Toll-like receptors-2 and -9 (TLR2 and TLR9) gene polymorphism in patients with type 2 diabetes and diabetic foot

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Abstract
Toll-like receptors (TLRs) are innate immune receptors that mediate the inflammatory response in diabetes mellitus (DM). The aim of this study is to evaluate the association of TLR2 and TLR9 gene polymorphism in patients with type 2 DM (T2DM) and diabetic foot (DF).

The study included 90 subjects divided into group I (30 patients with T2DM and DF), group II (30 patients with T2DM and no evidence of DF), and group III (normal control subjects). TLR2 (1350 T/C, rs3804100) and TLR9 (1237 T/C, rs5743836) genotyping was performed by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) technique for all subjects.

There was a statistically significant difference in the distribution of TLR9-1237 T/C genotypes between groups I and II (P < .029) as well as between groups I and III (P < .001). Calculated risk estimation revealed that TLR9-1237 polymorphism conferred almost 20 times increased risk of DF disorders in T2DM (OR = 20.95%, CI = 5.38–74.30). There was no statistical difference in the distribution of TLR2-1350 T/C genotypes between the 3 groups.

TLR9-1237 T/C gene polymorphism may be considered as a molecular risk for DF among patients with T2DM.

Abbreviations: BMI = body mass index, CI = confidence interval, DM = diabetes mellitus, DF = diabetic foot, OR = odds ratio, PCR = polymerase chain reaction, SD = standard deviation, T2DM = type 2 diabetes mellitus, TLR = toll-like receptor.

Keywords: diabetic foot, gene polymorphism, toll-like receptors, type 2 diabetes mellitus

1. Introduction
Persistent hyperglycemic microenvironment in type 2 diabetes mellitus (T2DM) leads to the development of complications like cardiovascular disease, neuropathy, nephropathy, and retinopathy. Wound healing impairment is a serious complication of T2DM which contributes to the high percentage of amputations performed worldwide. As per recent data, around 25% of T2DM patients develop nonhealing wounds once in their life. The reason could be that hyperglycemia in T2DM decreases the levels of cytokines and growth factors essential for wound healing. The immune system is also compromised in patients with T2DM which generally leads to prolonged inflammation and unresolved infection in the wound which either takes a long time to heal or does not heal at all.

Toll-like receptors (TLRs) are a family of genetically conserved transmembrane receptors involved in the innate immunity and pathogen recognition. Recognition of pathogen-associated molecular patterns by TLRs activates signaling events that induce the expression of effector molecules, such as cytokines and chemokines, controlling the adaptive immune responses.

Genetic variations within genes encoding these receptors have an important influence on the pathogenesis of inflammatory diseases. Variations within genes of the family of innate immune receptors may account, in part, for the inherited differences in the susceptibility to autoimmune or inflammatory diseases. The ability of certain individuals to respond properly to TLR ligands may be impaired by single nucleotide polymorphisms within TLR genes, resulting in an altered susceptibility to the disease. Considering the potential role of TLR pathway in the overall immune reconstitution, we assessed TLR2 and 9 gene polymorphism in patients with T2DM with and without diabetic foot (DF) as a herald to complete studying of other members of the TLR family in such a common multisystemic inflammatory condition in further studies.

2. Patients and methods
2.1. Study population
The present study included 90 Egyptian subjects. Group I included 30 patients (16 males and 14 females) with T2DM and DF, all patients were on insulin treatment and 13 of them were smokers. Their mean (standard deviation ±SD) age, diabetes mellitus (DM) duration, and body mass index (BMI)
were 58±9.3 years, 14.6±7.2 years, and 29.7±5.5 kg/m², respectively. Group II included 30 patients (9 males and 21 females) with T2DM with no evidence of DF, 13 patients were on insulin treatment, and 7 patients were smokers. Their mean (±SD) age, DM duration, and BMI were 33.1±9.3 years, 11.9±6.7 years, and 33.3±5 kg/m², respectively. Group III included 30 volunteer normal control subjects (7 males and 23 females), their mean (±SD) age and BMI were 38.7±12.6 and 30.2±5.2, respectively. The patients were recruited, from the internal medicine inpatient wards and outpatient clinics of Cairo University Hospitals during the period from January 2014 to January 2015. Patients who had history of cerebrovascular events, renal failure, or were on renal replacement therapy were excluded. The study protocol was approved by Cairo University ethical committee. All participants provided a written informed consent.

