Detection of toll like Receptor4 gene Polymorphism 896 A/G in Patient with Recurrent Urinary tract Infection

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Abstract

BackgroundThe numerous resident and recruited cells that make up the urinary tract's innate immune system include the Toll-like receptor 2 (TLR2), Toll-like receptor 4 (TLR4), Toll-like receptor 5 (TLR5), and Toll-like receptor 11 (TLR11). Early pathogen detection is made possible by these PRRs, which then translate this signal to start an immediate and potent pro-inflammatory immune response. TLR4 is encoded by the human TLR4 gene (Gene ID = 7099; 9q33.1), which produces a 224 amino acid protein. TLR4 activities in bladder cells include the stimulation of IL-6 and IL-8 production as well as the inhibition of bacterial invasion and encouragement of bacterial expulsion in bladder epithelial cells via the activation of MyD88-dependent or cAMP-dependent signaling pathways. Methods: The study comprised of 40 patients who were matched with 40 healthy control participants, ArMS PCR was used to detect the TLR4 gene polymorphism A/G (896) in patient and healthy control blood sample. Results: The 16S ribosomal RNA gene might be detected based on the findings of the PCR for 16SrRNA. Escherichia coli were found in 22 (55.0%) cases, Staph. aureus was found in 15 (37.5%) cases, and K. pneumonia and P. mirabilis were found in 2 (5.0%) and 1 (2.5%), respectively, using the 16S ribosomal RNA gene. By using the ARMS-PCR approach, the distribution of the TLR-4 (rs4986790) Polymorphism was discovered. There are three genotypes at this locus: AA, AG, and GG. The sole allele amplified by the wild type of homozygote genotype at a 406bp product size was the AA allele. Only the G allele was amplified at a 406bp product size in the mutant type of homozygote genotype. TLR-4 (rs4986790) genotypes AA, AG, and GG were distributed among the control group according to the Hardy Weinberg equation, whereas the heterozygote genotype demonstrated the amplification of the A and G alleles at 406bp product size, respectively. Conclusion: The distribution of TLR-4 (rs4986790) Polymorphism was detected by ARMS-PCR technique; accordingly, the homozygous genotype GG and heterozygous genotype AG were more frequent in patients' group and considered as risk factors for recurrent urinary tract infections

1. Introduction

Recurrence of at least three UTIs in a calendar year or at least two episodes in a period of six months is considered a recurrent urinary tract infection (rUTI) (1). Lower urinary tract infections (cystitis, urethritis) and upper urinary tract infections (pyelonephritis) are the two categories of urinary tract infections (2). A class of pattern recognition receptor molecules are known as toll-like receptors (TLR). It belongs to a group of innate immune receptors that are easily identifiable. It may start a chain of signal transductions that results in the production of inflammatory transmitters when paired with a, highly specialized immune molecule (3), (4). There are 10 TLRs in humans, numbered TLR1 to TLR10. A specific microbial PAMP, such as nucleic acids, flagella, lipopolysaccharides (LPS), and other chemicals making up microbial components, may be recognized by each receptor (5). A Considered to be a significant risk factor for developing a UTI is genetics (6). These genetic variables control the inflammatory response before, during, and after UTIs and are capable of interfering with any stage of the bacterial invasion process. These components include cytokines, receptors, and adhesion molecules, which are all separate gene products. TLRs are receptors that are found on the surface of numerous blood cells as well as epithelial cells like the uroepithelium. They are essential for the detection of infectious agents as well as the quick activation of signaling pathways for the destruction of microbial pathogens or the induction of adaptive immunity (7), (8). If the infection is left untreated, it results in intrauterine fetal death, increased prenatal mortality and morbidity, maternal problems include anemia, preeclampsia, renal failure, septicemia, and adult respiratory syndrome, as well as preterm labor and premature newborns (9). Researchers have looked at UTIs and single nucleotide polymorphisms (SNPs) of TLRs, however the results of these studies have been conflicting. Although urinary tract infections and *rs4986790* (TLR4 *896 A > G*) had been investigated in a previous meta-analysis (10).

