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Vascular Endothelial Growth Factor Gene Polymorphisms in Type 2 Diabetes Mellitus; Insertion/Deletion at -2549 is Associated with Retinopathy but not + 405 G/C Polymorphism

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Authors' contributions

This work was carried out in collaboration between all authors. Author HMA designed the study, wrote the protocol. Authors Nader El-Malky and AMK supplied the patients' samples. Authors ZT, Nadia El-Menshawy and HMA carried out all laboratories work and performed the statistical analysis. Author HMA wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Background: Retinopathy is a serious ocular complication of diabetes. Vascular endothelial growth factor (VEGF) is massively unregulated in diabetic retinopathy. The objective of this study was to investigate the G + 405 C polymorphism in the 5′-untranslated region and the I/D polymorphism at the – 2549 position of the promoter region of the VEGF gene in patients with type 2 diabetes mellitus.

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Methodology: In this study, 103 unrelated type 2 diabetic patients and 40 control subjects were involved in a case-control design. Genotyping of the I/D polymorphism at -2549 was analyzed by PCR and the G/C +405 polymorphism by PCR-RFLP.
Results: For +405 G/C polymorphism, there is no statistical significant difference in genotype distribution and allele frequencies in both diabetes groups when compared to control group or to each other. While I/D polymorphism at -2549 shows significant increase in ID genotype distribution in diabetes without and with retinopathy groups versus control group [OR (CI) =3.67 (1.27-10.8) & 5.5 (1.98-15.73) and P =0.01 & <0.001, respectively]. I allele is significantly increased in diabetes without and with retinopathy groups versus control group [OR (CI) = 3.99 (1.8-8.97) & 3.98 (1.84-8.71), respectively and P <0.001 for both]. There is no significant difference in genotype and allele frequencies in both diabetes groups when compared to each other (P =0.59 & 0.99, respectively).
Conclusions: Heterozygous form of I/D polymorphism and I allele at -2549 of VEGF gene might possibly be associated with a higher susceptibility to retinopathy as a complication of type 2 diabetes mellitus. While, no association was found with VEGF +405 G/C gene polymorphism.

Keywords: VEGF gene; diabetic retinopathy; -2549 I/D polymorphism; +405 G/C gene polymorphism; type 2 diabetes.

1. INTRODUCTION

Diabetic retinopathy (DR) is one of the most prominent vascular complications in diabetes and is the most common cause of blindness among diabetic adults [1]. It is characterized by increased vascular permeability, tissue ischemia and angiogenesis [2]. DR progresses from non-proliferative (background diabetic retinopathy) into proliferative (high-risk proliferative retinopathy) and finally advanced diabetic eye disease which is accompanied by recurrent hemorrhage and fibrosis, this results in tractional retinal detachment and neovascular glaucoma [1].

The pathogenesis of DR is influenced by environmental and genetic factors. Ethnic differences in the prevalence of DR may offer understandings into the relative importance of genetic or environmental risk factors. Therefore, it is important to identify molecular markers that may help in the diagnosis of DR in multiple populations [3].

Vascular endothelial growth factor (VEGF) is one of the most potent endothelial cell mitogens and plays a critical role in angiogenesis [4]. It is a highly conserved homodimeric glycoprotein [5], which specifically binds to two transmembrane VEGF tyrosine kinase receptors on endothelial cells to initiate intracellular signal transduction pathways that mediate angiogenesis and vascular permeability [4]. VEGF is massively unregulated in DR and not only promotes neovascularization but also increases vascular permeability and leakage [6]. It is produced from many cell types within the eye [5] and plays a key role in the pathogenesis of diabetic microvascular complications [6].

Several studies have shown that VEGF expression is increased in patients with DR [7,8] and others documented that VEGF levels are markedly elevated in vitreous of the eyes of individuals with proliferative DR [9]. The genetic variations in the VEGF gene can influence levels of VEGF protein expression [2].

