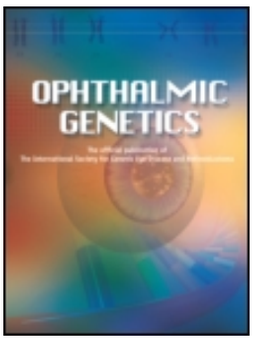

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RESEARCH REPORT

A Study of *VEGF* Gene Polymorphism in Egyptian Patients with Diabetic Retinopathy

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ABSTRACT

Background: There are subgroups of patients with diabetes mellitus (DM) in whom diabetic retinopathy (DR) does not develop despite poor long-term control of their disease, while others exercising fairly good control, develop retinopathy. So, we aimed to investigate the association of DR with −2578 polymorphism of the vascular endothelial growth factor (VEGF) gene, which has been reported to be associated with increased VEGF production, in Egyptian diabetic patients.

Materials and Methods: This is a case control study in which 148 diabetic patients were enrolled. Among them, 44 subjects had proliferative diabetic retinopathy (PDR), 30 had non-proliferative diabetic retinopathy (NPDR), and 74 individuals without retinopathy served as controls. A single nucleotide polymorphism (SNP) of the VEGF gene, a *C→A* transversion at −2578 (the *C/A* polymorphism), was investigated by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Results: We found a higher frequency of the polymorphic genotype in both the NPDR (66.7%) and PDR (72.7%) groups compared to the wild *C/C* genotype (33.3% in NPDR and 27.3% in PDR), but with no statistically significant difference from the control group. Significant association of the progression of DR to the polymorphic genotype was achieved at diabetes duration more than 20 years.

Conclusion: Despite of the higher frequency of both the polymorphic genotype and the *A* allele in cases with DR compared to the control group, there might be no significant association between the VEGF gene polymorphism and DR per se, unless it is longstanding.

Keywords: Diabetic retinopathy, Egypt, VEGF *C*-2578*A*

INTRODUCTION

Diabetic retinopathy (DR), a micro-vascular complication of diabetes mellitus (DM), is a main cause of blindness in adults.[1] However, there are subgroups of patients with DM in whom retinopathy does not develop despite poor long-term control of their disease, while others exercising fairly good control develop retinopathy. This is consistent with a genetic susceptibility to diabetic retinopathy. In addition, familial predisposition to retinopathy has also been noted in diabetes.[2]

The vascular endothelial growth factor (VEGF) gene is located on chromosome 6p21.3.[3] The functional property of single nucleotide polymorphism (SNP) –2578, which is located in the promoter region of this gene, has been shown to affect mRNA levels.[4] This SNP was first characterized by Brogan and co-authors in 1999.[5] Koukouakis and colleagues (2004) reported results suggesting that the allele A at SNP –2578 is associated significantly with increased VEGF expression.[6]

VEGF is a potent angiogenic and vascular permeability factor that is strongly implicated in type 2

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diabetes mellitus (T2DM) complications.[7] VEGF protein is more abundant in the eyes of diabetic patients, and is greatest in PDR cases.[8] VEGF is critically involved in the progression of retinopathy, and leads to the proliferative stage of the disease.[9]

Therefore, we suspected that the SNP –2578 might show a correlation with the progression of diabetic retinopathy through VEGF expression. So, the aim of the present study was to examine the –2578*C*/*A* polymorphism in the VEGF gene for an association with DR and its progression, in Egyptian patients.

MATERIALS AND METHODS

A total of 148 diabetic patients, of at least 8-year duration, were enrolled in this case-control study. Among them, 44 patients had proliferative diabetic retinopathy (PDR), 30 patients had non-proliferative DR (NPDR), and 74 individuals without retinopathy served as controls. All patients were recruited from the Ophthalmology and Internal Medicine outpatient clinics, Kasr Al Ainy Hospital. Kasr Al Ainy hospital is a central university hospital in Cairo and it is the centre for referral from most of the governorates in Egypt.

The diagnosis of DR was established based on clinical diagnosis (dilated fundus examination) and fundus fluorescence angiography (FFA) whenever needed. Peripheral blood samples (5 mL) were obtained from all subjects after they provided a written informed consent. The study was approved by Kasr Al Ainy ethical committee (according to the WMA Declaration of Helsinki), which also granted the Institutional Review Board approval.

