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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 TLR2-1350T/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.[1] The reason could be that hyperglycemia in T2DM decreases the levels of cytokines and growth factors essential for wound healing.[2] 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.[3]

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.[4,5]

Genetic variations within genes encoding these receptors have an important influence on the pathogenesis of inflammatory diseases.[6] 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.[7] Considering the potential role of TLRs 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)

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were 58 ± 9.3 years, 14.6 ± 7.2 years, and 29.7 ± 5.5 kg/m^2, 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 53.1 ± 9.3 years, 11.9 ± 6.7 years, and 33.3 ± 5 kg/m^2, 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). TLR2-1350 T/C and TLR9-1237 T/C genotyping were performed by polymerase chain reaction (PCR)–restriction fragment length polymorphism technique. Whole blood samples were collected in sterile vacutainer containing k_3EDTA 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 leucocytes was performed using Gene JET Whole Blood Genomic DNA purification Mini kit (Fermentas Life Sciences, Canada) following the manufacturer's instructions. TLR2-1350 T/C and TLR9-1237 T/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 TaqGreen 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 TLR2-1350 T/C (rs3804100) was performed according to Takahashi et al.[8] The primer set used was forward: 5′-TCATTTGGCATCATTGGAAA-3′ and reverse: 5′-GAGTTGCGGCAAATTCAAAG-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′-CTGCTTGCAGTTGACTGTGT-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 62 °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 BstNI 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 (χ^2) 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 T/C 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 ($P < .029$) and group III ($P < .001$).

The frequency of TLR9-1237 polymorphism (TC + CC) was significantly higher in group II 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[10] and their expression and activation is

Table 1

Demographic and laboratory data of the studied groups.

	Groups			All	Pairwise comparisons		
Mean ± SD	DM + DF (1)	DM only (2)	Control (3)	*P*	1*2	2*3	1*3
Age, y	58.0 ± 9.3	53.1 ± 9.3	38.7 ± 12.6	<.001	0.094	<0.001	<0.001
Sex	No (%)	No (%)	No (%)	.039			
Male	16 (53.3)	9 (30.0)	7 (23.3)				
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	0.054	0.947
Height, cm	163.7 ± 5.5	160.4 ± 7.0	161.4 ± 6.2	.037	0.022	0.114	0.130
BMI	29.7 ± 5.5	33.3 ± 5.0	30.2 ± 5.2	.012	0.005	0.037	0.382
Pulse, bpm	80.7 ± 5.5	79.7 ± 8.1	77.9 ± 5.6	.300	0.500	0.523	0.095
SBP, mm Hg	130.0 ± 20.5	132.0 ± 26.5	124.5 ± 18.7	.460	0.958	0.305	0.258
DBP, mm Hg	80.0 ± 12.3	83.7 ± 17.3	81.5 ± 13.3	.876	0.628	0.689	0.994
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 ± 8.8	<.001	0.085	<0.001	<0.001
2HPP, mg/dL	315.1 ± 107.2	274.7 ± 108.3	105.7 ± 16.4	<.001	0.077	<0.001	<0.001
HbA1c	8.2 ± 1.4	7.7 ± 0.7	6.4 ± 0.2	<.001	0.068	<0.001	<0.001
S cholesterol, mg/dL	223.5 ± 61.8	197.4 ± 50.3	167.0 ± 28.1	<.001	0.108	0.004	<0.001
TG, mg/dL	156.6 ± 35.0	138.2 ± 29.1	126.9 ± 26.7	<.001	0.058	0.035	<0.001
Blood urea, mg/dL	46.3 ± 27.2	40.5 ± 27.9	31.3 ± 11.3	.281			
S 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	<0.001	<0.001
AST, μ/L	35.5 ± 18.6	32.8 ± 13.4	23.2 ± 10.1	<.001	0.711	<0.001	<0.001
ALT, μ/L	35.3 ± 37.5	29.9 ± 11.9	19.6 ± 8.6	<.001	0.888	<0.001	<0.001
PT, s	13.2 ± 1.1	13.2 ± 1.0	12.0 ± 0.6	<.001	0.899	<0.001	<0.001
Hb, g/dL	10.7 ± 2.0	10.6 ± 1.7	12.0 ± 2.17	<.001	0.871	<0.001	<0.001
RBCs, $\times 10^6$	4.1 ± 0.8	3.9 ± 0.6	4.4 ± 0.4	.002	0.195	<0.001	0.042
WBCs, $\times 10^3$	7.2 ± 3.2	6.7 ± 2.9	4.7 ± 1.9	<.001	0.558	<0.001	<0.001
Platelets, $\times 10^3$	281.4 ± 91.3	266.5 ± 87.0	333.7 ± 51.2	.005	0.464	0.001	0.025

