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ASSOCIATION OF SNPS IN THE TLR4 GENE (RS4986790, RS4986791) WITH SUSCEPTIBILITY TO ACTIVE TB IN IRAQI POPULATION

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ABSTRACT

The main objective of this work was to assess the potential relation of (rs4986790 as well as rs4986791) SNPs, located at the TLR4 gene, and the susceptibility to developing active tuberculosis (TB). Approximately 78.9% of the samples demonstrated resistance to rifampicin. Testing of the TLR4 rs4986790 variant did not showed any significant differences in genotype frequencies between patients diagnosed with tuberculosis (TB) and the control group. The study revealed notable disparities in the genotypic and allelic frequencies observed between the groups in relation to TLR4 rs4986791. Individuals harboring the TT genotype showed an increased susceptibility to tuberculosis (TB), whereas those carrying the CC or CT genotypes revealed a decreased susceptibility. The results suggest that the TLR4 rs4986790 mutation may not exert a significant influence on the vulnerability to tuberculosis (TB) within this specific community. In spite of that, it had been suggested that the rs4986791 variant may apply a substantial impact on the susceptibility to tuberculosis.

INTRODUCTION

Mycobacterium tuberculosis (Mtb) is the bacterium known for its high pathogenicity and is known to be the main causative factor of tuberculosis, a severe disease infections that contributes to a substantial number of worldwide deaths. The primary mode of transferring for *Mycobacterium tuberculosis* is through the dissemination of respiratory droplets during coughing and sneezing. The main symptoms reported in persons suffering with tuberculosis include heightened appetite, elevated body temperature, reduced stamina, excessive fatigue, profuse night sweats, chronic cough, and weight loss (2). Globally, the annual number of deaths due to communicable diseases exceeds one million, making it the second leading cause of the death worldwide after HIV (7). Based on statistical data from Iraq, the incidence of tuberculosis (TB) in 2012 was reported to be 27 cases per 100,000 individuals annually, accompanied by a mortality rate of 11 cases per 100,000 individuals annually (15). This disease can be categorized into two distinct classifications: latent tuberculosis, which are characterized by the absence of disease progression, lack of

manifestation, and standard chest x-ray results; and active illness, that take place when the tubercle bacilli overpower the immunity and undergo multiplication. Latent infections represent the prevailing form of tuberculosis. Iraq has identified tuberculosis (TB) as a public health concern, primarily because it is among the seven nations in the region with a significant prevalence of this illness (16). The development of a tuberculosis infection is determined by many factors, including age, gender, societal circumstances, and the important contribution of host genetic factors to the development of tuberculosis (14). The efficient therapy of tuberculosis infection necessitates the existence of both innate and acquired immune responses. However, if the immune system gets weakened, the individual may experience the onset of active pulmonary or extra-pulmonary tuberculosis. The sensitivity of a host to tuberculosis has been found to be associated with genetic differences in molecules involved in innate host-defense systems (13).

Toll-like receptors (TLRs) is identified to be significant contributors to the initiation of innate immune responses following tuberculosis infection. The receptors are localized either at the cellular membrane or inside the cytoplasm, or otherwise, at the endosome membranes. Toll-like receptors (TLRs) have the ability to identify pathogen-associated molecular patterns (PAMPs), then triggering a protective response in the host organism. The existence of SNPs in genes in charge of production Toll-like receptors (TLRs) may have the power for modifying interactions between receptors and ligands or affect the expression of TLRs. As a result, this could impact the susceptibility of populations to infectious diseases (9).

Two distinct mutations Thr399Ile and Asp229Gly found in the TLR4 extracellular domain. These mutations had been associated with a reduced response to LPS in many cell types, including epithelial cells, alveolar macrophages and peripheral blood mononuclear cells (8).

Given the aforementioned discoveries, the primary objective of this work was to explore the possible correlation between (rs4986791 and rs4986790) SNPs in the TLR4 gene as well as the vulnerability for active tuberculosis (TB) among individuals in the Iraqi population residing in Al-Diwaniyah Governorate. Through this approach, our intention was to offer additional understanding regarding the intricate interaction between genetic and environmental elements that contribute to the susceptibility of tuberculosis in this distinct setting.