All participants underwent a complete screening panel, including medical history, clinical examination, and assessment of BMI. Biochemical profile included fasting and 2-hours postprandial glucose, glycated hemoglobin, creatinine clearance, total cholesterol, and triglycerides (data available at Table 1). TLR2-1350T/C and TLR9-1237T/C genotyping were performed by polymerase chain reaction (PCR)–restriction fragment length polymorphism technique. Whole blood samples were collected in sterile vacutainer containing K3EDTA to prevent blood clotting (BD, Becton, Dickinson and Company, South Carolina) from the patients. For quality control, genotyping was repeated with respect to case/control status from the control group.

2.2. Genotyping of TLR2 (1350T/C, rs3804100) and TLR9 (1237T/C, rs5743836)

Genomic DNA extraction from peripheral blood leukocytes was performed using Gene JET Whole Blood Genomic DNA purification Mini kit (Fermentas Life Sciences, Canada) following the manufacturer’s instructions. TLR2-1350T/C and TLR9–1237T/C genotyping was performed by PCR–restriction fragment length polymorphism technique. All PCR reactions were performed in a total volume of 25 μL containing 150 ng genomic DNA, 2X Dream Taq Green PCR Master Mix, 25pM of each forward and reverse primers (Fermentas, Lithuania). The PCR products were visualized by 3% agarose gel electrophoresis under UV light.

Genotyping of TLR2-1350T/C (rs3804100) was performed according to Takahashi et al.[8] The primer set used was forward: 5'-TACATTGGCATATTTGGAAA-3' and reverse: 5'-GAGTT-GCGGCAATTTCAAG-3'. The thermocycler program conducted was initial denaturation at 95 °C for 5 minutes followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 58 °C for 30 seconds, extension at 72 °C for 30 seconds, and a final extension step at 72 °C for 10 minutes. The generated amplicon is a 251 bp fragment, which was digested by MwoI enzyme (Fermentas-Lithuania). The wild type allele (T allele) produced a single band of 251 bp, while the polymorphic allele (C allele) produced 2 bands of 167 and 84 bp. For TLR9 1237T/C (rs5743836) genotyping, the primer set used was forward: 5'-ATGGGAGCAGAGACATAATGGA-3' and reverse: 5'-CTGGCTTGAGCTTGCTG-3'. The thermocycler program conducted was initial denaturation at 95 °C for 5 minutes followed by 36 cycles of denaturation at 94 °C for 40 seconds, annealing at 52 °C for 40 seconds, extension at 72 °C for 1 minute, and a final extension step at 72 °C for 10 minutes.[9] This generated a 135 bp fragment. The amplified material was digested by BstN1 enzyme (Fermentas-Lithuania).

The wild type allele (T allele) showed 2 bands of 108 and 27 bp, while the polymorphic allele (C allele) showed 3 bands of 60, 48, and 27 bp. For quality control, genotyping was repeated with respect to case/control status for 50 samples. Results of genotyping were interpreted blindly by 2 different observers, and were 100% concordant.

2.3. Statistical analysis

Data were analyzed using SPSS statistical package version 17. For numerical data, parametric data were expressed as mean, SD, and range, while nonparametric data were expressed as median and interquartile range. Qualitative data were expressed as frequency and percentage. Chi-square test or Fisher exact test were used to examine the relation between qualitative variables. Nonparametric numerical data were analyzed using Mann–Whitney U test. Unconditional logistic regression analysis was used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) for risk estimation. P-value less than .05 was considered significant. Chi-square (χ²) test was performed to assess deviation from Hardy–Weinberg equilibrium in controls.

