ORIGINAL ARTICLE

CONTENTS

Association of toll-like receptor 4 gene (Thr399Ile) polymorphism with type II diabetes mellitus patients

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ABSTRACT

Aim: This study investigates the association of the TLR4 Thr399Ile (rs4986791) polymorphism with T2DM in Iraqi patients and its influence on clinical parameters.

Materials and Methods: A case-control study was conducted on 200 participants (100 T2DM cases and 100 healthy controls). Phenotypic analyses included fasting blood glucose, HbA1c, lipid profiles, and insulin sensitivity indices. Genotyping of the TLR4 Thr399Ile polymorphism was performed using PCR-RFLP. Statistical analyses were conducted using SPSS.

Results: The CT and TT genotypes were significantly more frequent in T2DM patients, associated with higher odds ratios (OR=2.40, p=0.003; OR=5.33, p=0.04, respectively). HDL levels were significantly lower in variant genotypes (p = 0.01). Other clinical parameters showed no significant genotype-specific differences.

Conclusions: The TLR4 Thr399Ile polymorphism is significantly associated with increased T2DM risk and altered HDL levels in Iraqi patients, highlighting its potential as a genetic marker for early risk assessment.

KEY WORDS: Genetic susceptibility, Iraqi population, Type 2 diabetes mellitus (T2DM), TLR4 gene polymorphism, Thr399Ile (rs4986791)

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INTRODUCTION

Diabetes Mellitus (DM) is a chronic disease characterized by persistently high blood sugar due to issues with insulin production or action. It impairs the body's ability to metabolize fats, carbohydrates, and proteins, presenting a substantial global health challenge [1]. In 2021, an estimated 537 million adults worldwide were affected, with numbers expected to rise to 783 million by 2045. DM accounts for 10% of global healthcare spending, emphasizing its economic impact. In Iraq, over 2 million people are affected, with a 13.9% [2] prevalence of type 2 diabetes. DM includes type 1 (autoimmune destruction of insulin-producing cells), type 2 (linked to insulin resistance and lifestyle), gestational diabetes, and other less common forms. Type 2 diabetes, the most prevalent, is often associated with severe long-term complications, including cardiovascular diseases and organ damage [3]. Inflammation plays a role in several diseases like diabetes, cancer, and cardiovascular issues. Toll-like receptors (TLRs) are key components of the innate immune system, recognizing molecules associated with pathogens (PAMPs) and damaged cells (DAMPs) [4]. Eleven human TLRs exist; some are located on the cell surface, detecting microbial components, while others are inside the cell, recognizing nucleic acids [5]. TLR4 is unique because it activates two different signaling pathways: one leading to the production of pro-inflammatory cytokines, and the other resulting in interferon secretion, both of which influence inflammation and immune responses [6]. Toll-like receptors (TLRs), especially TLR4, play a critical role in the innate immune system by identifying molecular patterns from pathogens and initiate immune response.The location of TLR4 is on chromosome 9q33.1 [7], is expressed in organs such as adipose tissue and pancreas, and its genetic variations have been associated with type 2 diabetes (T2DM). Increased TLR4 activity and Inflammation are noticed in obesity, insulin resistance, and T2DM [8]. The genome wide association studies (GWAS) and other genetic studies are enhancing our competence to expect who is at risk for T2DM [9]. By understanding an individual's genetic susceptibility, we can develop personalized approaches to assist in prevent or delay the disease onset [10]. Using genetic information in conjunction with common diagnostic techniques such as glycated hemoglobin (HbA1c) and fasting blood glucose levels enable more comprehensive monitoring and management of diabetes risk [11]. The TLR4 gene

rs4986791single nucleotide polymorphisms (SNP), located in exon 3, lead to amino acid substitution at position 399: threonine to isoleucine (Thr399Ile) [12]. This substitution change the TLR4 protein's extracellular structure, possibly influencing how it interacts to ligands. Knowledge of these genetic polymorphisms permits for more specific diagnosis and the ability to expect when susceptible subjects might develop the disease [13].

AIM

This study investigates the association of the TLR4 Thr399Ile (rs4986791) polymorphism with T2DM in Iraqi patients and its influence on clinical parameters.

