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Polymorphism in the toll-like receptor 2 gene in patients with urinary tract infections

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Abstract--Urinary tract infection (UTIs) are considered to be the most common infections in human. The present study aimed to investigate the association between single nucleotide polymorphisms (SNPs) in the toll-like receptor 2 (*TLR2*) gene and the incidence of Urinary Tract Infection. A total of 100 urine specimens and venous blood sample from patients and (50) control collected from patients who are suffering from urinary tract infection who attended to Al-Imam Al-Sadiq Hospital/ Babylon, and Al-Hilla General Teaching Hospital during the period from February to October 2020. The aim of the present study was to determine *TLR2* expression during the UTI infection by measuring the *TLR2* in serum and urine. We also attempted to compare the *TLRs* expression with the immunological status of the patients to healthy. The concentration level of the *TLR-2* (systemic and mucosal) was determined by Enzyme Linked Immunosorbent Assay (ELISA) method. The concentration level of *TLR-2* mucosal molecule with mean value (3.877) pg./ml respectively while the control group with mean value (3.318) pg. /ml, The concentration level of *TLR-2* systemic molecule with mean value (5.023)pg./ml respectively while the control group with mean value (2.759)pg./ml. *TLR2* SNPs are suggested to influence susceptibility to develop UBC, and their potential in impacting *TLR2* serum and urine level is augmented.

Keywords---Urinary Tract Infection, toll-like receptor 2, single nucleotide polymorphism (SNP).

Introduction

Urinary tract infections (UTIs) are principally defined as the existence of bacteria in the urinary tract capable of producing functional and/or morphological

disorders Significant bacteriuria associated with infection is defined as the presence of $> 100,000$ CFU/mL in a urine culture (Grabe et al., 2015). Recurrent UTI (R-UTI) is diagnosed when patients manifest 3 or more episodes of UTI in 1 year, or 2 episodes in 6 months (NCBI - National Center for Biotechnology Information). Treatment of R-UTI presents a significant challenge to the professional due to the frequent appearance of symptoms and the high rate of anti-biotic resistance as a consequence of antibiotic prophylaxis guidelines aimed at preventing these infections(Geerlings, Beerepoot, & Prins, 2014).

Most paediatric UTIs are caused by Gram negative coliform bacteria arising from faecal flora colonising the perineum, which enter and ascend the urinary tract. *Escherichia coli* (*E. coli*) is the most common uropathogen, responsible for approximately 80% of paediatric UTIs. Uropathogenic *E.coli* strains possess specific properties, such as fimbriae to attach to the uroepithelial cell surface, to allow them to overcome host defences (Tullus, 2019) Other common uropathogens include *Klebsiella*, *Proteus*, *Enterobacter* and *Enterococcus* species (Edlin, Shapiro, Hersh, & Copp, 2013; Zorc, Kiddoo, & Shaw, 2005).

Toll-like receptors (TLRs) are trans membranous signaling receptors which play a key role in the innate and adaptive immune response, since they are involved in the regulation of inflammatory response and activation of the adaptive immune cells to reduce infectious pathogens and cancer cells (Bryant & Monie, 2012). The activation of the innate immune response in the urinary tract is dependent on recognition of bacterial components, products by TLRs (Chan, Mackenzie, Ng, & Leung, 2000) In recent years, it has become clear that the immune activation of bladder and kidney epithelial cells depends on TLRs, including TLR4, TLR5, and TLR11(Andersen-Nissen et al., 2007; Samuelsson, Hang, Wullt, Irjala, & Svanborg, 2004).

UTI appears to be more relevant to TLR1, TLR2, and TLR4 (Song & Abraham, 2008). They play a potential role in activating innate immunity with their early response against UTI and in protecting the mucosal barrier against attacks by bacteria. Uropathogenic *Escherichia coli* (*E. coli*) accounts for more than 85% of acute nonobstructive pyelonephritis and cystitis in uncompromised children (Mak & Kuo, 2006).

Single nucleotide polymorphisms (SNPs) among various TLR genes have been identified and maybe related to susceptibility/resistance to certain infections. This may lead to the development of TLR based gene therapy as well as the development of certain molecules to target these TLRs responsible for disease pathogenesis (Vidya et al., 2018).