ALT = alanine transaminase, AST = aspartate transaminase, BMI = body mass index, bpm = beats per minute, DBP = diastolic blood pressure, DM + DF = diabetes mellitus with diabetic foot (group 1), DM = diabetes mellitus without diabetic foot (group 2), FBS = fasting blood sugar, Hb = hemoglobin, 2HPP = 2-hours post-prandial, PT = prothrombin time, RBC = red blood corpuscle, SBP = systolic blood pressure, SD = standard deviation, TG = triglycerides, WBC = white blood cell.

increased in a plethora of inflammatory disorders including DM and its complications.[11–14] Persistent hyperglycemia in T2DM elicits chronic low grade inflammation and the innate immune system.[15]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.[16] 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.[18] Antiinfectious 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.[19] Also it has been shown that TLR2 is a potent player in the wound-healing mechanism.[20]

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 for the heteromutant and 7.9% for the homomutant genotypes in Japanese,[7] 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 TLR2-1350T/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	Group 1 (DM + DF) with group 3 (control)			Group 2 (DM) with GROUP 3 (control)		
	N	%	N	%	N	%	*P*	*P*	*P*	OR	95% CI	*P*	OR	95% CI
TLR2														
TT (wild)	29	96.7	29	96.7	26	86.7	.2	1	.353	1 (Reference)	–	.353	1 (Reference)	–
TC (hetero)*	1	3.3	1	3.3	4	13.3	ND	ND	ND	0.22	0.02–2.14	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)	–	.364	1 (Reference)	–
C allele	1	1.7	1	1.7	4	6.7				0.24	0.03–2.19		0.24	0.03–2.19

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.

* We could not correlate *TLC2* gene with other parameters within each group separately due to insufficient number.

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	Group 1 (DM + DF) with group 3 (control)			Group 2 (DM) with group 3 (control)		
	N	%	N	%	N	%	*P*	*P*	*P*	OR	95% CI	*P*	OR	95% CI
TLR9														
TT (wild)	6	20	15	50	25	83.3	<.001	.029	<.001	1 (Reference)	–	.013	1 (Reference)	–
TC (hetero)	22	73.3	15	50	5	16.7	<.001	.11	<.001	18.33	4.91–68.49		5	1.51–16.56
CC (homo)	2	6.7	0	0	0	0	.2	.492	.492	ND	–	ND		–
TC + CC (polymorphism)	24	80	15	50	5	16.7				20	5.38–74.30		5	1.51–16.56
TLR9 allele														
T allele	34	56.7	45	75	55	91.7	<.001	.054	<.001	1 (Reference)	–	.026	1 (Reference)	–
C allele	26	43.3	15	25	5	8.3				8.4	2.95–24.0		3.67	1.24–10.86

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.[28] Singh et al[29] 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 heteromutant genotype (TC), while none of them had homomutant 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%).[30] On the other hand, our results were not in agreement with the Chinese study reported by Junjie et al[23] 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 heteromutant genotype (TC) was 73.3% and the homomutant genotype (CC) was 6.7% while in group II the frequency of TLR9 heteromutant genotype (TC) was 50% and the homomutant 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 III ($P<.001$), 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[7] 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%).[31] In Japanese population, no TLR9-1237T/C polymorphism was present in 183 patients with systemic lupus erythematosus (SLE) and 198 controls.[32] But high frequency of the variant allele was reported in the Caucasian population (11%–16%).[33] Lazarus et al[34] 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 TLR variants would be related to the low incidence of diseases such as diabetes and coronary artery disease in the Oriental populations, compared with the western ones.[7]