METHODOLOGY

Sputum samples collection

A total of 800 participants, who were suspected tuberculosis patients at the Chest and Respiratory Disease Specialized Centre in Al-Diwaniyah Governorate, Iraq, provided 10 ml of sputum each in the early morning. The previous mentioned center is a reference laboratory that holds national accreditation and provides a range of services, one of which is diagnostic testing utilizing the Xpert® (MTB/RIF assay). The Xpert-MTB/RIF assay (Cepheid GeneXpert® System, USA) was utilized to conduct the assay.

GeneXpert

Samples were subjected to Xpert MTB/RIF testing in accordance with the recommendations presumed by the factory. The Xpert assay sample reagent, composed of NaOH and isopropanol, was introduced to the tubes in a 1:3 proportion in order to eradicate the mycobacteria and induce liquefaction of the sample. The combination was subjected to intense agitation and afterwards left sit for a duration of 15 minutes, after which it was agitated once more and left sit for an additional 5 minutes. Afterwards, a volume of 2 mL was carefully transferred into the Xpert assay cartridge and then introduced into the GeneXpert apparatus to facilitate the process of polymerase chain reaction (PCR) testing. The measurement and analysis procedures were performed in an automated manner and then reported using the GeneXpert Dx software.

Blood samples collection and DNA extraction

Aseptic approach was employed to obtain a 2 mL venous blood sample from each patient and control individual. The blood was collected into a vacutainer tube containing EDTA and then stored at a temperature of -80°C for subsequent processing. The genomic DNA was extracted from blood samples in the laboratory using the ReliaPrepTM Blood gDNA Miniprep System kit (Promega/ USA).

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Table 1: Restriction enzymes and primer utilized for genotyping of TLRs-4.

Tlr4 Snps	Primers Sequences	Restriction Enzymes
TLR4 (896 A / G)	Forward:5'-AGCATACTTAGACTACTACCTCCATG-3'	*NcoI*
	Reverse:5'-GAGAGATTTGAGTTTCAATGTGGG-3'	
TLR4 (1196 C / T)	Forward:5'-GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA-3'	*HinfI*
	Reverse:5'-GGAAATCCAGATGTTCTAGTTGTTCTAAGCC-3'	

RESULTS AND DISCUSSION

A comprehensive analysis was conducted on a sample size of 800 specimens using the GeneXpert MTB/RIF diagnostic instrument to detect the presence of Mycobacterium tuberculosis. Out of the total number of samples, 76 samples (9.5%) were found to be positive for the presence of the bacteria. Moreover, of the positive samples, 60 individuals (accounting for 78.9% of the positive cases) demonstrated resistance to rifampicin (RIF), which is a crucial first-line medication for TB

treatment. The observed prevalence rate is consistent with the anticipated frequency in the area, although it is noteworthy due to the significant percentage (78.9%) of cases exhibiting resistance to rifampicin among the samples that tested positive. The observed prevalence of rifampicin resistance is a matter of worry, as it may suggest the presence of wider challenges associated with drug resistance in the treatment of TB in the given geographical area. When examining these results in relation to other research, it is noteworthy that Al-Mussawi, (4) observed a somewhat lower prevalence of rifampicin resistance within a comparable population. Conversely, Selfegna and Alelign, (11) documented a greater incidence of rifampicin resistance in a distinct geographical region. The observed inconsistencies across these studies might potentially be ascribed to variances in the used sample methodologies, disparities in treatment approaches across different regions, or inherent genetic variables that influence the development of medication resistance.

