Annals of R.S.C.B., ISSN: 1583-6258, Vol. 25, Issue 1, 2021, Pages. 6497 – 6506
Received 15 December 2020; Accepted 05 January 2021.

Association of Toll-Like Receptor (TLR-4) Gene Polymorphisms in Diabetic Kidney Disease: Iraqi Cohort Study

Rawaa B. Al-Fatlawi[1], Bassim I. Mohammad[2], Hayder A. Al-Aubaidy[3], Najah R. Hadi[4]

[1] Faculty of Medicine, University of Kufa, Kufa, Iraq, Email: xxx
[2] College of Pharmacy, University of Al Qadisiyah, Iraq: Email: xxx
[3]School of Life Sciences, College of Science, Health and Engineering, La Trobe University, Bundoora, VIC, Australia 3086,
[4]Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Kufa, Iraq;
Email: [3]h.alaubaidy@latrobe.edu.au, [4]drnajahhadi@yahoo.com

Abstract: Aims: This study aims to examine the role of TLR-4 gene polymorphisms in Type 2 Diabetes Militias (T2D) and its complications. 400 T2D patients were enrolled in this study. Patients were distributed into three clinical groups: normal albuminuria (n=145), microalbuminuria (n=162), and macroalbuminuria (n=93) groups. Multi-nominal logistic regression analysis applied to study genotype and allele frequencies for gene polymorphism,for the (+194C/T). The genotypic frequencies of Micro albuminuria group were (n=116, 71.4%) normal CC and (n=32 20.0) heterozygous CT. Mutant homozygous was found inTT (n=14, 8.6%). The results in Normo albuminuria group demonstrate (n=73, %50.0) wild type CC, (n=45, 31.25%) heterozygous CT and mutant homozygous TT 18.75% (n=27). The odds ratio for the 194CT was 1.27; p=0.56. The TLR-4 gene of Thr399Ile polymorphism was correlated to the early onset of diabetic kidney disease, while Asp299Gly polymorphism of the TLR-4 gene seems to act as a protective factor for diabetic nephropathy.
Keywords: Diabetic Nephropathy; Type 2 Diabetes; Toll-Like Receptor-4; Gene polymorphisms.

INTRODUCTION

The Toll-like Receptors (TLRs) role in inherited and developing immunity suggests which genetic differences in the encoding genes have a significant impact on development of inflammation [1-2]. The TLR-4 gene is located on chromosome 9q32–33. Single nucleotide polymorphisms (SNPs) were identified in the coding region in exon 3 [3]. Previous studies investigated the potential ofreplacement of aspartic acid with glycine (D299G); others resulted in substitution of threonine with isoleucine [4-6]. Both mutations exist in the TLR-4 extracellular area. Several studies have indicated that the Asp299Gly genotype carriers have pro-inflammatory cytokines declined level, reactions of acute formand an increased susceptibility to infections. On the other hand, the TLR-4 Asp299Gly SNPswas associated with less risk of development of atherosclerosis and type 2 diabetes (T2D)[7]. Some studies have showed that TLR-4polymorphisms were affecting the progression of chronic inflammation and atherosclerosis diseases [8], decrease the development of diabetes& its cardiovascular progression. While some studies found that there is an association between TLR-4polymorphism and diabetes [7-9]. Also, it has been investigated that the D299G and T399I genotypes carriers have an importance decrease the prevalence of diabetic complications [10]. The previous studies proved a significant correlation between polymorphisms in TLRs 2 and 4 genes with regards to inflammatory response, and T2D pathogenesis. Other study showed that there was no association between TLR-4 polymorphism and diabetes or diabetes complications [11]. Some studies specified the correlation betweenTLR-4and diabetic nephropathy (DN)[12]. Considering the possible role

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of the TLR-4 pathway in diabetes, the present study was designed to examine the role of TLR-4 gene polymorphisms role in T2D and its complications.

MATERIALS AND METHODS

This case control study was approved by the Human Research Ethics, Faculty of Medicine - University of Kufa. Four-hundred participants were included in this study, age (35 – 65) years. Participants were excluded from this study if they had type 1 diabetes, heart failure, inflammatory disorders, liver disease, thyroid disease, malabsorption, or if they were taking steroids, anti-inflammatory, antihypertensive, or hypolipidemic drugs. Participants were divided into three study groups according to their albuminuria results: Normo albuminuria (145); Micro albuminuria (162); and Macro albuminuria (93).

BLOOD SAMPLES

Two milliliters of venous blood samples have been collected using a 5 ml EDTA test tube for further laboratory investigations. Laboratory tests were performed by AlfuratAlausatpathology Unit, Al-Najaf. Whole blood samples were collected in Eppendorf tubes for DNA isolation for genotype studies.