3. Results

The mean-fasting blood glucose, 2 hours postprandial blood sugar, glycated hemoglobin, serum cholesterol, and triglycerides were significantly higher in group I in comparison to groups II and III (P <.001). The mean creatinine clearance was significantly lower in group I in comparison to groups II and III (P <.001). (Data are summarized in Table 1).

Regarding TLR2-1350 there was no statistically significant difference noticed in the distribution of the wild type or the polymorphic TLR2-1350 genotypes between the different study groups. (Data are summarized in Table 2).

TLR9 polymorphic variants (TC and CC) were detected in 24/30 (80%) of group I, 22/24 were heterozygous (TC), and 2/24 were homozygous (CC). In group II, TLR9 polymorphic variants (TC and CC) were detected in 15/30 (50%), 15/15 were heterozygous (TC), and no patient was homozygous (CC). In group III, TLR9 polymorphic variants (TC and CC) were detected in 5/30 (16.9%), 5/5 of patients were heterozygous (TC), and no patient was homozygous (CC).

The frequency of TLR9-1237 polymorphism (TC+CC) was significantly higher in group I when compared to group II (P < .002) and group III (P < .001).

The frequency of TLR9-1237 polymorphism (TC+CC) was significantly higher in group I when compared to group III (P < .013).

The frequency of TLR9-1237 (TC) heterozygous genotype was significantly higher in group I when compared to group III (P < .001); however, it was nonsignificantly higher when compared to group II.

The frequency of TLR9-1237 (CC) homozygous genotype was nonsignificantly higher in group I when compared to groups II and III.

Calculated risk estimation revealed that TLR9-1237 polymorphism (TC+CC) conferred almost 20 times increased risk of DF disorders in T2DM (OR = 20, 95% CI = 5.38–74.30). (Results are summarized in Table 3).

4. Discussion

TLRs are pivotal innate immune receptors that induce inflammatory responses and their expression and activation is
Table 1

Demographic and laboratory data of the studied groups.

| Groups                | DM + DF (1) | DM only (2) | Control (3) | All P       | Pairwise comparisons       |
|-----------------------|-------------|-------------|-------------|-------------|---------------------------|
| **Groups**            |             |             |             |             |                           |
| **Age, y**            | 58.0±9.3    | 53.1±9.3    | 38.7±12.6   | <.001       | 0.094                     |
| **Sex**               |             |             |             |             | <.001                     |
| Male                  | 16 (53.3)   | 9 (30.0)    | 7 (23.3)    |             | <.001                     |
| Female                | 14 (46.7)   | 21 (70.0)   | 23 (76.7)   |             |                           |
| **Body weight**       | 79.7±15.1   | 86.0±14.4   | 78.4±12.8   | .083        | 0.054                     |
| **Height, cm**        | 163.7±5.5   | 160.4±7.0   | 161.4±6.2   | .037        | 0.022                     |
| **BMI**               | 29.7±5.5    | 33.3±5.0    | 30.2±5.2    | .012        | 0.005                     |
| **Pulse, bpm**        | 80.7±5.5    | 79.7±8.1    | 77.9±6.6    | .300        | 0.500                     |
| **SBP, mm Hg**        | 130.0±20.5  | 132.0±26.5  | 124.5±18.7  | .460        | 0.958                     |
| **DBP, mm Hg**        | 80.0±12.3   | 83.7±17.3   | 81.5±13.3   | .876        | 0.628                     |
| **DM duration**       | 14.6±7.2    | 11.9±6.7    | NA          | .149        |                           |
| **FBS, mg/dL**        | 161.1±49.9  | 146.3±52.8  | 85.0±6.8    | <.001       | 0.085                     |
| **2HPP, mg/dL**       | 351.5±107.2 | 274.7±108.3 | 105.7±16.4  | <.001       | 0.077                     |
| **HbA1c**             | 8.2±1.4     | 7.7±0.7     | 6.4±0.2     | <.001       | 0.068                     |
| **HDL cholesterol, mg/dL** | 223.5±61.8  | 197.4±50.3  | 167.0±28.1  | <.001       | 0.108                     |
| **TG, mg/dL**         | 156.6±35.0  | 132.8±29.1  | 126.9±26.7  | <.001       | 0.058                     |
| **Blood urea, mg/dL** | 46.3±27.2   | 40.5±27.9   | 31.3±11.3   | .281        |                           |
| **Creatinine, mg/dL** | 1.4±0.4     | 1.1±0.4     | 0.9±0.2     | .113        |                           |
| **Creatinine clearance** | 68.9±31.7   | 92.0±38.9   | 131.7±41.6  | <.001       | 0.005                     |
| **AST, μL**           | 35.3±18.6   | 29.8±15.4   | 23.3±10.1   | <.001       | 0.011                     |
| **ALT, μL**           | 35.3±37.5   | 29.9±11.9   | 19.6±6.6    | <.001       | 0.088                     |
| **PT, s**             | 13.2±1.1    | 13.2±1.0    | 12.0±0.6    | <.001       | 0.099                     |
| **Hb, g/dL**          | 10.7±2.0    | 10.6±1.7    | 10.2±1.7    | <.001       | 0.087                     |
| **RBCs, x10^6**       | 4.1±0.8     | 3.9±0.6     | 4.4±0.4     | <.002       | 0.195                     |
| **WBCs, x10^3**       | 7.2±3.2     | 6.7±2.9     | 4.7±1.9     | <.001       | 0.558                     |
| **Platelets, x10^3**  | 281.4±91.3  | 266.5±87.0  | 333.7±91.2  | .005        | 0.464                     |

