Role of toll like receptor -9 (1237 T/C) gene polymorphism in patients with type 2 diabetes and diabetic foot ulcer

Furqan Naeem Al-Karawi¹, Abeer Thaher Naji Al-Hasnawi¹* and Thekra Abd Jebur Al-Kashwan²

¹Medical Microbiology Department, College of Medicine, Kerbala University, Kerbala, Iraq.
²Medical Microbiology Department, College of Medicine, Kufa University, Najaf, Iraq.

*E-mail: abeer.zahir@uokerbala.edu.iq

Abstract. This study aimed to evaluate whether (1237 T/C) polymorphism is related with diabetic foot ulcer in type-2 diabetes mellitus of Iraqi patients in Najaf city. Diabetic foot ulcer (DFU) is one of the most prevalent and serious diabetic complications. DFU accounts for about 15% of the diabetic population. It is commonly associated with the increased diabetic morbidities and mortalities. The type 2 diabetes is recognized by aberrations of glucose, lipid and protein metabolism. It is increasingly appreciated that wound healing defect seen in diabetic patients is attributed to the altered protein and lipid metabolism. This study involved (180) subjects, between them, 60 were patients as group one suffering from type 2 diabetes and 60 were patient suffering from type 2 diabetes and foot ulcer as group two. In addition, 60 healthy group three as control subjects. Polymerase chain analysis was used for detection of TLR-9 genotypes followed by restriction analysis. We observed no significance differences in the distribution of the genotypes and alleles of (1237 T/C) polymorphism among the study groups.

Keywords. Type-2 diabetes mellitus, Diabetic foot ulcer, Toll Like Receptor-9 gene polymorphism, RFLP-PCR.

1. Introduction

Type 2 diabetes is considered the most prevalent metabolic aberration, and its consequences constituted the leading cause of morbidity and mortality. It comprises the majority of diabetic cases (85%) [1]. The macro- and micro-vascular complications, such as diabetic retinopathy, nephropathy and neuropathy are strongly associated with metabolic aberrations. The risk of hypertension, atherosclerosis and coronary artery diseases are also increasing. The underlying mechanisms, explaining the metabolic aberrations and its consequences are not fully clear [2]. It is increasingly demonstrated that most subjects diagnosed as overt type-2 diabetics showed relative diminution in insulin secretion and dysfunction of the β-cells of the pancreas. It probably preceded by compensative hyper-secretion of β-cells and peripheral resistance of insulin. Nevertheless, it is increasingly considered that the peripheral resistance and insulin hyper-secretion are just initiating events, whilst β-
cells dysfunction and insulin defects are considered the critical main event for development of overt type-2 diabetes [3]. Moreover, type-2 diabetes is multi-factorial. Environmental factors combined with the genetic component to explain the dramatic increase in the prevalence of type 2 diabetes. TLR4 and TLR9 genes as a molecular risk factors for T2DM and its complications such as foot ulcer in the discrete diabetic population [4]. Wound healing refers to the natural dynamic process which is responsible for removal and replacement of the devitalized skin structures in response to tissue injury to restore the normal skin integrity. It is characterized by a stepwise pattern of 4 consecutive phases. The first and second phase are inflammation and hemostasis. The ultimate goal of this phase is limitation of the tissue damage and conditioning of the field for reconstruction. The third phase comprises activation and proliferation of fibroblasts to form granulation tissue. The final step concerned about maturation of the granulation tissue to restore the tensile surface of the skin. Importantly, wound healing requires harmony between multiple factors to obtain its maximal benefits. Nevertheless, certain pathological, nutritional and metabolic aberrations may interfere with the process of wound healing [5]. Diabetes is one of the most common metabolic aberrations that interfere with wound healing. It has been reported that diabetic subjects show prolonged inflammatory and proliferative phases of wound healing. In addition, diabetic foot ulcer (DFU) occurs in 15% of the diabetic population, and responsible for 85% of the associated lower extremity amputations [6]. In both innate and adaptive immunity, the toll like receptor (TLR) have essential role. The different infectious diseases are related with genetic diversity in the toll like receptor genes. Different autoimmune disease is associated with toll like receptor 9, because it is contributes in the maturation of dendritic cells and proinflammatory cytokines production [7]. In European Americans, the high risk of asthma disease is associated with the genetic variation at position -1237 [8]. Further studies needed to confirmed the relationship between the very low frequency of these toll like receptor variants and the lower incidence of different diseases like diabetes.

