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Original article

Study of (TLR-4) Gene Polymorphism (rs4986791) in Type 2 Diabetes and Diabetic Nephropathy in Egyptian Patients

Dalia M. Abd El-Hassib [a], Fetnat M. Tolba [a], Mohamed E. Ibrahim [b], Sarah A. Elzamly [a], Dina S. Abdelmotaleb [a]

[a] Clinical and Chemical Pathology Department, Faculty of Medicine Benha University, Egypt.

[b] Internal Medicine Department, Faculty of Medicine Benha University, Egypt.

Corresponding to:
Dr. Sarah A. Elzamly.
Clinical and Chemical Pathology Department, Faculty of Medicine Benha University, Egypt.

Email: sarahelzamly@gmail.com

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Abstract:

Background: A member of pattern recognition receptors is the toll-like receptor 4 (TLR-4). It is a highly conserved receptor that can identify compounds like damage- and pathogen-associated molecular patterns. As inflammation is considered to have a very important role in Type 2 diabetes (T2D) and toll-like receptors play a central role in inflammation through immune responses, TLR-4 has a universal role in the T2D mechanism. Excessive amounts of glucose and free fatty acids lead to enlarged expression of TLR-4 mRNA and proteins in cases with T2D. **Aim of the study:** Is to determine the role of TLR-4 gene polymorphism (rs4986791), threonine 399 isoleucine (Thr399Ile) in the pathogenesis of T2D and diabetic nephropathy (DN) Egyptian patients. **Subjects and methods:** Sixty patients with T2D, thirty of them with DN and thirty without DN and twenty healthy controls had been genotyped for Thr399Ile polymorphisms of the TLR-4 gene by quantitative real-time PCR. **Results:** Toll-like receptor 4 polymorphism CT and TT genotypes and T allele found the relationship to T2D ($P<.05$), with a risk of T2D susceptibility ($OR>1$). But there had been no significant relationship regarding TLR-4 gene polymorphism among patients with and without nephropathy ($P=.674$ and $.296$) for CT and TT genotypes. **Conclusion:** This study elucidated that the distinguished distribution of genotypes CT and TT of TLR-4 gene polymorphism (rs4986791) among T2D patients and healthy subjects is allied with an increased risk of T2D. But there had been no significant relationship with diabetic nephropathy in Egyptian populations.

Keywords: Type 2 Diabetes, Diabetic nephropathy, Toll-like receptor 4, rs4986791, polymorphism.

Introduction

There is a global epidemic of diabetes mellitus. Diabetes affects 537 million individuals (twenty to seventy-nine years old). By 2030, there will be 643 million diabetics worldwide, and by 2045, there will be 783 million. About 240 million adults with diabetes are still undiagnosed. Additionally, type 2 diabetes is more likely to occur in 541 million adults [(1)].

The most prevalent form of diabetes, type 2, is related to an increased risk of developing a variety of comorbid conditions, containing coronary heart disease, stroke, peripheral vascular disease, and end-stage renal failure. It is also 5th greatest reason for death globally [(2)].

In T2D there is a decrease in the response of muscle, fat, and liver cells to insulin, so they cannot utilize glucose worthily from blood or store it. The pancreas initially produces extra insulin to compensate for this and over time the pancreas produces insufficient insulin leading to an increase in blood glucose level [(3)].

One of the severest chronic microvascular consequences of diabetes mellitus is diabetic nephropathy, and its prevalence is rising globally. Main patient burden in dialysis facilities around the world is caused by DN, which is the primary reason for end-stage renal failure and chronic kidney disorders [(4)].

Type 2 Diabetes and its consequences, diabetic nephropathy, atherosclerosis, and diabetic retinopathy are inflammatory diseases. Therefore, it may be a useful target for studying proteins associated with components of innate immune system [(5)].

Numerous mechanisms, including hypoxia, cell death in growing adipose tissue, activation of nuclear factor-kappa beta (NF-κB) and Jun N-terminal kinase pathways, activation of interleukin-1beta, and recruitment and activation of immune cells, are believed to oversee inflammatory state in type 2 diabetes [(6)].

