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Vascular endothelial growth factor +405G/C polymorphism as a predictor of diabetic retinopathy

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Abstract

Background: Diabetic retinopathy is a multistage event, and the most important of it is angiogenesis. The possible association between vascular endothelial growth factor (VEGF) +405G/C gene polymorphism and various diseases, in which angiogenesis might be critical in disease development, encourages many investigators to study its role in diabetic retinopathy (DR) development in diabetics. The aim of this work is to investigate +405G/C polymorphism of VEGF gene in Egyptian patients with type 1 diabetes mellitus (T1DM) and to assess its possible role as a predictor for the development and progress of diabetic retinopathy. A cross-sectional, observational study was undertaken in a sample of type I diabetic patients who attend diabetes polyclinic of RIO Hospital, Giza, Egypt, between October 2012 and December 2016 and who were willing to participate. Two hundred and sixty-six type 1 diabetic patients were studied (108 males and 158 females). All subjects were analyzed for VEGF +405G/C polymorphism by real-time PCR using TaqMan pre-designed single nucleotide polymorphism (SNP) genotyping assay.

Results: There were increased serum levels of VEGF in T1DM suffering from DR compared to those without. Also, there was increased +405 C/C of VEGF polymorphism and C allele frequency related to the severity of DR (non-proliferative retinopathy (NPR), proliferative diabetic retinopathy (PDR), and macular edema (ME)) and type C phenotype (ischemic) in T1DM suffering from DR.

Conclusion: Serum levels of VEGF and its +405G/C polymorphism could be used in the evaluation, development, and progression of DR.

Keywords: Diabetes mellitus, Diabetic retinopathy, Diabetic macular edema and vascular, Endothelial growth factor +405G/C polymorphism

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Background

Diabetes mellitus (DM) is a leading cause of blindness among adults aged 20–60 years (Shojaein and Mehri-Ghahfarrakh 2018). It is a chronic disease characterized by the presence of hyperglycemia symptoms and plasma glucose concentration ≥ 7 mmol/L (Al-Bahnasy et al. 2017).

There are two types of DM: type 1 DM (T1DM) and type 2 DM (T2DM). T1DM is one of the most common endocrine disorders affecting children and adolescents across the world (Katasaron et al. 2017) and is often accompanied by acute and chronic complications. The most common serious complication of DM is diabetic retinopathy (DR) as it may lead to loss of vision (Ockrim and Yorston 2010). Diabetic retinopathy is the primary cause of visual impairment in the working-age population (Fong et al. 2004).

Diabetic retinopathy is categorized based on the presence of visible vascular lesions by the fund examination. It is characterized by vascular tortuosity, retinal hemorrhage, micro-aneurysms, lipid exudates, and a proliferative stage where fragile new aberrant vessel development is more prevalent in T1DM where patients present at an earlier age.

It is noteworthy to mention that the susceptibility to DR has a genetic component independent of glycemic control and duration of diabetes (Liew et al. 2009). It would be useful to identify molecular markers that may predict the development of DR in the earlier stage of DM. One of these markers is vascular endothelial growth factor (VEGF) (Ajlan et al. 2016).

The role of VEGF gene polymorphism in DR is controversial. The possible association between VEGF gene polymorphism (+405 G/C) and various diseases in which angiogenesis might be critical in disease development encourage many investigators to study its role in the development of DR in diabetics (Hicklin and Ellis 2005; Lu et al. 2005).

The VEGF, a mitogen that promotes vascular endothelial cell proliferation and angiogenesis, is a 45-KDa glycoprotein secreted by endothelial cells and smooth muscle cells (Adamis and Shima 2005). It is encoded by VEGF gene that is located on chromosome 6p21.3 and comprises a 14 Kb coding region with 8 exons and 7 introns exhibiting alternate splicing to form a family of proteins (Vincent et al. 1996). It is a polymorphic gene with several single nucleotide polymorphisms (SNPs) in a regulatory region (Watson et al. 2000).

Recently, there is a growing interest in investigating VEGF SNPs that may affect the inheritable susceptibility to DR such as +405 G/C (rs: 2010 963).

Aim of the work

The aim of this work is to predict DR among Egyptian patients attending with type I diabetes with phenotype classification (A–C) to investigate the genetic polymorphisms of vascular endothelial growth factor (VEGF) +405G/C genes in Egyptian patients attending diabetes polyclinic of Research Institute of Ophthalmology with type 1 diabetes mellitus and to assess their possible role as predictors for the development and progression of DR.