TLR-4 GENOTYPING/ALLELIC DISCRIMINATION

Analysis of gene polymorphism of TLR-4 was performed in two selected single-nucleotide polymorphism (SNPs), (Asp299Gly) TLR-4 (+986 A/G) exon 3, and (Thr399Il3) TLR-4 (+1196 C/T) exon 3, that were common and associated with altering the level of TLR-4 expression and varied frequency in the general population. These SNPs were genotyped by real time polymerase chain reaction (PCR) allelic discrimination. Primers and probes were designed using Primer Express ^{TM} software (ABI, USA), table (1) [13].

Table 1: Primers and Probes used in the allelic discrimination study

Primer/probe	Sequence (5′ →3′ direction)
TLR-4 exon3 Chromosome 9 Thr399Ile (rs4986791)	
Forward	CAACAAAGGTGGGAATGCTTT
Reverse	TGTTTCAAATTGGAATGCTGGA
FAM- probe	GACAACCAGCCTAAAGTAT
VIC-probe	GACAATCAGCCTAAAGTATT
TLR-4 exon3 Chromosome 9 Asp299Gly (rs4986790)	
Forward	TCTGCTCTAGAGGGCCTGT
Reverse	AGTCACACTCACCAGGGAAA
FAM- probe	CTACCTCGATGATATTATTGACT
VIC-probe	CCTCGATGGTATTATTGACT

STATISTICAL ANALYSIS

Mean ± standard error (SE) was measured for quantitative variables. Multi-nominal logistic regression analysis applied to study genotype and allele frequencies for gene polymorphism.Genetic equilibrium test using the Hardy-Weinberg equilibrium (HWE) for

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assessment of genotype and allele frequencies by the chi square test was also performed. The significance level for statistical tests was 0.05 ($p<0.05$), Odd's ratio (OR) at 95 % confidence intervals (CI) was determined. For HWE; P-value >0.05 is considered to be consistent with HWE.

RESULTS

1.1.Thr399il3 (rs4986791), (+1196C/T)

The distribution of genotype and allele frequencies among nephropathy groups compared with controls (Normo albuminuria) for the (+1196C/T) is shown in table (2). The three genotypes of the TLR-4 Thr399il3 site were CC, CT and TT. The genotypic frequencies of Micro albuminuria were (n=l 16, 71.4%) normal CC and (n=32%20 .0) heterozygous CT. Mutant homozygous was found in TT (n= l4, 8.6%). The ratios of repeat alleles in this score are not identical to the adjusted Hardy- Weinberg Equilibrium (HWE) with a significantly differences (P=0.05), Table 2.The distribution of Cl I96T genotype frequencies of Macro albuminuria group is 63% (n=59) individuals with wild type CC and 26.3% (n=42) with heterozygous CT. Mutant homozygous TT was seen in 10.5% (n= 10). For controls (Normo albuminuria), the results demonstrate (n=73, %50.0) wild type CC, (n=45, 31.25%) heterozygous CT and mutant homozygous TT 18.75% (n=27). The ratios of repeat alleles in this score are not identical to the adjusted Hardy-Weinberg Equilibrium (HWE) with a significant difference (P=0.08), however, in genetic disorders, Different factors could disturb Hardy-Weinberg balance either by the impact of genes distribution within the population or by changing the gene frequencies. These factors include: Small population size Mutation, Gene flow (migration), Selection and Non-random mating [5], Table 2.The T and C alleles has significantly difference between control and Micro albuminuria groups with OR=3.2, there are also significantly difference between control and Macro albuminuria groups with OR= 2.63, and the significantly difference of the two-allele frequency for both T and G was P=0.02.In studying polymorphism of Cl l96T, the result showed that the carriers of the homo-mutant genotype ll96TT were significantly at higher risk of DN for Micro albuminuria than the wild type and heterozygote (with OR=2.71) and (Macro albuminuria with OR= 2.50) than the wild type and not for heterozygote l196CT (with OR=2.27). The carriers of the heterozygote genotype l l96TT were significantly at higher risk of DN for Micro albuminuria than the wild and mutant types, the T allele frequency in Micro albuminuria. Macro albuminuria patients and controls were (0.24%), (0.19%) and (0.34%) respectively,the ratio of odds for the l196CT genotype have a second risk of NP after l l96TT and association with high risk than the wild type l l 96CC.