Toll-like receptors (TLRs), a type one transmembrane receptor family, are included in the first identification of pathogens via various pathogen-associated molecular patterns (PAMPs). These PAMPs trigger signalling cascade of events, which in turn triggers the host's immune response [(7)].

Pancreatic islet cells (i.e., beta-cells and resident macrophages) express toll-like receptor 4 (TLR-4), which when activated may lead to insulin resistance, pancreatic cell malfunction, and changes in glucose homeostasis [(8)].

TLR-4 has also been shown to detect endogenous molecules produced by damaged tissues and necrotic cells. These molecules, termed damage-associated molecular pattern molecules and it is involved in mediating chronic inflammatory [(9)].

In cases with untreated T2D, excess glucose and free fatty acids increase TLR-4 mRNA and protein production. Free fatty acids; activate inflammatory response and metabolic signalling in insulin resistance, primarily through TLR-4 NF-κB signalling pathway in pancreatic beta cells and macrophages [(10)].

Studies on the underlying mechanisms of intra-renal inflammation can offer novel therapeutic targets for anti-inflammatory regimens against DN, because toll-like receptors are linked to the pathogenesis of both acute and chronic renal illness [(11)].

In the context of excessive hyperglycaemia, hypoxia, and hyperlipidaemia, that are considered endogenous ligands, they form the foundation of the pathophysiology of DN. TLR-4 is triggered by these endogenous ligands that is increased in the pathogenesis of DN [(12)].

Cases with DN had higher levels of high-mobility group box 1, TLR-4 endogenous ligand, in their proximal tubular cells [(13)].

On chromosome 9q33.1, the toll-like receptor 4 gene, which produces the TLR-4 protein, is found. Mutations in this gene

may change how TLR-4 recognizes and interacts with its environment [14].

Human TLR-4 gene contains 2 important single nucleotide polymorphisms, rs4986790 and rs4986791. One of which causes amino acid aspartate to be replaced by glycine on the 299th location of encoded peptide (Asp299Gly), and other causes replacement of threonine by isoleucine on 399th location of peptide structure, described by C and T allele [15].
Involvement of TLR-4 polymorphisms, particularly rs4986790 and rs4986791, in the course of T2D and related complications, such as nephropathy, is gaining attention, as the results of previous studies are controversial [16].

Subjects and methods:

Subjects:

Sixty T2D cases (Group I and Group II) had been recruited from the Internal Medicine Department and Outpatient Clinic at Benha University Hospital. Their mean age was (56±5.46) years; there were 17 males and 43 females.

Group I: Comprised thirty cases complicated with DN and their diagnosis depended on fulfilling the diagnostic criteria of the American Diabetes Association which include albuminuria and low estimated GFR.

Group II: Comprised 30 cases without DN, and their diagnosis depended on their medical records and fulfilling diagnostic criteria of American Diabetes Association, that specifies fasting plasma glucose ≥ 126 mg/dl or 2-hour postprandial plasma glucose ≥ 200 mg/dl or random blood sugar ≥ 200 mg/dl.

Group III: Comprised twenty apparently healthy persons serving as control group selected from the general population, their mean age was (55±6.28) years and range (of 42-65) and they were 5 males and 15 females.

This case-control study was carried out from February 2022 to August 2022. Approval of the study protocol was granted by the Ethical Committee of the Faculty of Medicine, Benha University. Study code (MS: 23-1-2021), and all subjects signed a written informed consent before joining the study.

Sample Collection:

After fasting for 10 hours seven millilitres of venous blood were drawn under complete aseptic conditions from each participant and divided as following:

1. Two milliliters of whole blood had been taken in tri K ethylene diamine tetra acetate (1.2mg/ml) vacutainer, then separated into two aliquots 1 had been used for: the determination of glycosylated haemoglobin (HbA1c), and the other stored at -80°C for subsequent DNA extraction.
2. Five milliliters of blood were taken in plain test tubes (without anticoagulant), left to clot for 30 minutes at room temperature, and then centrifuged (at 1500 rpm for 15 minutes) and separated serum had been used for clinical chemistry tests.

A twenty-four-hour urine collection has been done by collecting urine in an aseptic container over a full twenty-four-hour period and used to measure microalbumin.

