

Association of vascular endothelial growth factor – 634G/C and receptor for advanced glycation end products G82S gene polymorphisms with diabetic retinopathy

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Abstract

• **AIM:** To investigate the association of receptor for advanced glycation end products (RAGE) G82S and vascular endothelial growth factor (VEGF) –634 G/C gene polymorphisms with diabetic retinopathy (DR).

• **METHODS:** Our cross –sectional study included 61 diabetic patients, 12 of them had proliferative diabetic retinopathy (PDR), 15 had non proliferative diabetic retinopathy (NPDR), 34 had no diabetic retinopathy (NDR) and 61 healthy controls. Participants were tested for RAGE G82S and VEGF –634 G/C polymorphisms by polymerase chain reaction –restriction fragment length polymorphism.

• **RESULTS:** We found a significant association between VEGF –634 G/C polymorphism and PDR as PDR patients had increased incidence of VEGF –634 CC genotype compared to NDR patients [odds ratio for CC vs (GC+GG)=6.5, 95% CI=1.5–27.8, $P=0.021$]. Also VEGF –634 CC genotype and C allele were significantly higher in the PDR than in NPDR patients, which is a novel finding in our study ($P=0.024$, 0.009 respectively). The mean triglycerides level was significantly higher in diabetic patients with CC genotype ($P=0.01$) as compared to patients with other genotypes. All cases and control subjects were of the same heterozygous RAGE 82G/S genotype.

• **CONCLUSION:** Patients carrying VEGF –634 C polymorphism have a higher risk of PDR development, so VEGF –634 G/C polymorphism could be used as a

predictive marker for PDR in diabetic patients. We could not find a significant association between RAGE G82S polymorphism and DR.

• **KEYWORDS:** diabetic retinopathy; vascular endothelial growth factor; receptor for advanced glycation end products; gene polymorphism

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INTRODUCTION

Diabetic retinopathy (DR) is an important microvascular complication of diabetes mellitus (DM) and is considered a frequent cause of new cases of blindness among adults^[1].

Although uncontrolled blood glucose and duration of diabetes are important risk factors in the development of DR, some patients with longstanding uncontrolled DM develops the retinopathy later and less severe than those with well controlled DM^[2-3]. This can be explained by the presence of genetic factors which have an important role in the pathogenesis of DR^[4].

The inheritance pattern of DR is complex and polymorphic markers including the receptor for advanced glycation end products (RAGE) and vascular endothelial growth factor (VEGF) have been investigated. Advanced glycation end products are produced from non-enzymatic glycation and oxidation of protein, nucleic acids and lipids. The expression of RAGE in the retina is unregulated in DR patients due to sustained interaction of the advanced glycation end products with RAGE leading to activation of the proinflammatory transcription factor-nuclear factor- κ B and this can be altered by the genetic polymorphism in RAGE^[5]. RAGE gene is present on chromosome 6p21.3 at the major histocompatibility complex locus in the class III region^[6]. A quite large number of single nucleotide polymorphisms in the RAGE coding and noncoding regions (including G82S polymorphism) have been identified^[7-8].

VEGF has been implicated in the development of DR. Multiple ocular cell types produce VEGF, which is increased in the vitreous and aqueous fluids of patients with proliferative diabetic retinopathy (PDR)^[9-10]. VEGF causes the initial changes in DR including leukostasis, breakdown of the blood-retinal barrier, and macular edema as well as neovascularization in the eye^[11]. The VEGF gene is composed of eight exons separated by seven introns and is located on chromosome 6^[12]. Variants in the VEGF gene are suggested to play role in the levels of VEGF protein expression^[13-14] and several studies have investigated the associations between polymorphisms in the VEGF gene and DR. However, the results are contradictory. The VEGF-634 G/C gene polymorphism is in the 5'-untranslated region and has been associated with an increase in VEGF promoter activity^[15-18]. We studied the association of RAGE G82S and VEGF-634 G/C gene polymorphisms with DR in Egyptian patients.