2. Materials and Methods

2.1. Subjects

This study included 180 Iraqi subjects. Group 1 including 60 patients with T2DM and DF, their age mean, disease duration, and body mass index were 56.9±9.2year, 10.7 ±4.8year, and 27.1± 3.8kg/m2, respectively. Group 2 including 60 patients with T2DM and without DFU, their mean of age, duration of disease and body max index were 47.9± year 8.6, 2.9±year 3.0, 30.1± 4.1kg/m2, respectively. Group 3 including 60 the healthy control their age mean and BMI were 45.0±8.9, 45.0±8.9 respectively. Also, the gender and smoking history were performed for three groups. The patients were recruited to Al-Sader hospital in Najaf city and the study protocol was approved by Kufa University ethical committee. The medical history, clinical examination, and assessment of BMI are taken from all participants. The parameters like fasting and glycated hemoglobin, Urea and creatinine, total cholesterol, and triglycerides data as shown in (Table 1). Polymerase Chain Reaction-Restriction Fragment Length Polymorphism analysis was used for detection the genotypes of TLR-9(1237 T/C) polymorphism.

2.2. Genotyping of TLR-9 (1237 T/C)

From peripheral blood leukocytes, the genomic DNA extracted by using Flexi Gene DNA kit (Qiagen), Restriction fragment length polymorphism technique used for detection the genotypes of TLR-9 polymorphism. The total volume of PCR reactions consist of 25mL includes 2 μl of DNA, 12.5X of Master Mix, 0.3 μl of each forward and reverse primers for detection of TLR-9(1237 T/C) polymorphism. The set of primer used was included the forward primer: 5’ATGGGAGCAGACATAATGGA-3’ and reverse primer: 5’CTGCTTGCAGTTGACTGTGT-3’ [4].
3% agarose gel electrophoresis was performed and run at 60 volt for 3hrs for visualized the PCR products under UV light by using ethidium bromide dye.

2.3. **The cycling conditions**

The conditions includes 5 minutes of initial denaturation at 95°C followed by 40 second of 35 cycles of denaturation at 95°C, in addition to 40 seconds of annealing at 62°C and 1 minute of extension at 72°C with a final extension step at 72°C for 10 minutes. The created is about 135 bp fragments. The PCR product was then restricted with BstNI enzyme. The T allele represent the wild type consist of 108 and 27 bp fragments while the C allele represent the mutant type and composed of 60, 48 and 27 bp fragments.

2.4. **Statistical Analysis:**

ANOVA test, and student t test was used to test the level of significance (p-value) for all parameters between the study groups. Chi-square test, Odds ratios (ORs) and 95% confidence intervals was used also to determine the significant level of the categorical variables. The Statistical Package of Social Science software (Version 22, SPSS Inc., Chicago, IL, USA) was used to performed for all data statistical analysis [9].

3. **Results and Discussion**

There were no significant differences between study groups regarding gender, as shown in Figure 1. About smoking patients, there was highly associated between diabetic foot ulcer group (P<0.001) comparing with diabetic patients and control, as shown in Figure 2. The mean-fasting blood glucose, glycated hemoglobin, urea and creatinine were highly significant in group I, as in Table 1. There was a non-significant association in the allocation of the polymorphic TLR-9 genotypes or allele frequency between different study groups, as shown in Table 2. The TLR-9 gene polymorphism was done by using the BstNI restriction enzyme. The (TT) homozygous allele, (CT) heterozygous and (CC) homozygous mutant alleles were result from digestion by restriction enzyme, as shown in Figure 3.

