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Toll-Like Receptor 9 Serum Level and Genetic Polymorphism Linked with Type Ii Diabetes Mellitus Among Iraqi Patients

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Abstract: The rising incidence of Type 2 diabetes mellitus (T2DM) globally attaining epidemic levels and is emerging as a significant public health concern. Toll-like receptors (TLRs) are innate immune system receptors that facilitate the inflammation in diabetic mellitus disorder. The study evaluates the relationship between rs352139 SNP in *TLR9* gene in patients with T2DM in Iraq, and its effect on TLR9 level in those patients. This study included 60 T2DM cases and 40 apparently healthy controls. The SNP were identified using Sanger sequencing for rs352139. Current findings indicated a significant elevation in *TLR9* levels in T2DM patients compared to healthy people (1.182± 0.76 *versus* 0.836 ± 0.74 ng /ml, $p \leq 0.05$). T2DM risk factors include AA, GG genotypes in comparison with controls (40% *versus* 22.5%; P = 0.009; OR = 2.30 and 40% *versus* 20%; p = 0.005; OR 2.67). Patients carrying the AG and GG genotypes, had higher Serum concentrations of TLR9 in comparison with the control group (1.13 ± 0.26 *versus* 0.79 ± 0.18 ng/ml; 1.22 ± 0.18 *versus* 0.71 ± 0.16 ng/ml) respectively. This study indicate a risk association between rs352139 and T2DM. Also, rs352139 increase TLR9 serum level in patients with T2DM.

Keywords: diabetes mellitus, T2DM, toll-like receptors,TLR9, gene polymorphism, rs352139.

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Introduction

Diabetes mellitus (DM) is a prevalent chronic condition worldwide, with a notable increase in its prevalence over the past thirty years, it causes hyperglycemia by reducing insulin excretion, action, or both, Leading to disturbances of carbohydrate and fat metabolism and resulting in hyperglycemia (1,2). It is a persistent metabolic disorder marked through increased blood glucose grades (3). Diabetes mellitus type 2 is a hormonal and metabolic disorder resulting from a confluence of hereditary and environmental influences that lead to diverse alterations in insulin functionality in peripheral tissues and pancreatic beta cells. The primary variables contributing to the onset of T2DM include preexisting problems like overweight and obese, which are regarded as the principal determinants in the development of T2DM (5). Insulin resistance has become recognised as a

significant risk factor for the onset of T2DM (4,6). Toll-like receptors (TLRs) defined as a first line of defence, they are present on several cells, including immune cells, epithelial cells, microglia, and astrocytes inside the Central Nervous System, they detect pathogen-associated molecular patterns (PAMPs) (7), including host-derived chemicals generated by injured or dying cells known as damage-associated molecular patterns (DAMPs). Multiple signalling molecules, including important pro-inflammatory mediators, are induced when stimulation triggers the binding of TLRs, which in turn triggers the production of a number of adaptor proteins and downstream kinases. Truly, remarkable advancements in the field of immunology have hinted that TLRs may hold therapeutic promise in the treatment of inflammatory disorders, autoimmune disorders, microbial infections, and malignancies in humans (8). TLR9 is connected with autoimmune disorders and correlated with high-danger asthma and low-danger DM (9). Remarkably, it was found that TLR9 inactivity enhanced insulin susceptibility and ability to tolerate glucose in diabetic people. The interaction of infection and diabetes is linked to foot ulcer infection in individuals with diabetes (10). There are several gene variations and single nucleotide polymorphism (SNPs) that have been associated to T2DM. In 2003, SNPs were found in the human genome. Since then, genome-wide association studies found links among these genetic locations as well as diseases (11). Recent research finds many SNPs within the genetic material that encode human TLRs (TLR1–10) as well as demonstrate that these genetic variants may have an effect on various auto-immune disorders in humans (12). TLR9 polymorphism enhances susceptibility to T2DM and the related immunological responses, including inflammation. A study genotyped three TLR9 Polymorphisms (rs352140, rs187084, and rs5743836) within Saudi individuals, concluding that rs187084 and rs5743836 exhibited a high association with T2DM in patients from Saudi Arabia. The research offers more proof how TLR9 gene polymorphism significantly contribute to the development of T2DM in a this population (13). A separate study examined the influence of TLR-9 (1237 T/C) gene polymorphism in individuals with T2DM and diabetic foot ulcers, finding no significant variations in the distribution of genotypes and alleles of the (1237 T/C) polymorphism across the study groups (9). Another study evaluate how TLR9 polymorphism (rs352139) affects the likelihood of acquiring a cytomegalovirus (CMV) after renal transplantation, they conclude that rs352139 could reveal the likelihood of an infection with CMV following renal transplantation (14). For the same SNP, (15) Investigated the association between (rs352139) and Systemic Lupus Erythematosus (SLE), These findings indicated that these SNP could not play a significant role in determining susceptibility to SLE. (16) sought to determine if the TLR9 gene polymorphism (rs352140 and rs352139) act as a marker for the likelihood to development and growing severity of membranous nephropathy (MN) among Taiwanese individuals, and their findings indicate that TLR9 polymorphisms may be play a role in the advancement of MN.