SUBJECTS AND METHODS

We recruited 61 Egyptian patients with type 2 diabetes and 61 Egyptian healthy controls. Among diabetic patients, twelve patients had PDR, fifteen patients had non proliferative diabetic retinopathy (NPDR) and thirty-four patients without retinopathy. All patients were recruited from Kasr Al-Ainy Hospitals Outpatient Clinics (Diabetes and Endocrinology Clinic, and Ophthalmology Clinic) Cairo University, Egypt. The diagnosis of type II diabetes was in accordance with the American Diabetes Association while the diagnosis of DR was confirmed by fundus examination.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the Declaration of Helsinki and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Sample Collection Eight milliliters venous blood were withdrawn and divided into 3 tubes: 2 mL blood were collected into a sterile ethylenediaminetetraacetic acid (EDTA) vacutainer tube and stored at -20°C for the assay of RAGE G82S and VEGF -634 G/C genotypes, 2 mL blood were collected into a fluoride vacutainer tube for measurement of plasma glucose, and the other 4 mL were collected in a plain tube, serum was separated for determination of lipid profile [total cholesterol, triglycerides, high density lipoproteins (HDL) and low density lipoproteins (LDL)].

Laboratory Investigations Plasma glucose and serum lipid profile tests including total cholesterol, triglycerides, HDL and LDL were assayed on Beckman Coulter AU 680 auto-analyzer (Beckman Coulter, Brazil).

DNA Preparation Genomic DNA was extracted from

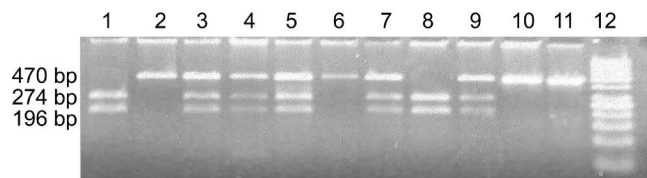


Figure 1 Agarose gel showing RFLP analysis of the VEGF -634 G/C polymorphism Lane 12 shows 50 bp DNA ladder. Lanes 10 and 11 show the amplified product; lanes 3, 4, 5, 7 & 9 show the (GC) genotype, lanes 1 & 8 show (GG) genotype, and lanes 2 & 6 show (CC) genotype.

peripheral blood leukocytes using QIA-amp DNA blood Mini Kit (Qiagen). Enzymatic amplification was performed by PCR using Taq polymerase enzyme and Hybaid thermal cycler (Promega Corporation, USA).

Determination of Receptor for Advanced Glycation End Products G82S Gene Polymorphism by Polymerase Chain Reaction -restriction Fragment Length Polymorphism

According to Gu *et al*^[19] RAGE G82S polymorphism was detected using the following primers: forward, 5'-GTAAGCGGGCTCCTGTTGCA-3'; reverse, 5'-GGCCAAGGCTGGGGTTGAAGG-3' PCR conditions were as follow: after an initial denaturation at 95°C for 5min, the DNA was amplified by 35 cycles of 94°C for 30s, 62°C for 40s, and 72°C for 45s, with a final elongation at 72°C for 10min. The PCR products were digested by the restriction enzyme *Afu* I (Thermo), followed by electrophoresis on a 3% agarose gel.

Determination of Vascular Endothelial Growth Factor -634 G/C Gene Polymorphism by Polymerase Chain Reaction -restriction Fragment Length Polymorphism

According to Sfar *et al*^[20], A 470 bp PCR amplification fragment was generated using the primers 5'-TTGCTTGCC ATTCCCCACTTGA-3' (forward) and 5'-CCGAAGCGAGA ACAGCCAGAA-3' (reverse). After an initial denaturation at 96°C for 2min, thirty-five PCR cycles were performed (96°C for 30s, 61°C for 40s, and 72°C for 40s), followed by an elongation of 72°C for 10min. The product was digested with BsmFI (thermo). The -634G allele was cut into two fragments of (196 and 274 bp) while the -634C allele remained uncut (470 bp) (Figure 1).