ALT = alanine transaminase, AST = aspartate transaminase, BMI = body mass index, FBS = fasting blood sugar, Hb = hemoglobin, 2HPP = 2-hours post-prandial, DM = diabetes mellitus, DF = diabetic foot, 2DF = diabetes mellitus without diabetic foot (group 2), DM + = diabetes mellitus with diabetic foot (group 1), DM + DF = diabetes mellitus without diabetic foot (group 2), **P** = pair-wise comparison, SD = standard deviation, TG = triglycerides, WBC = white blood cell.

Increased in a plethora of inflammatory disorders including DM and its complications. Persistent hyperglycemia in T2DM elicits chronic low grade inflammation and the innate immune system. Genetic susceptibility to secondary complications of T2DM like DF ulcer is multifactorial and risk may involve factors which are related to the activation of the immune system. Previous studies showed that increased TLR expression, signaling, and activation may contribute to the increased inflammatory status in the human diabetic wounds, since TLR1, 2, 4, and 6 mRNA expression were found to be significantly increased in wounds of diabetic patients compared with those in nondiabetic subjects (P < .05). [17]

TLR2 is an important extracellular member in mammalian toll family of leucine rich receptors. It is known to be a signaling receptor for many microbial products including whole gram-positive bacteria and mycoplasma. Antimicrobial property of TLR2 is evident from the fact that the TLR2 deficient mouse strain is more prone to infection with gram-positive bacteria Staphylococcus aureus and shows defective clearance of spirochaetes after infection by Borrelia burgdorferi as compared to their wild type counterparts. [17] Also it has been shown that TLR2 is a potent player in the wound-healing mechanism. [23]

The assessment of TLR2-1350 T/C gene polymorphism in our Egyptian control subjects revealed that the frequency of wild genotype (TT) was 87.7%, and the heteromutant genotype (TC) was 13.3%, while none of them had the homomutant genotype (CC). These results were close to those reported in Caucasians being 10.2% to 15% and in Norway being 15% for the TC heteromutant genotype and 0% for the CC homomutant genotype. [21] Purdue et al. [22] showed that the frequencies of the TC and CC genotypes in the United States of America were 12.9% and 0.5%, respectively. On the other hand, these frequencies were not in accordance with that reported in the far east being 47.4% for the heteromutant and 4.7% for the homomutant genotypes in Chinese, [23, 41] 1.1% for the heteromutant and 7.9% for the homomutant genotypes in Japanese, [24] and 38% for the heteromutant and 10.6% for the homomutant genotypes in Koreans. [24] The difference in results could be attributed to the ethnic differences between the studied populations.