The human VEGF gene is located on chromosome 6 (6p21.3) and is highly polymorphic. It includes eight exons and seven introns. Of particular interest is an insertion/deletion (I/D) polymorphism of the 18 base pair (bp) fragment at – 2549 position of the promoter region that has been implicated in a number of diseases, especially those with angiogenic basis [10,11]. Also, at least 30 single-nucleotide polymorphisms (SNP) in this gene have been described. The G + 405 C (rs2010963) polymorphism in the 5′-untranslated region is a common SNP and is related to VEGF protein production [12,13]. The +405 G/C polymorphism alters the activity of the internal ribosomal entry site B enhancing initiation of translation at the AUG start codon. Also, regulates the production of the large VEGF isoform translated at an alternative CUG codon. Therefore, it is believed that the +405 G/C polymorphism likely affects expression at the post-transcriptional level [14,15].

The purpose of this study was to investigate the G + 405 C polymorphism in the 5′-untranslated region and the I/D polymorphism of the 18 bp fragment at the – 2549 position of the promoter

region of the VEGF gene in patients with type 2 diabetes mellitus and to assess their possible role in the risk of developing diabetic retinopathy.

2. METHODS

A case-control study enrolled 143 subject; 103 unrelated type 2 diabetic patients and 40 controls was conducted. Diagnosis of type 2 diabetes based on the current American Diabetes Association Criteria for the Diagnosis and Classification of Diabetes [16]. Diabetic patients were classified into two groups. Diabetes without retinopathy group (46 patients) recruited from Diabetic & Endocrinology Unit at Specialized Medical Hospital, Mansoura University, Egypt. After carful fundus examination to exclude any retinopathy. Diabetes with retinopathy group (57 patients), that are proved to have retinopathy and recruited from Fluorescein Angiography Unit and Argon Laser Clinic, Mansoura Ophthalmic Centre, Mansoura University, Egypt.

Control subjects were unrelated healthy volunteers with matched age and sex with no history of diabetes or any major clinical disorders and had normal glycosylated hemoglobin (HbA1c). Also, carful fundus examination was done to exclude any retinopathy. Also, HbA1c was measured for all diabetic patients. Fully informed and written consent was obtained from all participants. The protocol of the study was approved by the Institutional Ethics Committee and in accordance with the guidelines of the Declaration of Helsinki.

2.1 Samples Collection and DNA Extraction

Venous blood samples (6 ml) were obtained on EDTA containing tubes from all patients and control subjects; 1ml of the sample was used for HbA1c estimation (COBAS, INTEGR, Roche Diagnostics, USA). Genomic DNA was isolated from peripheral blood leukocyte using the QiaAmp kit (Qiagen, GmbH, USA). The extracted DNA was stored at -20℃ until analysis.

2.2 VEGF Gene I/D Polymorphism at -2549 Analysis

Genotyping of the I/D polymorphism was analyzed by polymerase chain reaction (PCR) using the following primers: forward 5'-GCTGAGAGTGGGGCTGACTAGGTA-3' and reverse 5'-GTTTCTGACCTGGCTATTTCCAGG-3'. PCR amplification was performed using Dream Tag Green PCR Master Mix (Fermentas, USA). Genomic DNA (300 ng) was amplified in a final volume of 30 µl using the following conditions: denaturation at 95℃ for 6 min followed by 35 cycles at 94℃ for 1 min, 57℃ for 1.5 min and 72℃ for 2 min. A final extension was at 72℃ for 10 min. The amplification products were separated by electrophoresis on 2.5% agarose gel stained with ethidium bromide; GeneRuler 50 bp DNA ladder (Germentas, USA) was used as a marker. For the VEGF I/D polymorphism two bands were observed, 211 bp for D allele and 229 bp for I allele (Fig. 1).