MATERIALS AND METHODS

A case-control study was conducted over a nine-month period, from February to October 2024, at a private clinic and the Diabetic Center of Al-Sadder Medical City in Al-Najaf, Iraq. Two hundred participants were enrolled,the case group (n=100; 52 males and 48 females, aged 36-62 years) comprised individuals diagnosed with type 2 diabetes mellitus. These participants were randomly selected from patients attending the Diabetic Center for routine checkups. The control group (n=100; 49 males and 51 females) consisted of apparently healthy individuals, also randomly selected

INCLUSION CRITERIA

The study enrolled participants with a fasting blood glucose level of 126 mg/dL or higher and who exhibited classic diabetes symptoms such as frequent urination, nighttime urination, increased hunger, and weight loss. Additionally, only those previously diagnosed with type 2 diabetes by a endocrinologist were included.

EXCLUSION CRITERIA

Those excluded from the study included individuals aged 34 or younger, those with type 1 diabetes or requiring insulin therapy. Also excluded were individuals with autoimmune diseases, cancer, severe kidney or liver disease, pregnant women, and those currently taking glucocorticoids.

LIMITATION

However, A key limitation of this study is the small size of the comparison groups A larger sample size will increase statistical power.

MATERIALS

This study utilized a variety of materials, including: agarose (Condalab, Spain); Diamond nucleic acid dye (Promega, USA); distilled water (Pioneer, Iraq); 2x Taq Plus PCR smart mix kit (SolGent, South Korea); kits for cholesterol, DNA extraction, glucose, HbA1c, HDL, human insulin, and triglycerides (Linear, Spain; Favorgen, Taiwan; i-sens, South Korea; Tosoh, Japan; and Linear, Spain, respectively); primers (Alpha DNA, USA); proteinase K (Peakendness, China); restriction enzymes (Promega, USA); Safe-Green Opti-DNA Marker (ABM, Canada); and Tris BE buffer (10X) (Promega, USA).

METHODOLOGY

Participants in this study included patients diagnosed with type 2 diabetes mellitus and healthy control. After excluding individuals who did not respond or met the pre-defined exclusion criteria, all participants provided written informed consent. Blood samples were collected, and phenotypic and genotypic analyses were performed at the Clinical Laboratory Sciences Department, Faculty of Pharmacy, university of Kufa.

PHENOTYPIC ANALYSIS

Blood samples were collected from all participants and divided into two aliquots. One portion was used to measure fasting blood glucose, serum lipid profile, and serum insulin levels. The other portion was used for genetic analysis of the TLR-4 gene(Thr399Ile) polymorphism. HbA1c levels were determined using the Finecare™ HbA1c Rapid Quantitative Test. Serum measurements were taken immediately following sample collection. Data regarding age,sex, body mass index, smoking status, family history of T2DM, and chronic medication use were also collected from each participant.

GENOTYPING MEASUREMENTS

Genomic DNA was extracted from blood samples of both T2DM patients and control group using the FavorPrep™ Blood Genomic DNA kit (Favorgen). DNA concentration and purity were measured with a Nanodrop spectrophotometer. A specific genomic region was amplified via polymerase chain reaction (PCR) using a T-professional thermocycler (Biometra, Germany). Primers (Alpha-DNA, lyophilized powder) with their sequences detailed in Table 1.

The PCR reaction utilized the 2x Taq plus PCR Smart Mix kit (SolGent, South Korea). The PCR conditions were optimized to ensure efficient DNA amplification. The polymerase chain reaction (PCR) protocol for am-

Table 1. Sequences of primers used for detecting the toll-like receptor 4 (TLR4) Thr399Ile (rs4986791) single-nucleotide polymorphisms

SNP	Primers
Thr399Ile rs4986791	F: 5'-GGT TGC TGT TCT CAA AGT GAT TTT GGG AGAA-3' R: 5'-CC TGA AGA CTG GAG AGT GAG TTA AAT GCT-3'

Table 2. Comparison of Demographic and Serum Lipid Profiles between Type 2 Diabetes Mellitus (T2DM) Patients and Healthy Controls

	Patients n = 100	Control n = 100	P-value
	Age(year)		
Mean ±SD	53.04 ± 9.77	51.37 ± 8.41	0.19
	BMI kg/m^2		
Mean ±SD	28.42 ± 4.27	27.48 ± 3.74	0.09
	Family history for T2DM n(%)		
	65(65%)	59(59%)	----------
	Triglyceride (mg/dl)		
Mean ±SD	249.90± 31.65	128.56 ± 28.73	0.0001
	Cholesterol (mg/dl)		
Mean ±SD	223.63 ± 36.30	164.15 ± 26.74	0.0001
	HDL (mg/dl)		
Mean ±SD	37.35 ± 6.92	54.93 ± 10.39	0.0001
	VLDL (mg/dl)		
Mean ±SD	49.98 ± 6.33	25.71 ± 5.74	0.0001
	LDL (mg/dl)		
Mean ±SD	136.30 ± 29.75	83.51 ± 28.16	0.0001