A case-control population research design did employ to investigate the potential linkage of SNPs in genes encoding TLR-4 and the heightened vulnerability to developing active TB in the Iraqi people residing in Al-Diwaniyah Governorate. Table 2 displays the demographic features of both tuberculosis (TB) cases and controls. There were no statistically significant differences seen between cases and controls in terms of age, gender, smoking status, and residency in a rural area ($p > 0.05$). Age and gender as a non-significant determinant for TB susceptibility in our study aligns with findings from Zhang et al., (17) who also did not observe association between age and sex with TB risk. However, different study in Iraq, Baghdad Governorate by AL-Kaisse et al., (3) found age to be associated with increased susceptibility, but gender was not. Our observation that age, gender and smoking did not have a significant association with TB deal with study in Duhok Province conducted by Abdulkareem et al., (1), who also reported that, there is no association between age, gender, smoking, alcohol consumption, diabetes mellitus and immunosuppressive therapy with tuberculosis. Finally, regarding living in rural areas, our results show there is no significant association with TB, our results were consistent with the results of Ortega et al., (10), who also found no significant association between living in rural areas with TB as well as there is no significant association with age, smoking except for gender they reported there was a significant association with gender.

Table 2: Demographic features of cases and controls

	Control n = 76 (%)	(TB) cases n =76 (%)	OR	95% CI	P-value
Age					
Mean ± SD	37.6 ± 9.8	40.2 ± 10.2	-	-	0.11
Gender					
Male	54 (71.1%)	51 (67.1%)	1.2	0.6 – 2.39	0.59
Female	22 (28.9%)	25 (32.9%)			
Smoking					
Yes	33 (43.4%)	30 (39.5%)	0.85	0.44 – 1.62	0.62
No	43 (56.6%)	46 (60.5%)			
Living in a rural area					
Yes	19 (25%)	26 (34.2%)	0.641	0.37 – 1.295	0.215
No	57 (75%)	50 (65.8%)			

The study examined the genotypic and allelic distributions of TLR4 SNPs D299G (rs4986790) and T399I (rs4986791) in both control subjects and patients with tuberculosis (TB). The results of this analysis are presented in Table 3. The genotypic frequencies of TLR4 rs4986790 (896A/G) (Figure 1) did not exhibit statistically significant variations between the groups ($P > 0.05$). The AA genotype was found to be the most prevalent genotype among both the control group and patients with PTB. In relation to the frequency of alleles, it was noticed that the G allele was major allele in patients with PTB. However, not at all statistically significant difference was identified

between the frequencies of the G and A alleles in both the control and the TB patients. Both the control and the TB group demonstrated adherence to the Hardy-Weinberg equilibrium, with chi-square values of 0.34 and 0.07, respectively and the corresponding p-values were 0.84 and 0.96, respectively as shown in the Table 4. These results indicate that the genotyping data used in this study is of high quality. The maintenance of this uniformity enables the establishment of a robust basis for future genetic investigations. The dominant genetic model evaluating the SNP rs4986790 (AA+AG vs. GG) reveals that individuals possessing the AA or AG genotype might exhibit a slightly decreased susceptibility to TB. On the other hand, the recessive genetic model, (AA vs. AG+GG), suggests that individuals with the AA genotype may have a slightly reduced risk of catching TB. This association, similar to the dominant model, was not statistically significant as clarified by the Table 5. Both genetic models showed that the A allele having a protective role against TB.

The TLR4 gene variant (1196C/T) rs4986791 (Figure 2) showed statistically significant variations in both genotypes and alleles, as seen in Table 3. TLR4 SNP rs4986791 investigation highlights a notable correlation between the C and T alleles and susceptibility to tuberculosis among the examined group. C allele prevalence in the control group was determined to be 64.5%, in controls and 40.1% in individuals diagnosed with tuberculosis (TB). Similarly, the T allele was seen in 35.5% of controls and 59.9% of TB patients with odd ratio (OR) of 0.36, this indicates that the existence of the C allele in this group may be associated to a reduced risk of tuberculosis (TB) compared to the T allele ($p<0.0001$), suggesting that this single nucleotide polymorphism (SNP) may play a significant duty to determine susceptibility or resistance against tuberculosis. The genotypic distributions of the TLR4 SNP rs4986791 are shown significant differences between individuals without tuberculosis (TB) and those diagnosed with TB. Individuals who possess the CC genotype may exhibit a decreased susceptibility to tuberculosis (TB). The observed frequency of this genotype exhibited a significant decrease in tuberculosis patients (26.3%) as compared to the control group (42.1%).