2.3 VEGF Gene G + 405 C Polymorphism Analysis

VEGF G + 405 C polymorphism was analyzed by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). PCR amplification performed as described above using 5′-ATTTATTTTTGCTTGCCATT-3′ and 5′-GTCTGTCTGTCTGTCCGTCA-3′ as the forward and reverse primer pairs, respectively. Then the PCR product was digested with FastDigest BsmFI (Thermo Scientific, USA) restriction nuclease at 37℃ overnight. The digestion products were separated by electrophoresis through 2.5% agarose gel stained with ethidium bromide; GeneRuler 50 bp DNA ladder (Germentas, USA) was used as a marker. The uncut fragment was 304 bp (C allele) and digestion products were 193 and 111 bp (G allele) (Fig. 2).

2.4 Statistical Analysis

Statistical analyses of data were done by Statistical Package for Social Science version 22 (SPSS, Inc., Chicago, IL). Qualitative variables expressed as number and percentage. Quantitative variables expressed as mean ± standard deviation. Statistical significance was defined as P value ≤0.05. For quantitative date, student t-test was used to compare between two groups. Repeated measure ANOVA to compare same group in different times followed by post Hoc test for intra-time comparison. Chi square test was used for qualitative data and odds ratio & 95% confidence interval (OR & 95% CI) for risk assessment.

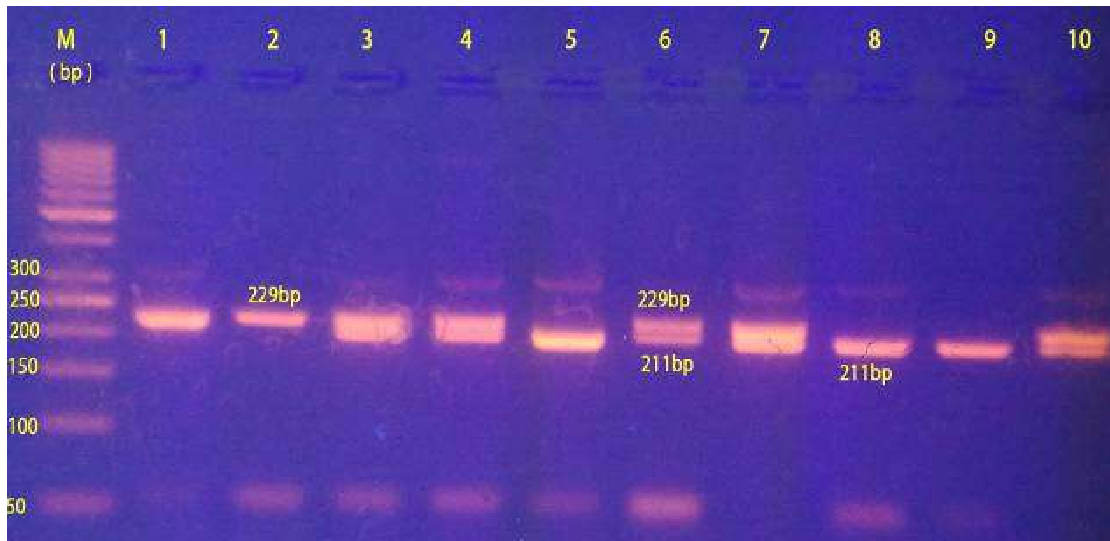

Fig. 1. VEGF gene I/D polymorphism at -2549. marker ladder, lane M; II genotype (229 bp), for example lane 2; DD genotype (211 bp), for example lanes 5, 8 and ID genotype (211 and 229 bp), for example lanes 3, 4, 6 & 10

