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Serum Level and Single-nucleotide Polymorphism (rs1927911) of Toll-like Receptor 4 Among Type 2 Diabetes Mellitus Iraqi Patients

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Abstract

Toll-like receptor 4 (TLR4), a member of the TLR family, plays a key role in innate immunity. Type 2 diabetes mellitus (T2DM) is a chronic condition characterized by impaired insulin sensitivity and abnormal immune responses. This study aimed to examine the association of the TLR4 single-nucleotide polymorphism (SNP) rs1927911 with T2DM susceptibility and to evaluate the SNP's effect on TLR4 levels. The study included 60 T2DM patients and 40 healthy controls. Blood samples were collected for serum analysis and DNA extraction. TLR4 levels were significantly elevated in T2DM patients carrying the AG genotype compared to controls (0.48 ± 0.10 vs. 0.30 ± 0.02 ng/ml, p = 0.03). Conversely, the controls with AA and GG genotypes had significantly higher levels of TLR4 compared to the patients (0.50 ± 0.11 versus 0.32 ± 0.08 and 0.43 ± 0.08 versus 0.35 ± 0.03 ng/ml, respectively), T2DM risk factors include AA genotype with an elevated incidence of T2DM (OR = 4.1), GG genotype (OR = 1.04), and the A allele (OR = 1.42). However, the AG genotype was not considered a risk factor for T2DM (OR = 0.47, 95% CI: 0.21–1.06). In conclusion, the AA and GG genotypes and the A allele contribute to T2DM susceptibility, while the AG genotype influences TLR4 levels without significantly increasing disease risk.

Keywords: T2DM, TLR-4, Single-nucleotide polymorphism, ELISA, DNA-sequencing.

مستوى المصل وتعدد أشكال النوكليوتيدات المفردة (rs1927911) لمستقبل شبيه التول 4 بين مرضى السكري من النوع الثاني العراقيين

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الخلاصة

يُعد مستقبل شبيه التول 4 (TLR4) ، أحد أعضاء عائلة مستقبلات شبيه التول (TLRs) ، ومن العناصر الأساسية في المناعة الفطرية. داء السكري من النوع الثاني (T2DM) هو حالة مزمنة تتسم بضعف

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حساسية الإنسولين واستجابات مناعية غير طبيعية. هدفت هذه الدراسة إلى فحص العلاقة بين تعدد الأشكال النوكليوتيدي المفرد rs1927911 في جين TLR4 وقابلية الإصابة بداء السكري من النوع الثاني، بالإضافة إلى تقييم تأثير هذا التعدد على مستويات TLR4 . شملت الدراسة 60 مريضًا مصابًا بالسكري من النوع الثاني و40 شخصًا سليمًا كمجموعة ضابطة. تم جمع عينات الدم لتحليل المصل واستخلاص الحمض النووي. أظهرت النتائج أن مستويات TLR4 كانت مرتفعة بشكل ملحوظ لدى مرضى السكري من حاملي النمط الجيني AG مقارنةً بالأصحاء (0.10 ± 0.48) مقابل (0.02 ±0.30) نانوغرام/مل، (p = 0.03). في المقابل، أظهر الأشخاص الأصحاء من حاملي الأنماط الجينية AA و GG مستويات أعلى بشكل ملحوظ من TLR4 مقارنةً بالمرضى (0.11 ± 0.50 مقابل 0.08 ± 0.32 و0.08 ± 0.43 مقابل 0.03 ± 0.35 نانوغرام/مل، على التوالي). وشملت عوامل الخطر للإصابة بالسكري النمط الجيني AA مع ارتفاع ملحوظ في نسبة الإصابة (OR = 4.1) ، والنمط الجيني GG (OR = 1.04) ، والأليل A (OR = 1.42)ومع ذلك، لم يُعتبر النمط الجيني AG عامل خطر للإصابة بداء السكري من النوع الثاني (OR = 0.47, 95% CI: 0.21–1.06). في نتيجة هذه الدراسة، اظهر النمطين الجينيين AA وGG، بالإضافة إلى الأليلA ، مساهمة في قابلية الإصابة بداء السكري من النوع الثاني، بينما يؤثر النمط الجيني AG على مستويات TLR4 دون أن يزيد خطر الإصابة بشكل ملحوظ.