The objective of this investigation was to assess TLR9 levels in the serum

of T2DM patients. Furthermore, to evaluate rs352139 and its association with T2DM, researchers concentrated on specific TLR9 gene SNP (rs352139) and its relationship with several autoimmune diseases. This study is the first investigation assessing (rs352139) and how it impacts on the development of T2DM, as well as its effect on TLR9 serum levels in T2DM patients and non-DM individuals in Baghdad, Iraq.

Materials and methods

Sample collection. One hundred Iraqi individuals were recruited, including sixty patients with T2DM, from attendees at Al-Tarmiah General Hospital, the National Centre for Diabetes Treatment, and Research private clinics in Baghdad, Iraq. The group consisted of 29 males and 31 females. The mean was 46.63 years of age, with a range of ages from (22-71), as determined by physicians using fasting blood sugar assessments. The control samples consisted of 20 healthy males and 20 healthy females. The average age was 44.9 years, spanning from 22 to 66 years. All subjects randomly picked, ensuring they were genetically unrelated. Five millilitres was collected of peripheral blood from each participant (diabetic patients and apparently healthy control). The blood was separated into two parts after collection. One part was put into a plain tube to be used for serum collection, and the other part was put into an EDTA tube to be used for DNA extraction. Both parts were then refrigerated at -20 degrees Celsius till the DNA extraction procedure for genomic tests involving the TLR9 gene polymorphisms rs352139.

DNA extraction. The Norgen® blood DNA extraction kit from NEB® (USA) was used to extract genomic DNA from EDTA blood. carefully following the directions provided by the maker. The DNA samples was kept at -20 °C until analysis.

Serum level of TLR9. To ascertain the serum concentration of TLR9, an enzyme-linked immunosorbent test kit obtained from Fine Biotech in China was employed, according to the manufacturer's instructions.

TLR9 gene SNP selection and genotyping. The single-nucleotide polymorphism (SNP) rs352139 in the TLR9 gene was examined. The SNP genotypes of individuals were determined using carefully created forward (5′-CTGGTTCTGAAGCCTAATTCT- 3′) and reverse (5′-TGTTGTTGTAGCTCAGGTTTA -3′) primers table (1), intended for amplifying the intronic section of the human chromosome. containing the rs352139 SNP (chr3:52224356) using PCR. Twenty-five microlitres of the PCR amplification reaction Table (2), comprised 12.5 µl of OneTaq (NEB®) mastermix, 3 µl of DNA sample, 1.5 µl of each primer, and 6.5 µl of nuclease-free water. The reaction was conducted under optimum PCR conditions for this gene, as indicated in Table (3).

Table (1): Primer sequences for detection (rs352139 in TLR9) gene.

Primers	Sequence (5`-3`)	Product size (bp)
TLR9 F	CTGGTTCTGAAGCCTAATTCT	622 bp
TLR9 R	TGTTGTTGTAGCTCAGGTTTA	622 bp

Table (2): Components of PCR amplification reaction for detection of (rs352139 in TLR9) .

Material	Volume (µl)
Master Mix	12.5
Forward primer	1.5
Reverse primer	1.5
D.W	6.5
Template DNA	3
Total	25

Table (3): PCR parameters for amplification of TLR9 gene.

Stage	Temperature	Time	Cycle No.
Initial Denaturation	94 °C	3 mins	1
Denaturation Annealing Extension	94 °C 53°C 68°C	30 sec. 45 sec. 30 sec.	30x
Final Extension	68 °C	5 mins.	1

The analysis of FASTA sequence files was performed utilising Geneious Prime software and aligned with the RefSeq of TLR9 gene, accession codes NG_033933 and NC_000003.1.