Methods:

All participants were subjected to:

1- Full History taking including name, age, socio-economic status, duration of disease, and family history of diabetes mellitus.
2- Blood pressure measurement.
3- Body mass index had been calculated as weight in kilograms divided by squares of height in meters (kg/m^2).
4- Estimated glomerular filtration rate had been calculated using Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) Creatinine Equation (2021). Calculator available from eGFR Calculator National Kidney Foundation.

Routine Tests:

All biochemical parameters (FPG, triglycerides (TG), Total Cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and serum

creatinine) had been measured by Dialab chemical analyzer, SN:4714209, Austria. While HbA1c and Microalbumin were measured by Architect c4000 chemical analyzer, SN: C462233, Japan.

DNA Extraction and PCR Methods:

Genomic DNA had been extracted using whole blood genome DNA extraction kits GF-1 Nucleic Acid Extraction Kit fifty columns, Cat #GF-BD-050, lot No (40249-22) and (40202-21), vivantis, Malaysia according to manufacturer instructions.

Enzymatic amplification of the extracted DNA was performed by TaqMan® Universal Master Mix II kit, no Uracil-N-Glycosylase, containing components wanted to perform PCR, particularly planned for real-time PCR by using TaqMan® probe, specific primers, and Applied Biosystem Step One ® Real-Time PCR Thermal Cycling Block, SN:271003648, Singapore.

The specific primers used for TLR-4 gene Single Nucleotide polymorphism rs4986791 were: Forward (5′-CAACAAAGGTGGGAATGCTTT-3′), Reverse(5′TGTTTCAAATTGGAATGCTGGA-3′) and TaqMan probes used for the detection of SNP were: FAM-probe (GACAACCAGCCTAAAGTAT) and VICprobe(GACAATCAGCCTAAAGTATT). The Real-time cycler conditions showed in (table 1).

Representative allelic discrimination plot from the genotyping assay of TLR-4 gene polymorphism rs4986791 showed in the following figure **(**Figure 1**).**

Table (1): The Real-time cycler conditions.

Stage	Cycle	Time	Temperature
Pre-Holding stage	1	30sec	60°C
Holding stage	1	10min	95°C
Denaturation	40	15 sec	92°C
Annealing		1min	60°C
Post- Holding stage	1	30sec	60°C

Sec: seconds, min: minutes.