Materials and Methods

Subjects of the Study

Clinical samples were collected from patients admitted to the out-patient clinics and two hospitals of Babylon Province: who attended to Al-Imam Al-Sadiq Hospital/ Babylon, and Al-Hilla General Teaching Hospital during the period from

February to October 2020. The study involved (100) patients were subjected for sampling which include both urine and venous blood sample from patients and (50) control. The age of patients & controls ranged from 18 to 48 years. A valid consent was achieved from hospitals administration and from patients and controls before their inclusion in the study. The procedure had been informed before the samples were collected, making absolutely sure that they understood the procedure that was to be carried out. The subjects were sentient that they had the right to reject to be included in the study without any detrimental effects.

Blood collection

Five milliliters of venous blood were collected from each participant. The blood was divided into two aliquots; the first was dispensed in a plain tube to collect serum, while the second was drawn in EDTA tube and stored at -20 °C until DNA isolation.

Serum and urine level of TLR2

Enzyme-linked immunosorbent assay kit (MyBioSource, Canada) was used to determine serum level of TLR2, and instructions of the manufacturer were followed.

Molecular Study

DNA Extraction

Genomic DNA from white blood cells (WBCs) for both Hemodialysis patients and control group were extracted by using DNA extraction kit (Favorgen)

Sample preparation

About 200 µl blood was added to a 1.5 ml microcentrifuge tube. About 40 µl proteinase K (10 mg/ml) was added to the 1.5 ml microcentrifuge tube and briefly mixed. The mixture is incubated at 60 °C for 15 minutes.

Cell Lysis

About 200 µl of FABG Buffer was added to the 1.5 ml microcentrifuge tube and mixed by shaking vigorously. The mixture was incubated in a 70 °C water bath for 15 minutes. During incubation, the tube was inverted every 3 minutes. At this time the required volume of Elution buffer was pre-heated (100 µl/sample) to 70 °C (for Step 5 DNA Elution).

DNA binding

About 200 µl of absolute ethanol (96-100%) was added to the sample lysate and immediately mixed by shaking vigorously for 10 seconds. If precipitate appeared, it was broken up by pipetting. The FABG Column was placed to a 2 ml collection tube and the entire mixture (including any precipitate) was transferred carefully to FABG Column. The samples were centrifuged at 14,000 × g

for 5 minutes and 2 ml collection tube containing the flow-through was discarded and the FABG column placed in a new 2 ml collection tube.

Wash

About 400 µl of W1 Buffer was added to the FABG column and centrifuged at 14,000×g for 30 seconds and the flow-through was discarded and the FABG Column was placed back in the 2 ml collection tube. About 600 µl of Wash Buffer was added to the FABG column and centrifuged at 14,000×g for 30 seconds and the flow-through was discarded and the FABG column was placed back in the 2 ml collection tube and centrifuged again at 14,000×g for 3 minutes without any addition to dry the column matrix.

DNA Elution

The dried FABG column was transferred to a new 1.5 ml microcentrifuge tube. About 100 µl of pre-heated Elution buffer or TE buffer was added to the membrane center of the FABG column matrix. Stand FABG Column for 3-5 min or until the buffer is absorbed by the membrane. The tube was centrifuged at 14,000×g for 30 seconds to elute the purified DNA.

Pure DNA The DNA was stored at -4°C or -20°C

Polymerase Chain Reaction (PCR)

Conventional PCR was used to amplify a target DNA using specific primer pairs. PCR typically consists of three consecutive steps (denaturation, annealing, and elongation) of repeated cycles to get PCR product (amplicon). The PCR thermal cycling conditions are mentioned in the table (2-5). The size of PCR products (5 µl) were analyzed in 1.5% (w/v) agarose gel by electrophoresis using 1× TBE buffer and visualized by staining with simply safe dye. Product size was determined by comparison with Gene Ruler 100 bp DNA ladder (Intronbio, Korea)

Table 1 : primers used in this study

Gene	Primer sequences 5'→3'	Amplicon size (bp)	References
<i>TLR2</i> rs3804099	Forward outer: 5'ATTGCAAATCCTGAGAGTGGGAA-3	Common product size: 349 bp TT: 228 bp	Ehsan <i>et al.</i> , 2018
	Reverse outer: 5'-CAAACCTTCATCGGTGATTTTCACA-3		
	Forward inner (T allele): 5-CCAAAAAGTTTGAAGTCAATTCAGAAT-3	CC: 173 bp	
	Reverse inner (C allele): 5-TCATATGAAGGATCAGATGACTTCCG-3	CT: 228 bp and 173 bp	

Statistical Method

The Statistical Package for Social Science (SPSS), version 23 was employed to the analysis of different biomarkers in the study. Chi-square analysis was utilized to a comparison between percentage and analysis of variance (ANOVA), least significant difference and Duncan test or t-test was employed to a comparison between means.