Similarly, the CT genotype demonstrates a discernible association with a diminished vulnerability to tuberculosis. As evidenced by Table 3, the incidence of this ailment was observed in 44.7% of the control cohort, but it was detected in merely 27.6% of individuals diagnosed with tuberculosis. In contrast, it has been shown that individuals with the TT genotype exhibit significant increase in susceptibility to TB. The genotype in question exhibited a statistically significant association with tuberculosis patients ($p<0.0001$). The prevalence of TT genotype was found to be higher among tuberculosis patients (46.1%) compared to the control group (13.2%). The genotypic distribution of the control group exhibited no significant deviation from Hardy-Weinberg equilibrium (Chi-Square= 0.040, P value= 0.98). This observation indicates that the frequencies of alleles and genotypes in the control population remain constant and are not experiencing any substantial evolutionary changes. A notable deviation from the Hardy-Weinberg equilibrium was detected within the TB group, as shown by a Chi-Square value of 13.74 and P value of 0.001 (Table 4). The potential causes for this deviation in equilibrium include non-random mating, mutations, genetic drift, and selection. The observed disparity implies the potential presence of genetic variables or biases within the TB group, which may be impacting the distribution of genotypes. The dominant genetic model (CC+CT vs. TT), showed that, individuals possessed CC or CT genotype may exhibit a decrease susceptibility to tuberculosis, while recessive genetic model (CC vs. CT+TT) showed that, individuals with TT genotype showed increased susceptibility to tuberculosis as shown in Table 5. The aforementioned results underscore the possible involvement of the rs4986791 single nucleotide polymorphism (SNP) in the susceptibility and resistance to tuberculosis (TB). The observed genotypes seem to be associated with varying levels of risk, indicating a multifaceted interplay that potentially encompasses other genetic and environmental elements.

Figure 1: Gel electrophoresis using 2% agarose gel (1X TBE buffer, 90 V) for one hour of TLR 4 (896A/G) polymorphisms after digestion PCR products by *NcoI*. Lanes 1, 2, 6, 7 and 10: indicate AA genotype, Lanes 3, 4 and 5: indicate AG genotype and Lanes: 8 and 9: indicate GG genotype.

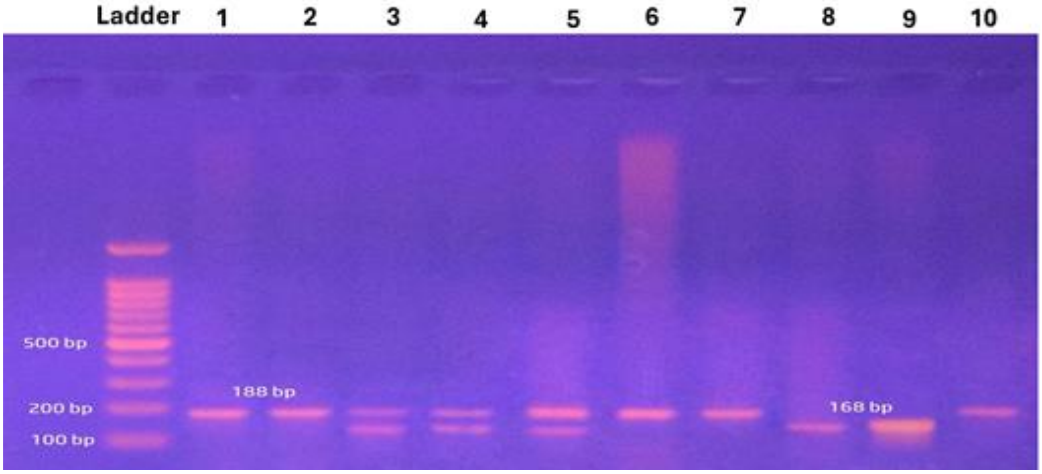

Figure 2: Gel electrophoresis using 2% agarose gel (1X TBE buffer, 90 V) for one hour of TLR 4 (1196C/T) polymorphisms after digestion PCR products by *HinfI*. Lanes 1, 2, 3, and 4: indicate CC genotype, Lanes 6, 7 and 10: indicate CT genotype and Lanes: 5 and 9: indicate TT genotype.

