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# ROLE OF TLR-4(896A/G) GENE POLYMORPHISMS IN PATIENTS WITH DIABETIC FOOT ULCER

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**Abstract:** One of the most prevalent and dangerous consequences of diabetes, diabetic foot ulcers enhance bacterial resistance to a variety of antibiotics while also causing substantial morbidity and mortality in patients. The study aims to confirm whether whether single nucleotide polymorphisms of TLR-4(896A/G) genes are associated with Diabetic foot ulcer in terms of risk, and severity.

**Methods**: Eighty eight samples were included in the current study ranging in their age from 20 to 67 and comprising 22 control subjects, 22 diabetic patients, and 44 diabetic foot ulcer patients who attended to Central Diabetic Foot) in Najaf governorate between December 2021 and March 2022.

**Result**s: According to this study, male are more likely to get diabetic foot ulcers than female, and people in the age range of 51 to 60 are more likely (39%).The genotyping of Toll-like receptors-4 (TLR-4) 896A/G (rs4986790) gene revealed three genotypes; the wild homozygous AA type, the heterozygous AG and the homozygous GG type. The frequency for these three types in diabetic foot patients were (2.3%, 65.9% and 31.8%) respectively, while diabetes patients were (27.3%, 13.6 % and 59.1%) respectively. Finally, in control group they were (63.63%, 36.37% and 0%) respectively, and the AG was common in diabetic foot patients, while GG genotype more frequent in diabetes and AA genotype was more frequent in healthy control. The study concluded that the presence of AG genotype and G allele from TLR4 896A/G(rs4986790) in the DFU patients may predict the probability of developing severity of this disease.

***Keywords :Diabetic foot ulcer, TLR-4, SNP***

## 1. Introduction

Diabetes patients frequently get devastating foot infections. A wound, most frequently a neuropathic ulceration, is where diabetic foot infections (DFIs) generally start. While microbes infiltrate every wound ,then, infections are categorized as light (superficial, modest in magnitude and depth), intermediate (greater depth, wider distribution), or serious (together with systemic symptoms or metabolic disturbances) [1]. This classification strategy, along with a circulatory examination, assists in determining which patients require hospitalization, which require specific imaging tests or surgical procedures, and which require limb amputation [2].

The principal pattern recognition receptors (PRRs) of the innate immune system are toll-like receptors (TLRs) [3]. The receptor has evolved significantly and identifies pathogen associated molecular patterns (PAMPs), which comprise lipids, lipopeptides, proteins, and nucleic acids as limited molecular components of invading pathogens [4]. TLR stimulation and the following sterile infection caused by TLR identification of endogenous ligands on both immune and non-immune skin cells create alarm signals that warn of tissue damage. TLR activation, on the other hand, has an influence on the wound healing process that extends beyond the first detection of cellular damage. The process of wound healing and tissue regeneration appears to be enhanced or hindered depending on the location, timing, and degree of activation [5].

Through the creation of a persistent subclinical inflammatory process, TLR4 is heavily engaged in the pathogenic process of type-2 diabetes, which further results in pancreatic B cell dysfunction. Poor healing is positively connected with the ensuing uncontrolled hyperglycemia [6]. Differential TLR4 expression in human diabetic wounds impairs the wound healing cascade, which ultimately results in chronic non-healing ulcers. Due to single nucleotide polymorphisms (SNPs) in the TLR4 extracellular domain, the dysregulation of TLR4 signaling may change the ligand binding capacity and upset the pro- and anti-inflammatory cytokines, altering the risk of chronic inflammation and postponing wound healing. The co-segregating SNP responsible for the shift in amino acids in the TLR4 outer domain has been found [7]. the study aims to confirm whether the impact of gene polymorphisms with some immunological markers and bacterial infection associated with Diabetic foot ulcer. In particular, the researcher aims find whether single nucleotide polymorphisms of TLR-4 (896A/G) genes are associated with Diabetic foot ulcer in terms of risk, and severity.

## 2. Methodology

Eighty eight individuals were recruited in this investigation, which included three groups: DFU (n = 44), D without FU (n = 22), and Controls (n = 22). This study was carried out in AL-Sader hospital in Al-Najaf City, Iraq (December 2020 -March 2021). The information from each subject's physical and clinical exams was recorded in a data sheet from clinicians. The patients included in this study were those who only suffering from diabetic foot ulcer and patients suffering from any complication diseases were excluded. Blood specimens were collected for TLR-4(896A/G) gene polymorphism PCR determination.

Two milliliters of venous blood sample were taken from all subjects. For extraction of DNA, has been transferred from both groups to an anticoagulant EDTA tube, the blood was collected in triplicates in sterile Eppendorf tubes and stored frozen at -20 ºC.