Our study revealed that the frequency of TLR2-1350 T/C polymorphism in both group I and group II was 96.7% for the wild genotype (TT), 3.3% for the heteromutant genotype (TC), and no one was homomutant (CC). Statistical analysis revealed that there was no significant difference encountered in the distribution of the polymorphic genotypes of TLR2 between the 3 groups.

Dasu and Martin [17] found that TLR2 mRNA expression was increased significantly in wounds of patients with diabetes compared with nondiabetic wounds. Mohammad et al. [25] reported an increased TLR2 expression in bone marrow derived macrophage of nonobese diabetic mice. Creely et al. [26] showed increased TLR2 expression in the adipose tissue of type 2 diabetes (T2DM) patients. TLR9, expressed primarily by the subsets of B cells and myeloid cells in humans, is involved in both systemic as well as local inflammatory responses. [27] TLR9 is an important endosomal member of TLR family which causes wound necrosis by
### Table 2

**TLR2 results in the 3 study groups.**

|       | Group 1 (G1) N = 30 | Group 2 (G2) N = 30 | Group 3 (G3) N = 30 | All groups | G1 with G2 | G1 with G2 (DM + DF) with group 3 (control) | G2 (DM) with GROUP 3 (control) |
|-------|---------------------|---------------------|---------------------|------------|------------|---------------------------------------------|-------------------------------|
|       | N %                 | N %                 | N %                 | P          | P          | OR 95% CI                                   | OR 95% CI                     |
| **TLR2** |                      |                      |                      |            |            |                                            |                              |
| TT (Wild) | 29 96.7            | 29 96.7             | 26 86.7             | .2         | 1          | 353 1 (Reference)                           | ND                             |
| TC (hetero) | 1 3.3              | 1 3.3               | 4 13.3              | ND         | ND         | ND 0.22                                    | 0.02–2.14                     |
| **TLR2 allele** |                  |                      |                      |            |            |                                            |                              |
| T Allele | 59 98.3            | 59 98.3             | 56 93.3             | .2         | 1          | 364 1 (Reference)                           | ND                             |
| C Allele | 1 1.7              | 1 1.7               | 4 6.7               |            |            | 0.24 0.03–2.19                             | 0.24 0.03–2.19                 |

|       | Group 2 (DM) with GROUP 3 (control) | Group 3 (control) |
|-------|-------------------------------------|-------------------|
| **CI** |                                     |                   |

* = We could not correlate TLR2 gene with other parameters within each group separately due to insufficient number.

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### Table 3

**TLR9 results in the 3 study groups.**

|       | Group 1 (G1) N = 30 | Group 2 (G2) N = 30 | Group 3 (G3) N = 30 | All groups | G1 with G2 | G1 with G2 (DM + DF) with group 3 (control) | G2 (DM) with GROUP 3 (control) |
|-------|---------------------|---------------------|---------------------|------------|------------|---------------------------------------------|-------------------------------|
|       | N %                 | N %                 | N %                 | P          | P          | OR 95% CI                                   | OR 95% CI                     |
| **TLR9** |                      |                      |                      |            |            |                                            |                              |
| TT (Wild) | 6 20                | 15 50               | 25 83.3             | <.001      | .029       | <.001 1 (Reference)                         | –                              |
| TC (hetero) | 22 73.3             | 15 50               | 5 16.7              | <.001      | .11        | <.001 18.33                                | 4.91–68.49                    |
| CC (homo) | 2 6.7               | 0 0                 | 5 16.7              | ND         | ND         | .492                                        | ND                             |
| TC + CC (polymorphism) | 24 80             | 15 50               | 5 16.7              | ND         | ND         | ND 20                                      | 5.38–74.30                    |
| **TLR9 allele** |              |                      |                      |            |            |                                            |                              |
| T allele | 34 56.7             | 45 75               | 55 91.7             | <.001      | .054       | <.001 1 (Reference)                         | –                              |
| C allele | 26 43.3             | 15 25               | 5 8.3               | ND         | 8.4        | 2.95–24.0                                   | 3.67 1.24–10.86               |