1. Introduction

Diabetes mellitus (DM) is a multifactorial disease characterized by high plasma glucose levels and irreversible small- and large-artery problems. These concerns mostly cause high mortality, morbidity, and loss of quality of life. Diabetes affected 537 million 20-79-year-olds in 2021, and 783 million by 2045. Type 2 DM (T2DM) has long been thought to affect middle-aged to late-aged people, with adolescents and younger persons rarely diagnosed. According to the report, young people are developing early-onset T2DM at an exponential rate. Between 2002 and 2012, this demographic's prevalence rose approximately 5% yearly [1]. The U.S. anticipated $412.9 billion in 2022 for diagnosed diabetes, including $306.6 billion in direct medical expenses and $106.3 billion in indirect costs. People with diabetes spend 2.6 times more on medical care than those without diabetes. Glucose-lowering drugs and diabetic supplies comprise 17% of diabetes-related direct medical expenses [2]. The genetic correlation with T2DM is more pronounced than that with type 1 DM (T1DM) [3]. Metabolic illness is caused by an insufficient response of insulin-sensitive tissues and insufficient insulin production by pancreatic cells, which affects glucose homeostasis and leads to disease [4]. T2DM can lead to microvascular problems such as retinopathy and nephropathy, and macrovascular issues such as cardiovascular comorbidities due to hyperglycemia and hypothyroidism complications [5]. T2DM is caused by insulin resistance and decreased insulin production; however, other pathophysiological disorders also dysregulate glucose metabolism [6]. Obesity, inactivity, urban living, and poor diet contribute to the onset of T2DM, which has quadrupled globally and affects 90% of adults [7,8]. Asia, the Middle East, and North Africa are experiencing rapid epidemiological shifts, attributable to a confluence of urbanization, dietary shifts, and genetic predispositions. Urbanization promotes sedentary lives, which, when coupled with inadequate dietary practices, heightens the risk of chronic diseases. In Asia, dietary modifications have been noted, accompanied by escalating rates of obesity and diabetes attributed to heightened consumption of high-calorie, low-nutrient foods. Moreover, certain individuals in these areas are genetically predisposed to metabolic diseases like T2DM and hypertension. Environmental and lifestyle factors often raise hereditary risks, multiplication illness prevalence. These trends highlight the need for public health strategies that include urban planning, food education, and genetic research to reduce the rising illness burden in these locations [9, 10]. Genetic indicators called Toll-like receptors (TLRs) recognize microbial molecular patterns and protect tissue from hemostasis