Statistical Analysis Data were coded and entered using the software Statistical Package for Social Science (SPSS) Version17. Comparison between groups was done using the Chi-square test and the Fischer exact test for qualitative variables. *t* test and non-parametric Mann-Whitney *U* test were used to compare two groups, whereas analysis of variance and nonparametric test (Kruskal-Wallis test) were used to compare multiple groups. *P*-values presented were adjusted using Bonferroni correction for multiple comparison and values <0.05 were considered significant.

Table 1 Descriptive data of diabetic cases

Parameters	All diabetics (n=61)	NDR (n=34)	DR			n (%)
			All (n=27)	NPDR (n=15)	PDR (n=12)	
Age (a)	54.98±7.99	53.9±8.6	56.3±7.1	54.9±6.8	58.1±7.5	
Male	16 (26.2)	6 (17.6)	10 (37.0)	8 (53.3)	2 (16.7)	
DM duration (a)	8.0 (4.0-13.0)	4.5 (2.0-6.5)	10 (10-15)	10 (10-14)	13 (10-20)	
Patients on insulin	32 (52.5)	17 (50.0)	15 (55.6)	8 (53.3)	7 (58.3)	
Glucose (mg/dL)	240.93±107.75	258.4±127.5	218.9±72.6	192.8±55.1	251.7±80.5	
Cholesterol (mg/dL)	195.38±46.70	195.4±51.6	195.4±40.7	187.0±42.6	205.9±37.2	
TG (mg/dL)	191.87±79.15	199.9±88.4	181.8±65.9	164.3±60.6	203.7±68.4	
HDL (mg/dL)	39.93±9.80	42.7±9.0	36.4±9.8	35.7±10.8	37.4±8.6	
LDL (mg/dL)	125.92±54.89	128.9±64.5	122.2±40.6	117.7±41.2	127.9±40.8	
T.chol/HDL	4.8 (3.8-6.6)	4.3 (3.5-5.7)	5.4 (4.3-7.0)	4.9 (4.3-7.4)	5.4 (4.2-6.9)	
VEGF						
CC	14 (22.9)	6 (17.7)	8 (29.6)	1 (6.6)	7 (58.3)	
GC	30 (49.2)	20 (58.8)	10 (37.1)	7 (46.7)	3 (25.0)	
GG	17 (27.9)	8 (23.5)	9 (33.3)	7 (46.7)	2 (16.7)	

DM: Diabetes mellitus; TG: Triglycerides; HDL: High density lipoproteins; LDL: Low density lipoproteins; T.chol: Total cholesterol; VEGF: Vascular endothelial growth factor. Variables with normal distribution are presented as mean±standard deviation. Skewed variables are presented as median (interquartile range).

Table 2 Genotype and allele frequency of VEGF-634 G/C polymorphism

Genotype or allele	Healthy controls (n=61), %	All diabetics (n=61), %	NDR (n=34), %	DR						
				All (n=27), %	P	NPDR (n=15), %	P	PDR (n=12), %	P	OR (95% CI)
CC	24.6	30.0	17.7	29.6	0.269	6.6	0.414	58.3	0.021	6.5 (1.5-27.8)
GC plus GG	75.4	70.0	82.3	70.4		93.4		41.7		
C allele	48.4	47.5	47.1	48.1	1.000	30.0	0.115	70.8	0.135	2.7 (1.0-7.4)
G allele	51.6	52.5	52.9	51.9		70.0		29.2		

VEGF: Vascular endothelial growth factor; DR: Diabetic retinopathy; NDR: No diabetic retinopathy; NPDR: Non proliferative diabetic retinopathy; PDR: Proliferative diabetic retinopathy. P values versus NDR patients are shown.

RESULTS

Clinical and Laboratory Background of Diabetic Patients The characteristics of diabetic patients are listed in Table 1. We compared between DR and no diabetic retinopathy (NDR) patients regarding different clinical and laboratory characters. There was a significant difference in DM duration, HDL and total cholesterol/HDL ratio between the two groups where DR patients had a higher DM duration, lower HDL cholesterol and higher total cholesterol/HDL cholesterol ratio (P=0.0001, 0.013, 0.024, respectively).