2. Patients and Method

The current study was designed as a case control study include 100 samples; these samples were collected during the period between November 2021 to March 2022. From men and women aged 5

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to 50 years in the Alnumaniyah General Hospital and Haj Jalal hospitals in Wasit, Iraq. The appearance of urine sample was recorded as clear, yellow, turbid or bloody. All of the samples were then grown on Blood Agar, MacConkey Agar and Mannitol salt agar. with a sterile standard loop (0.001ml) at 37°C for 24 hours. patients were stratified as 60 patients with a diagnosis of Negative who were excluded from the study, and other 40 patients with initial biochemical prove of Recurrent urinary tract infection who were included in the study among them, there were (13) male and (27) female, these patients were submitted to further blood sample. The study comprised of 40 patients who were matched with 40 healthy control participants, Healthy individuals 40 with (19) male and (21) females, ArMS PCR was used to detect the TLR4 gene polymorphism *A/G* (*896*) in patient and healthy control blood sample.

ARMS-PCR Method

Blood samples from healthy controls and patients with the TLR-4 rs4986790 gene polymorphism A/G were tested using the ARMS-PCR (Amplification Refractory Mutation System) assay. This procedure was followed as described (11). Genomic DNA was isolated from blood samples using the gSYAN DNA extraction kit (Frozen Blood) Geneaid. USA, in accordance with manufacturer's instructions. Using a Nanodrop spectrophotometer (THERMO, USA), which measures DNA content (ng/L) and checks DNA purity by measuring the absorbance at (260/280 nm), the isolated blood genomic DNA was examined.

ARMS-PCR master mix preparation

The GoTaq® G2 Green Master Mix kit was used to produce the ARMS-PCR master mix, and this master mix performed two reactions for each sample in accordance with the manufacturer's instructions.

1- Wild type allele ARMS PCR reaction Mix.
2- Mutant type allele ARMS PCR reaction Mix.

Table (1): Wild type allele ARMS PCR Master mix protocol

ARMS PCR Master mix	Volume
DNA template	5µl
Wild type Forward primers (10pmol)	2µl
Common Reverse Primer (10pmol)	2µl
G2 Green Master Mix	12.5µl
PCR water	3.5µl
Total volume	25µl

Table (2): Mutant type allele ARMS PCR Master mix protocol

ARMS PCR Master mix	Volume
DNA template	5µl
Mutant type Forward primers (10pmol)	2µl
Common Reverse Primer (10pmol)	2µl
G2 Green Master Mix	12.5µl
PCR water	3.5µl
Total volume	25µl

ARMS-PCR Thermocycler Conditions

PCR thermocycler conditions were done for each gene independent as following Table: 3

Table (3): ARMS-PCR Thermocycler Conditions

PCR step	Temp	Time	repeat
Initial Denaturation	95°C	5min	1
Denaturation	95°C	30sec.	35 cycle
Annealing	58°C	30sec.	
Extension	72°C	1 min	
Final extension	72°C	5min	1
Hold	4°C	Stop	_

Statistical Analysis

Use Microsoft Office Excel 2010 with SPSS version 25. After running the Kolmogorov-Smirnov test and deciding whether the numerical data were distributed normally or not, the mean and standard deviation were reported. If the variable is regularly distributed, an independent sample t-test was employed to analyze the mean difference between any two groups. When a variable is normally distributed, an Anova test may be used to analyze the mean difference between more than two groups. Any two category variables were studied for association using the chi-square test. To evaluate risk, the odds ratio and 95% confidence interval were used. P-values of less than 0.05 were deemed significant, while P-values of less than 0.01 were regarded highly significant (12).

3. Results

The mean age of the patients was 28.65 ± 9.97 and the mean age of the control subjects was 27.57 ±18.83; there was no statistically significant difference in the mean ages of the two groups (P = 0.729). 13 (32.5%) men and 27 (67.5%) females made up the sample, compared to 19 (47.5%) males and 21 (52.5%) females in the control group.