Result

Genotyping of *TLR2* rs3804099 Gene Polymorphisms

The amplified of *TLR-2* (rs3804099) target sequences of studied groups were detected by ARMS technique are summarized in (table 1-1) and figure (1-2). by electrophoresis on a 2% agarose gel, the product sizes of the PCR reaction was determined. Respectively, the allele-specific products were 228 bp and 173 bp for the presence of T and C alleles and the common PCR product was 349 bp, as shown in figure (NCBI - National Center for Biotechnology Information).

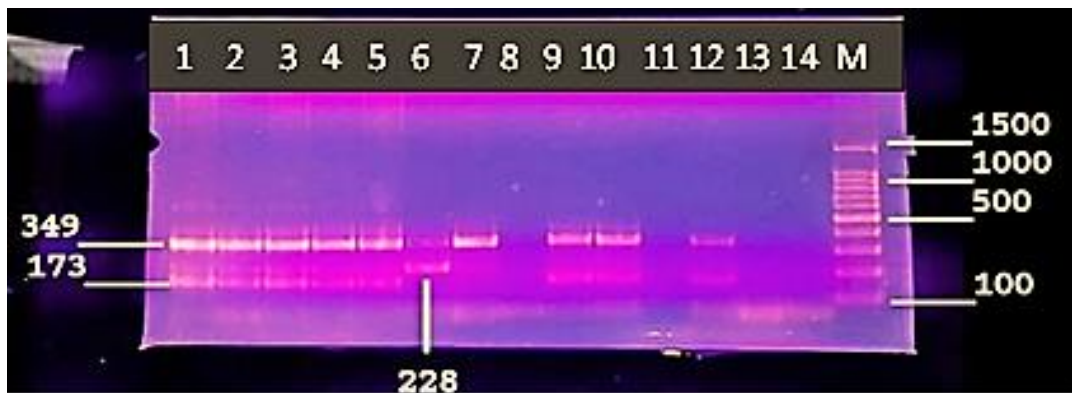


Figure (1-2): Determination of *TLR2* rs3804099 gene polymorphism by ARMS-PCR method; The M column: a ladder pattern (100bp); the columns (8, 11, 13 and 14) are blank; the columns (1, 2, 5, 9, 10 and 12) represent the TT genotype, the columns (3 and 4) represent the TC genotype, the column (6) represent the CC genotype.

It can be seen that the frequency of TT genotypes in patients and control groups (which reached 80% and 76%, respectively) it was significantly increased in patients than control. While, the frequency of TC genotypes in patients and control groups which reached 2% and 0%, respectively. It was significantly increased in patients than control. On the other hand, the frequency of CC genotype in patients and control groups was 17.4% and 24%, respectively, that decreased in patients compared with control group. Analyses showed no significant variations between study groups in the *TLR 2* (rs3804099) polymorphism (TT vs TC: OR=2.02, CI (95%) 0.0938 to 43.7262, P=0.6527). also no significant variations between study groups in the *TLR 2* (rs3804099) polymorphism (TT vs CC :OR=5.2000, CI (95%): 0.6442 to 41.9722, P=0.121. However high risk values have been detected in the homo dominant, hetero

codominant model, as well as allele frequency (OR = 2.02 .5.2and 11.01 respectively).

Table 2: Genotype distribution and odd ratio of ARMS of TLR2 gene rs3804099polymorphisms between the patients and healthy control

Genotype rs3804099	Patients No.(%)	Control No.(%)	P value	O.R	CI (95%)
TT^a	60(80%)	24 (76%)			
TC	2 (2.6%)	0 (0%)	0.6527	2.0248	0.0938 to 43.7262
CC	13(17.4%)	1 (24%)	0.1218	5.2000	0.6442 to 41.9722
Total No.	75 (100%)	25 (100%)			
Allele	Frequency	Frequency			
T	0.81	0.96			
C	0.19	0.04	0.0201	11.0164	1.4578 to 83.2492