1.2.Asp299Gly (rs4986790) (896A/G)

The distribution of genotype and allele frequencies among groups compared with controls (Normo albuminuria) for the (+299A/G) is shown in table (3). The three genotypes of the TLR-4 Asp299Gly site were AA, GA and GG. The base distribution frequencies of G and A were (0.48) and (0.52) in the control group (Normo albuminuria). They were 0.41 and (0.59) in the micrNP and Macro Albuminuria groups 0.32 and 0.68, respectively. The A and G alleles has no significantly difference in all groups. The genotypic frequencies of Micro albuminuria patients were 21% (n=34) normal GG and 39.39% (n=64) heterozygous GA and mutant homozygous AA 39.39 (n=64). In controls the result showed 16.3 % (n=23) wild type GG, 64.52% (n=94) heterozygous GA and mutant homozygous AA in 19.35% (n=28). The A and G alleles has significantly difference between control and Micro albuminuria groups with OR=3.2, there are no significantly difference between control and Macro albuminuria groups, and the significantly difference of the two-allele frequency for both T and G was p=0.02,

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Table 3.In studying polymorphism of G299A, the result showed that the carriers of the homo-wild genotype 299AA were significantly protective from DN for both Micro albuminuria with OR=2.71 and Macro albuminuria with OR= 2.50 than the mutant type and heterozygote (with OR 0.40 and 0.82, 0.96 and 0.83 respectively). And the G and A allele frequency has been deferring between control and Micro Albuminuria with P=0.03, table (3).

Table 2: The genotyping and allele frequencies of (rs4986791), (+1196C/T) in the studied groups

Groups	HW frequency	Genotypes				*P*-HWE	Allele frequency	
		CC	CT	TT	Total		C	T
Normo albuminuria (1)	Observed frequency (%)	73 (50.0)	45 (31.25)	27 (18.75)	145 (100.0)	0.08	0.66	0.34
	Expected frequency (%)	30.2 (43.1)	32.5 (45.1)	8.8 (11.8)	74 (100.0)			
Micro albuminuria (2)	Observed frequency (%)	116 (71.4)	32 (20.0)	14 (8.6)	162 (100.0)	0.05	0.81	0.19
	Expected frequency (%)	74.4 (66.3)	23.2 (30.2)	2.7 (3.5)	81 (100.0)			
Macro albuminuria (3)	Observed frequency (%)	59 (63.2)	24 (26.3)	10 (10.5)	93 (100.0)	0.24	0.76	0.24
	Expected frequency (%)	25.5 (58.2)	15.8 (36.2)	2.5 (5.6)	44 (100.0)			
1 *vs.* 2	OR	0.40	1.82	3.46			0.46	2.2
	P	0.09	0.40	0.29			0.02	0.02
1 *vs.* 3	OR	0.58	2.27	2.96			0.61	2.63
	P	0.40	0.76	0.69			0.16	
2 *vs.* 3	OR	0.46	0.70	0.80			1.35	0.74
	P	0.55	0.73	1.0			0.49	

HW: Hardy-Weinberg; P-HWE: probability of Hardy-Weinberg equilibrium; OR: odd ratio; P: Fischer exact probability (two tailed)

Table 3: The genotyping and allele frequencies of (rs4986790) (896A/G) in the studied groups

Groups	HW frequency	Genotypes				*P*-HWE	Allele frequency	
		AA	AG	GG	Total		A	G
Normo albuminuria (1)	Observed frequency (%)	28 (19.35)	94 (64.52)	23 (16.3)	145 (100.0)	0.10	0.52	0.48
	Expected frequency	19.7 (26.64)	35.8 (49.95)	16 (23.41)	72 (100.0)			

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	(%)							
Micro albuminuria (2)	Observed frequency (%)	64 (39.39)	64 (39.39)	34 (21.22)	162 (100.0)	0.29	0.59	0.41
	Expected frequency (%)	26.7 (34.92)	36.9 (48.35)	12.7 (16.74)	76.7 (100.0)			
Macro albuminuria (3)	Observed frequency (%)	46 (50.0)	34 (36.36)	13 (13.64)	93 (100.0)	0.45	0.68	0.32
	Expected frequency (%)	10.2 (46.49)	22 (43.39)	5.1 (10.12)	51 (100.0)			
1 *vs.* 2	OR	2.71	0.96	0.40			1.33	0.75
	P	0.104	0.051	0.752			0.393	0.393
1 *vs.* 3	OR	2.50	0.83	0.82			1.96	0.51
	P	0.215	0.055	1.0			0.03	0.03
2 *vs.* 3	OR	0.65	0.14	0.71			0.68	1.48
	P	0.580	1.0	0.723			0.240	0.240