Detection of TLR-4 (*rs4986790*) (A/G) Polymorphism

The distribution of TLR-4 (*rs4986790*) Polymorphism was detected by ARMS-PCR technique Figure (1).

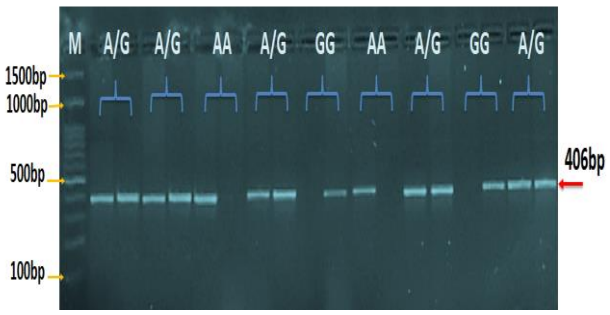

Figure (1): The lane (AA) wild type homozygote was showed as A allele only. The lane (GG) mutant type homozygote was showed as G allele only, whereas the (A/G) heterozygote were showed as both A and G allele.

The distribution of the TLR-4 (rs4986790) genotypes AA, AG, and GG within the control group was

calculated using the Hardy Weinberg equation, and the results are displayed in table (4).

Table (4): Hardy Weinberg equation				
Genotypes	Observed	Expected	χ2	P
Homozygote reference AA	34	33.306	2.552	0.279 ¥ NS
Heterozygote AG	6	6.387		
Homozygote variant GG	0	0.306		

The association between TLR-4 (*rs4986790*) POLY gene polymorphism and risk of recurrent urinary tract infection is shown in table (5).

Table (5): Genotypes frequency of TLR-4 (rs4986790) POLY in patients and control group							
S TLR-4	Patients n = 40	Control n = 40	P1	P2	OR	95% CI	EF
GG	2	0	0.215 ¥ NS	0.126 ¥ NS	2.214	1.683 – 2.913	0.431
AG	10	6		0.216 ¥ NS	2.02	0.654 – 6.25	0.505
AA	28	34		Reference	Reference	Reference	Reference

The association between TLR-4 (*rs4986790*) allele polymorphism and risk of recurrent urinary tract infection is shown in table (6).

Table (6): Alleles frequency of TLR-4 (rs4986790) in patients and control group							
TLR-4	Patients n = 80	Control n = 80	P	OR	95%CI	EF	PF
G	14	6	0.048 ¥ S	2.606	0.95 -7.19	0.617	-
A	66	74		0.382	0.148 – 1.05	-	0.617

The frequency distribution of genotype according to age, the mean age and gender was showed in tables (7) and (8) respectively.				
Characteristic	Genotype			p
	GG	AG	AA	
Age (years)				
< 15, n (%)	0 (9.1 %)	1 (0%)	3 (10.7%)	0.534 ¥ NS
15-29, n (%)	2 (100.0 %)	5 (60.0 %)	13 (39.3%)	
≥ 30, n (%)	0 (0%)	4 (40.0 %)	12 (50.0 %)	
Mean ±SD	31.00 ± 8.61	28.87 ± 8.57	29.60 ± 11.03	0.776 †NS
Range	22 - 40 years	20-43 years	5 -50 years	

Tables (8)				
Characteristic	Genotype			*P*
	GG	AG	AA	
Gender				
Male, n (%)	1 (50.0 %)	3 (30.0%)	9 (32.1 %)	0.907 ¥ NS
Female, n (%)	1 (50.0 %)	7 (70.0 %)	19 (67.9%)	

The frequency distribution of genotype according to species of bacteria was showed in tables (9).