It should be noted that the occurrence of diabetes and/or its complications depends on the interaction among multiple risk factors, like the presence of different risk alleles, environmental factors, and the lifestyle. The contribution of any single-gene polymorphism is rather small and the interactive effect of several factors may lead to an under- or overestimation of the role of a given gene polymorphism in determining the phenotype.[7] Therefore, the results might not apply to groups with different genetic or environmental backgrounds. Moreover, studying the association between *TLRs* gene variant and susceptibility to T2DM and/or its complication will strengthen our understanding of the link between innate immunity and T2DM.

The small number of studied subjects and lack of appropriate sample size quantification are among the limitations of our study. 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 TLR9-1237T/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.

References

[1] Singh K, Agrawal Nk, Gupta SK, et al. Association of variant rs7903146 (C/T) single nucleotide polymorphism of TCF7L2gene with impairment in wound healing among north Indian type 2 diabetes population: a case-control study. Int J Lower Extrem Wounds 2013;12:310–5.

[2] Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. Physiol Rev 2003;83:835–70.

[3] Singh K, Agrawal NK, Gupta SK, et al. Differential expression of Toll like receptor 4 in type2 diabetic patients with impaired wound healing. J Diabetes Metab 2013;4:260.

[4] Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2001;2:675–80.

[5] Andreakos E, Foxwell B, Feldmann M. Is targeting Toll like receptors and their signaling pathway a useful therapeutic approach to modulating cytokine-driven inflammation? Immunol Rev 2004;202:250–65.

[6] Schröder NWJ, Schumann RR. Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease. Lancet Infect Dis 2005;5:156–64.

[7] Liu F, Lu W, Qian Q, et al. Frequency of TLR2 4, and 9 gene polymorphismsin Chinese population and their susceptibility to type 2 diabetes and coronary artery disease. J Biomed Biotechnol 2012;2012: 373945.

[8] Takahashi M, Chen Z, Watanabe K, et al. Toll-like receptor 2 gene polymorphisms associated with aggressive periodontitis in Japanese. Open Dent J 2011;5:190–4.

[9] Rahman HAA, Khorshied MM, Khorshid OMR, et al. Toll-like receptor 2 and 9 genetic polymorphisms and the susceptibility to B cell non-Hodgkin lymphoma in Egypt. Ann Hematol 2014;93:1859–65.

[10] Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004; 4:499–511.

[11] Rosa Ramirez S, Ravi Krishna Dasu M. Toll-like receptors and diabetes complications: recent advances. Curr Diabetes Rev 2012;8:480–8.

[12] Acosta JB, del Barco DG, Vera DC, et al. The pro-inflammatory environment in recalcitrant diabetic foot wounds. Int Wound J 2008; 5:530–9.

[13] Dasu MR, Thangappan RK, Bourgette A, et al. TLR2 expression and signaling-dependent inflammation impair wound healing in diabetic mice. Lab Invest 2010;90:1628–36.

[14] Dasu MR, Jialal I. Amelioration in wound healing in diabetic toll-like receptor-4 knockout mice. J Diabetes Complications 2013;27:417–21.

[15] Bergmann C, Bachmann HS, Bankfalvi A, et al. Toll-like receptor 4 single-nucleotide polymorphism Asp299Gly and Thr399Ile in head and neck squamous cell carcinoma. J Transl Med 2011;9:139–47.

[16] Amoli MM, Hasani-Ranjbar S, Roohipour N, et al. VEGF gene polymorphism association with diabetic foot ulcer. Diabetes Res Clin Pract 2011;93:215–9.