during inflammation. They draw leukocytes to infected tissues and trigger innate and adaptive immune responses. Subfamilies of TLRs are intracellular or on the cell surface [11,12]. TLRs have an ectodomain that recognizes pathogen-associated molecular patterns (PAMPs), a transmembrane domain, and a cytoplasmic TIR domain that activates additional signaling pathways. They are produced in response to damage and non-physiological cell death [13,14]. TLRs activate NF-κB, a transcription factor that facilitates the generation of proinflammatory cytokines. Inflammatory cytokines, including TNF-α, IFN-γ, and IL-6, disrupt insulin signaling by decreasing IRS-1 phosphorylation, hence diminishing glucose absorption. TLR-induced inflammation elevates oxidative stress, further compromising insulin receptor functionality, and TLRs activate specific immune responses that may be involved in autoimmune diseases such as rheumatoid arthritis (RA) [15, 16]. Research by Al-Humairi *et al.,* [17] demonstrated that the downregulation of some PRRs, such as TLR7, plays a role in the etiology and pathogenesis of urinary bladder cancer. Single nucleotide polymorphisms (SNPs) are genetic variations where a single nucleotide in the DNA sequence differs among individuals. These variations are important for identifying mutations linked to cancer and genetic disorders. SNPs can affect gene function by altering splicing sites and regulatory sequences. They are widely used in pharmacogenomics, disease risk assessment, and genetic screening. Common detection methods include RFLP-PCR, real-time PCR, and sequencing techniques [18,19]. Recent studies have identified several SNPs in human TLR genes, suggesting that they may affect the functionality of various human diseases, demonstrating that individuals with the CT genotype of TLR4 (rs1927911) exhibited a considerably higher susceptibility to tract infections [20]. Peng *et al.,* identified that TLR4 polymorphisms increased susceptibility to T2DM [21]. In contrast, four TLR4 SNPs were genotyped in Asian patients, indicating that rs1927911 appears to have no impact [22]. This study aimed to determine TLR4 levels in T2DM patient sera and correlate them with other variables, focusing on a specific gene SNP (rs1927911), their involvement in T2DM, and its impact on TLR4 serum levels.

2.Subjects and Methods

2.1. Studied groups.

The participants in this case-control study were categorized into a patient group and a control group, recruited from individuals attending private clinics in Baghdad, Iraq, from October 2023 to April 2024. The patient group comprised 60 individuals, including 29 males and 31 females, diagnosed with T2 DM as determined by physicians using fasting blood sugar tests. The average age was 46.63 years (range: 22–71 years). The control group comprised 20 healthy males and 20 healthy females. The average age was 44.9 years (range: 22–66 years). Data for each sample, including sex, age, and weight, were collected (Table 3).

2.2. Blood collection

Five millilitres of venous blood were obtained from each participant. The blood was partitioned into two aliquots: the first was placed in a gel tube for serum collection, while the second was collected in an EDTA tube and preserved at −20 °C until DNA extraction.

2.3. Serum level of TLR4

To determine the serum level of TLR4, an enzyme-linked immunosorbent assay kit (Fine Biotech, China) was used according to the manufacturer's instructions.

2.4. TLR4 gene SNP

Genomic DNA was extracted from blood using a Norgen® blood DNA extraction kit (NEB®, USA). The recovered DNA was subjected to PCR amplification using specifically

designed forward (5′-GTAGCGGGCTTTTAAATAAAC-3′) and reverse (5′-CGAACAAAAGAAAAACCTCAG-3′) primers to amplify the intron region of the human chromosome encompassing the rs1927911 SNP (chr9:117707776). Twenty-five microliters of the PCR amplification reaction were utilized as described in Table 1. The reaction was conducted under optimum PCR conditions for this gene, as indicated in Table 2 [23]. The amplified PCR fragments were sent to Macrogen, Korea, for Sanger sequencing to determine nucleotide polymorphism. FASTA sequence files were analyzed using Geneious Prime software and aligned with the RefSeq of TLR4 genes (accession numbers NG_011475 and NC_000009.12 (Figure 1).

Table 1: Components of PCR amplification reaction for detection of (rs1927911in TLR4).

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 2: PCR parameters for amplification of TLR4 gene