Statistical analysis. A statistical analysis was conducted to ascertain the importance of group variance. Data were expressed as mean ± standard deviation, evaluated using independent t-test and ANOVA (Duncan test), with statistical significance assessed by IBM SPSS Version 27.0. The genotypes of TLR9 were represented as percentage occurrences and notable disparities in their distribution between patient and control groups were evaluated using two-tailed Fisher's exact probability (p), odds ratio (OR), and 95% confidence intervals (CI) to determine the link between SNP and the disease, area under the curve (AUC) of TLR9 levels and we calculate the best cut off values for sensitivity and specificity using the receiver operating characteristic (ROC) curve. The estimations were performed using WinPepi 11.65 software. A probability (p) value of < 0.05 was deemed significant following the use of the rate of false discoveries modifications.

Results and discussion

Serum level of TLR9. The result showed significant increased in serum level mean of TLR-9 in T2DM patients 1.182± 0.76 compared to control 0.836 ± 0.74 with significant difference ($p \leq 0.05$), Table (4), Figure (1). ROC analysis results showed that predictive value of TLR 9 was statistically significant (AUC: 0.665; $p<0.05$). For 3.93 TLR9 cut off value, sensitivity was 0.0 and specificity was 0.98, Table (5), Figure (2). This result agree with (19) which hypothesised that the sustained hyperglycemia and tissue damage in T2DM patients may induce chronic inflammation via the activation of endosomal TLRs, such as TLR9. TLR9 gene is linked to β-cells, specialised pancreatic cells that manufacture insulin and contribute to high blood glucose levels. Immune cells are not directly involved. The innate immune system may be affected by TLR9 (17). Therefore, our findings align with the concept that TLR expression correlates with illness severity or the extent of inflammation, as indicated.

Consequently, TLR expression is elevated in numerous inflammatory illnesses such as T2DM (18). TLR9 was considerably upregulated in the wounds of patients with T2DM complexations compared to the controls.

Table (4): Serum level of TLR-9 in T2DM patients and control groups

Characteristic	Serum level of TLR9 (Mean ± S.D ng /ml)		p- value
Groups	Patients (n=60)	Control (n=40)	
Total	1.182± 0.76	0.836 ± 0.74	0.02

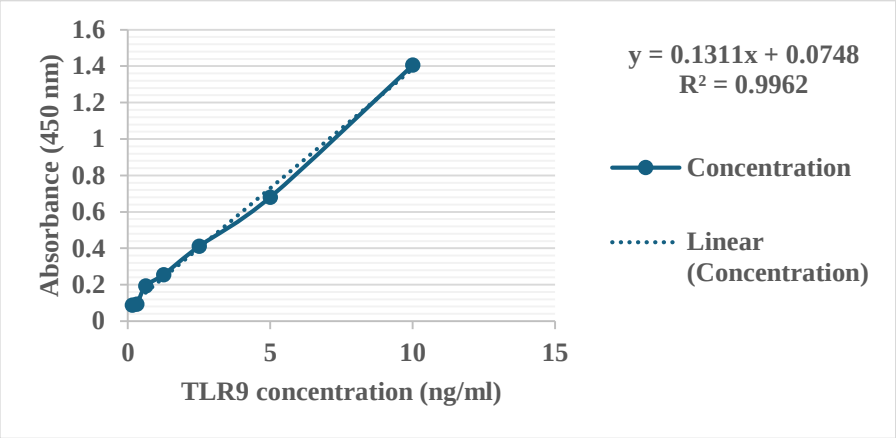

Figure (1): standards curve of Toll-like receptor 9.