The phenotype classifications are as follows:

- Type A: slow with mild blood-retinal barrier (BRB) breakdown.
- Type B: leaking type with severe BRB breakdown.
- Type C: ischemic type with vascular occlusion.

Subjects and method

Subjects

A cross-sectional, observational study was undertaken in a sample of type I diabetic patients who attend diabetes polyclinic of the Research Institute of Ophthalmology (RIO's Hospital, Giza, Egypt) between October 2012 and December 2016 and who were willing to participate. The sampling procedure consisted of randomly selecting 2 days each week (Sunday and Wednesday) and recruiting all the diabetic patients (T1DM) who attended on those days to be the study population.

- A comprehensive data were collected from patients with T1DM with the aim of identifying genetic and environmental risk factors for diabetes complications.

In the first visit:

- A detailed history followed by baseline blood samples for fasting blood sugar (FBS), post prandial blood sugar (PPBS), and glycosylated hemoglobin (HBA1c) were obtained to confirm DM.
- Once confirmed, all patients were referred to the Ophthalmic Department of the Diabetic Polyclinic.
- Written informed consent was obtained from all subjects in accordance with the principles of the Declaration of Helsinki (WMA Declaration of Helsinki 2013).
- The protocol of this study was approved by the RIO Ethics Committee, and the venous blood samples were collected from each subject after the consent form had been signed.

Inclusion criteria:

- Participants diagnosed to have diabetes mellitus without diabetic retinopathy were included in this study.

- Participants diagnosed to have diabetes mellitus with diabetic retinopathy were also included.

Exclusion criteria:

- Participants with known other systemic diseases which could manifest as retinal pathological lesions such as hypertensive retinopathy.
- Participants with very hazy ocular media which obscure the ocular fundus.
- Gestational diabetics.
- Participants not accepting informed consent.

1. Full medical examination:

- Full personal, family, and medical history including a standardized questionnaire for cardiovascular diseases, gender, age, age of onset of diabetes, and duration of diabetes (Cambell and Lynn 1990).
- Medical examination.
- ECG and blood pressure.
- Monthly follow-up of patients with uncontrolled blood sugar curve.
- Investigations for other diabetic complications as neuropathy and nephropathy.
- Monthly follow-up of patients with uncontrolled blood sugar levels.

2. Ophthalmic examination:

- Absolute visual acuity (visual acuity is the symptom of macular edema and is important in a complete ophthalmological examination).
- Intraocular pressure using slit-lamp applanation to no meter.
- Pin torch external eye examination to screen for extraocular abnormalities.
- Anterior segment biomicroscopic examination using Haag *Streit slit lamp.*
- Fundus examination.
- Fluorescein fundus angiography (FFA) using fundus camera (Trc50ex) and intravenous injection of 5-ml sodium fluorescein solution. Serial pictures were taken for each eye every few seconds for 1 min and then after 3 min. The pictures were studied to categorize the different phenotypes of DR according to FFA as being mentioned in the phenotype classification of DR (A.B.C) (Cunha-Vas 2007).
- OCT to estimate retinal and macular thickness.

3. Genetic study
   Full medical history including name, age, sex, family history, parental consanguinity, and complaints as well as the family pedigree was constructed

(Alessandro 2010). A complete medical genetic examination was done for all participants and an analysis of the genetic factor effect.

Methods
Laboratory investigations
Sample collection
Venous blood samples (10 ml) were withdrawn from all subjects and distributed in the following tubes:

1. Sterile plain vacutainer tube (6 ml) was centrifuged, and the serum was stored at − 20 °C to measure the following:

- Lipid profile (cholesterol, high-density lipoprotein, low-density lipoprotein, and triglycerides) and kidney function tests (urea and creatinine) using regular commercial kits (Bunn (1981)).
- VEGF using immune-sorbent assay (ELISA) kits according to the manufacturer's recommendations with a commercially available ELISA kit (R&D, Systems, MN, USA).