P≤0.05;OR=(95%CI);^a reference

The (C) wild allele was associated with the protection against bacterial vaginosis by *Gardnerella vaginallis* among HIV-infected women (Mehta et al., 2020)TLR2 genotype rs3804099 was associated with susceptibility to Tuberculous meningitis TBM.The association was found with meningeal rather than pulmonary TB and was strongest when miliary TB was found on chest radiography. Furthermore, the association increased with the severity of neurologic symptoms . These results demonstrate a strong association of TLR2 SNP T597C with the development of TBM and miliary TB and indicate that TLR2 influences the dissemination of M. tuberculosis. The allele (C) variant was associated with a risk of 3 to 6 times greater for the individual to contract tuberculous meningitis (Thuong et al., 2007). Table (1-2) shows the comparison of clinical characteristics according to TLR2 rs3804099genotypes in patients, The study showed a higher correlation between TLR2 (serum) level with TLR2 rs3804099 (P = 0.028), TLR2 in serum level was 207±283, 60.16±26 and 147±145 in CC,CT and TT (respectively). There are no significant correlation between age and TLR2 (urine) level with TLR2 rs3804099.

Table 3: Comparison of clinical characteristics according to TLR2 rs3804099 genotypes in patients.

Parameters	TLR2rs3804099 genotypes			P value
	CC: 13(17.4%)	TC: 2 (2.6%)	TT: 60(80%)	
TLR2 in serum, mean±SD	207±283	60.16±26	147±145	0.028
TLR2 in urine, mean±SD	133.04±63	173±0.9	151.89±78.08	0.731
Age mean±SD	37±10.4	29±12.7	34.9±8	0.440

Discussion

A number of biological proteins that reduce inflammation adversely influence TLR signaling pathways. TLR2 is primarily responsible for identifying Gram-positive bacteria's cell wall features (Kuroda et al., 2003). TLR2 mutations, according to detectives, are linked to a decreased response to Gram-positive lipoproteins, putting them at an increased risk of Gram-positive infections (Tavares, Maldonado, Ojeda, & Minano, 2005). The SNP rs3804099 is found in the exonic region of the TLR2 gene. TLR2 gene expression regulation is influenced by variations in these areas. As a result, SNPs may impact TLR2 expression and activity by affecting transcription factor binding and mRNA stability (Thomas et al., 2006).

The synonymous polymorphism +597T>C (rs3804099) is found in the third exon of the TLR2 gene on chromosome 4q32 (Uno et al., 2014). Previous research has linked the SNP rs3804099 to a variety of infectious diseases, including bacterial meningitis and pulmonary tuberculosis (Zheng, Hu, & Gao, 2017). Another study found that the TLR2 gene mutation rs3804099 was substantially linked to the development of proteinuria following kidney donation. Their findings can help anticipate proteinuria and could lead to more personalized treatment for people who have had a kidney transplant. After kidney transplantation, this result suggested that the risk of proteinuria was toughly associated with the rs3804099 (Elbrolosy et al., 2022). Other authors discovered that the rs3804099 SNP has been associated to a number of illnesses, including polycystic ovarian syndrome and *H. pylori* infection and peptic ulcer, when compared to non-significant SNPs (Filus, Trzmiel, Kuliczowska-Paksej, Tworowska, & Milewicz, 2006).

For the SNP rs3804099, the allele (T) variant was associated with the susceptibility of APN (acute pyelonephritis) and ALN (acute lobar nephronia) (Chen et al., 2015). the variant (C) allele was associated with susceptibility to infectious endocarditis (Golovkin et al., 2015). After 24 h of *L. pneumophila* stimulation, the mRNA expression level of MyD88 was significantly lower with TLR2 (C597T) CT/TT ($p = 0.0482$). The (T) variant allele provided higher protection against infection by *Legionella pneumophila*, with the level of MyD88 mRNA expression (myeloid differentiation protein) significantly lower (Wang, Zhang, Yue, & Wang, 2013).

TLR2 expression may be influenced by the SNP rs3804099. After stimulation with peptidoglycan, PBMCs from individuals with Behcet's disease who held the rs3804099 TT genotype produced larger quantities of TLR2 mRNA than those who carried the CC or CT genotypes (Qi et al., 2013). rs3804099 (genotypes CC, CT, and TT) carriers of the T allele produced higher serum levels of IL-17, and this difference was kept significant by pooling CT and TT individuals and comparing against CC individuals, the carriers of the T allele are higher producers of IL-6, especially when they combine CT and CC with TT genotype (Santana et al., 2017).

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