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

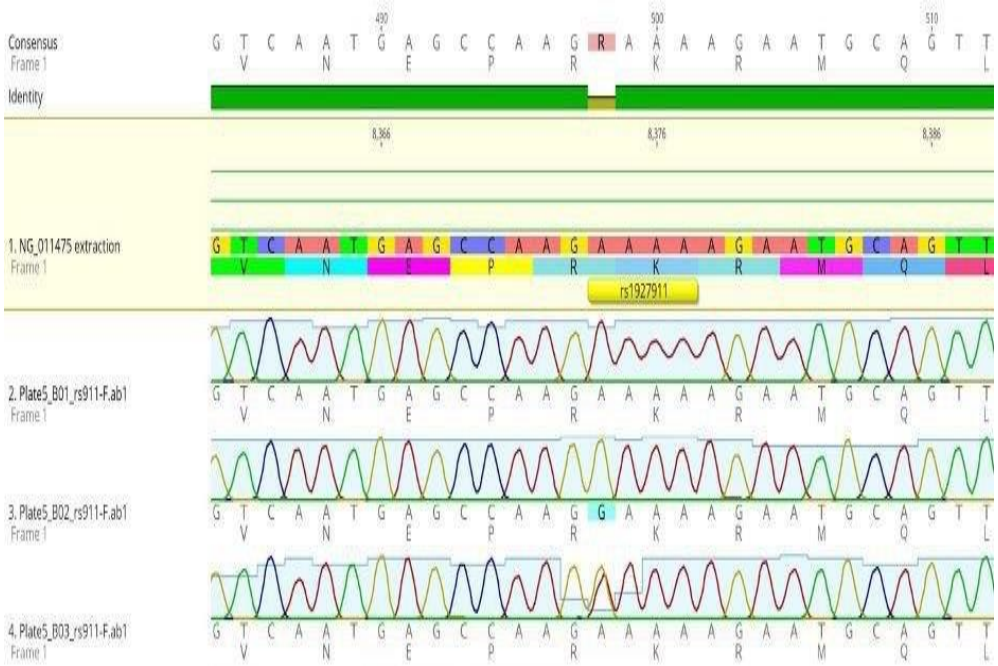

Figure 1: DNA sequence chromatogram of TLR4 gene SNP (A>G: rs1927911) showing three genotypes: AA (sequence 2), GG (sequence 3) and AG (sequence 4), in addition to the reference sequence (rs1927911), as revealed by Geneious software analysis.

2.5. Statistical analysis

A statistical study was conducted to determine the significance of the group variance. Data are presented as mean ± standard deviation and were analysed using an independent t-test and ANOVA (Duncan test), with statistical significance determined by IBM SPSS Version 27.0. The genotypes of TLR4 were expressed as percentage frequencies, and significant differences 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 establish the association between SNP and disease. These estimations were conducted using WinPepi 11.65. A probability (p) value of ≤ 0.05 was deemed significant following the use of false discovery rate adjustments.

3. Results

Patients with T2 DM exhibited a higher mean age (46.63 years) than the control group (44.9 years). The age range of (41 – 60) years was the most prevalent among T2DM patients, comprising 68.1%, with a statistically significant difference ($p \leq 0.05$), while the other age groups didn't show significant differences ($p > 0.05$). Regarding sex, 51.7% of T2DM patients were female, whereas 48.3% were male. The analysis of Body Mass Index (BMI) revealed that the control group had lower proportions of individuals with the normal weight (18-25 kg/m²), overweight (26-30 kg/m²), and obese (BMI > 30 kg/m²) categories compared to the patient group (34.5% versus 65.5%, 46.2% versus 53.8%, and 37.5% versus 62.5%, respectively, as shown in Table 3. BMI was measured once for each participant during the study period.

Table 3: Baseline characteristics of T2DM patients and controls

Parameters	T2DM patients No. (%)	Control No. (%)	p-value
Age (years)			
20-40	16 (53.3%)	14 (46.7%)	0.71 NS
41 – 60	32 (68.1%)	15 (31.9%)	0.01 *
> 60	12 (52.2%)	11(47.8%)	0.84 NS
Age mean ± SE (Years)	46.63 ± 1.63	44.90 ± 2.08	0.51 NS
SEX			
Male	29 (48.3%)	20 (50%)	0.87 NS
Female	31(51.7%)	20 (50%)	
Body mass index (BMI)			
18– 25	19 (65.5%)	10 (34.5%)	0.09 NS
26 – 30	21(53.8%)	18 (46.2%)	0.63 NS
> 30	20 (62.5%)	12 (37.5%)	0.16 NS
BMI mean ± SE (Kg/m^2)	27.47 ± 0.63	26.93 ± 0.75	0.58 NS