Table 3. Comparison of Fasting Plasma Glucose, HbA1c, Insulin Levels, and Insulin Resistance Indices between T2DM Patients and Healthy Controls

	Patients (n = 100)	Control (n = 100)	P-value
	FPG (mg/dl)		
Mean ±SD	218.71 ± 30.37	81.27 ± 16.14	0.0001
	HbA1c		
Mean ±SD	9.22 ± 1.44	5.34 ± 0.53	0.0001
	Plasma insulin level mIU/L		
Mean ±SD	22.94 ± 6.51	14.06 ± 4.23	0.0001
	HOMA-IR index		
Mean ±SD	12.38 ± 5.27	2.80 ± 0.91	0.0001
	QUICKI		
Mean ±SD	0.27 ± 0.012	0.33 ± 0.02	0.0001

plifying the Thr399Ile (rs4986791) single nucleotide polymorphism (SNP) involves a series of temperature changes over time. The process begins with an initial denaturation step at 95°C for 5 minutes. Following this, the sample undergoes 30 cycles of a three-step process: denaturation at 95°C for 30 seconds, annealing at 60 °C for 30 seconds and extension at 72°C for 30 seconds Finally, the reaction concludes with a prolonged extension step at 72°C for 10 minutes, ensuring complete synthesis of the target DNA fragment. This specific temperature cycling regime is optimized to amplify the Thr399Ile SNP region for subsequent analysis.

PCR-RESTRICTION FRAGMENT LENGTH POLYMORPHISM

(PCR-RFLP) ANALYSIS FORTLR4 GENE SNP (THR399ILE) ANALYSIS

The restriction enzyme HinfI, originating from the bacterium *Haemophilus influenzae*, plays a crucial role in the analysis of the Thr399Ile single nucleotide polymorphism (SNP) amplicons resulting from the amplification reaction. This enzyme specifically targets and cleaves DNA at the recognition sequences. The enzyme's optimal activity is achieved at a temperature of 37°C, requiring an incubation period of one hour. The

Table 4. Association between Toll-like Receptor 4 (TLR4) Thr399Ile Polymorphism and Type 2 Diabetes Mellitus

(rs4986791)	T2DM, n(%) (n=100)	Control , n (%) (n=100)	OR(95% CI)	P- value
Genotypes				
CC	44(44%)	67 (67%)	Reference	
CT	49 (49%)	31 (31%)	2.40(1.33 - 4.33)	0.003
TT	7 (7%)	2(2%)	5.33(1.05 - 26.84)	0.04
Dominant Model				
CC	44(44%)	67 (67%)		
CT+ TT	56(56%)	33(33%)	2.58 (1.45 - 4.59)	0.001
Alleles				
C	137(68.5%)	165(82.5%)		
T	63(31.5%)	35(17.5%)	2.17(1.35 - 3.47)	0.001
Total (2N)	200	200		

Table 5. Association Between TLR4 Thr399Ile (rs4986791) Gene Polymorphism Genotypes and Clinical Parameters in T2DM Patients

Clinical Characteristic	Genotype M ± SD			P-Value
	CC (n=44)	CT (n=49)	TT (n=7)	
BMI	28.15±4.5	26.42±4.3	29.78±4.9	0.06
FBS (mg/dl)	217.27±30.33	227.48±33.55	220.98±30.18	0.31
HbA1c	9.44±1.48	8.93±1.40	8.88±1.50	0.21
Insulin(µIU/mL)	22.88±5.51	21.57±6.43	23.67±6.25	0.47
Cholesterol (mg/dl)	224.54±41.03	233.27±39.35	215.27±35.09	0.39
HDL (mg/dl)	39.11±4.77	35.22±7.17	36.6±8.29	0.01*
Triglycerides(mg/dl)	250.25±32.61	245.75±28.25	250.13±30.18	0.76
VLDL (mg/dl)	50.05±6.52	49.15±6.24	50.02±5.55	0.77
LDL (mg/dl)	135.38±33.20	148.90±29.27	128.65±29.82	0.06
HOMA IR	12.26±5.42	12.12±4.56	12.91±5.01	0.92
QUICKI	0.270±0.012	0.271±0.011	0.269±0.016	0.87

digested products were separated on a 2% agarose gel (Condalab, Canada) through electrophoresis. The gel was stained with Diamond Nucleic Acid Dye to enable visualization under a UV apparatus.

PATIENT CONSENT

All participants were informed about the study procedures, and those who agreed to participate provided both verbal and written consent prior to sample collection.