2. Sterile fluoride vacutainer tube for blood sugar (2 ml)
3. Sterile ethylene diamine-tetra-acetate "EDTA" vacutainer (4 ml) tubes used for the following:

- HbA1c was estimated using Lobona system Cunha-Vas (2007).
- Analysis of VEGF +405G/C genotype (rs: 2010963) by real-time PCR using TaqMan® Pre-Designed SNP Genotyping Assays, ID assay No 8311614 (applied biosystems) performed on Step One Real-time PCR system (Fig. 1). It was done in two steps:
- Step 1: Extraction of genomic DNA from peripheral blood leucocytes using Spin column DNA isolation kit QIA amp Mini spin column according to the manufacturer's recommendations (QIAGEN, USA) and then detection of DNA concentration and stored at − 20 °C till used.
- Step 2: Amplification of the extracted DNA and genotypes were determined by real-time PCR. Two TaqMan MGB (minor groove binding) probes for distinguishing between the two alleles; one probe labeled with VIC dye detected the allele C sequence and one probe labeled with FAM dye detected the allele G sequence (Table 1).

Statistical analysis
All data were analyzed using the statistical package for social studies software (SPSS version 20). Quantitative
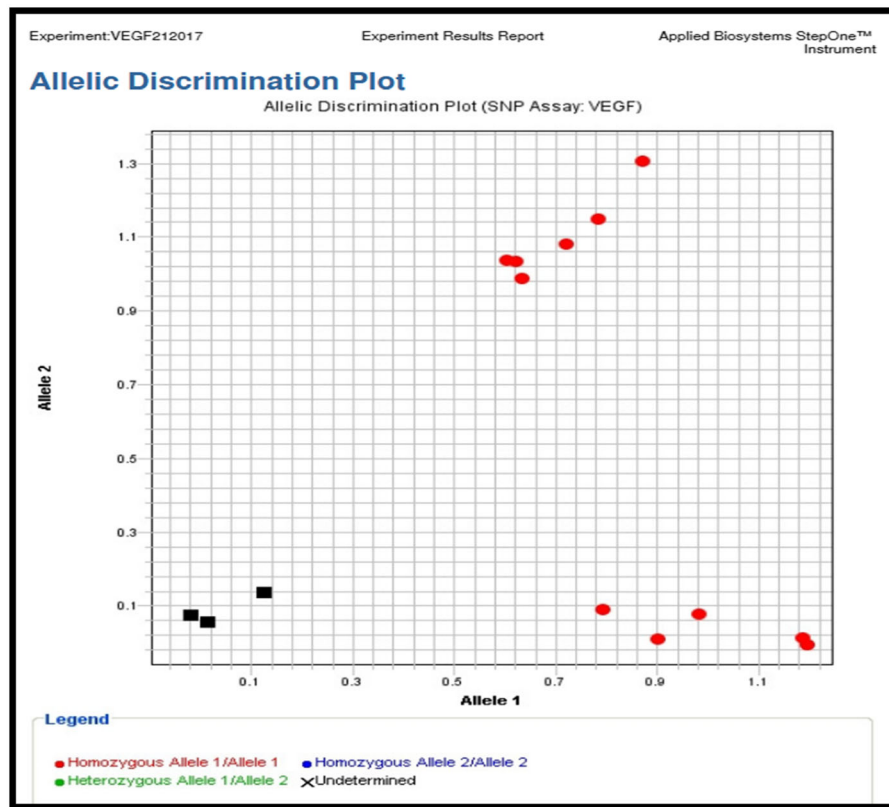
Fig. 1 Allelic discrimination plot (SNP assay: VEGF). Taqman SNP, genotyping assay user guide. Thermofisher Scientific, applied biosynthesis Taqman (2017)

data were expressed as mean values ± standard deviation. To ensure adequate statistical power, we included the allele frequency in the statistical study. The frequency of distributions was estimated for quantitative variables. Normally distributed data were compared using the ANOVA test for more than two groups. The significance of differences between proportions was tested by the chi-square test (X^2). Odds ratio and 95% CI were calculated. Differences were considered significant with P value < 0.05. Allele and genotype differences between groups and deviations from Hardy–Weeinberg equilibrium were tested by chi-squared test. Univariate logistic regression analysis was used to test the association between diseases and gene polymorphism and presented as unadjusted odds ratios (OR) with a confidence interval (95% CI).

Results

A total of 266 type 1 diabetic patients (40.6% males and 59.4%) were studied; the male-to-female ratio was 1: 1.46(108:158). The mean age at ophthalmologic examination was 41.7 ± 12.5 years. Fifty-six percent of the studied patients had a duration of DM > 10 years. The overall proportion of any DR was 71.4% (n = 190).

