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Is Gene polymorphism of Advance Glycation End Product receptors related to severity of neuropathy among Iraqi type II diabetic patients: Case control study

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Abstract;

Objective: To detect any correlation between ***rs1800624*** **(374 A/T)** RAGE gene polymorphism with the development and/or severity of diabetic peripheral neuropathy. **Patients and method:** The cross sectional case controlled study include 149 type 2 diabetic patients, patients recruited from diabetic center in Marjan medical city and from diabetic center in Al-Sadr medical city in the Middle Euphoarates area of Iraq during the period from June 2019 to April 2020 .The patients is divided into 2 group according to clinical finding and results of neurophysiologic studies; group I with diabetic neuropathy (DNP) and group II without neuropathy. Genetic study using polymerase chain reaction (PCR) and Restriction fragment length polymorphism (RFLP) were done for all participants looking for RAGE (receptor of advanced glycation end products) gene by determining RAGE gene rs1800624 polymorphism for both groups**. Results:** The three genotypes of RAGE gene, AA (wild-type) and the two RAGE ***rs1800624*** polymorphic genotypes (AT and TT) were analyzed for both groups. Majority with DPN group (72.4 %) carry the wild type gene (AA). Heterozygous polymorphic RAGE gene (AT) was present in (55 %) of patients with DPN, homozygous RAGE gene polymorphisms(TT) were also more common in patients with DPN (29%).The study found significant association of TT genotype and severity of DNP.TT genotype was dominant in 63% of patients with severe DNP, while TT genotype was observed only in 17.6% of patients with mild and in 19.1% of patients with moderate peripheral neuropathy. **Conclusions:** There was significant association between ***rs1800624*** **(374 A/T)** RAGE gene polymorphism and with occurrence and/or severity of diabetic peripheral neuropathy.

Keywords: Receptors, advanced glycation end products, gene polymorphism, diabetic peripheral neuropathy

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Introduction

Diabetes mellitus is a clinical syndrome characterized by hyperglycemia caused by absolute or relative deficiency of insulin. Hyperglycemia has many causes but is most commonly due to type 1 or type 2 diabetes[1]. Insulin deficiency affects the metabolism of carbohydrate, protein, fat and can cause significant disturbance of water and electrolyte homeostasis; death may result from acute metabolic decompensation[2]. Longstanding metabolic derangement is associated with functional and structural changes in many organs, particularly those of the vascular system, which lead to the clinical 'complications' of diabetes. These characteristically affect the eye, the kidney and the nervous system[1]**.** The molecular mechanisms involved in the development of Diabetic peripheral neuropathy is a complex process that includes activation of the polyol pathway, exaggerated oxidative stress, over activity of protein kinas C and increased formation of advanced glycation end products in the presence of hyperglycemia. In addition, there is increasing evidence that genetic factors could also contribute to the development of Diabetic peripheral neuropathy [3]. Long-term hyperglycemia accelerates the formation and accumulation of advanced glycation end products and leads to the over-expression of their cellular receptors in diabetes [4]. The receptor for advanced glycation end products, a multi-ligand member of the immunoglobulin superfamily of transmembrane cell surface molecules, is the best characterized receptor for advanced glycation end products [5]. The activation of the receptor by advanced glycation end products plays a major role in the pathogenesis of diabetic vascular complications [6].

The gene for receptor is located on chromosome 6p21.3 near the HLA locus [7], and at least 30 polymorphisms have been identified [8]. Three polymorphisms have been highlighted in studies that include the most common functional polymorphisms (_374 T/A) in the promoter region and a common coding change of a Gly to Ser at amino acid 82. This functional polymorphism plays a role in the development of diabetic complications [8].

Aim of study:

The present study aim to:

Detect any relation between RAGE gene polymorphism and the development and/or severity of diabetic peripheral neuropathy.