The TARMS-PCR, which may identify known SNPs, consists of three complimentary reactions: one that amplifies normal DNA at a particular locus but cannot amplify mutant DNA, and the other two that amplify mutant DNA but cannot amplify normal DNA.
DNA was extracted from blood using G-spin™ Total DNA Extraction Kit (Intron-Bio, USA) stored at -20 ºC until it was required for use. TLR-4(896A/G) polymorphism was detected by PCR. PCR with site-specific primers (TARMS-PCR) consist of three complementary reactions, two of which include a primer specific for the mutant DNA sequence , and one of which has a primer specific for the normal DNA sequence and cannot amplify mutant DNA at a particular locus. Forward outer:
5′-TGAACCCTATGAACTTTATCC-3′ ,
Reverse outer:
5′GTTAACTAATTCTAAATGTTGCCATC-3′,
Forward inner (A allele):
5′GCATAC TTAGACTACTACCTCGATGA3′
and Reverse inner(G allele):
5′CAAAC AATTAAATAAGTCAATAATAC3 ,
with an amplicon product of Common product size: 383 bp , A: 147 bp , G:287 bp ,AG:147 bp and 287 bp [8]. Amplification was performed on 95 ºC for 5 minutes, followed by 30 cycles each of 95 ºC for 30 seconds, 51 ºC for 35 seconds, 72 ºC for 45 seconds, followed by final extension of 72 ºC for 10 minutes. PCR products were electrophoresed on 1.5% agarose gels, stained with ethidium bromide and observed using a Cleaver gel documentation system (Biometer, Germany).

According to Promega Biosystem the Corporation's instructions, which are outlined in the table, amplification reactions were carried out in 0.2 ml tubes of Accu Power PCR Premix tubes (1).

**Table (1)**: Promega protocol of PCR reaction mixture volumes for Tetra –ARMS-PCR

| Component | Volume in a 25 µl reaction |
|---|---|
| **GoTaq®Promega Green Master Mix 2X** | 12.5 µl |
| **Forward outer primer (10 µM)** | 1 µl |
| **Reverse outer primer (10 µM)** | 1 µl |
| **Forward inner primer (10 µM)** | 1 µl |
| **Reverse inner primer (10 µM)** | 1 µl |
| **Template DNA** | 7 µl |
| **Nuclease Free water** | 1.5 µl |

**Statistical analysis**

Statistical analysis was carried out using a GrafPad prism 5 computer software. The statistical significance of the measured odds ratio (OR), the frequency of a given genotype and the presence or absence of illness was determined using a customized $\chi 2$ formula. P-value of 0.05 was considered significant.

**3. Results**

**Sex distribution of patients**

According to the study's findings, male patients outnumbered female patients by a ratio of 66% to 34% for DFU and 60% to 40% for D without FU, respectively, as shown in figure (1).

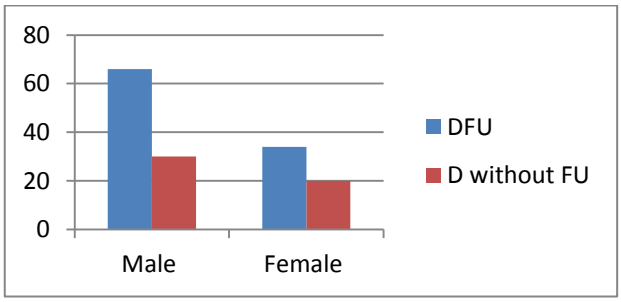


**Figure (1):-** Patienst distribution by gender for those with and without foot ulcers

**Distribution of the patients with and without foot ulcer according to age group**

Based on their age ranges, patients were divided into two groups within each of five categories. Figure (2) shows the distribution of DFU by age ranges, with the frequency occurring in those between the ages of 51 and 60. This was accompanied by the lowest frequency occurring in those between the ages of 21 and 30, with values of 39, 20, 20, 16 and 5, respectively. The distribution of diabetes by age groups also shows that those between the ages of 51 and 60 had the highest frequency, followed by those between 61 and 70, 31 to 40, and those between the ages of 21 and 30.

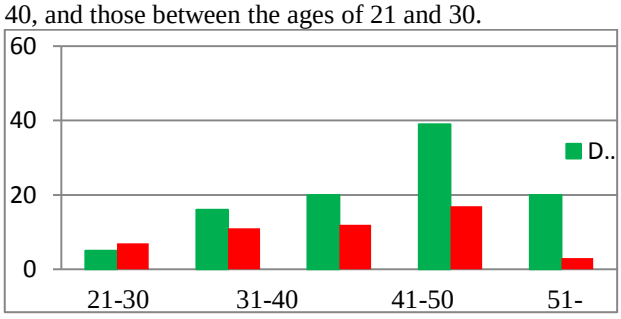


**Figure (2):-** Patients are distributed according on their age groups.

**Distribution of TLR4 Gene 896A/G(rs4986790) polymorphism in DFU**

The results of detection polymorphism in TLR4 896A/G(rs4986790) locus shows there are three genotypes GG, GA and AA with different bands , common product size: 383 bp , AA: 147 bp , GG:287 bp and AG:147 bp and 287 bp, as shown in Figure (3) for DFU patients , D without FU and control groups.