Clinical Examination

All subjects underwent complete ophthalmic examination, including best corrected visual acuity, slit-lamp examination, and dilated fundus examination using non-contact and contact fundus lenses with the slit lamp. The diagnosis of diabetic retinopathy or its absence was confirmed by FFA when needed.

DR was classified as NPDR or PDR. NPDR denoted signs of microaneurysm, hemorrhage, hard exudate, retinal edema, venous abnormality, soft exudate, peripheral ischemia on fluorescein angiography, or intraretinal microvascular abnormality (IRMA). PDR denoted signs of new vessels on or within 1 DD of the disc, new vessels elsewhere, vitreous hemorrhage, fibrovascular proliferation, and rubeosis iridis.[10]

Diabetic maculopathy, resulting from diabetic retinopathy, is defined as the presence of retinal thickening within one disc diameter or two of the macula.[11] Diabetic maculopathy was diagnosed by dilated fundus examination using contact fundus lens with the slit lamp, and the diagnosis was confirmed by FFA and optical coherence tomography (OCT).

Cases excluded from this study were those with media opacities dense enough to obscure fundus view which would affect the clinical diagnosis of DR. Also cases who had undergone previous ocular surgery were excluded, as this would affect the DR stage.

Genotyping

Total genomic DNA of patients and healthy controls was extracted from about 2 mL anti-coagulated whole blood on EDTA using Qiagen extraction kit (catalog number 51104, USA). The genotyping of *C* −2578*A* (rs 699947) single nucleotide polymorphism (SNP) was performed by using a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) assay. For quality control, genotyping was repeated for random samples in each group to confirm our results.

DNA was amplified by PCR using 5′-GCCTTAGG ACACCATACCGATG–3′ (sense) and 5′-GCTGCCCC AGGGAACAAAGTTG-3′ (antisense) primers spanning the vascular endothelial growth factor (VEGF) *promoter region containing C* −2578*A* site. PCR reactions were carried out in a 10-μL reaction mixture containing 100 ng of genomic DNA, 0.5 μM of each primer, and 1× of GoTaq Colorless Master Mix (Fermentas, Germany).

The PCR reaction tubes were then placed in the thermal cycler (Perkin Elmer 9600, Singapore). The PCR cycles were as follows: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 60 °C of annealing temperature for 30 s, and 72 °C for 30 s; a final extension step of 72 °C for 5 min was included.

PCR products were digested with Bgl II using the conditions recommended in the manufacturer's instructions (Fermentas, Germany). After digestion, the PCR products were electrophoresed on ultraviolet-trans-illuminated, ethidium bromide-stained 3.5% agarose gel for the identification of VEGF genotypes, yielding PCR products of 285, 206, and 79 bp for the heterozygotes (C/A), while the wild homozygous genotype (C/C) appeared as a single band of 285 bp and the homo-mutant (A/A) appeared as two bands of 206 and 79 bp.[12]

Statistical Analysis

Data were statistically described in terms of mean ± standard deviation (±SD), or frequencies (number of cases) and percentages when appropriate. Fisher's exact and Chi square tests were used to evaluate associations between SNP and retinopathy. Odds ratio (OR) and 95% confidence interval (CI)

were calculated for the association of the polymorphic genotype and the *A* allele with the presence of DR and maculopathy. All *p* values less than 0.05 were considered statistically significant. All statistical calculations were done using computer programs SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows.

RESULTS

The characteristics of the studied subjects are described in Table 1. Genotype distributions and allele frequencies of $C-2578A$ polymorphism in the *VEGF* gene of the studied groups regarding the grade of DR are shown in Table 2. Using Fisher's exact test, there was no statistically significant association regarding the genotype distributions ($p=0.361$) (OR = 1.612, 95% CI = 0.816–3.184) or the allele frequencies ($p=0.825$) (OR = 1.128, 95% CI = 0.697–1.827) of $-2578C/A$ polymorphism to the susceptibility to or progression of DR.

Also the genotype distributions and allele frequencies of *C*-2578*A* polymorphism in the *VEGF* gene regarding the presence or absence of diabetic maculopathy are shown in Table 3. The association of genotype distributions and allele frequencies of $C-2578A$ polymorphism in VEGF gene and diabetic maculopathy showed no statistical significance ($p=0.724$ and $p=0.526$, respectively) (For $C/A+A/A$: OR = 0.877, 95% CI = 0.437–1.763; for *A*: OR = 0.849, 95% CI = 0.513–1.407).