Patients and method

This case control study include 149 type 2 diabetic patients, those patients recruited from diabetic center in Marjan medical city 96 (64%) patients and from diabetic center in Alsadr medical city 54 (36%) during the period from June 2019 to April 2020 .Where both centers are main diabetic patients drainage in the middle Euphrates area of Iraq. The average age of the patients was between 37-75 years (mean 56.13 ± 8.60 years), 82 (55 %) patients were male and 67 (45 %) were female.All participants underwent full assessment including detailed history and comprehensive physical examinations done by board certified expert neurologist looking especially for sings of peripheral neuropathy. All participants underwent neurophysiologic assessment (conductive velocity, distal latency, amplitude.) including both

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sensory and motor nerves of upper and lower limbs. All patients were sent for biochemical assessment random blood sugar and glycosylated hemoglobin. The patients were divided in to 2 groups: group I with neuropathy as study group and group II without neuropathy as control group. Group 2 patients (with DPN) classified in to mild, moderate and severe DPN depending on Toronto clinical neuropathy score (TCNS), the system was first adopted by a research group in Toronto for the screening of DPN. The TCSS (Table 1) composed of three main parts: symptom scores, reflex scores, and sensory test scores. The minimum score is 0 point while the maximum score is 19 points. The criteria of classification for DPN have also been proposed according to the TCSS score: 0-5 points, no DPN,6- 8 points, mild DPN; 9- 11 points, moderate DPN; and 12 - 19 points, severe DPN [9].

Table 1 Toronto clinical scoring system (TCSS)

TCSS items		Description
Symptoms score	Pain	0 = absent, 1 = present
	Numbness	0 = absent, 1 = present
	Tingling	0 = absent, 1 = present
	Ataxia	0 = absent, 1 = present
	Upper-limb symptoms	0 = absent, 1 = present
Reflex score	Knee reflexes	Score for each side: 0 = normal, 1 = reduced, 2 = absent
	Ankle reflexes	Score for each side: 0 = normal, 1 = reduced, 2 = absent
Sensory test score	Pinprick	0 = normal, 1 = abnormal
	Temperature	0 = normal, 1 = abnormal
	Light touch	0 = normal, 1 = abnormal
	Vibration sense	0 = normal, 1 = abnormal
	Position sense	0 = normal, 1 = abnormal

Genetic study

DNA extraction the preparation of high-molecular weight genomic DNA from whole blood for all participants was carried out by using G-spin TM total DNA Extraction kit, which contains:
Buffer CL 25ml.; Buffer BL 25ml.; Buffer WA 40ml. ; Buffer WB 10ml.
Buffer CE 20ml. ; Spin column-collection Tube ; RNase A 3mg in 1vil proteins K 22mg in 1vil.
Quantification of DNA (µg/ml) was measured by adding 50 µl of stock DNA into the plastic disposable cuvette (Eppendorf UVette) and assessed at 260 nm wavelength. On the other hand, a wavelength of 280 nm was used for measurement of protein contamination. The purity of DNA was assessed by measuring the ratio of A260/A280.

Primer designing:

Target gene		Sequence (5'-3')	Tm (°C)	Product size
AGER	FH	5'-CTTTCACGAAGTTCCAAACAGG-3'	64	211 bp
	RH	5'-CTAGGGTCTCATTCCCTCAGA-3'	62	
AGER	RA	5'-CATTAAAGATCCGGGCAGGAC-3'	66	334 bp
	RA	5'-AGTTGCATCAATAGGGTTCAGG-3'	64	

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Polymerase Chain Reaction (PCR):A PCR kit provided by ABM, Canada was used. All PCRs was done in duplicates to minimize the variations. PCR reaction was run in a thermal cycler with following conditions: 94°C for 3 minutes followed by 35 cycles of 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 5 minute with a final extension
of 5 minute at 72C.