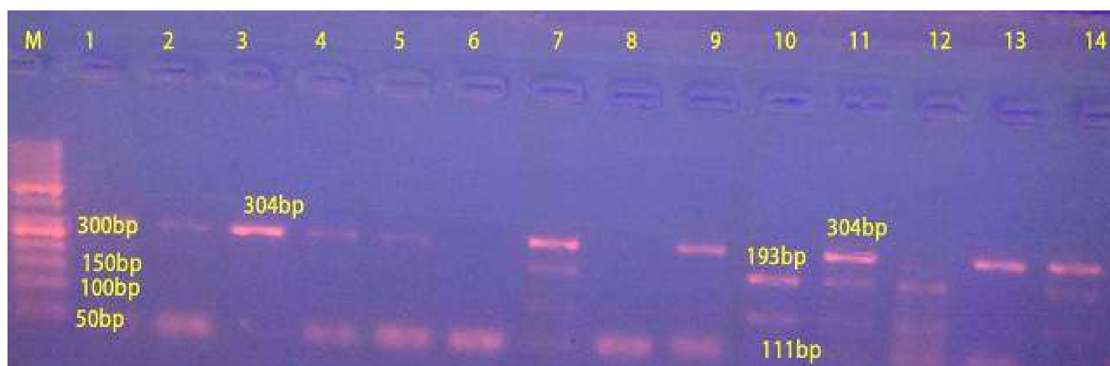

Fig. 2. VEGF gene G + 405 C polymorphism. Marker ladder, lane M; CC genotype (304 bp), for example lane 3; GG genotype (193 and 111 bp), for example lane 10; and GC genotype (304, 193 and 111 bp), for example lanes 7 & 11

3. RESULTS

The characteristics of patients and control subjects are listed in Table 1. There is significant difference in HbA1c in the three groups (*P* <0.001). Pairwise comparison revealed significant increase in HbA1c in both diabetes groups versus control group (*P* <0.001, for both) and in diabetes with retinopathy versus without retinopathy (*P* = 0.002) (not shown in the table).

3.1 VEGF Gene G + 405 C Polymorphism

There is no significant difference in genotype distribution and allele frequency in the diabetes groups (without and with retinopathy) when compared to the control group or to each other (Table 2).

3.2 VEGF Gene I/D Polymorphism at -2549

There is significant decrease in DD genotype distribution in both diabetes groups versus control group [OR (CI) =0.18 (0.06-0.5) & 0.14 (0.05-0.39) for diabetes without and with retinopathy groups, respectively and *P* <0.001, for both]. While, there is significant increase in ID genotype distribution in both diabetes without and with retinopathy groups versus control [OR (CI) =3.67 (1.27-10.8) & 5.5 (1.98-15.73) and *P* =0.01 & <0.001, respectively]. However, there

is no significant difference for II genotype distribution in both diabetes groups versus control group. I allele is significantly increased in diabetes without and with retinopathy groups versus control group [OR (CI) =3.99 (1.8-8.97) & 3.98 (1.84-8.71), respectively and P <0.001 for both]. Meanwhile, D allele is significantly increased in controls. There is no significant difference in genotype distribution and allele frequency in both diabetes groups (without and with retinopathy) when compared to each other (P =0.59 & 0.99, respectively) (Table 3).

4. DISCUSSION

Retinopathy is a serious ocular complication of diabetes that causes loss of vision in adults [17]. DR is characterized by increased vascular permeability, tissue ischemia and neovascularization. VEGF can stimulate angiogenesis and increases the permeability of the microvasculature [18] and has been implicated in the development of diabetic retinopathy [11]. It has been suggested that VEGF protein expression is affected by genetic variant in the VEGF gene [18].

In the present study, no significant association was found between VEGF G + 405 C gene polymorphism and DR. This result is in accordance with Watson et al. [12], Ray et al. [19] and Buraczynska et al. [11] who found no significant association between retinopathy status and the VEGF G + 405C polymorphism. Uthra et al. [20] and Petrovič et al. [21] reported no significant association between the rs2010963 (G + 405 C in this study) and DR. In addition, Bleda et al. [22] analyze the VEGF gene +405 polymorphism and found no association with DR but when analyze allele frequency; they observed that the presence of the G allele is a risk marker for developing retinopathy. Lu et al. [18] in their meta-analysis found that VEGF rs2010963 (G + 405 C) polymorphism was not associated with diabetic retinopathy in both Europeans and East Asians ethnity. Also, Han et al. [3] in their met-analysis confirm the non-significant association between VEGF rs2010963 polymorphism and DR.