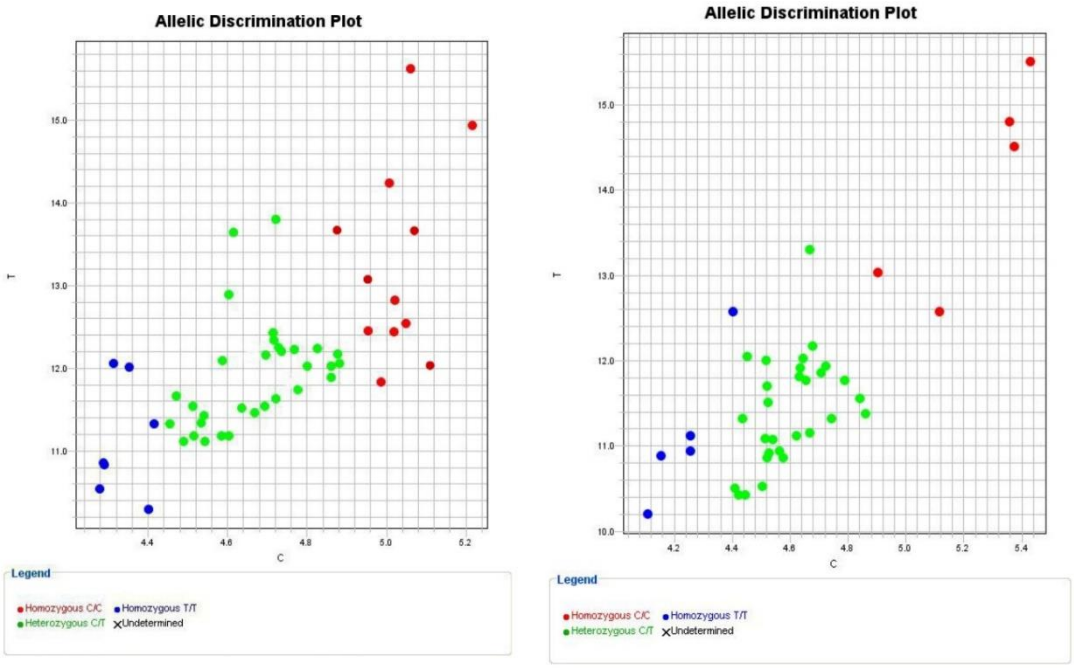

Figure (1): Allelic discrimination blot for different genotypes of TLR-4 gene polymorphism rs4986791 of the studied subjects.

Statistical Methods:

Statistical Package for Social Science had been used to update, code, and tabulate obtained data (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.). Mean, Standard deviation, median, and range for numerical data, as well as frequency and percentage for non-numerical data, had been used to portray categorical data. To investigate the connection between 2 qualitative variables, a Chi-Square test had been performed. To compare observed and anticipated genotype frequencies, Hardy-Weinberg equilibrium analyses had been carried out. Odds ratio is a metric for the relationship between exposure and result. Its precision is estimated using a ninety-five per cent confidence interval. P-value is deemed significant if it is <0.05 at a ninety-five per cent confidence level.

Results:

Patient Demographic, Clinical, and Laboratory Data: There had been no statistical variation among studied groups regarding age, sex, BMI, smoking, TC, HDL and LDL (P>.05) and there had been a variation among the studied groups regarding; Family history of diabetes mellitus, Hypertension, FPG, HbA1c and TG (P<.01) (Table 2).

Type 2 diabetes with DN group had been significantly related to higher creatinine, 24hr microalbuminuria, and lower eGFR when compared to T2D without DN (P<.001 for each) (Table 3).

Table (2): Comparison between T2D and control groups regarding demographic, clinical, and laboratory data.

	T2D n = 60	**Control n = 20**	**P-value**
Age (years)	56.38 ± 5.46	55.2± 6.28	P=.402
Male (n)	17 (28.3%)	5 (25%)	P=.772
Female (n)	43 (71.7%)	15 (75%)	
BMI (kg/m^2)	33.67 ± 5.41	31.84 ± 3.83	P=.165
Smoking (n)	14 (23.3%)	4 (20%)	P=1.0
Family history of D M (n)	53 (88.3%)	7 (35%)	*P<.001**
Hypertension (n)	45 (75%)	0 (0%)	*P<.001**
FPG (mg/dL)	189.42 ± 53.32	89.55 ± 7.39	*P<.001**
HbA1c (%)	8.31 ± 1.48	4.76 ± 0.42	*P<.001**
TC (mg/dL)	212.52 ± 47.72	194.70 ± 36.06	P=.131
HDL (mg/dL)	48.25 ± 13.64	48.90 ± 12.85	P=.852
LDL (mg/dL)	120.70 ± 32.14	111.60 ± 33.47	P=.281
TG (mg/dL)	158.0 (60.0 – 323.0)	118.0 (64.0 – 237.0)	*P=.007**
Duration (years)	10.77 ± 0.62	-	-
Nephropathy (n)	30	-	-

BMI; body mass index, FPG; fasting plasma glucose, HbA1c; glycosylated haemoglobin, TC; total cholesterol, HDL; high-density lipoprotein, LDL; low-density lipoprotein, TG; triglyceride, * statistically significant.

Table (3): Comparison of renal function tests among T2D patients with nephropathy and without nephropathy.

	T2D with nephropathy n = 30	**T2D without nephropathy n = 30**	**P value**
Serum creatinine (mg/dL) Median (Range)	4.4 (1.8 – 8.7)	0.9 (0.7 – 1.2)	*P<.001**
24hr urinary microalbumin (mg/24hr) Median (Range)	272.00 (45.00 -956.00)	12.00 (5.70 – 25.00)	*P<.001**
eGFR (mL/min/1.73 m^2) Median (Range)	19.55 (9.80 – 51.90)	108.70 (87.10 – 167.80)	*P<.001**

eGFR: estimated glomerular filtration rate.