![Figure 1. Distribution of study groups according to gender.](image-url)
Figure 2. Distribution of study groups according to smoking history.

Table 1. Mean differences of age, duration, clinical and biochemical parameters according to study groups.

| Parameter                      | (mean ±SD) | Group 1       | Group 2       | Group 3       | P-value          |
|--------------------------------|------------|---------------|---------------|---------------|-----------------|
| Age (years)                    | 56.9±9.2*  | 47.9±8.6      | 45.0±8.9      |               | <0.001*         |
| Duration (years)               | 10.7±4.8   | 2.9± 3.0      |               |               | <0.001*         |
| Body Mass Index (kg/cm2)       | 27.1± 3.8  | 30.1± 4.1*    | 27.8±3.3      |               | <0.001*         |
| Fasting Blood Sugar (mg/dl)    | 223.6 ± 83.1* | 212.2± 69.7* | 101.9± 17.5  |               | <0.001*         |
| HbA1c (%)                      | 9.0 ± 2.8# | 8.0 ± 1.9     | 5.3± 0.5      |               | <0.001*         |
| Total cholesterol(mg/dl)       | 210.7±65.1 | 214.5±51.6    | 201.9±58.1    |               | 0.48            |
| HDL(mg/dl)                     | 42.8±12.1  | 42.8±9.3      | 40.1±10.9     |               | 0.30            |
| LDL(mg/dl)                     | 123.2±50.2 | 135.5±36.5    | 125.3±44.3    |               | 0.44            |
| Triglycerides(mg/dl)           | 170.7±96.7 | 181.9±87.0    | 157.8±80.6    |               | 0.33            |
| Blood urea(mg/dl)              | 42.7±21.7* | 32.2±11.0     | 29.1±7.1      |               | <0.001*         |
| Serum creatinine (mg/dl)       | 6.5±2.8*   | 4.5±3.2       | 0.6±0.15      |               | <0.001*         |

*P value is of statistical significant, # significant group by post hoc tests for one-way ANOVA.

Group 1= diabetic patient with foot ulcer, Group 2= diabetic patient without foot ulcer, Group 3= healthy control, Hb= hemoglobin, SD= standard deviation, HDL= high density lipoprotein, LDL= low density lipoprotein.

Table 2. TLR-9 gene polymorphism and their allele frequencies in the studied groups.

| TLR-9 gene polymorphism results | Group1(G1) | Group2(G2) | Group3(G3) | All groups | G1 with G2 | G1 with G3 | G2 with G3 |
|--------------------------------|------------|------------|------------|------------|------------|------------|------------|
|                                | N=59 | N=60 | N=59 | N=59 | P | OR(95%CI) | P | OR(95%CI) | P |
| TLR-9 genotypes                |       |       |       |       |   |           |   |           |   |
| TT                             | 45 (76.3) | 38 (63.3) | 45 (76.3) |               | 0.1 | 1.0 (0.4-2.3) | 0.5 (0.2-1.2) |
| TC                             | 13 (22.0) | 21 (35.0) | 14 (23.7) | 0.4 | 1.9 (0.8-4.1) | 1.0 (0.4-2.3) | 0.1 |
| CC                             | 1 (1.7) | 1 (1.7) | 0 (0.0) |               |           |           |           |
| TLR-9 allele                   |       |       |       |       |   |           |   |           |   |
| C allele                       | 15 (12.7) | 23 (19.2) | 14 (11.9) | 0.2 | 1.6 (0.8-3.3) | 0.9 (0.4-2.0) | 0.6 (0.3-1.2) |
| T allele                       | 103 (87.3) | 97 (80.8) | 104 (88.1) |               | 0.2 | 0.8 | 0.1 |