PCR- based RFLP analysis Restriction endonucleases (High Fidelity when possible) and buffers for the DNA manipulations were acquired from New England Biolabs (NEB). Restriction digests were prepared in a final volume of 40 µl, or multiples thereof, according to the appropriate purposes of digests. For preparative and analytical purposes, samples were prepared to meet the following criteria: 1-3 µg of PCR product was digested with a 1X appropriate restriction buffer and 10 units of the needed restriction enzyme(s). The digests then were incubated at 37°C (or enzyme appropriate temperature) for 3 hours.

Statistical Analysis:

Statistical analysis was carried out using SPSS version 23. Categorical variables were presented as frequencies and percentages. Continuous variables were presented as (Means ± SD). Student t-test was used to compare means between two groups. ANOVA test was used to compare means between three groups or more. Mann-Whitney test was used to compare means between two groups when variable was not normally distributed. Chi-square test was used to find the association between categorical variables. Correlation coefficient (r) was used to assess the relationship between two continuous variables. A *p*-value of ≤ 0.05 was considered as significant.

Results

Demographic data of the study patients

Table 1 show the demographic characteristics of patients in both studied group. The mean age of all patients in our study was 56.13 ±8.60 years; there were no significant differences in the mean age between both groups (P > 0.05). Statistical analysis showed there were no significant differences regarding gender, smoking and residence between both studied group (group I and II). (P > 0.05). The only statistically significant finding in demographic data between both group was the presence of family history of DPN. (P < 0.05)

Table 4.1. Demographic data of the study patients

Study variables	Group I (N=91)	Group II (N=58)	Total (N=149)	P-value
Age (years)	56.67 ± 8.79	55.29 ± 8.31	56.13 ± 8.60	0.343

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Gender **Male** **Female** **Total**	49 (53.8) 42 (46.2) 91 (100.0)	33 (56.9) 25 (43.1) 58 (100.0)	82 (55.0) 67 (45.0) 149 (100.0)	0.715
Smoking **Yes** **No** **Total**	18 (19.8) 73 (80.2) 91 (100.0)	13 (22.4) 45 (77.6) 58 (100.0)	31 (20.8) 118 (79.2) 149 (100.0)	0.699
Residence **Urban** **Rural** **Total**	60 (65.9) 31 (34.1) 91 (100.0)	43 (74.1) 15 (25.9) 58 (100.0)	103 (69.1) 46 (30.9) 149 (100.0)	0.291
Family history **Yes** **No** **Total**	63 (69.2) 28 (30.8) 91 (100.0)	30 (51.7) 28 (48.3) 58 (100.0)	93 (62.4) 56 (37.6) 149 (100.0)	0.031*

* significant differences at P≤0.05
Group I = Patients with diabetic neuropathy.
Group II = Patients without diabetic neuropathy.

Basal characteristics of all patients in the study

Table 2 shows non-significant difference in the duration of diabetes between patients with diabetic neuropathy in comparison with patients without diabetic neuropathy (P < 0.06)

There was highly significant difference in the HbA1C level between patients with diabetic neuropathy in comparison with patients without diabetic neuropathy (P < 0.01).

There was non-significant difference in the body mass index (BMI) between patients with diabetic neuropathy in comparison with patients without diabetic neuropathy (P > 0.05)

Regarding other complications of diabetes, the study found that although there were no significant differences between both group regarding hypertension and diabetic nephropathy (p. > 0.05), however there was highly significant difference in diabetic retinopathy between patients with diabetic neuropathy and patients without diabetic neuropathy (p. < 0.01). Table 2

Table 4.2. Basal characteristics of all patients in the study

Study variables	Group I (N=91)	Group II (N=58)	Total (N=149)	P-value
Duration (years)	11.73 ± 7.39	10.60 ± 5.07	10.90 ± 6.65	0.06
BMI (Kg/m^2)	29.09 ± 2.66	29.07 ± 2.64	29.08 ± 2.65	0.966
HbA1C	8.15 ± 1.37	7.27 ± 1.24	7.80 ± 1.39	<0.001**
Hypertension **Yes** **No** **Total**	60 (65.9) 31 (34.1) 91 (100.0)	43 (74.1) 15 (25.9) 58 (100.0)	103 (69.1) 46 (30.9) 149 (100.0)	0.291