TABLE 1. Clinical characteristics of cases with non-proliferative diabetic retinopathy (NPDR), proliferative diabetic retinopathy (PDR) and control groups.

Groups	Control	NPDR	PDR
Number	74	30	44
Mean age (years)	48.57 ± 11.09	53.13 ± 8.44	51.18 ± 15.31
Female sex (no.)	42	28	22
Mean DM duration (years)	12.0 ± 3.24	14.53 ± 3.82	16.36 ± 5.55
Diabetic maculopathy [No. (%)]	0 (0 %)	14 (46.67 %)	40 (90.91 %)

DM, diabetes mellitus

TABLE 2. Genotype distributions and allele frequencies of $C-2578A$ polymorphism in the vascular endothelial growth factor (VEGF) gene of the studied groups regarding the grade of diabetic retinopathy (DR).

	Control (74)		NPDR (30)		PDR (44)		
Genotype or allele	No.	Frequency	No.	Frequency	No.	Frequency	*p* Value
C/C	30	40.5%	10	33.3%	12	27.3%	0.361
$C/A+A/A$	44	59.5%	20	66.7%	32	72.7%	
				*1.612 (0.816–3.184)			
C	100	67.7%	40	66.7%	56	63.6%	0.825
A	48	32.4%	20	33.3%	32	36.4%	
				*1.128 (0.697–1.827)			

Significant *p* value <0.05
*Odds Ratio and 95% confidence interval were calculated for the presence or absence of DR

TABLE 3. Genotype distributions and allele frequencies of $C-2578A$ polymorphism in the vascular endothelial growth factor (VEGF) gene regarding the presence or absence of diabetic maculopathy.

	No diabetic maculopathy		Diabetic maculopathy		
Genotype or allele	No.	Frequency	No.	Frequency	*p* Value
C/C	32	34%	20	37%	0.724
$C/A+A/A$	62	66%	34	63%	
			*0.877 (0.437–1.763)		
C	122	64.9%	74	68.5%	0.526
A	66	35.1%	34	31.5%	
			*0.849 (0.513–1.407)		

Significant *p* value <0.05
*Odds Ratio (95% CI)

TABLE 4. Genetic contribution of the vascular endothelial growth factor (VEGF) gene to the grade of diabetic retinopathy (DR), by adjusting for the influence of diabetes mellitus (DM) duration.

			DR			
			No DR	NPDR	PDR	*p* Value
DM duration >20 yrs	*C/C*	Count (% within gene)	2 (33.3%)	4 (66.7%)	0 (0%)	<0.001
		% within DR	100.0%	100.0%	0%	
	C/A + A/A	Count (% within gene)	0 (0%)	0 (0%)	8 (100.0%)	
		% within DR	0%	0%	100.0%	

NPDR, Non-proliferative diabetic retinopathy; PDR, Proliferative diabetic retinopathy
Significant *p* value <0.05

We compared subjects with DM less than 15 years, more than 20 years and a third group of 15–20 years duration, regarding the genetic contribution of the VEGF gene to the grade of DR, by adjusting for the influence of DM duration, using Fisher's exact test, which was statistically significant at DM duration more than 20 years ($p < 0.001$) (Table 4).

DISCUSSION

Several common polymorphisms in the *VEGF* gene have been described. In this study, we focused on −2578*C/A* polymorphism in the promoter region of the *VEGF* gene for genetic analysis, as this polymorphism has been studied in different ethnic groups, but not previously in the Egyptian population. Also it has been found to be associated with several diseases such as amyotrophic lateral sclerosis in a Russian population.[13]

It has been suggested that polymorphisms of the *VEGF* gene influence susceptibility to proliferative diabetic retinopathy (PDR).[14]

In our work, analysis of *VEGF* −2578 genotype distribution showed higher frequency of the polymorphic genotype (both *C/A* and *A/A*) in each of the non-proliferative diabetic retinopathy (NPDR) (66.7%) and the PDR (72.7%) groups compared to the wild *C/C* genotype (33.3% in NPDR and 27.3% in PDR). However, these frequencies carried no statistically significant difference from the control group. Also regarding the allele frequencies, we had a higher frequency of the *A* allele in each of the PDR group (36.4%) and the NPDR group (33.3%) compared with the control group (32.4%). This was also statistically insignificant.