All clinical characteristics of patients grouped according to the presence of DR are summarized in Table 2.

There is a significant increase in the following: HbA1c between groups with and without DR and between NPDR and PDR groups, total cholesterol, HDL, and TG in the group without DR compared to the PDR group (Table 3).

Patients with no DR had a shorter duration of DM and were younger than patients with DR. Gender

Table 1 Relation between fluorescence signals and sequences in a sample

A substantial increase in	Indicates
VIC-dye fluorescence only	Homozygosity for allele C (mutant allele)
FAM-dye fluorescence only	Homozygosity for allele G (wild allele)
Both VIC- and FAM-dye fluorescence	Allele C-allele G heterozygosity

Table 2 Clinical characteristics of patients grouped according to the presence of DR

	Retinopathy		No retinopathy		P value
	No.	%	No.	%	
Gender					
Male	80	74.1	28	25.9	0.430
Female	110	69.6	48	30.4	
Age					
< 30 years	30	56.6	23	43.4	0.004
30–40 years	43	65.2	23	34.8	
41–60 years	104	78.2	29	21.8	
> 60 years	13	92.9	1	7.1	
Education					
Illiterate	121	73.3	44	26.7	0.343
Middle	37	63.8	21	36.2	
High	32	74.4	11	25.6	
Duration of diabetes					
≤ 10 years	41	53.2	36	46.8	< 0.001
11–20 years	65	77.4	19	22.6	
> 20 years	84	80.0	21	20.0	
Control of diabetes					
Controlled	64	51.6	60	48.4	< 0.001
Uncontrolled	126	88.7	16	11.3	
Family history of DR					
Positive	37	82.2	8	17.8	0.039
Negative	66	65.3	35	34.7	
Unknown	120 patients did not know				
History of Cons.					
Positive	27	64.3	15	35.7	0.246
Negative	163	72.8	61	27.2	
Hypertension					
No	114	68.3	53	31.7	0.034
Yes	58	81.7	13	18.3	
IHD					
No	169	72.8	63	27.2	0.737
Yes	4	66.2	2	33.3	
Nephropathy					
No	159	71.3	64	28.7	0.198
Yes	13	86.7	2	13.3	
Neuropathy					
No	88	69.8	38	30.2	0.375
Yes	84	75.0	28	25.0	

Table 3 Laboratory investigations of diabetics grouped according to DR types

		No.	Mean	SD	P value
HbA1c	No	76	7.65	1.58	0.003
	NPDR	85	7.81	1.53	
	PDR	105	8.00	1.61	
Total cholesterol	No	76	230.67	41.03	0.001
	NPDR	85	231.67	45.16	
	PDR	105	239.36	47.46	
Triglycerides	No	76	172.47	26.44	0.002
	NPDR	85	177.81	43.27	
	PDR	105	179.98	33.29	
HDL	No	76	49.18	11.72	0.001
	NPDR	85	47.64	11.61	
	PDR	105	51.34	10.41	
LDL	No	76	152.72	43.19	0.028
	NPDR	85	148.84	45.86	
	PDR	105	151.28	47.10	
FBS	No	76	223.4	76.4	0.835
	NPDR	85	223.5	79.1	
	PDR	105	217.4	84.0	
PPBS	No	76	286.3	81.2	0.437
	NPDR	85	306.2	105.1	
	PDR	105	295.8	103.1	
Creatinine	No.	76	1.0	0.4	0.003
	NPDR	85	1.0	0.2	
	PDR	105	0.9	0.2	
Urea	No.	76	42.0	10.2	0.001
	NPDR	85	40.2	10.8	
	PDR	105	36.9	6.6	
Serum VEGF	No.	76	189.9	67.05	0.678
	DR	190	193.87	57.09	

Diabetes mellitus was considered uncontrolled when the level of HbA1c ≥ 6.5%

DR, whereas the family history of DR and hypertension was correlated to retinopathy and there was a highly significant correlation of duration of diabetes to retinopathy. Microangiopathy (nephropathy and neuropathy) may not be clinically detected; however, further investigations are needed.

In Table 4, the CC genotype and C allele are more frequent among patients with DR compared to diabetics without DR (18.0% vs. 10.5% and 45.1 vs 43.0, respectively).

In Table 5, there is an increased CC genotype frequency related to the severity (10.5%, 14.8%, and 20.8%).