Toll-like receptor 4 gene polymorphism had been assessed between all studied subjects. Among the T2D group, 20% had CC, 61.7% had CT, and 18.3% had TT, while, among the control group, 50% had CC, 45% had CT, and 5% had TT. the gene genotypes, CT, TT, and T allele found to be associated with T2D (P<.05), with risk of T2D susceptibility (OR>1) (Table 4).

Among T2D with the DN group, 16.7% had CC, 60% had CT, and 23.3% had TT, while, among T2D without the DN group, 23.3% had CC, 63.3% had CT, 13.3% had TT. No significant association was found regarding TLR-4 gene polymorphism between studied cases with and without nephropathy (Table 5).

Table (4): Comparison of TLR-4 gene polymorphism among T2D patients and control group.

TLR-4 gene polymorphism		T2D n = 60		Control n = 20		P value	OR (95 % CI)
		N.	%	N.	%		
Genotypes	**CC**	12	20	10	50		Reference
	CT	37	61.7	9	45	*.30**	3.426 (1.128 – 10.409)
	TT	11	18.3	1	5	*.49**	9.167 (1.003 – 83.77)
Dominant model	**CC**	12	20	10	50		Reference
	CT + TT	48	80	10	50	*.12**	4.0 (1.357 – 11.790)
Recessive model	**CC + CT**	49	81.7	19	95		Reference
	TT	11	18.3	1	5	.179	4.265 (0.515 – 35.341)
Alleles	**C**	61	50.8	29	72.5		Reference
	T	59	49.2	11	27.5	*.019**	2.550 (1.168 – 5.567)

N; number, OR; odds ratio, CI; confidence interval, Reference, according to NCBI database; C, Cysteine; T, thymine; P<.05 is considered significant; OR<1 has been considered protective; OR>1 has been considered risky.

Table (5): Comparison of TLR-4 gene polymorphism between studied cases with and without nephropathy.

TLR-4 gene polymorphism		T2D with nephropathy n = 30		T2D without nephropathy n = 30		P value	OR (95 % CI)
		N.	%	N.	%		
Genotypes	**CC**	5	16.7	7	23.3		Reference
	CT	18	60.0	19	63.3	.674	1.326 (0.356 – 4.947)
	TT	7	23.3	4	13.3	.296	2.450(0.456 – 13.161)
Dominant model	**CC**	5	16.7	7	23.3		Reference
	CT + TT	25	83.3	23	76.6	.520	1.522 (0.423 – 5.472)
Recessive model	**CC + CT**	23	76.7	26	86.7		Reference
	TT	7	23.3	4	13.3	.322	1.978 (0.513 – 7.635)
Alleles	**C**	28	46.7	33	55		Reference
	T	32	53.3	27	45	.362	1.397 (0.681 – 2.865)

N; number, OR; odds ratio, CI; confidence interval, Reference, according to NCBI database; C, Cysteine; T, thymine; P<.05 has been considered significant; OR<1 has been considered protective, OR>1 has been considered risky.

Discussion:

It is considered that TLR-4 plays an important role in the development of T2D and its consequences like DN, as abnormal immune response had been connected to Type 2 diabetes and DN [8].

TLRs involving TLR-4 have been innate Immune genes that encode membrane proteins and they play an important role in modulating innate immunity [17].

Numerous coding and non-coding variants at the TLR-4 locus are linked to corresponding illnesses, supporting the idea that TLR-4 plays a significant functional role in the evolution of inflammatory disorders [18].

TLR-4 gene SNPs are linked to a variety of inflammatory and autoimmune illnesses, and they can interfere with intracellular signaling in mononuclear cells, triggering immunological reactions [19].

Different illness susceptibilities can be linked to differences in variant allele frequencies between various ethnic regions [20].

In this regard, we concentrated on evaluating the impact of TLR-4 polymorphisms on the risk of DN and T2D in the Egyptian population.

In the present work there had been no statistical variation among studied groups regarding age, gender, BMI, smoking, TC, HDL and LDL.