Table (5): Area Under the Curve of TLR9

Area	SE	Probability	95% CI		Sensitivity	Specificity	Cut-off value
			Lower Bound	Upper Bound			
0.665	0.055	0.005	0.556	0.774	0.0	0.98	3.93

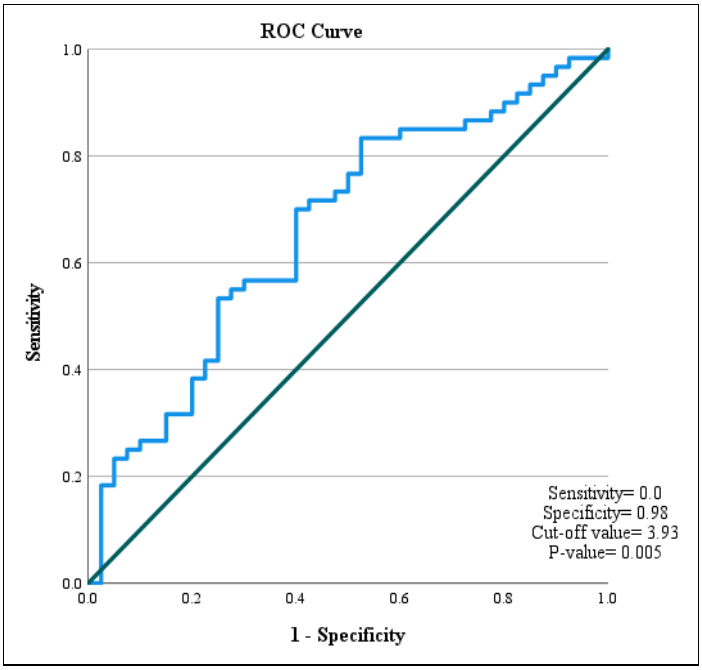

Figure (2): Receiver Operating Characteristic (ROC) Curve for TLR 9.

TLR9 gene SNP RS352139. The patient and control groups were evaluated for SNP in the TLR9 gene. According to NCBI, the TLR9 variant (6808 A>G) is located inside the TLR9 gene (chr3:52224356). To determine the SNP, the Sanger method was used to evaluate allelic variation for the SNP (rs352139), Figure (3). The FASTA sequence data were aligned to the RefSeq of TLR9 and analysed using Geneious Prime software. The frequency distribution of genotypes and alleles is shown in Table (6). In current results, the rs352139 seems to be associated with T2DM in patients carrying the homozygous wild and mutant genotypes AA, GG. A notable difference ($p \leq 0.05$) was noted in the frequency of the AA wild genotype between those with T2DM and those who were healthy, with 40% versus 22.5% (OR = 2.30, 95% CI: 0.94-5.61; P = 0.009). The GG genotype was also more common in T2DM patients (40% vs. 20% in the controls), and this difference was shown to be significant in statistics ($p \leq 0.05$) (OR = 2.67, 95% CI: 1.06-6.70; p = 0.005). 20% of T2DM and 57% of the control group had the AG genotype (OR = 0.18, 95% CI: 0.08-0.45; p = 0.06). The allele frequencies for A and G were (50%, 50%) in T2DM patients, and (51%, 49%) in the control group, respectively. the risk is increased in carriers of allele G compared to carriers of ancestral allele A (OR = 1.05, 95% CI: 0.60-1.85; OR = 0.95, 95% CI: 0.54-1.67), respectively. The rs352139 in the TLR9 gene was examined for association analysis due to the following reasons: (1) is reported to have a strong correlation with susceptibility to various autoimmune disorders, including systemic lupus erythematosus (SLE) and membranous glomerulonephritis (MGN), in Asian populations. (2) Lack of studies about the genetic polymorphisms rs352139 of TLR9 in patients with T2DM in Iraq. Future studies will need to conduct experiments to confirm the theory. Consequently, additional research is necessary to ascertain whether the polymorphism found in this study is linked to T2DM patients. Current study indicates that TLR9 polymorphism rs352139 has a substantial impact in the risk of developing T2DM in the Iraqi population. The finding of TLR9 polymorphisms as genetic risk factors for T2DM susceptibility in Iraq indicates the potential advantage of assessing this polymorphism as prognostic markers in predictive clinical testing for T2DM globally.