*** (P≤0.05), NS: Non-Significant.**

Serum levels of TLR4

Serum levels of TLR4 showed no significant difference between T2DM patients and controls (0.39 ± 0.04 *versus* 0.36 ± 0.04; $p > 0.05$). Some of the characteristic subgroups tended to have a higher level (females 0.438 ± 0.392, age group 41–60 years 0. 477 ± 0.405 and BMI group 26-30 (0.494 ±0.442), but without significant difference compared to the corresponding subgroups, the TLR4 serum levels were approximated, and moreover, there were no significant variations between their means in their characteristic subgroups (Table 4).

Table 4: Serum levels of TLR4 in T2DM patients and controls distributed according to some characteristics.

Characteristic	Serum level of TLR4 (Mean ± S.D ng /ml)		p- value
groups	Patients (n=60)	Control (n=40)	
Total	0.39 ± 0.04	0.36 ± 0.04	0.611
Gender			
Male	0.341 ± 0.213	0.415 ± 0.285	0.19
Female	0.438 ± 0.392	0.307 ± 0.106	0.37
Age group			
20-40	0.323 ± 0.115	0.426 ± 0.321	0.5
41-60	0.477 ± 0.405	0.318 ± 0.085	0.32
>61	0. 252 ± 0. 134	0.337 ± 0.184	0.39
BMI			
18-25	0.290 ± 0.129	0.485 ± 0.366	0.08
26-30	0.494 ± 0.442	0.338 ± 0.136	0.52
>30	0.378 ± 0.272	0.294 ± 0.112	0.39

TLR4 gene SNP

Single nucleotide polymorphisms of the TLR4 gene were analysed in both the patient and control groups. The TLR4 (-8374A A/G) polymorphism is located within the TLR4 gene, as reported by NCBI. Allelic variation for SNP (rs1927911) was assessed using the Sanger technique to identify the SNP. FASTA sequence data were analysed using Geneious Prime software and aligned to the RefSeq of TLR4. Table 5 illustrates the distribution of the genotypes and allele frequencies. Regarding the AA wild genotype, a significant difference ($p \leq 0.05$) was observed between T2 DM patients and healthy individuals (25% vs. 7.5%), suggesting a potential association with increased disease risk. The prevalence of the GG genotype was 38% in T2 DM patients compared to 37.5% in the control group, with no statistically significant difference ($p > 0.05$). The AG genotype was more common among controls (55%) than patients (36.7%). The A allele was more frequent among T2DM patients (43%) than in controls (35%), corresponding to an odds ratio (OR) of 1.42. Although this difference was not statistically significant, it may suggest a trend towards increased T2 DM risk.

Table 5: Single nucleotide polymorphism (rs1927911) of TLR4 gene in T2DM patients and control and allele frequency

TLR4 SNP rs1927911 genotyping and allele frequencies	**Patient group (n=60)**	**Control group (n=40)**	**Odd ratio**	**95% confidence intervals**	**Fisher's exact probability**
AA	15 (25.0)	3 (7.50)	4.11	1.12 – 15.06	0.03
AG	22 (36.77)	22 (55.0)	0.47	0.21 – 1.06	0.1
GG	23 (38.33)	15 (37.50)	1.04	0.46 – 2.34	1.0
Total	60 (100.0)	40 (100.0)			
Alleles frequencies					
A	52 (43.0)	28 (35.0)	1.42	0.79 – 2.54	0.302
G	68 (57.0)	52 (65.0)	0.70	0.39 – 1.26	0.302

TLR4 SNP-impact on TLR4 serum level

Table 6 illustrates the impact of the rs1927911 SNP on serum TLR4 concentrations in the studied groups. Although the overall difference in serum TLR4 levels between patients and controls was not statistically significant, as shown in Table 4, subgroup analysis by genotype revealed significant differences. Among individuals with the AG genotype, T2DM patients exhibited significantly higher serum TLR4 levels than controls (0.48 ± 0.10 vs. 0.30 ± 0.02 ng/ml, $p \leq 0.05$). In contrast, for the AA and GG genotypes, the control group showed higher TLR4 levels than the patient group, but the differences were not statistically significant (AA: 0.50 ± 0.11 vs. 0.32 ± 0.08 ng/ml; GG: 0.43 ± 0.08 vs. 0.35 ± 0.03 ng/ml; $p > 0.05$ for both comparisons).