The result is contradictory with Awata et al. [2] where +405 polymorphism was strongly associated with DR in Japanese subjects. Suganthalakshmi et al. [23] found the heterozygous form is significantly higher in patients with diabetic retinopathy compared with diabetic without retinopathy. While, Szaflik et al. [24] found that the C allele of VEGF +405 polymorphism was associated with an increased risk of DR. This discrepancy might be due to different ethnicity of the patients or may be related to other contributing factors among those populations that need further exploration.

Also, the same patients and controls have been screened for VEGF gene I/D polymorphism of the 18 bp fragment at -2549 position of the promoter region. The current study showed a significant association of ID genotype and I allele with retinopathy in diabetic patients when compared to healthy control group, while no significant difference was found when compared to diabetes without retinopathy group. The results in accordance with that obtained by Bleda et al. [22] where the ID genotype was identified as a potential risk marker of diabetic retinopathy. Moreover, the result is partly in accordance with Foad et al. [25] who found no significant difference in genotype distribution and allele frequency in diabetic patients with retinopathy, diabetics without retinopathy and control subjects.

Table 1. Characteristics of the control, diabetes with and without retinopathy groups

	Control (n= 40)	Diabetes without retinopathy (n=46)	Diabetes with retinopathy (n=57)	*P*
Age (years)	47.88±7.1	46.72±4.58	48.75±6.13	0.23
Sex:				
Male	18 (45)	20 (43.5)	28 (49.1)	
Female	22 (55)	26 (56.5)	29 (50.9)	0.84
Diabetes duration (years)	-	10.13±3.92	11.63±4.7	0.2
HbA_{1c} (%)	5.24±0.86	7.69±0.65	8.24±1.0	0.000*

Data are number (%) & Mean ± SD.
**: Significant (P = 0.05)*

Table 2. Genotype and allele frequencies of the G + 405C polymorphism in the control, diabetes without and with retinopathy groups

	Control (n= 40)	**Diabetes without retinopathy (n= 46)**	**Diabetes with retinopathy (n= 57)**	**Diabetes without retinopathy versus control**			**Diabetes with retinopathy versus control**		
				OR (CI)	**Pearson x^2 (*df.*=2)**	***P***	**OR (CI)**	**Pearson x^2 (*df.*=2)**	***P***
Genotype									
CC	32 (80)	28 (60.9)	39 (68.4)	0.39 [0.13-1.14]	2.86	0.09	0.46 [0.16-1.3]	1.94	0.16
GC	6 (15)	14 (30.4)	14 (24.6)	2.48 [0.76-8.33]	2.06	0.15	1.72 [0.54-5.66]	0.59	0.44
GG	2 (5)	4 (8.7)	4 (7.0)	1.81[0.26-15.21]	0.06	0.8	2.51 [0.44-18.59]	0.62	0.43
		P' = 0.73							
Allele: C	70 (87.5)	70 (76.1)	92 (80.7)	0.45 [0.18-1.1]	2.97	0.08	0.6 [0.025-1.43]	1.12	.28
G	10 (12.5)	22 (23.9)	22 (19.3)	2.2 [0.91-5.41]			1.67 [0.7-4.07]		
		P' = 0.42							

*Data are number (%); OR, odd ratio; CI, confidence interval. * : Significant (P = 0.05).*
P': For diabetes without versus with retinopathy groups

Table 3. Distribution of I/D polymorphism at – 2549 position of the promoter reg ion in the control, diabetes without and with retinopathy groups