HW: Hardy-Weinberg; P-HWE: probability of Hardy-Weinberg equilibrium; OR: odd ratio; P: Fischer exact probability (two tailed)

DISCUSSION

The results of our study refer to that the Asp299Gly polymorphism could be a protecting agent for nephropathy in patients with T2D, while the Thr399il3 polymorphism could be a risk indicator for the development of nephropathy in T2D independent of the features of another laboratory and clinical. But the mechanism which underlying this association demands to be further investigated. These findings were described earlier by some studiesbut they did not discuss their correlation to diabetes and/or nephropathy [14-16]. In addition, previous studies showed a link between DN and TLR-4 Thr399Ile polymorphism [17]. Additionally, the TLR-4-Thr399Ile polymorphisms were notified to be not public in most of Asian ethnic population studies. In subjects of Chinese, they couldn't recognize any heterozygous or homozygous varied genotypes of TLR-4-Thr399Ile polymorphisms [18]. However, this association was observed for nephropathy in a high prevalence renal disease [10]. Maldonado-Bernal et al., 2011 found that the Asp299Gly polymorphism not associated with T2D itself, but the TLR-4 variants could be related indirectly to diabetes type 2 [19]. Nevertheless, this might increase the risk of T2D in patients who suffer from increasing of TC/HDL-C [10]. Similar to our findings, Buraczynska et al., reported that the Thr399 allele was related to a decreased risk of clinical diabetes [10]. Many studies have shown that *TLR-4-Thr399Il e* polymorphism is associated with reduced susceptibility to T2D, while other reports revealed no association between this polymorphism and T2D in Mexican patients [20]. In a Russian study, the investigation did not demonstrate the significance related to the

*TLR-4*Thr399Ile polymorphism with risk of coronary artery disease [10]. Only the study of Rudofsky et al. reported the Asp299Gly polymorphism affects diabetic complications, that study was involving 530 T2D patients. In their main findings, they highlighted thatTLR-4 genotype is strongly related to decreasing distribution of diabetic neuropathy in T2Dpatients[17].It has been shown by our result that TLR-4 participated in the DN pathogenesis. Devaraj et al. reported that TLR-4 expression and its functional activation, legend, and signaling were increased detected and participated in the proinflammatory state of diabetes type 2. Furthermore, in animal models, the knockout of TLR-4 leads to decline in the proinflammatory status of diabetes [21].At position 299 for Asp299Gly and position 399 for the Thr399il3, because of the amino acid exchange, the functional influence of TLR-4 will be large. Still, the molecular mechanisms of how to increases or decreaseTLR-4 polymorphism could affect the risk of early diabetic nephropathy haven't determined yet. Butthis could be that the innate immune responses affect the pathogenesis of diabetic nephropathy. The information about polymorphisms and diabetic elaboration of TLR-4 genetic was limited. Rudofsky et al., in a German population, reported that Thr399Ile and Asp299Gly genotypes of the TLR-4 were related to DN in T2D, but it wasn't related to DN itself [17]. While Buraczynska et al., in the population of Poland, reported that early onset of diabetic early beginning of diabetic retinopathy type-2 retinopathy in patients with T2D was associated with Asp299Gly therefore the current study aimed to study the relationship between diabetic nephropathy and TLR-4 polymorphisms[10].Our study addressed the knowledge gap of the correlation between TLR-4-Asp299Gly code and the early and/or later development of DN in patients with T2D. This polymorphism is strongly related with the early onset of T2D rather than susceptibility to the development of DN,in addition, therewas a correlation between TLR-4 Thr399Ile genotype and the prevalence DN. Thus, according to previously discussed [10], the data suggested that the pathogenesis of DN might partly differ in T2D patients[10]. By using an interactive term model, the type of relationship effect between T2D and TLR-4 was lindear. So, the results of the current study addressed the question of whether the innate immune response could contribute to the development of nephropathy in T2D. In positions 299 and 399, the polymorphisms of amino acids define the variation in the extracellular domain structure, as a result, the pattern of the isoforms of the TLR-4 receptor will recognition. (Lin et al., 2013) provided evidence which TLR-4 inhibition gives reno-protective effects in eNOS knockout of mice diabetic with the development of diabetic nephropathy[22]. Nevertheless, there is rare information on the effects of these polymorphisms on the bonding which ligands to the receptor [16].

CONCLUSION

The TLR-4 gene of Thr399Ile polymorphism was correlated to the early onset of diabetic kidney disease, while Asp299Gly polymorphism of the TLR-4 gene seems to act as a protective factor for diabetic nephropathy.

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