 8 | 12 | 1 |
| 4-6 Months | 3 | 8 | 0 |
| Above 6 Months | 3 | 9 | 0 |
| Total | 41 | 57 | 2 |
| $\chi^2$=4.924, *p*=0.55 | | | |
| Duration of Symptoms | NC_000004.12:g.153705165 Polymorphism | | |
| | G>G | G>A | A>A |
| 0-2 Months | 49 | 7 | --- |
| 2-4 Months | 16 | 5 | --- |
| 4-6 Months | 9 | 2 | --- |
| Above 6 Months | 9 | 3 | --- |
| Total | 83 | 17 | --- |
| $\chi^2$=2.049, *p*=0.56 | | | |

**Table 6.** Association between Symptoms Duration and TLR4 Polymorphisms

| Duration of Symptoms | NC_000009.12:g.117713024 Polymorphism | | |
|---|---|---|---|
| | A>A | A>G | G>G |
| 0-2 Months | 15 | 40 | 1 |
| 2-4 Months | 7 | 14 | 0 |
| 4-6 Months | 2 | 9 | 0 |
| Above 6 Months | 2 | 9 | 1 |
| Total | 26 | 72 | 2 |
| $\chi^2$=4.416, *p*=0.62 | | | |
| Duration of Symptoms | NC_000009.12:g.117713324 Polymorphism | | |
| | C>C | C>T | T>T |
| 0-2 Months | 40 | 15 | 1 |
| 2-4 Months | 15 | 6 | 0 |
| 4-6 Months | 7 | 4 | 0 |
| Above 6 Months | 9 | 1 | 2 |
| Total | 71 | 26 | 3 |
| $\chi^2$=10.78, *p*=0.095 | | | |

### 3.4. Haplotype Analysis

Linkage disequilibrium (LD) between TLR-2 NC_000004.12:g.153703800, TLR-2 NC_000004.12:g.153703946, TLR-2 NC_000004.12:g.153704936, and TLR-2 NC_000004.12:g.153705165 and TLR-4 NC_000009.12:g.117713024 ,TLR-4 NC_000009.12:g.117713324 was calculated using SNPstats online tool (Table 7). In case of TLR2 polymorphisms, moderate LD was observed between NC_000004.12:g.153703800 and NC_000004.12:g.153704936 (D'=0.54) and NC_000004.12:g.153704936 and NC_000004.12:g.153705165 (D'=0.63). While the remaining possible combinations of TLR 2 polymorphisms NC_000004.12:g.153703800 and NC_000004.12:g.153703946 (D'=0.2), NC_000004.12:g.153703800, NC_000004.12:g.153705165 (D'=0.2), NC_000004.12:g.153703946, NC_000004.12:g.153704936 (D'=0.3), NC_000004.12:g.153703946 and NC_000004.12:g.153705165 (D'=0.2) showed no LD. Similarly, no LD was observed between two TLR4 polymorphisms (D'=0.16). The haplotype frequencies of two loci (TLR2 and TLR4) were no different from one another in healthy subjects and patients. Haplotype C-A-C-G of TLR2 and

haplotype C-A of TLR4 had the highest frequency in both controls and patients. No significant difference was observed in any of the haplotype combinations between the two groups. None of the haplotype combinations were found to be significantly associated with tuberculosis.