Diabetic Retinopathy and Receptor for Advanced Glycation End Products G82S Polymorphism All cases and control subjects were of the heterozygous 82G/S genotype.

Diabetic Retinopathy and Vascular Endothelial Growth Factor -634 G/C Polymorphism The genotyping results of -634 G/C polymorphism of cases and controls are summarized in Table 2. The CC genotype was significantly higher in PDR patients as compared to NDR patients (OR=6.5, 95% CI=1.5-27.8, P=0.021).

Table 3 demonstrates the comparison between the PDR and NPDR patients regarding the VEGF -634 G/C genotypes distribution. Both CC genotype and C allele were significantly associated with increased risk of PDR

development (P=0.024, 0.009 respectively).

We studied the association of VEGF -634 G/C polymorphism with different laboratory characteristics of diabetic patients (Table 4). Patients with CC genotype had significantly higher triglycerides level as compared to those with other genotypes (P=0.01).

DISCUSSION

Several studies have studied the association between VEGF -634 G/C polymorphism and DR. However, our study is the first one that found a significant difference between the PDR and NPDR patients, highlighting the association between VEGF -634 C polymorphism and PDR development in diabetic patients. The second novel finding in our study is the significant association between VEGF -634 CC genotype and hyperlipidemia in diabetic patients.

We studied 34 diabetic patients without retinopathy, 15 with NPDR, 12 with PDR and 61 healthy controls to identify DNA polymorphisms associated with DR in Egyptian patients. RAGE G82S and VEGF -634 G/C genotype and allele frequencies were compared between diabetic patients (all patients, NPDR or PDR) and healthy controls; also, they were compared between patients with retinopathy (all retinopathy, NPDR or PDR) and NDR patients (Table 2).

Table 3 Genotype and allele frequency of VEGF -634 G/C polymorphism among diabetic retinopathy patients

VEGF genotype	PDR (n=12)	NPDR (n=15)	P	OR (95% CI)
CC	58.3	6.6	0.024	19.6 (1.9-201.6)
GC+GG	41.7	93.4		
C allele	70.8	30.0	0.009	5.7 (1.8-18.4)
G allele	29.2	70.0		

DR: Diabetic retinopathy; NDR: No diabetic retinopathy; NPDR: Non proliferative diabetic retinopathy; PDR: Proliferative diabetic retinopathy.

Table 4 Association of VEGF -634 G/C genotypes with different laboratory parameters in diabetic patients (n=61)

Parameters	CC (n=14)	GC (n=30)	GG (n=17)	P
Glucose (mg/dL)	265 (213-360)	221 (152-306)	199 (134-226)	0.05
Cholesterol (mg/dL)	203.3±42.4	186.1±52.9	205.2±36.4	0.32
TG (mg/dL)	243.4±76.8	183.9±76.1	163.5±70.0	0.01 ^a
HDL (mg/dL)	35.9±8.7	39.9±10.4	43.3±8.7	0.11
LDL (mg/dL)	118.9±47.6	121.1±62.1	140.3±46.5	0.45
T.chol/HDL ratio	5.7 (4.7-7.6)	4.3 (3.6-6.6)	4.7 (4.0-5.9)	0.12

TG: Triglycerides; HDL: High density lipoproteins; LDL: Low density lipoproteins; T.chol: Total cholesterol. ^aP-value of CC vs GG=0.01, P=0.04 for CC vs GC. Variables with normal distribution are presented as mean±standard deviation. Skewed variables are presented as median (interquartile range).

There was no significant difference in VEGF -634 G/C genotype and allele frequencies, neither between healthy controls and diabetic patients nor between controls and NPDR patients. However the C allele was significantly higher in PDR patients than in the control group (OR=2.6, 95% CI=1.0-6.7, P=0.044) suggesting that VEGF -634 G/C polymorphism is not associated with diabetes itself but it might be associated with PDR. We postulate that this association is related to the mechanisms of VEGF as a key angiogenic factor in the process.