Table 6: The impact of rs1927911 SNP on TLR4 concentrations in the studied groups.

Genotype for rs1927911	Serum level of TLR4 (Mean±SD) (ng/ml)		P-value
	T2DM patients	Control	
GG	0.35 ± 0.03 ^{A}	0.43 ± 0.08 ^{A}	0.39 NS
AA	0.32 ± 0.08 ^{A}	0.50 ± 0.11 ^{A}	0.32 NS
AG	0.48 ± 0.10 ^{A}	0.30 ± 0.02 ^{A}	0.03 *

Means with different letters in the same column differ significantly, * ($P \leq 0.05$), NS: non-significant.

4.Discussion

These findings indicate a risk association with age, female sex, and obesity. Research indicates that age-related changes in insulin sensitivity and metabolic processes raise the risk of T2DM, especially in individuals aged 41- 60. This aligns with previous study conducted by Peng *et al*., [24]. This could happen because of reduced physical activity, altered fat distribution, and decreased metabolic flexibility. The prevalence of T2DM among males and females is somewhat equitable, with a slight increase in females compared with males. This observation resembled research done by Vasanthakumar and kambar, the survey indicated that the prevalence of diabetes among women was 60.26% compared to 39.74% in men [25]. This elevation may result from hormonal fluctuations, especially estrogens, which are important in regulating metabolic processes associated with energy balance and can affect inflammatory responses. Numerous inflammatory elements, including macrophages and monocytes, are stimulated by estrogen via estrogen receptors present in these cells. Moreover, a correlation exists between diminished estrogen levels in post-menopausal women and an elevated inflammatory condition. Post-menopausal women have elevated lymphocyte and monocyte numbers, heightened release of proinflammatory cytokines, and a rise in senescent inflammatory cells, typically indicative of impaired immunologic function, in contrast to premenopausal women. Estrogens may safeguard against the onset of insulin resistance by modifying metabolic processes related to energy balance and downregulating or inhibiting inflammation [26]. A greater proportion of T2DM patients were classified as normal weight and obese in comparison to the control group. This demonstrates that although obesity is a recognized risk factor for T2DM, this aligns with Sati *et al*., and Abed *et al.*, which found that obesity (body mass index [BMI] ≥30 kg/m²) is the most significant risk factor for T2DM [27, 28]. Strong evidence suggests that obesity treatment may prolong the progression from prediabetes to T2DM. Substantial evidence suggests that treating obesity may slow the development of T2DM from prediabetes. Moderate and sustained weight loss is an important factor for improving glucose management and decreasing the requirement for medicines with lower glucose levels [25, 29]. Individuals with a normal BMI may also be substantially