**Table 7.** Haplotype Frequency Distribution of TLR2 and TLR4 Polymorphisms in Patients and Controls

| TLR Haplotypes | Controls | Patients | *p*-value | Odd Ratio (95% CI) |
|---|---|---|---|---|
| TLR-2 (893C/T) –TLR-2 (1039A/G)-TLR-2 (2029C/T) –TLR-2 (2258G/A) | | | | |
| C-A-C-G | 0.5498 | 0.6462 | --- | 1.00 |
| C-A-T-G | 0.2691 | 0.2319 | 0.065 | 1.84 (0.97-3.51) |
| C-A-T-A | 0.0713 | 0.0506 | 0.13 | 2.28(0.79-6.55) |
| C-A-C-A | 0.0189 | 0.031 | 0.89 | 0.88(0.15-5.17) |
| C-G-C-G | 0.0354 | 0.0177 | 0.32 | 1.93(0.53-7.02) |
| C-G-T-A | 0.0258 | 0.0034 | 0.11 | 12.33(0.56-271.70) |
| Rare | | | 0.26 | 2.43(0.52-11.38) |
| Global haplotype association p-value: 0.11 | | | | |
| TLR-4 (896C/T) –TLR-4 (1196A/G) | | | | |
| C-A | 0.4979 | 0.5391 | --- | 1.00 |
| C-G | 0.2871 | 0.3009 | 0.79 | 1.10(0.53-2.29) |
| T-A | 0.1121 | 0.0809 | 0.43 | 1.59(0.50-5.06) |
| T-G | 0.1029 | 0.0791 | 0.52 | 1.41(0.49-4.09) |
| Global haplotype association p-value: 0.56 | | | | |

## 4. DISCUSSION

The expression pattern of TLRs in different types of cells, such as macrophages, dendritic cells, and epithelial cells, is an important regulatory mechanism of innate immune response against pathogens, particularly MTB. TLR-ligand complex initiates host immune response to combat the MTB infection through the production of cytokines, leading to granuloma formation [17]. Genetic mutations are the most common source of error which results in human phenotype variations. These variations may predispose to disease progression. Single nucleotide polymorphism (SNP) involves the change of a single nucleotide at a specific position. This change may code for the same or different amino acids, resulting in synonymous and non-synonymous mutation respectively, which may or may not alter the function of a protein. Genetic variations can be accountable in inhibiting the potential role of TLRs in fighting against tuberculosis by altering its

signaling pathway [18].

Despite the wide range of therapies available for disease management, different subgroups of patients may fail to find the cure for a particular disease. This is because of the different molecular mechanisms which form the basis of inter individual response to therapy. Advances in personalized genetic medicine attempt to overcome these differences by dissecting this molecular mechanism (at the single nucleotide level) affecting the clinical and epidemiological outcomes as evident from studies involving polymorphism. Patient care has been potentially revolutionized through personalized medicine. The genetic profile of the patient aids the physician to provide personalized medicine for disease prognosis [19].

In the current study, the influence of TLR2 and TLR4 polymorphisms affecting the intra cellular and extra cellular domains of human Toll-Like Receptors and their susceptibility to pulmonary tuberculosis in the Pakistani population was investigated. As the population was randomly distributed, no significant difference was observed between healthy subjects and patients regarding TLR2 and TLR4 polymorphisms genotype and allele distribution. In terms of TLR 2 polymorphism, only wild-type genotype C/C of NC_000004.12:g.153704936 (NP_001305716.1:p.Arg677Trp) polymorphism showed a statistically significant association ($p<0.05$) with the disease. The wild-type genotype C/C of NC_000004.12:g.153704936 (NP_001305716.1:p.Arg677Trp) polymorphism performed a protective role in controls with an odd ratio of 2.2, which reflects that controls have two times less risk of developing tuberculosis. To date, such an association between homozygous wild-type genotype and disease phenotype has yet to be reported for tuberculosis. Therefore, the detection of this polymorphism provides a strong basis to assess the risk profiles regarding susceptibility to tuberculosis.

Similarly, Schröder et al. observed TLR2 NC_000004.12:g.153704936 (NP_001305716.1:p.Arg677Trp) polymorphism as a predisposing risk factor in developing tuberculosis [20]. However, Xue et al. and Schröder et al. failed to identify any individual carrying NC_000004.12:g.153704936 (NP_001305716.1:p.Arg677Trp) polymorphism in south eastern Chinese population [15] and in Germans [20], respectively. Ben-Ali et al. also reported the association of this respective polymorphism with developing tuberculosis [20].