A gradual increase in CC genotype from types A to B and C in both genders is observed (Tables 6 and 7).

proportion did not differ significantly among groups. The study revealed no significant correlation of gender, education, family history of diabetes, consanguinity, ischemic heart disease, nephropathy, and neuropathy to

Table 4 Genotype and allele frequency of +405 G/C VEGF polymorphism in patients with and without DR

VEGF gene +405 Polymorphism		With retinopathy (133)		Without retinopathy (*n*=57)		OR	(95% CI)	*P* value
		No.	%	No.	%			
Genotype	GG	37	27.8	14	24.6	0.84	(0.41, 1.72)	0.64
	CG	72	54.1	37	64.9	1.57	(0.83, 2.98)	0.17
	CC	24	18.0	6	10.5	0.53	(0.21, 1.39)	0.20
Alleles	G	146	54.9	65	57.0	1.09	(0.70, 1.70)	0.70
	C	120	45.1	49	43.0	0.92	(0.59, 1.43)	

A gradual increase in CC genotype from types A to B and C in both genders is observed (Table 8).

Discussion

The discovery of VEGF has revolutionized our understanding of vasculogenesis and angiogenesis during development and physiological homeostasis (Apte and Chen 2019). As the development of DR is a multistage event, the most important of it is angiogenesis so it could be suggested that the VEGF play an important role in the pathogenesis of DR (Stitt et al. 2013; Duh et al. 2017; Rubsam et al. 2018).

The results of this study demonstrated increased VEGF serum levels in type 1 diabetics suffering from DR than those without. These results, no doubt call for the possibility that increased serum VEGF could be involved in the development of DR. This is in accordance with other investigators (Mironidou-Tzouveleki et al. 2011; Gonzalez-Salinas et al. 2017; Zhou et al. 2019).

The pathogenesis of DR is extremely complicated. The regulating process involves multiple retinal cells such as retinal astrocytes, Muller, microglia, and pigment epithelial cells, and VEGF is expressed in all of these cells (Grigsby et al. 2012; Abcouwer 2017). Vascular endothelial growth hormone (VEGF) is the most potent vasoactive factor; the normal expression of which is necessary for maintaining the structural and functional homeostasis of the retinal cells, but whose overexpression could lead to retinal angiogenesis in the presence of pathological factors such as hypoxia and hyperglycemia (Kennedy and Frank 2011; Sorrentino et al. 2016).

Much work has been done to study the mechanisms of VEGF in the hope of reducing neovascularization and vessel leakage by blocking its effects. Although the pathophysiological mechanisms for increased VEGF expression which leads to the development and progression of DR is not yet clear, there are several acceptable explanations, one of them shows that since VEGF is a strong inducer of the inflammation, hence it plays a significant role in the development of DR as inflammation plays a key role in its development (Rubsam et al. 2018; Ramakrish-man et al. 2014). Pursuing the same subject there is also evidence that Muller cell-derived VEGF plays an essential and causative role in the retinal inflammation (Wong et al. 2010).

Another explanation is the induction and overexpression of VEGF by persistent hyperglycemia which can lead to the increase of vascular permeability and decrease the inhibition of pro-apoptotic protein disruption of the vascular homeostasis and succeeded by neovascularization in the retina (Ajlan et al. 2016). Finally, vascular endothelial growth factor (VEGF) is able to induce matrix metalloproteinase (MMP) expression which is one of the major causes of DR by remodeling of extracellular matrix, inducing apoptosis in the retinal cells, and promoting neovascularization of retinal cells (Giebel et al. 2005; Solanki et al. 2018).

It falls within the interest of this study that the CC genotype and the C allele of +405 G/C polymorphism of VEGF gene are increased related to the severity of DR (PDR and DME) and type C phenotype (ischemic) in Egyptian type 1 diabetic patients suffering from DR than those without. This is in accordance with other results where the SNP +405 G/C (rs: 2010963) has previously been associated to PDR in other populations (Buracznska et al. 2007; Badre et al. 2013; Fan et al. 2014).