STATISTICAL ANALYSIS

Data for continuous variables are presented as mean ± standard deviation (SD). To compare means between the T2DM and control groups, Student's t-tests were performed using SPSS version 26 and Microsoft Excel 2019. The Hardy-Weinberg equilibrium was assessed with a chi-square goodness-of-fit test. Logistic regression analysis was used to calculate odds ratios. Statistical significance was defined as a p-value below 0.05.

ETHICAL APPROVAL

The study protocol received ethical approval from the Scientific Committee for Research Ethics at the Faculty of Pharmacy, Kufa University (Approval No: 211#16-12-2022).

RESULTS

A comparison of demographic and lipid profiles between individuals with type 2 diabetes mellitus (T2DM) and healthy controls, Table 2. Age and body mass index (BMI) were not significantly different between the two groups ($p = 0.19$ and $p = 0.09$, respectively), those with T2DM exhibited significantly elevated levels of triglycerides, total cholesterol, low-density lipoprotein (LDL), and very-low-density lipoprotein (VLDL), and lower levels of high-density lipoprotein (HDL) (all $p^1 < 0.0001$). A family history of diabetes was also more common in the T2DM group (65%) compared to the control group (59%).

The study compared metabolic markers between individuals with type 2 diabetes mellitus (T2DM) and

a control group. Table 3 shows significant differences in glucose metabolism and insulin sensitivity. T2DM patients had much higher fasting plasma glucose, HbA1c, insulin levels, and HOMA-IR, while their QUICKI values were lower (all $p < 0.0001$). These results clearly demonstrate the impaired glucose regulation and insulin resistance characteristic of T2DM compared to the control group.

The distribution of the TLR4 (Thr399Ile) polymorphism genotypes and alleles, and their relationship with the risk of developing type 2 diabetes (T2DM) shown in Table 4. A notable association was observed between the TLR4 Thr399Ile (rs4986791) polymorphism and T2DM. Specifically, the CT and TT genotypes were found more frequently in T2DM patients (49% and 7%, respectively) compared to the control group (31% and 2%). This increased prevalence was linked to a higher risk of T2DM, with odds ratios (OR) of 2.40 ($p = 0.003$) for the CT genotype and 5.33 ($p = 0.04$) for the TT genotype. Both dominant and allelic genetic models also demonstrated statistically significant associations with T2DM ($p = 0.001$).

The study investigated the impact of the Toll-like receptor 4 gene polymorphism (rs4986791) on clinical parameters in type 2 diabetes (T2DM) patients. Table 5 summarizes the results, showing the relationship between the TLR4 Thr399Ile (rs4986791) genotypes and clinical parameters under a Codominant model. High-density lipoprotein (HDL) levels varied significantly among genotypes ($p = 0.01$), with the CC genotype exhibiting the highest levels. Body mass index (BMI), fasting blood sugar (FBS), glycated hemoglobin (HbA1c), insulin, cholesterol, triglycerides, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), homeostatic model assessment of insulin resistance (HOMA-IR), and quantitative insulin sensitivity check index (QUICKI) did not show statistically significant differences ($p > 0.05$), suggesting a limited association with this polymorphism.