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Diabetic retinopathy **Yes** **No** **Total**	76 (83.5) 15 (16.5) 91 (100.0)	10 (17.24) 48 (82.76) 58 (100.0)	86 (57.7) 63 (42.3) 149 (100.0)	0.005**
Diabetic nephropathy Yes No Total	5 (5.5) 86 (94.5) 91 (100.0)	1 (1.7) 57 (98.3) 58 (100.0)	6 (4.0) 143 (96.0) 149 (100.0)	0.405

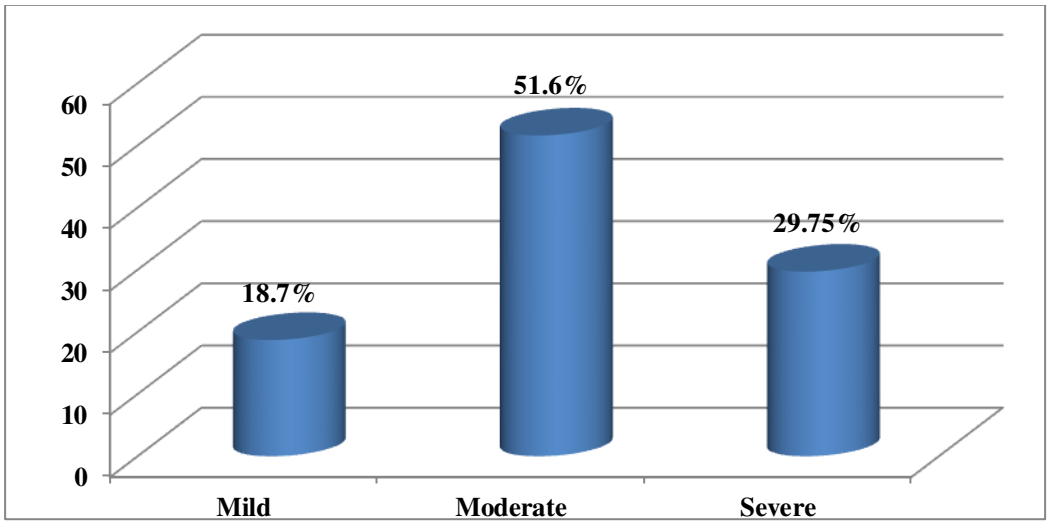
** Highly significant differences at P≤0.01

Group I=- Patients with diabetic neuropathy.

Group II = Patients without diabetic neuropathy.

Distribution of patients with diabetic neuropathy according to severity

Figure 1 shows that the majority (51.6%) of patients presented with moderate diabetic neuropathy and severe diabetic neuropathy presents in 29.75 % of patients while only 18.7% of patients presents with mild form of diabetic neuropathy.



(Figure 1) Distribution of patients with diabetic neuropathy according to severity

The association between genotype and study group

The frequency of rs1800624 (374 A/T) SNP in the human RAGE gene for the tow study groups were examined using molecular approaches like conventional PCR technique and the RFLP protocol for genotyping the DNA nucleic acids polymorphism. The results are shown in figure(2) and Table (3). The three genotypes of RAGE gene, AA (wild-type) and the two RAGE geners1800624 polymorphic genotypes (AT and TT) were analyzed for both group (Group1) and (group II). Wild genotype was present in (72.4 %) in patients without DPN were carry the wild genotype (AA) while it was only 13.2 % in patients of group I.

Heterogenic type (AT) of RAGE gene was present in (55 %) of group I patients, while only 11 (19 %) of group II patients were TA genotype. Homogeneous RAGE gene polymorphisms (TT) were also more common in group I (31.8 %) than in group II (8.6 %).