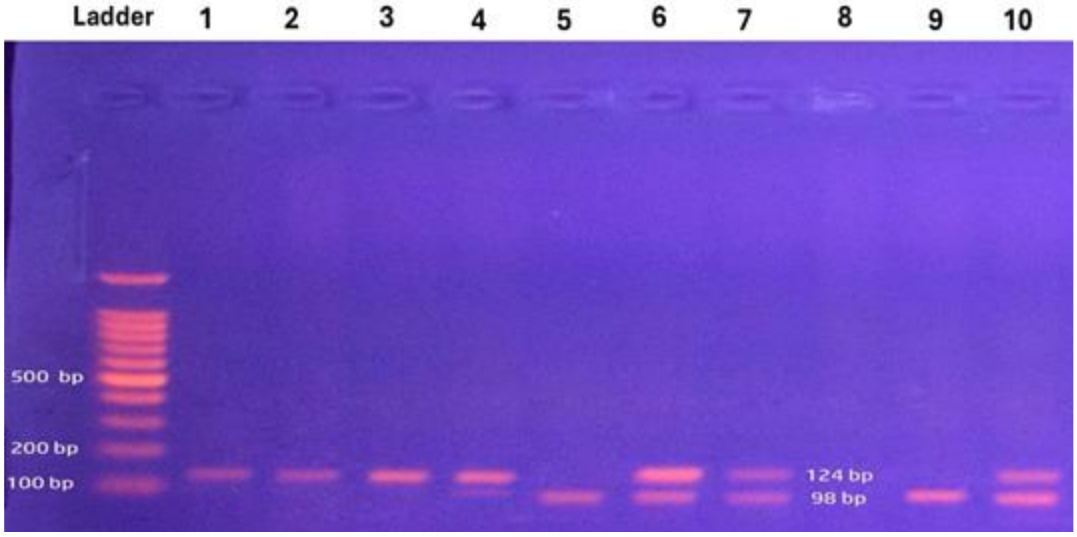

Table 3: Presents the genotypic and allelic frequencies of TLR-4 gene SNPs at positions 896A/G and 1196C/T between both control subjects as well as individuals diagnosed with tuberculosis (TB).

SNP	Alleles Genotypes	Control	TB patients	OR	95% CI	P value
TLR4 (896A/G) D299G (rs4986790)	AA	38 (50%)	29 (38.1%)	0.61	0.32 – 1.17	0.14
	AG	30 (39.5%)	35 (46.1%)	1.3	0.68 – 2.49	0.81
	GG	8 (10.5%)	12 (15.8%)	1.59	0.61 – 4.15	0.34
	A Frequency	106 (69.7%)	93(61.2%)	0.68	0.42 – 1.1	0.11
	G Frequency	46 (30.3%)	59 (38.8%)			
	CC	32 (42.1%)	20 (26.3%)	0.4911	0.24 – 0.97	0.04

TLR4 (1196C/T)	CT	34 (44.7%)	21 (27.6%)	0.4717	0.23 – 0.92	0.02
	TT	10 (13.2%)	35 (46.1%)	5.6341	2.52 – 12.58	P<0.0001
T399I (rs4986791)	C Frequency	98 (64.5%)	61 (40.1%)	0.3694	0.2322 – 0.5876	P<0.0001
	T Frequency	54 (35.5%)	91 (59.9%)			

Table 4: Hardy-Weinberg Equilibrium Analysis for the Observed and Expected Genotypic Frequencies in Control and TB Groups.

rs4986790	**Control**	AA	AG	GG	Chi-Square	P value
	Observed genotype	38	30	8	0.34	0.84
	Expected genotype	37	32.1	6.9		
	TB	AA	AG	GG		
	Observed genotype	29	35	12	0.07	0.96
	Expected genotype	28.4	36.1	11.5		
rs4986791	**Control**	CC	CT	TT		
	Observed genotype	32	34	10	0.04	0.98
	Expected genotype	31.6	34.8	9.6		
	TB	CC	CT	TT		
	Observed genotype	20	21	35	13.74	0.001
	Expected genotype	12.2	36.5	27.3		