|       | Group 2 (DM) with GROUP 3 (control) | Group 3 (control) |
|-------|-------------------------------------|-------------------|
| **CI** |                                     |                   |

* = confidence interval, DM + DF = diabetes mellitus with diabetic foot (group 1), DM = diabetes mellitus (without diabetic foot [group 2]), ND = not determined, OR = odds ratio, TLR = toll-like receptor.
promoting inflammation. The increased expression of TLR9 with the increase in the wound grade suggests that the TLR9 expression directly modulates the severity of diabetic wounds. Singh et al. found that TLR9 message and protein were higher in diabetic wounds compared to control wounds. In our study, the frequency of TLR9-1237T/C polymorphic genotypes in the control group was 16.7% for the heterozygous genotype (TC), while none of them had homozygous genotype (CC). These frequencies were close to that reported in Americans (TC = 28.3% and CC = 2.4%), Portuguese (TC = 18.7% and CC = 0.8%), and Italians (TC = 17.3% and CC = 1.7%). On the other hand, our results were not in agreement with the Chinese study reported by Junjie et al. being 46.9% and 9% for the polymorphic genotypes TC and CC, respectively. This could be due to the ethnic differences between the studied populations.

Our study showed that in group I, the frequency of TLR9 heterozygous genotype (TC) was 73.3% and the homozogous genotype (CC) was 6.7% while in group II the frequency of TLR9 heterozygous genotype (TC) was 50% and the homozogous genotype (CC) was 0%.

The statistical analysis revealed that the frequency of TLR9-1237T/C polymorphic variants (TC and CC) was significantly higher in group I compared to group II (P < 0.001), but it was nonsignificantly higher in group I compared to group II. The calculated risk estimation in our study revealed that TLR9-1237 polymorphism (TC+CC) conferred almost 20 times increased risk of DF in patients with T2DM (OR = 20, 95% CI = 5.38–74.30).

Liu et al. studied the frequency of TLR2, 4, and 9 gene polymorphisms in Chinese population and their susceptibility to type 2 diabetes and coronary artery disease. They examined several TLR family gene variants that have been commonly reported in Western countries, 2 of them were TLR9 promoter-1237T/C polymorphism, and TLR2 Arg677Trp and Arg753Gln polymorphisms in all subjects. However, they did not detect the presence of any variant for these single nucleotide polymorphisms in their subjects.

It was reported that the Korean population had low frequency of TLR9-1237T/C (0.3%). In Japanese population, no TLR9-1237T/C polymorphism was present in 183 patients with systemic lupus erythematosus (SLE) and 196 controls. But high frequency of the variant allele was reported in the Caucasian population (11%–16%). Lazarus et al. showed that the genetic variation at position-1237 is associated with an increased risk of asthma in European Americans. It is not clear whether the very low frequency of these TLR9 variants would be related to the ethnic differences between the studied populations.

A multicenter trial could improve the credibility of the results and help in building a regression model. Studying TLR2 and 9 only is another limitation of our work. Studying other TLR members could widen the scope of our understanding of the pathogenesis of such diabetic complication.

In conclusion, our results suggest a potential role for the host genetic background in DF susceptibility and demonstrate that there is an association between TLR9-1237 T/C polymorphism and the risk of DF. Deeper insights into the pathogen mechanism underlying TLR9 mediated mechanisms in DF are needed. Ultimately, functional studies dissecting the role of this gene polymorphism may allow the identification of potential therapeutic targets.

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