There was significant association between genotype and study group. **(X^2=149.0, P=<0.001*)**

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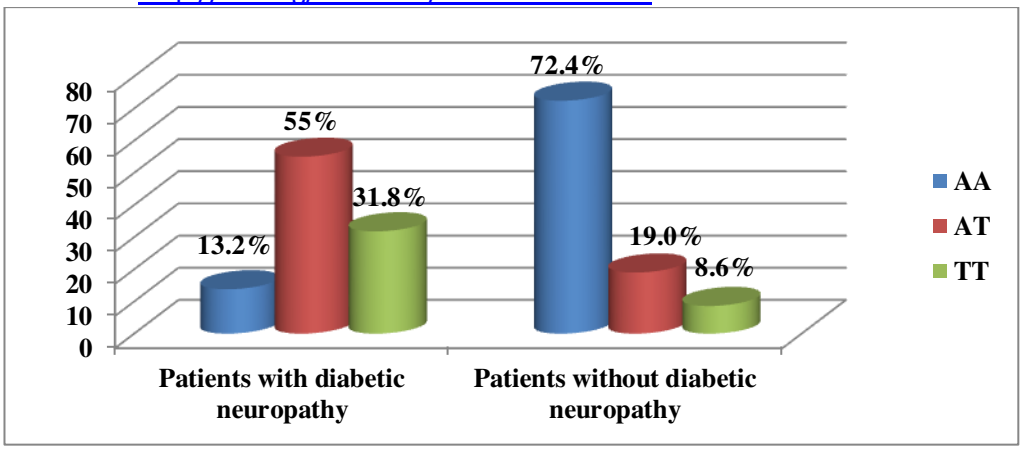

Figure (2) The association between genotype and study group.

** Highly significant differences at P≤0.01

Table (3) The association between genotype and study group

Study variables	Study group		Total	χ2	P-value
	I	II			
	No. (%)	No. (%)	No. (%)		
AA	12 (13.2)	42 (72.4)	54 (36.3)	47.02	<0.001*
AT	50 (55)	11 (19.0)	61 (41)		
TT	29 (31.8)	5 (8.6)	34 (22.7)		
Total	91 (100.0)	58 (100.0)	149 (100.0)		

** highly significant differences at P≤0.01
Group I = Patients with diabetic neuropathy.
Group II = Patients without diabetic neuropathy.

Association between genotype and severity of diabetic neuropathy

The study found that TT genotype was dominant in 63% of patients with severe diabetic peripheral neuropathy. However, the TT genotype was observed only in 17.6% of patients with mild and in 19.1% of patients with moderate peripheral neuropathy as shown in table 4 and figure 3. In contrast, the wild type AA genotype was present in 41.2% in patients with mild peripheral neuropathy, in 6.4% of patients with moderate peripheral neuropathy, and in 7.4% in patients with severe peripheral neuropathy. Heterogenic type (AT) was present in 74.5% in patients with moderate peripheral neuropathy, in 41.2% in patients with mild neuropathy , and in 29.6% in patients with severe peripheral neuropathy.

(N=91, X^2=77.38,P=<0.001*).

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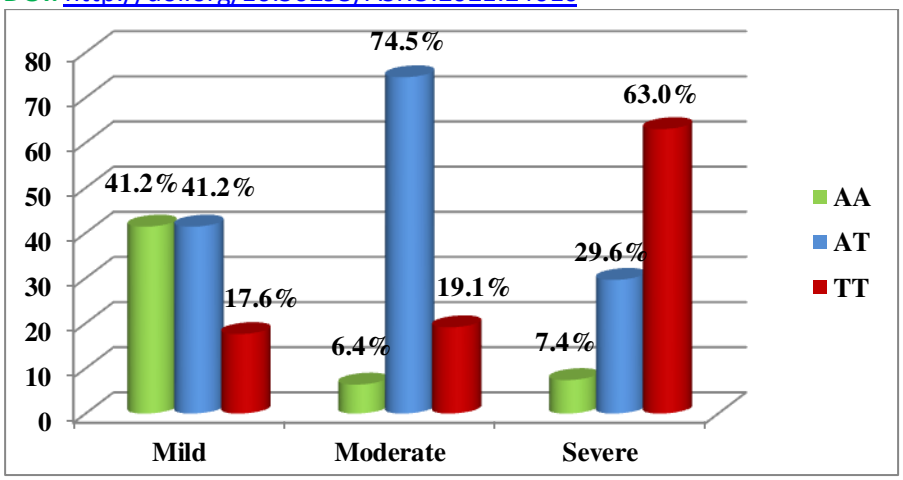