[17] Dasu MR, Martin SJ. Toll-like receptor expression and signaling in human diabetic wounds. World J Diabetes 2014;5:219–23.

[18] Flo TH, Halaas O, Trop S, et al. Differential expression of Toll-like receptor 2 in human cells. J Leukoc Biol 2001;69:474–81.

[19] Kuo IH, Mendini AC, Yoshida T, et al. Activation of epidermal Toll-like receptor 2 enhances tight junction function: Implications for atopic dermatitis and skin barrier. J Invest Dermatol 2013;133:988–98.

[20] Xu Y, Zhou Y, Lin H, et al. Toll-like receptor 2 in promoting angiogenesis after acute ischemic injury. Mol Med 2013;31:555–60.

[21] Dumhur A, Zibar L, Wagner J, et al. Association studies of gene polymorphisms in toll-like receptors 2 and 4 in Croatian patients with acute myocardial infarction. Scand J Immunol 2012;75:517–23.

[22] Purdue MP, Lan Q, Wang SS, et al. A pooled investigation of toll-like receptor gene variants and risk of non-Hodgkin lymphoma. Oxford Univ Press Carcinog 2009;30:275–81.
[23] Junjie X, Songyao MS, Yanyan S, et al. The association between Toll-like receptor 2 single-nucleotide polymorphisms and hepatocellular carcinoma susceptibility. BMC Cancer 2012;12:57.
[24] Kim MK, Park SW, Kim SK, et al. Association of toll-like receptor 2 polymorphisms with papillary thyroid cancer and clinicopathologic features in a Korean population. J Korean Med Sci 2012;27:1333–8.
[25] Mohammad MK, Morran M, Slotterbeck B, et al. Dysregulated Toll-like receptor expression and signaling in bone marrow-derived macrophages at the onset of diabetes in the non-obese diabetic mouse. Int Immunol 2006;18:1101–13.
[26] Creely SJ, McTernan PG, Kusminski CM, et al. Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. Am J Physiol Endocrinol Metab; 2007;292: E740–7.
[27] Guiducci C, Tripodo C, Gong M, et al. Autoimmune skin inflammation is dependent on plasmacytoid dendritic cell activation by nucleic acids via TLR7 and TLR9. J Exp Med 2010;207:2931–42.
[28] IIiev AI, Stringaris Ak, Nau R, et al. Neuronal injury mediated via stimulation of microglial Toll-like receptor-9 (TLR9). FASEB J 2004; 18:412–4.
[29] Singh K, Agrawal NK, Gupta SK, et al. Increased expression of TLR9 associated with pro-inflammatory S100A8 and IL-8 in diabetic wounds could lead to unresolved inflammation in type 2 diabetes mellitus (T2DM) cases with impaired wound healing. J Diabetes Complications 2016;30:99–108.
[30] Carvalho A, Osório NS, Saraiva M, et al. The C allele of rs5743836 polymorphism in the human TLR9 promoter links IL-6 and TLR9 upregulation and confers increased B-cell proliferation. PLoS One 2011;6:e28256.
[31] Hur JW, Shin HD, Park BL, et al. Association study of Toll-like receptor 9 gene polymorphism in Korean patients with systemic lupus erythematosus. Tissue Antigens 2005;65:266–70.
[32] Tao K, Fujii M, Tsukumo SI, et al. Genetic variations of Toll-like receptor 9 predispose to systemic lupus erythematosus in Japanese population. Ann Rheum Dis 2007;66:905–9.
[33] Hold GL, Rabkin CS, Gammon MD, et al. CD14159C/TandTLR9-1237T/C polymorphisms are not associated with gastric cancer risk in Caucasian populations. Eur J Cancer Prev 2009;18:117–9.
[34] Lazarus R, Klimecki WT, Rabyetal BA. Single-nucleotide polymorphisms in the Toll-like receptor 9 gene (TLR9): frequencies, pairwise linkage disequilibrium, and haplotypes in three U.S. ethnic groups and exploratory case-control disease association studies. Genomics 2003; 81:85–91.