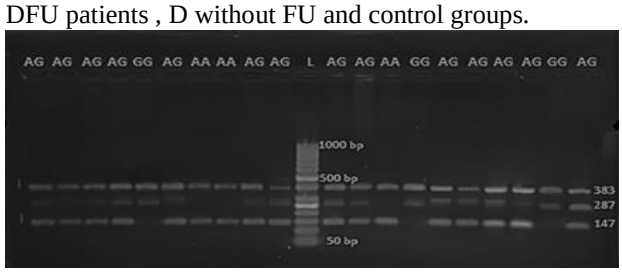


**Figure (3)**:- Ethidium bromide stained agarose gel showing, the TARMS-PCR products for TLR4 Gene 896A/G(rs4986790) polymorphism,3 (AA genotype), 14(AG) and 3 (GG genotype).

The following genotypes were shown to be prevalent in DFU patients; AA(2.3%), GA(65.9%) and GG(31.8%) ; while in the D without FU;AA (27.3%) ,GA (13.6%) and GG (59.1%), Table (2). In the same table explain that allele G (64.77%) was high frequency than D without FU and show higher significant differences between all genotype at P-value $\leq$ 0.05, while not found significant differences between allele frequency at P-value $\leq$ 0.05.

Table (2): TLR4 896A/G promoter Variant Genotype and Allele Frequency in DFU and D without FU

| TLR4 genotype | DFU n=44 | D without FU n=22 | OR(95 % CI) | P-value |
|---|---|---|---|---|
| **A/A** | **(1) 2.3%** | **(6) 27.3%** | 0.062(0.0069 to 0.5560) | 0.011* |
| **A/G** | **(29) 65.9%** | **(3) 13.6%** | 12.244(3.1178 to 48.0877) | 0.000*** |
| **G/G** | **(14) 31.8%** | **(13) 59.1%** | 0.323(0.1119 to 0.9331) | 0.036* |
| **Allele frequency** | | | | |
| **A allele** | **31** | **15** | 1.051(0.4910 to 2.2517) | 0.897ns |
| **G allele** | **57** | **29** | | |
| ***(P<0.05): significant , **or***( P<0.05) higher significant** | | | | |

**OR : Odds Ratio , CI: Confidence Interval**

The following genotypes were shown to be prevalent in DFU patients; AA(2.3%), GA(65.9%) and GG(31.8%) ; while in the healthy subject ;AA (63.63%) ,GA (36.37%) and GG (0%), Table (3). In the same table explain that allele G (64.77%) was high frequency and show higher significant differences between all genotype

at P-value ≤ 0.05 and found significant differences between allele frequency at P-value ≤ 0.05.

**Table (3):** TLR4 896A/G promoter Variant Genotype and Allele Frequency in DFU and Control

| TLR4 | DFU n=44 | Control n=22 | OR(95 % CI) | P-value |
|---|---|---|---|---|
| A/A | (1) 2.3% | (14) 63.63% | 0.013(0.0015 to 0.1158) | 0.000*** |
| A/G | (29) 65.9% | 8 (36.37%) | 3.383(1.1614 to 9.8558) | 0.025* |
| G/G | (14) 31.8% | (0) 0% | 21.393(1.2113 to 377.8380) | 0.036* |
| Allele frequency | | | | |
| A allele | 31 | 36 | 0.120(0.0500 to 0.2920) | 0.000*** |
| G allele | 57 | 8 | | |
| *(P<0.05): significant , **or***( P<0.05) higher significant | | | | |

**OR : Odds Ratio , CI: Confidence Interval**

The following genotypes were shown to be prevalent in D without FU patients; AA(27.3%), GA(13.6%) and GG(59.1%) ; while in the healthy subject ;AA (63.63%) ,GA (36.37%) and GG (0%), Table (4). In the same table explain that allele G (81.81%) was high frequency and show significant differences between only AA and GG genotype at P-value ≤ 0.05 and not found significant differences between AG genotype, while show higher significant differences allele frequency at P-value ≤ 0.05. Results of control were agreed with expected Hardy- Weinberg equilibrium results which not found deviation in control and there was not found significant difference between observed and expected frequencies ($\chi$= 1.086). Since the homozygous (A/A) is represent the common type in control groups so it common in the Iraqi population.