Figure (3) Association between genotype and severity of diabetic neuropathy

** Highly significant differences at P≤0.01

Table (4) Association between genotype and severity of diabetic neuropathy

Study variable	Severity of diabetic neuropathy			Total	P-value
	Mild	Moderate	Severe		
	No.(%)	No.(%)	No.(%)	No.(%)	
AA	7 (41.2)	3 (6.4)	2 (7.4)	12 (13.2)	<0.001*
AT	7 (41.2)	35 (74.5)	8 (29.6)	50 (55.0)	
TT	3 (17.6)	9 (19.1)	17 (63.0)	29 (31.8)	
Total	17 (100.0)	47 (100.0)	27 (100.0)	91 (100.0)	

** highly significant differences at P≤0.01
Group I = Patients with diabetic neuropathy.
Group II = Patients without diabetic neuropathy.

Discussion

Although RAGE genes has been topic of interest in many studies involving various ethnic populations to explore its link with Type II diabetes and post diabetic manifestations but results observed were conflicting, genetics being an important player for the progression of this disease identifies individuals at risk of developing T2DM [10].Three polymorphisms have been highlighted in studies that include two common functional polymorphisms (_429 T/C and

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_374 T/A) in the promoter region and a common coding change of a Gly to Ser at amino acid 82. The _429 T/C and _374 T/A polymorphisms were shown to have a marked effect on transcriptional activity [8], with the G82S occurring in the AGE binding domain. In present study, the frequency of rs1800624 (374 A/T) SNP in the human RAGE gene for the two study groups were examined using molecular approaches like conventional PCR technique and the RFLP protocol for genotyping the DNA nucleic acids polymorphism. To our knowledge, this is the first study to investigate the association between *RAGE* polymorphisms and DPN in Iraqi populations. The three genotypes of RAGE gene, AA (wild-type) and the two RAGE geners1800624 polymorphic genotypes (AT and TT) were analyzed for both group (Group1) and (group II). In present study found that majority of patients without DPN (72.4 %) were carry the wild genotype (AA) while it was only 13.2 % of patients with DPN were AA genotype. Heterozygous genotype (AT) of RAGE gene was present in (55 %) of group I patients, while only 11 patients (19 %) of group II were AT genotype. Homozygous RAGE gene (TT) polymorphisms were also more common in group I (31.8 %) than in group II (8.6 %). Although there were drawbacks on this study, including the small sample size, which therefore may not be truly representative of all members of society in Iraq, the results that we reached in this study indicate the possibility of a role for this gene polymorphism in the occurrence and/or severity of a DPN in patients with type 2 diabetes. Although there are some studies of the of genetic role of long term diabetic complications (retinopathy, neuropathy and nephropathy), in different population of the world, however the results were different and conflicting. Pettersson-Fernholm *et al*; in 2003 found that the association between the RAGE _374 T/A polymorphism and albumin excretion in diabetic patients with poor metabolic control in Finland, which may suggests a gene-environment interaction in the development of diabetic nephropathy[11]. Zulfiqar S. *et al*; in 2018 found non-significant association between -429T>C site and T2DM complication groups in Pakistani populations[12].

Conclusions

There was significant association between ***rs1800624* (374 A/T)** RAGE gene polymorphism and the occurrence and/or severity of diabetic peripheral neuropathy**.**

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