	Control (n= 40)	**Diabetes without retinopathy (n= 46)**	**Diabetes with retinopathy (n= 57)**	**Diabetes without retinopathy versus control**			**Diabetes with retinopathy versus control**		
				OR (CI)	**Pearson x^2 (*df.*=2)**	***P***	**OR (CI)**	**Pearson x^2 (*df.*=2)**	***P***
Genotype									
DD	30 (75)	16 (34.8)	17 (29.8)	0.18 [0.06-0.5]	12.34	<0.001*	0.14 [0.05-0.39]	17.4	<0.001*
ID	8 (20)	22 (47.8)	33 (57.9)	3.67 [1.27-10.8]	6.12	0.01*	5.5 [1.98-15.73]	12.3	<0.001*
II	2 (5)	8 (17.4)	7 (12.3)	4 [0.71-29.3]	2.1	0.14	2.66[0.46-19.74]	0.74	0.38
		P' = 0.59							
Allele: D	68 (85)	54 (58.7)	67 (58.8)	0.25 [0.11-0.56]	13.11	<0.001*	0.25 [0.11-0.54]	14.07	<0.001*
I	12 (15)	38 (41.3)	47 (41.2%)	3.99 [1.8-8.97]			3.98 [1.84-8.71]		
		P' = 0.99							

Data are number (%); OR, odd ratio; CI, confidence interval, Significant (P<0.05);
P': For diabetes without versus with retinopathy groups

In contrast, Awata et al. [2] and Buraczynska et al. [11] found an association between the DD genotype and increase in the risk of DR. The discrepant findings might be due to differences in the statistical power, the recruitment of studied population, the type of retinopathy, and the genetic and environmental backgrounds.

It is well established that DR is determined by both genetic and environmental factors. It would be useful to identify molecular markers that may help to predict the development of retinopathy at earlier stages of diabetes [18]. In this study, we have focused on the 5'-untranslated region and promoter region of VEGF gene for genetic analysis as they have been shown to be highly polymorphic. In addition, most of the hypoxia responsive elements are present in these regions and retinal hypoxia has been observed in DR. During hypoxia, hypoxia-inducible factors bind to the hypoxia-response elements and induce the expression of VEGF, which leads to the stimulation of angiogenesis [23]. Moreover, VEGF could induce the earliest changes in retinopathy including leukostasis, blood-retinal barrier breakdown, and macular edema & neovascularization in progression of DR. In addition, VEGF inhibition has been indicated to cause a marked reduction in retinal neovascularization and prevention of the blood–retinal barrier breakdown [18].

The current study also showed that diabetes with retinopathy group is associated with higher level of HbA_{1C} than in controls and diabetes without retinopathy group. This result is in harmony with Stratton et al. [26] who confirmed the steep association between HbA1c exposures and both incidence and progression of DR in type 2 diabetes. Longo-Mbenza et al. [27] found significant association of poor control of HbA1c (poor glycemic control) and long-term microvascular complications of diabetes as DR in African patients. Moreover, Foad et al. [25] found that Glycosylated hemoglobin was significantly higher in diabetic patients (with and without retinopathy) than in controls.

Hyperglycemia has been recognized as the primary pathogenic factor in the development and progression of diabetic retinopathy [28]. Several biochemical pathways are supposed to be activated secondary to hyperglycemia. These abnormal pathways in turn, may influence several vasoactive factors that are likely to create functional and morphological changes in the retina of patients with diabetes [17]. Furthermore, improving control of blood glucose reduced development of retinopathy [29].

5. CONCLUSION

The present study suggests that, the heterozygous form of I/D polymorphism and I allele at -2549 of VEGF gene might possibly be associated with a higher susceptibility to retinopathy as a complication of type 2 diabetes mellitus. While, no association is found with VEGF G + 405 C gene polymorphism. Further well-designed large-scale studies with the consideration of gene-gene and gene-environment interactions should be conducted to investigate the association is recommended.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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