Two novel SNPs (NC_000004.12:g.153703800 (NP_001305716.1:p.Ser298Phe) and NC_000004.12:g.153703946 (NP_001305716.1:p.Lys347Glu)) in TLR2 gene were also analyzed in the current study. The investigation of these SNPs showed them to be unlikely candidates for any significant change in the structure and function of proteins. In contrast to these findings, Ogus *et al* reported the association of NC_000004.12:g.153705165 (NP_001305716.1:p.Arg753Gln) polymorphism with tuberculosis in Turkish population [21]. Similarly, Xue et al. showed NC_000004.12:g.153705165 (NP_001305716.1:p.Arg753Gln) polymorphism in patients at a very low frequency (15). Whereas, Biswas et al. suggested that TLR2 NC_000004.12:g.153705165 (NP_001305716.1:p.Arg753Gln) polymorphism is not responsible for the increased prevalence of tuberculosis in Indian population [22].

Najmi *et al* and Schröder *et al* reported the importance of TLR4 polymorphisms (NC_000009.12:g.117713024 (NP_612564.1:p.Asp299Gly) and NC_000009.12:g.117713324 (NP_612564.1:p.Thr399Ile)) in developing tuberculosis [23, 24]. However, in the current study, these two polymorphisms showed no significant association with tuberculosis in Pakistani population. Likewise, no association was found for NC_000009.12:g.117713024 (NP_612564.1:p.Asp299Gly) with tuberculosis in Mexican [25] and Gambian populations [25, 26]. In contrast, Singh *et al* reported a significant association of TLR4 polymorphisms (NC_000009.12:g.117713024 (NP_612564.1:p.Asp299Gly) and NC_000009.12:g.117713324 (NP_612564.1:p.Thr399Ile)) in Indian population [27].

Linkage Disequilibrium (LD) is characterized on a continuous stretch of genomic sequence within a population and describes the extent of inheritance or correlation of an allele of one SNP with an allele of another SNP. $D'$ is a measure of population genetics and is linked to the recombination events occurring between two markers in genome. Its value ranges between 0 and 1. A $D'$ value of 1 specifies complete LD. This represents the absence of any recombinational event between two SNPs. Whereas, a $D'$ value of 0 specifies frequent recombination between two SNPs within a population. The current study showed no association of any haplotype combination with pulmonary tuberculosis, as LD observed in two loci haplotype frequencies of TLR2 and TLR4 polymorphisms remained

relatively weaker than that reported among Indian (D'=0.8) [28] and South Indian (D'= 0.64) populations [29].

Similarly, no direct evidence of a relationship was observed between bacillary load and TLR 2 and 4 polymorphisms and duration of symptoms in patients (Figure 1 and 2). This suggests that the severity of the disease in patients is independent of the grades of bacillary load. Although most of the patients presented with a longer duration of symptoms had a low bacillary load (1+) because of the non-specific treatment they had been receiving from some general physician. On the other hand, the patients who recently developed disease symptoms had a high bacillary load (2+, 3+). Hence, the non-specific treatments tend to reduce the virulence of the mycobacterium.

## 4.1. Conclusion

This study highlights the importance of genetic screening and testing for infectious diseases, as it may help to find novel genetic markers for early diagnosis or monitor the treatment response. In the current study, out of the 6 SNPs only one SNP of the TLR2 gene, that is, NP_001305716.1:p.Arg677Trp was found to be significantly associated in the homozygous wild-type form. Moreover, no association was found between the bacillary load and duration of the disease. The findings suggest that this study needs to be conducted on a much larger scale to establish the association of NP_001305716.1:p.Arg677Trp with tuberculosis in the general Pakistani population.

**Author Contribution**

**Nireeta Yousaf:** data curation, methodology. **Saffa Maqsood:** formal analysis. **Shandana Khan:** writing - original draft. **Mamoona Zaheer:** methodology. **Muhammad Umair Hanif:** conceptualization supervision, writing- review & editing.

**Conflict of Interest**

The authors of the manuscript have no financial or non-financial conflict of interest in the subject matter or materials discussed in this manuscript.

**Data Availability Statement**

The data associated with this study is not available due to ethical, legal, or commercial restrictions.

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