Group 1= diabetic patients with foot ulcer, Group 2= diabetic patients without foot ulcer, Group 3= healthy control, CI= confidence interval, OR= odd ratio, TLR= toll-like receptor.
Figure 3. Gel electrophoresis of RFLP-PCR product of TLR-9 (1237 T/C) gene polymorphism digested with BstNI restriction enzyme were separated by using 3% agarose gel at 60 volt for 3hrs. Homozygous TT genotype and Heterozygous TC genotype results. M: DNA molecular marker 50bp size by ethidium bromide stained bands in the gel.

4. Discussion

The current study showed a considerable difference (P<0.001) of age on the development of DFU. A similar observation was reported in the studies of [10, 11, 12], whose found that the prevalence of DFU increases with rising age. This is reasonable because the old age patients have higher risk for development of DFU. According the gender, our study revealed to a non statistical association (P=0.6). This result was related with study by [13, 14], whose found a non statistical association in pervasiveness of DFU in patients gender. In contrast to [9, 15], show that male sex has been recognized as a riskiness factor to the development of diabetic foot ulcer. This may be due to a small female population in the study. Additionally, this study refers to enormous difference about smoking on the prevalence of DFU and these results was compatible with the results of [16, 17] whose proved a connection of cigarette smoking with the development of DFU. In the present study, the finding that the mean duration of diabetes in patients with DFU was highly significant (P<0.001) compared with non-DFU patients. The present study in agreement with a study conducted by [18, 19, 20, 21] whose found that the long duration of diabetes was the main factor causing DFUs. About BMI (P<0.001) and these results were in agreement with a number of studies conducted by [22, 23], significant difference between BMI and development of DFU in T2DM. Previous studies have also shown that HbA1c was a contributory factor for DFU [13, 24]. But the current study differ from other studies conducted by [15, 25], whose demonstrated that poor glycemic control is an independent risk factor for the development of ulcers. This may be due to the method of assessing sample collection and patients number. There was no significant association between DFU group and metabolic factors that includes, cholesterol, triglyceride, HDL and LDL, this consequence was correlated with a study accomplished by (25). In regard to serum urea and creatinine there was high significant differences in DFU (P<0.001) compared with other studied groups. The result of this study agrees with other studies conducted by [15, 26], whose demonstrated that the possibility of kidney dysfunction associated with the diabetic foot disease. According to the several studies, the development and complications of type 2 diabetes are associated with different types of toll like receptors [27, 28]. Genetically susceptibility
to diabetic complication such as a foot ulcer involving several factors that are associated with the stimulation of immune response. TLR-9 is a necessary endosomal member of the TLRs family [4] reported that TLR-9 was associated with severity of diabetic injury. Unfortunately, in Iraq there are no studies involved the effect of TLR9-1237T/C polymorphism on development of DFU and T2DM. Our study demonstrated a non correlation among all groups of TLR-9 (1237T/C) genotype frequencies (P=0.5). Also, allele frequencies showed non significant difference among all groups for TLR-9 (1237T/C) gene polymorphism (P=0.5) and these results were close to the results of [8], who found that the recurrence of TLRs-2, 4 and 9 gene polymorphism don’t detected in T2DM as well as coronary artery disease (CAD), otherwise, it is still not full clarified correlation of the very relatively low-frequencies of these variants of TLRs with the incidence of T2DM and CAD in the or population. Furthermore, the present study results were disagreement with the studies conducted by [4, 29], whose reported that the (TC and CC) polymorphic genotypes frequencies have high significant among DFU and T2DM groups compared with healthy individuals. Ethnic diversity may be linked with the variation of above results.

5. Conclusion

Current study have shown that the TLR-9 gene polymorphism may not have a role in diabetic foot ulcer disease. There is no significant association between this polymorphism gene and development of diabetic foot ulcer disease in T2DM.

6. References

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