**Table (4):** TLR4 896A/G promoter Variant Genotype and Allele Frequency in D without FU and Control

| TLR4 | D without FU n=22 | Control n=22 | OR(95 % CI) | P-value |
|---|---|---|---|---|
| A/A | (6) 27.3% | (14) 63.63% | 0.214(0.0597 to 0.7697) | 0.018* |
| A/G | (3) 13.6% | 8 (36.37% ) | 0.276(0.0619 to 1.2331) | 0.091ns |
| G/G | (13) 59.1% | (0) 0% | 63.947(3.4392 to 1189.0154) | 0.005** |
| Allele frequency | | | | |
| A allele | 15 | 36 | 0.114(0.0428 to 0.3086) | 0.000*** |
| G allele | 29 | 8 | | |
| *(P<0.05): significant , **or***( P<0.05) higher significant | | | | |

**OR : Odds Ratio , CI: Confidence Interval**

**Table(5):Expected frequencies of genotypes of the TLR4 SNP using Hardy-Weinberg equilibrium.**

| | Genotype | A/A | G/A | G/G | Chi-squared value | P-value |
|---|---|---|---|---|---|---|
| Control | Observed genotype | 14 | 8 | 0 | 1.086 | 0.297ns |
| | Expected genotype | 14.7 | 6.5 | 0.7 | | |
| DFU | Observed genotype | 1 | 29 | 14 | 8.683 | 0.003** |
| | Expected genotype | 5.5 | 20.1 | 18.5 | | |
| D without FU | Observed genotype | 6 | 3 | 13 | 10.674 | 0.004** |
| | Expected genotype | 2.6 | 9.9 | 9.6 | | |

## 3. Discussion

By comparing the frequency of men to women, it was found that men made up 72.2% of the population [9]. Male predominance in DFU may be attributed to factors like poor adherence to foot care procedures and gender-related disparities in living styles and occupational activities that place greater demands on the feet. The wounds heal more quickly in females than in males; this may due to hormonal differences, which also explain why in females, increased estrogen receptors act as endogenous enhancers of the healing process, while in males,Because androgenic species impede down dermal regeneration, higher levels of androgen were considered to be harmful to wound healing [10].

The findings from this research's findings were consistent with those from study [11] ,they reported that 50% prevalence of DFU in the 48–57 age range. Consistent with recent research that showed that older age groups to have a greater incidence of DFU [12,13] showed that 32 (31%) of people in the age range of 51 to 60 had DFU. Among diabetic patients, age was a risk factor for amputation, peripheral vascular disease (PVD), and neuropathy [14]. Elderly diabetic patients typically have peripheral neuropathy and vascular lesions due to their advancing years and disease history, which causes diabetic foot ulcers that heal slowly and require extensive medical care [15].

TLRs play a vital role in mammalian innate immunity as the pattern - recognition receptors. They are activating by pathogen-associated molecular patterns and endogenous molecules,, triggering the activated of signal transduction pathways, that stimulate dendritic cell maturation and the production of cytokine [16]. These receptors play an important role in activating innate immunity [17]. TLR4 polymorphism has been suggested to be associate with atherosclerosis and chronic inflammatory disease in Caucasians[18]. Furthermore, a

study conducted by [19] who reported that the TLR4 (Thr399Ile) gene polymorphism have strong association between diabetic neuropathy is more common in those with T2DM. As far as we are aware, no investigation has shown a link between the TLR4 (Thr399Ile) gene polymorphism and the emergence of DFU and T2DM in Iraq.[21,22]

The current study found a significant correlation in the distribution of TLR4 gene polymorphism across the three groups, which contradicted the findings of [20], other studies that found a non-significant association of TLR4 polymorphism in T2DM patients. This may be explained by the fact that the T2DM the patient is generally unable to provide a sufficient inflammatory response due to the compromised ability to fight infection. On the other hand, the result of present study was agreement with [23,24,25] who reported a significant association between TLR4-Thr399Ile gene polymo -rphism and development of DFU in T2DM might be associated with impaired expression and action of TLR4 in T2DM patient resulting in impairment the healing of wound and developing of DFU in patient withT2DM.

**Conclusion**

The study concluded that the diabetic foot ulcer is more frequent in males than Females and appears that higher frequency was 39 % in the age 51-60 years . The presence of AG genotype and G allele from TLR4 896A/G (rs4986790) in the DFU patients may predict the probability of developing severity of this disease.

Ethical Approval:
All patients and healthy participants in this study completed the questionnaire and provided all required approves for the research to proceed. I have secured the necessary permissions from Al-Kufa University and established health centers

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