Table 5: Distribution of TLR4 SNPs under dominant and recessive genetic model

	Genotype	Control	TB	OR	95% CI	P value
rs4986790 (A>G)	**Dominant genetic model**					
	AA+AG	68	64	0.6275	0.24-1.63	0.34
	GG	8	12			
	Recessive genetic model					
	AA	38	29	0.617	0.32-1.17	0.14
	AG+GG	38	47			
rs4986791 (C>T)	**Dominant genetic model**					
	CC+CT	66	41	0.1775	0.07-0.39	P < 0.0001
	TT	10	35			
	Recessive genetic model					
	CC	32	20	0.49	0.24-0.97	0.04
	CT+TT	44	56			

The objective of our research was to assess the potential correlation between the genetic variations of TLR4 1196C/T and 896A/G with the likelihood of developing TB among the Iraqi population. The findings of the current research indicate that there were no statistically significant disparities observed. in the allelic frequency of TLR 4 at position 896A/G between the A and G alleles, as well as between the three genotypes, in both the control group and tuberculosis (TB) patients. However, TLR-4 1196C/T allelic frequency showed significant difference between the C and T alleles, as well as between the CT, CC, and TT genotypes. In contrast to our findings, Ortega et

al., (10) mentioned that there was statistically significant correlation between the frequency of the G allele and those infected with Pulmonary Tuberculosis (PTB). Nevertheless, the previous study mentioned that the C and T alleles occurrences among individuals infected by tuberculosis (TB) showed a significant variation in comparison to the control group, specifically in respect to the TLR-4 1196C/T variant.

In contrast, the study accomplished by Fouad et al. (6) did not mention any significant variations in allele frequency or genotypic frequencies associated with the rs4986790 variant. Nevertheless, the researchers mentioned that there was a significant association between tuberculosis (TB) and rs4986791 genetic variant, explicitly in relation to the frequencies of the C and T alleles, as well as the CT, CC, and TT genotypes. In contrast to a study in South India by Selvaraj et al., (12), our observation did not show any significant differences between mis-sense changes in the TLR-4 (1196C/T) TLR-4 (896A/G) extracellular domain and patients diagnosed with pulmonary tuberculosis (PTB). Mhmoud, (8) conducted a study in which noticed that the presence of TLR4 genetic variants, (rs4986790 and rs4986791) in Sudanese population was significantly more prevalent ($p<0.0001$) in individuals with pulmonary tuberculosis (PTB) compared to the control group. The observed divergence might potentially be due to differences in population genetics, the methodology used in the research, or the statistical analysis conducted. Moreover, a comprehensive meta-analysis accomplished by Zhou and Zhang (18) including various research investigating the same SNPs polymorphism shown a multifaceted association that exhibits differences across diverse ethnic groups. The rs4986790 G allele was shown to have statistical significance in certain groups, while not showing the same level of significance in others. Similarly, rs4986791 T alleles showed a similar pattern of significance over different populations.

Differences in population samples, methodology used, or underlying genetic and environmental variables might be the factors that result in these differences in outcomes. The complex correlation between TLR4 polymorphisms and vulnerability to TB are clarified by these results. The comparisons highlight the complicated genetic connections to pulmonary tuberculosis (PTB) and ensuring to carefully consider factors when interpreting the results. The differences found in the studies mean we need to look deeper; this could involve using larger sample sizes, including people from different ethnic backgrounds, and enhanced analytical methods.

CONCLUSION

We conducted a comprehensive analysis about the connection among TLR4 polymorphisms (896A/G and 1196C/T) and TB susceptibility within the Iraqi population. The TLR4 896A/G polymorphism was now not shown to be associated with TB danger, while the TLR4 1196C/T version became. In this place, the C allele discovered to have a defensive impact that lowering tuberculosis susceptibility. The T allele and the TT genotype, conversely, there has been a correlation observed between the preceding elements and a heightened susceptibility to the development of the situations. These findings spotlight the importance of genetic changes in tuberculosis susceptibility as well as help future diagnostic and treatment procedures within the place. More research to investigate the genetic and environmental factors to determine susceptibility to tuberculosis is needed for the future.

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