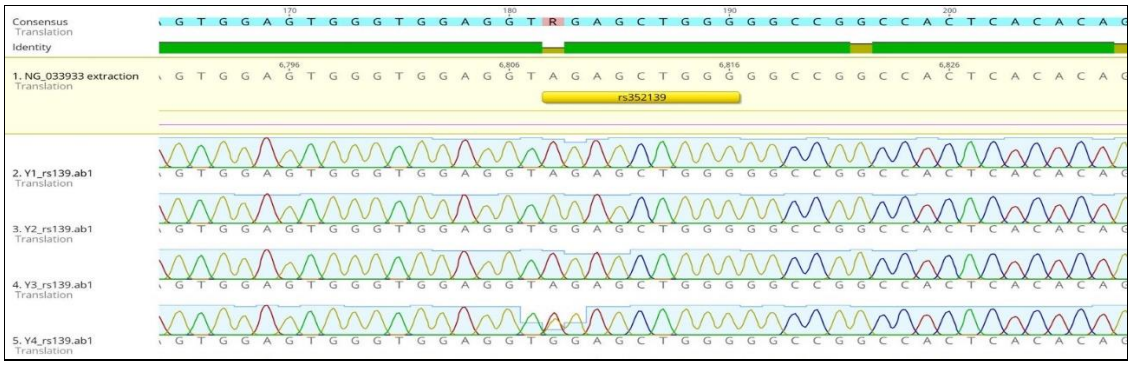

Figure (3): Chromatogram of DNA sequence for TLR9 gene Polymorphism (A>G : rs352139) demonstrating three different genotypes: AA (sequence 2), GG (sequence 3) and AA (sequence 4), AG (sequence5) as well the sequence of reference (rs352139), as reported via Geneious software analysis.

Table (6): Single-nucleotide polymorphism (rs352139) of TLR9 gene in T2DM patients and apparently healthy control and allele frequency.

TLR9 SNP rs352139 genotyping and alleles frequencies	Patients No. (%)	Control No. (%)	Odd ratio (OR)	95% confidence intervals (CI)	p-value
AA homozygous wild	24 (40.0)	9 (22.5)	2.30	0.94-5.61	0.009 *
AG heterozygous mutant	12 (20.0)	23 (57.5)	0.18	0.08-0.45	0.06 NS
GG homozygous mutant	24 (40.0)	8 (20.0)	2.67	1.06-6.70	0.005 *
Alleles frequencies					
A	60 (50.0)	41 (51.0)	0.95	0.54-1.67	0.886 NS
G	60 (50.0)	39 (49.0)	1.05	0.60-1.85	0.886 NS

*** (P ≤ 0.05) Significant, NS: Non-Significant.**

TLR9 SNP-impact on TLR9 serum level. The concentration of TLR9 according to the (6808 A>G) SNP (rs352139) between groups is shown in Table (7). The results showed that T2DM patients with the AG, GG genotypes had a considerably higher blood TLR9 level in comparison with the controls (1.13 ± 0.26 versus 0.79 ± 0.18 ng/ml; 1.22 ± 0.18 versus 0.71 ± 0.16 ng/ml), respectively, (P > 0.05). whereas the individuals with T2DM who carry the AA wild genotype had slightly higher serum levels of TLR9 (1.17 ± 0.12 ng/ml) compared to the controls (1.08 ± 0.16 ng/ml, P > 0.05). So, rs352139 may influence the inflammation via increase TLR9 production which was also associated with T2DM complexations(22).

Table (7): Concentration of TLR-9 in patients group and control group according to (rs352139) polymorphism

Genotyping of TLR9 rs352139	TLR9 level mean±SE (ng/ml)		p- value
	T2DM patients	Control	
AA	1.17 ± 0.12 ^{A}	1.08 ± 0.16 ^{A}	0.748 NS
AG	1.13 ± 0.26 ^{A}	0.79 ± 0.18 ^{A}	0.204 NS
GG	1.22 ± 0.18 ^{A}	0.71 ± 0.16 ^{A}	0.109 NS

The similar letters referred to a non-significant difference (P > 0.05) among the genotyping of the same group; NS: Non-Significant.

Conclusion

The SNP (rs352139) of this study enhanced the TLR9 production and influence the inflammation. The present study is the first examination of TLR9 polymorphism rs352139 in T2DM patients, revealing for the first time. The results indicate that the TLR9 gene, an important inflammation-related gene, could be linked to the rise of T2DM among Iraqi individuals. Patients with AG and GG genotypes had significantly elevated serum levels of TLR9 in comparison to the control group. The AA and GG genotypes and allele G demonstrated elevated in T2DM (odds ratios of 2.30 ,2.67 and 1.05) respectively, suggesting their potential as risk factors. Conversely, the AG genotype and allele A exhibited lower odds ratios of 0.18, 0.95, respectively. indicating their protective characteristics.

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