Similarly, Nakamura and colleagues in their 2009 study on the Japanese population, found a higher frequency of the *A* allele in the group with PDR than in the control group at –2578*C/A* polymorphism, but this was statistically significant in their study. They speculated that the *A* allele at SNP −2578 in the *VEGF* gene may be associated with PDR by up-regulating mRNA levels of the *VEGF* gene through increasing the intraocular synthesis of VEGF.[15]

Another meta-analysis indicated that the rs699947 polymorphism might be associated with the risk of DR among Europeans but not among East Asians. The frequency of rs699947 *C/A* in European populations (combination of the four European studies: *CC* 26.84%, *CA* 51.07% and *AA* 24.47%) is significantly different from the frequency in East Asian populations (combination of the four East Asian studies: *CC* 55.34%, *CA* 36.68% and *AA* 7.98%).[3]

Also, in concordance to our study, Abhary and co-authors (2009) and Chun and co-authors (2010), in their studies on Australian and Korean subjects respectively, found increased incidence of the A allele in patients with NPDR as well as PDR. However, this increased incidence in their studies was statistically significant.[16,17]

Furthermore, Yang and colleagues (2011), in their study on the Chinese population, observed a significant association of DR with the homozygous genotype of the minor allele for promoter SNP rs699947. However, in their study they analyzed eight SNPs in the *VEGF* gene using a mass-array genotyping system.[18]

The duration of diabetes mellitus (DM) has been recognized as an important factor in the development and progression of DR as reported in the Diabetes Control and Complications Trial (DCCT).[19] So we conducted a sub-analysis to evaluate the genetic effect more clearly, adjusted to the effect of the duration of DM. We compared subjects with DM less than 15 years, more than 20 years and a third group of 15–20 years duration. This sub-analysis yielded a significant association of the progression of DR to the polymorphic genotypes (both C/A and A/A) as adjusted to the duration of DM at a more than 20-year duration.

In the study by Nakamura and co-authors (2009), the subjects were grouped according to the duration of DM and status of DR (a first group consisting of subjects with longer duration (>20 y) of DM without DR, and a second group of those with shorter DM (<15 y) but having DR). They observed a positive association of the A/A genotype at SNP-2578 with DR weighted for duration.[15]

Besides the association of genotype distribution and allele frequency of the *VEGF* gene to DR, we also studied its association to diabetic maculopathy, and we did not find any significant association.

This is in concordance with Awata and colleagues (2005) who compared the frequency of the SNP –2578C/A polymorphism genotype and allele with DR, and DR (NPDR and PDR) with and without macular edema, and found no significant association.[20]

CONCLUSION

So to conclude, we found a higher frequency, in Egyptian patients, of both the polymorphic genotypes (both *C/A* and *A/A*) and the *A* allele in cases with DR (whether NPDR or PDR) compared to control, but this increase did not reach the level of statistical significance. However, when we adjusted our results to the duration of DM, we achieved a significant association at longer DM duration (more than 20 years). So there might be no association between the *VEGF* gene polymorphism and DR per se, unless the DM was longstanding. This could explain why not all diabetic patients develop DR, even if longstanding, and why not all of them progress to PDR.

Background factors (genetic and otherwise) differentiating populations can modify the expression of a gene and lead to different levels of association. The reduced sample size might be a limitation to our study. It is necessary to reproduce allelic association studies in many ethnically diverse populations to evaluate the real importance of this gene in DR. In the future, genetic testing may identify individuals at increased risk of PDR, although additional large-scale studies must be performed to clarify this issue.

Bevacizumab (Avastin; an anti-VEGF) has been used as an essential tool for the treatment of proliferative diabetic retinopathy. However, its use is not associated with regression of retinal neovessels in all treated cases.[21] So, our paper may give a clue for this differential response to therapy and may be a good guide for the proper selection of cases who may benefit from this modality of treatment.

We suggest performing the following analyses to further clarify the effect of −2578 *C/A* polymorphism on VEGF expression: VEGF mRNA expression analysis according to VEGF −2578 *C/A* genotypes, VEGF protein levels in the serum of patients by enzyme-linked immunosorbent assay (ELISA) according to VEGF −2578 *C/A* genotypes, cloning the promoter region of the *VEGF* gene containing the polymorphism −2578 *C/A* and performing a reporter assay to confirm that this nucleotide variation in the promoter region influences transcriptional activity.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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