DISCUSSION

The TLR4 gene variation (Thr399Ile) refers to a single nucleotide polymorphism (SNP) in the Toll-like receptor 4 gene, resulting in an amino acid change from threonine (Thr) to isoleucine (Ile) at position 399. This variation has been studied for its potential role in increasing susceptibility to type 2 diabetes mellitus (T2DM) [14]. To best our knowledge our study represents one of the earlier comprehensive case-control investigation examining the association between the Toll-like receptor 4 (TLR4) polymorphism (rs4986791) and the risk of developing type 2 diabetes mellitus (T2DM). Our results provide insight into the potential role of TLR4 genetic variants in the pathogenesis of T2DM. While our findings support the association between the TLR4 polymorphism (rs4986791) and T2DM risk, they also reveal some contrasting evidence with previous studies. A clear association between the TLR4 gene (Thr399Ile) polymorphism and risk of T2DM, where the CT and TT alleles were observed more frequently in patients group 49% and 7%, respectively; in comparison to the controls (31% and 2%), indicating increased type 2 diabetes risk with odds ratios (OR) of 2.40 and 5.33 for the CT and TT genotype respectively. These results provide evidence of genetic predisposition for T2DM linked with TLR4 gene polymorphisms, in agreement with the idea that TLR4 plays important role in metabolic disturbances that increase the susceptibility to T2DM [15]. Despite, earlier studies have shown opposite findings. For instance, [16] found that the TLR4 gene (Asp299Gly) polymorphism was linked with a decreased risk of T2DM and atherosclerosis, implying that this variation may play a protective effect in some societies. On the other hand, our finding suggests that the TLR4 gene Thr399Ile (rs4986791) polymorphism is associated with an increased risk of T2DM, indicating potential variations in the ways that different polymorphisms in TLR4 gene may influence diabetes mellitus risk. Variations in study population, sample size, and genetic background, along with various methodologies used to evaluate the associations, may be the cause of the differences in findings. Additionally, our findings show a significant relationship between TLR4 gene rs4986791 polymorphism and lipid metabolism, particularly high-density lipoprotein cholesterol (HDLc) levels. The CC genotype of TLR4 Thr399Ile (rs4986791) was linked with higher HDLc levels comparison with other genotypes $p=0.01$. This finding is consistent with previous research suggesting that activation of TLR4 can change lipid metabolism, resulting in alterations in HDLc levels and participating in the development of metabolic disorders like T2DM [16]. It has been demonstrated that the inflammatory response triggered by TLR4 activation affects lipid metabolism, as well as the regulation of total cholesterol and other lipid profiles. [16]. These findings further support the idea that TLR4 gene polymorphisms can influence lipid metabolism and, as a result, play a role in the pathophysiology of type 2 diabetes [17]. On the other hand, some studies have shown no significant relationship between TLR4 gene polymorphisms and lipid metabolism. Following Bonferroni corrections, Taís Silveira Assmann1 et al., [18] found no difference in lipid profiles of patients classified by the presence of the 299Gly and Thr399Ile variant. Similarly, Yin et al., [19] found no significant association between TLR4 gene polymorphisms (including

rs4986791) and lipid levels, particularly HDLc. These discrepancies may arise from differences in the types of cohorts studied, the presence of other confounding factors such as medication use, or the specific metabolic pathways involved. Our study found significant differences in glucose metabolism and insulin sensitivity between T2DM patients and the control group. Specifically, T2DM patients had significantly higher fasting plasma glucose, HbA1c, insulin levels, and HOMA-IR, while their QUICKI values were lower (all $p<0.0001$). These results clearly highlight the impaired glucose regulation and insulin resistance characteristic of T2DM. The relationship between TLR4 polymorphisms and insulin resistance remains an important area of investigation [20]. Our findings are further supported by earlier studies [21-23] that indicated TLR4 is important in the inflammatory mechanisms underlying insulin resistance. Nevertheless, inconsistent findings have been reported by certain studies. For instance, Yin et al.'s meta-analysis [19] found no significant correlation between the TLR4 rs4986791 polymorphism and the risk of type 2 diabetes, especially in terms of insulin resistance and glucose metabolism. This discrepancy could be due to differences in genetic variation across populations, sample sizes, and environmental factors that may influence the function and expression of TLR4. Furthermore, the interpretation of these relationships is complicated by the intricate interactions of genetic, metabolic, and environmental factors in the pathophysiology of type 2 diabetes [24]. Several studies have explored the association between TLR4 gene polymorphisms and T2DM complications. For example, Buraczynska et al., [25] reported that the G allele of the Asp299Gly variation was linked with an increased risk of diabetic nephropathy, while Rawaa et al., [26] found that the TLR4 Thr399Ile variation was associated to the early onset of diabetic kidney disease. These findings suggest that TLR4 may influence the development diabetes mellitus complications such as nephropathy, but the polymorphisms implicated may vary across studies, [27]. Although the exact mechanisms remain to be elucidated, our findings add to the growing body of evidence, indicating that TLR4 polymorphisms may be involved in the risk and progression of T2DM and its related complications. Our finding provides new evidence supporting the link between the TLR4 gene rs4986791 polymorphism and T2DM risk, in addition to its influence on glucose regulation and lipid metabolism. While our findings consistent with some earlier studies [28-30], they also highlight inconsistencies in the literature, suggesting that the association between TLR4 gene polymorphisms and T2DM is complex and influenced by different factors. Larger sample sizes, diverse populations, and more accurate methodologies are required to confirm these associations and explain the role of TLR4 in the pathophysiology of T2DM.

CONCLUSIONS

This study revealed a significant association between TLR4 gene (Thr399Ile, rs4986791) polymorphism and the likelihood of developing type 2 diabetes mellitus in Iraqi population. T2DM was more common in people with CT and TT genotypes. These findings refer that TLR4 gene polymorphisms may play a key role in the development of T2DM and suggest that genetic testing could be a helpful method for early diagnosis and assessment the risk of the disease.

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CONFLICT OF INTEREST

The Authors declare no conflict of interest

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