affected, potentially due to the familial history and genetic influences that can contribute. In the present investigation, the serum level of TLR4 was slightly elevated in T2DM patients compared to that in the control group. This aligns with the results obtained by Taha *et al.*, who reported that Patients with T2DM exhibited markedly elevated levels of TLR4 mRNA and protein in monocytes compared to control subjects [30]. Researchers have determined that TLR4 expression and functional activation are elevated in patients with recently diagnosed T2DM. The elevation of TLR4 expression in T2DM patients is attributed to the essential function of TLR4 in the immune response [31]. Increased TLR4 activity exacerbates chronic inflammation and insulin resistance in T2DM [32]. To further understand the role of TLR4 in the etiology and pathophysiology of T2DM, SNP were examined. Disease-association studies have indicated that SNPs in TLR genes correlate with an elevated risk of developing various forms of T2DM. Among these, TLR4 SNPs, which have been proposed to confer a protective effect against T2DM, are posited to predict the therapeutic response to anti-inflammatory treatments [33]. Several studies have assessed the correlation between TLR4 polymorphisms and the risk of T2DM. One of them focused on (rs4986790, rs4986791, and rs11536889), the data indicate that these SNPs may increase the risk of T2DM, but rs1927911 may have no effect [22]. Conversely, [21] concluded that (rs10759932 and rs1927911) are related to T2DM in the Chinese population, particularly among female participants. Furthermore, Jiang *et al*., indicated that (rs1927914 and rs1927911) were correlated with T2DM in a Han Chinese population [34]. Various investigations of this polymorphism have been published in recent years. Based on our results, the rs1927911 appears to be associated with T2DM [35]. Here, it was found that homozygous wild AA and homozygous mutant GG were considered risk factors for T2DM. The heterozygous mutant AG had no risk, but the patients who carried it had higher levels of TLR4 (Mean±SD) compared with the control group. The G allele was present at low frequency in patients compared with controls, and the frequency of the A allele among patients was higher than that of controls, with no significant differences between groups. Our findings disagree with Rykov *et al*., which revealed that the GG genotype frequency in T2DM was 61%, the heterozygous genotype was 31%, and the AA genotype was 20%. The G allele frequency was observed in 77% of patients and 69% of controls, whereas the A allele frequency was found in 22% of patients and 31% of controls. They found that the risk was reduced (OR = 0.42; 95% CI, 0.23–0.77) in carriers of allele A compared to carriers of ancestral allele G [36]. However, in our study, the risk was increased in carriers of allele A compared to carriers of ancestral allele G. The rs1927911 may influence susceptibility to T2DM by modulating TLR4-mediated immune responses. TLR4 is known to activate the Nuclear Factor Kappa B (NF-κB) pathway and promote the release of proinflammatory cytokines such as Tumor Necrosis Factor-α (TNF-α) and Interleukin-6 (IL-6), which interfere with insulin signaling by inhibiting IRS-1 phosphorylation, thereby contributing to insulin resistance [37, 38]. Polymorphisms in TLR4 may alter receptor expression or signaling efficiency, influencing the chronic low-grade inflammation characteristic of T2DM. The AG genotype may balance this response, maintaining immune regulation while avoiding excessive activation. Further functional and longitudinal studies are needed to confirm these potential mechanisms and their therapeutic implications. It should be noted that this study focused solely on the rs1927911 SNP and did not include analysis of other TLR4 polymorphisms or haplotypes. Future studies involving multiple SNPs and haplotypic assessments would be beneficial to further elucidate potential cumulative or interactive genetic effects on TLR4 expression and T2DM susceptibility.

Conclusion

Individuals aged 41–60 years are at a heightened risk for T2DM, and both normal-weight and obese individuals exhibited an increased prevalence of the disease. The study suggests

that the AA and GG genotypes of rs1927911, as well as the A allele, are associated with an increased risk of T2DM. In contrast, the AG genotype may contribute to elevated TLR4 serum levels without directly increasing disease susceptibility. These findings highlight the potential role of TLR4 in the pathogenesis of T2 DM, particularly through genetic variations. Future studies are warranted to explore the functional mechanisms by which rs1927911 polymorphisms modulate TLR4 expression and contribute to T2DM development. Larger, multi-center studies across different populations, as well as investigations into the therapeutic potential of targeting TLR4 pathways, could provide deeper insights and pave the way for personalized treatment strategies.

Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. The study protocol, subject information, and consent form were reviewed and approved by a local ethics committee according to document number (Ref.: CSEC/0923/0088) on September 25, 2023.

Conflicts of interest

There are no conflicts of interest.

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