Similarly, Awata *et al*^[17] and Yang *et al*^[21] found no difference in the distribution of VEGF -634 G/C alleles between normal controls and diabetic group.

We did not find a significant difference in VEGF -634 G/C genotype and allele frequencies neither between NDR and DR patients nor between NDR and NPDR patients. However the VEGF -634 G/C polymorphism differs significantly between NDR and PDR patients where the CC genotype was significantly increased in the PDR patients (OR=6.5, 95% CI= 1.5-27.8, P=0.021).

In harmony to this study, Yang *et al*^[21] found a significant difference in the frequency of VEGF -634 polymorphism between NDR and PDR groups (P=0.0265). However, they also found a significant difference between NDR and DR groups (P=0.0253).

In contrast, Awata *et al*^[17], Petrovic *et al*^[22], Nakamura *et al*^[23] and Chun *et al*^[24] found no statistically-significant difference in VEGF -634 G/C distribution between the PDR and NDR groups. However, Awata *et al*^[17] found that the C allele was significantly higher in the NPDR patients when compared to

NDR group (P=0.0026).

Our study and that of Yang *et al*^[21] demonstrate that, VEGF -634 G/C polymorphism is associated with PDR. However, our study is the first study, according to our knowledge, that found a significant difference between the PDR and NPDR patients (Table 3) where the CC genotype was significantly higher in PDR patients [OR of CC vs(GC+GG)=19.6, 95% CI=1.9-201.6, P=0.024]. Also, the C allele was significantly higher in the PDR than in NPDR patients (OR=5.7, 95% CI=1.8-18.4, P=0.009). This is reasonable because VEGF induced vascular permeability and angiogenesis, which are important factors in PDR development.

In harmony with this study, Yang *et al*^[21] found that PDR patients have greater plasma VEGF levels than NDR or NPDR patients and also they found that, the fasting plasma VEGF levels were significantly greater in subjects with -634 CC genotype.

On the contrary, Szaflik *et al*^[25] and Uthra *et al*^[26] found no significant difference between PDR and NPDR patients regarding VEGF -634 polymorphism; however Szaflik *et al*^[25] observed that the C allele was significantly higher in NPDR than NDR patients.

We further assessed the relation between VEGF -634 G/C genotypes and laboratory features (Table 4), the mean triglycerides level was significantly higher in diabetic patients with CC genotype (P=0.01) as compared to patients with other genotypes, so the CC genotype could be a risk factor for hyperlipidemia in diabetic patients which needs further assessment in future studies.

As there is no statistical significant difference between the

diabetic patients and the normal controls regarding VEGF -634 G/C polymorphism, we postulate that this polymorphism mostly synergizes with diabetic conditions to cause retinopathy.

The RAGE G82S polymorphism was reported to be associated with DR risk [27-28]. In our study, we failed to find a significant association between G82S polymorphism and DR as all patient and control subjects were of the same heterozygous 82G/S genotype. Similarly Yoshioka *et al* [29] found that, G82S of the RAGE gene was not related to DR in Japanese diabetic patients.

In conclusion, our findings suggest that diabetic patients carrying VEGF -634 C polymorphism have an increased risk of PDR development. The most likely mechanism is that the -634 C allele is associated with increased VEGF level, therefore; VEGF -634 G/C polymorphism could be used as a marker to predict PDR risk in diabetic patients and may indicate patients who need more frequent monitoring and could also suggest anti VEGF treatment to these patients. However, a large scale study is needed to reach a solid conclusion.

Future research would benefit from the use of a larger sample of DR patients with measurement of plasma and vitreal VEGF and correlation of their levels to the VEGF -634 G/C polymorphism and the severity of DR. Also future studies could correlate these findings to the response to therapy offered such as anti-VEGF therapy and intravitreal corticosteroid injections.

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