Table (9):					
Characteristic	PCR (16SrRNA) results				p
	E. coli	S. aureus	K. pneumonia	P. mirabilis	
Genotype					
GG, n (%)	1 (4.5 %)	1 (6.7%)	0 (0 %)	0 (0 %)	0.893 ¥ NS
AG, n (%)	6 (27.3%)	3 (20.0%)	1 (50.0%)	0 (0%)	
AA, n (%)	15 (68.2 %)	11 (73.3 %)	1 (50.0%)	1 (100.0%)	

4. Discussion

The present results show the mean age of patients are 28.65 ± 9.97 years and 27.57 ± 18.83 years for control group (P= 0.729), these results agree with results of (13) which showed the mean age of the patients at diagnosis time was 26.49±4.623 years (range 19-43 years). The TLR has both an intracellular and an external domain, with the extracellular domain operating to recognize the molecular pattern associated with the pathogen and initiate the immune response. The intracellular region of proteins is where the TLR and adaptor protein interact (14)(15) proposed that the G-A transition at position 896 resulted in the substitution of glycine for aspartic acid, affecting the TLR4 extracellular domain's functionality. As a consequence, TLR4 loses part of its activities as a result of these polymorphisms that change how it interacts with its ligands. The genes that code for TLR4 have many genetic variants. With varied genotype frequencies within the same group, these polymorphisms are distributed variably across human populations.

The human TLR4 A>896G (Asp299Gly), which is located on chromosome 9q33.1(6). TLR4 A>896G

(Asp299Gly) has been identified in a number of global populations and ethnic groups (16) ;(17); hence, the findings of the current research serve as a fresh replication and strengthen the gene's link with disease. The TLR4 gene has numerous well recognized SNPs. The (896 A > G) SNP (rs4986790) polymorphism and the (1196 C>T) SNP (rs4986791) polymorphism are found in signal peptide sequence of TLR4 gene and affect the serum concentration of TLR4(18).

To determine if genetic polymorphisms in TLR4 may be a possible genetic marker to predict the susceptibility to UTI, 896 A > G SNP (rs4986790) were chosen, and their genetic connections with UTI risk were investigated using ARMS-PCR analysis. In this investigation, the genotype frequencies of TLR4 (rs4986790) were assessed in 40 patients with UTI and 40 healthy controls. Statistically, there were no changes between the groups (P = 0.215) in the distribution of the genotype frequencies of TLR4 (rs4986790). The present findings are consistent with result (18), which showed that there was no statistically significant correlation between rs4986790 and UTI (p=0.866). Between UTI patients and controls, the frequencies of the GG, AG, and AA genotypes were 2, 10, 28, and 0, 6, 34, respectively (P=0.215). The heterozygous AG genotype was not a significant risk factor (OR=4.329), and the homozygous GG genotype was more common in the patient group compared to the control group, 2 versus 0, respectively. This genotype may be considered a significant risk factor with an OR of 2.214, which indicates that patients with the homozygous GG genotype are about two times more likely to develop UTI disease than patients with other genotypes.

The current results confirm earlier research by demonstrating the rarity of homozygous mutations across all groups. According to the current findings, which were comparable to those of (19), just one patient (2.04%) had a mutant heterozygous type, while all other patients and control individuals had a wild homozygous type. (20), which demonstrated that individuals with acute cystitis and urethritis were more likely than controls to have the AG genotype (p = 0.02). According to statistical analysis, the frequency of allele G was significantly higher in the sick group than in the group of healthy controls, at 14 vs 6, respectively (p = 0.048). similar to the current discovery (21), showed G allele conferred high risk for UTI. The present findings also show non-significant association between TLR4 896 A/G (Asp299Gly) and age groups, gender and type of bacteria (p < 0.05).

5. Conclusion

The TLR-4 distribution (*rs4986790*) Polymorphism was discovered using the ARMS-PCR method, and genotype G was associated with a higher frequency of the allele G in the sick group compared to the control group. With an OR of 2.214, the homozygous genotype GG may be regarded as a significant risk factor since it was more prevalent in the patient group compared to the control group. This means that patients with the homozygous GG genotype are roughly twice as likely to develop UTI disease as patients with other genotypes.

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