The study done in 2018 [21] found no statistical variation in age and sex between the studied groups. Our result of BMI agreed with data obtained from this work [22] who found no statistical difference between patients with T2D and control group. Meta-analysis study [23] disagreed with our result regarding smoking as they suggested that smoking is related to enhanced risk of T2D. Other study showed that all lipid derivatives (TG, HDL, LDL, and TC) were Higher in the diabetic group indicating a higher risk of developing T2D [24].

There had been a variation among the studied groups regarding; Family history of diabetes mellitus, Hypertension, FPG, HbA1c, and TG. In agreement with these results the Chinese study [25] found that family history risk categories of diabetes have significant and independent relation to prevalence of T2D. Regarding hypertension the systolic blood pressure and diastolic blood pressure levels had been higher in individuals with diabetes [26]. In addition, diabetes cases had been having high FPG and HbA1c when compared to the control group [27].

Type 2 diabetes with DN group had been significantly higher creatinine, 24hr micro albuminuria, and lower eGFR when compared to T2D without DN. Similarly, other study showed a significant increase in creatinine levels in diabetic nephropathy patients [28]. Regarding eGFR result, it was consistent with other study [29], which reported that in most of the T2D patients who had albuminuria (micro and macro), their eGFR was significantly reduced.

Regarding TLR-4 gene polymorphism CT, TT genotypes, dominant model, and T allele were found to have higher frequency associated with T2D, with increased risk of T2D susceptibility.

The findings had been in keeping with the meta-analysis study [30], which reported that rs4986791 had been associated with increased T2D risk under homozygous, heterozygous, and allelic models.

These findings had been also consistent with this research [31], which revealed that the genotypic distribution of rs4986791 polymorphism of TLR-4 in the patient group with T2D was homozygous CC genotype (63.6%), the heterozygous CT genotype (25.25%), and recessive homozygous TT (11.11%). In the control group, genotype frequencies had been (67% CC, 17.5% CT, and 6.5% TT), and T- allele frequency had been higher in T2D studied cases group with (P=.003).

Another study done by a group of scientists [32] proved that SNP (rs4986791) CT genotype is associated with increased

insulin levels and insulin resistance, which are risk factors for metabolic syndrome and diabetes. In this context another study, support our results [33] who reported that TLR-4 SNPs (Asp299Gly) and (Thr399Ile) had been related to cytokine storm (increased inter leukin-6) which has an important role in T2D diabetes pathogenesis. Also, study [34] performed on two different TLR-4 SNP (rs5030717 and rs5030718) showed that TLR-4 polymorphisms can be useful in expecting those with type 2 diabetes who have been at risk of hypertension and dyslipidaemia.

Conversely, other research [35] demonstrated that the distribution of TLR-4 Thr399Ile genotypes in patients with T2D was CC genotype (83.3%), CT genotype (16.7%) and in the control group was CC genotype (83.3%), CT genotype (11.7%) with no statistically significant difference between them (P=.4). Furthermore, TLR-4 rs4986790 and rs4986791 polymorphisms were found to be similarly distributed in both non-diabetic and type 2 diabetic individuals [36]. This could be as the two polymorphisms rs4986790 and rs4986791 have been related to the rise in TLR-4 Mediated IL-10 production and reduction in proinflammatory cytokines [37].

Our results were against this study [38] that showed minor alleles of Asp299Gly and Thr399Ile polymorphisms had been related to protection for T2D, this may be due to Asp299Gly and Thr399Ile polymorphisms had blunted responses to inhaled lipopolysaccharide in humans [39].

The relation of the studied SNP (Thr399Ile) to DN in our study showed no significant association among patients with and without nephropathy. This was consistent with the Iranian research [40] that failed to identify any homozygous variant genotypes of *Thr399Ile* polymorphisms in Iranian subjects and so, they could not discover any relationship between this polymorphism and diabetic nephropathy.

Conclusion :

To conclude, rs4986791 of TLR-4 gene polymorphism appears to be important in the pathogenesis of T2D. As this study elucidated the distinguished distribution of TLR-4 gene genotypes (CT and TT) between type 2 diabetic studied cases and healthy subjects allied with increased risk of T2D. But no significant association was found between these variants and diabetic nephropathy.

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