Table 5 Genotype frequency of +405 G/C VEGF polymorphism in patients related to severity (NPDR&PDR) of retinopathy

		+405 VEGF gene polymorphism						
		GG (%)		CG (%)		CC (%)		*P* value
Retinal state	No	14	24.6	37	64.9	6	10.5	0.372
	NPDR	20	32.8	32	52.5	9	14.8	
	PDR	17	23.6	40	55.6%	15	20.8	

Table 6 Genotype frequency of +405 G/C VEGF polymorphism in patients with retinopathy phenotype (A–C)

+405 VEGF gene polymorphism								
		GG (%)		CG (%)		CC (%)		*P* value
Retinophyphenotypes	Non	13	22.8	38	66.7	6	10.5	0.381
	A	9	28.1	20	62.5	3	9.4	
	B	12	27.3	24	54.5	8	9.4	
	C	17	29.8	27	47.4	13	22.8	

There was increased CC genotype VEGF frequency in patients with macular edema (20.3% vs. 13.5%) (Table 7)

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Table 7 Genotype frequency of +405G/C genotype VEGF polymorphism in patients with macular edema

+405 VEGF gene polymorphism								
		GG		CG		CC		P value
Macular edema	No	36	28.6	73	57.9	17	13.5	0. 430
	Yes	15	23.4	36	56.3	13	20.3	

However, to our knowledge, there are no studies regarding the behavior of this SNP in the Egyptian diabetic population suffering from DR.

Some SNPs of VEGF gene such as +405 G/C polymorphism influences VEGF protein expression and has functional significance on VEGF protein production (Watson et al. 2000). These data have no doubt call for a possibility that +405 C/C VEGF in diabetics could be involved in DR development and severity through increasing VEGF expression and hence production.

It is noteworthy to mention that this SNP is implicated in the risk of several disorders in which vascular injury and acceleration of inflammation are critical in disease development (Hon et al. 2004; Hamedian et al. 2012; Atousa et al. 2014; Samira and Shayan 2016).

On the other hand, Watson et al. found that the G allele at position +405 affects the transcriptional activity and increases VEGF production in peripheral blood mononuclear cells in response to lipopolysaccharide. They also showed a dose-dependent effect of the G allele. The highest VEGF protein production was recorded for GG genotype, intermediate for GC, and the lowest for CC genotype.

Emerging data show that anti-VEGF therapy, which is less destructive than laser, can reverse the diabetic retinopathy. This supports the suggestion of the role of this SNP in the pathogenesis of DR (Gupta et al. 2013; WyKoff et al. 2017). Moreover, the American Academy of Ophthalmology (AAO) preferred practice pattern committee now stated that there is sufficient evidence for the treatment of DR with anti-VEGF treatment (American Academy of ophthalmology 2017).

Conclusion

This study suggested that VEGF +405G/C polymorphism may be considered as a genetic marker for predicting DR in type 1 Egyptian diabetics and its severity.

Authors' contributions
AKA contributed to the molecular genetic laboratory studies. NAK contributed to the medical analysis work and molecular genetic laboratory studies.SHA, MSE, and MRA contributed to the medical analysis work. AAA and SRN contributed to the clinical genetic examination. SR contributed to the medical examination of patients. LAM, SAM, and MAA contributed to the biochemical analysis. MK contributed to the biochemical analysis and carried out the immunoassays. MT participated in the sequence alignment. MHA participated in the design of the study and performed the statistical analysis. MAF, OAH, SMS, and ZMO contributed to the medical ophthalmological examination of patients. All authors read and approved the final manuscript.

Availability of data and materials
Medical equipment

Equipment	Source
XI300 Chemical Autoanalyzer	Research Institute of Ophthalmology
Elisa Reader	Research Institute of Ophthalmology
Lobna System	Research Institute of Ophthalmology
Step One Real-Time PCR System	Research Institute of Ophthalmology

Kits

Kit	Source
Blood sample	Research Institute of Ophthalmology
Glucose	Erba XL System Pack
Glycosylated hemoglobin	Erba XL System Pack
Urea	Erba XL System Pack
Creatinine	Erba XL System Pack
Total cholesterol	Erba XL System Pack
HDL cholesterol	Erba XL System Pack
LDL cholesterol	Erba XL System Pack
Triglyceride	Erba XL System Pack

Table 8 Genotype frequency of +405 G/C VEGF in patients with retinopathy phenotypes in both genders

+405 VEGF genotype								
		GG		CG		CC		P value
Retinopathy type (males)	No	2	13.3	12	80.0	1	6.7	0.539
	A	5	29.4	10	58.8	2	11.8	
	B	6	42.9	6	42.9	2	14.3	
	C	9	34.6	13	50.0	4	15.4	
Retinopathy type (females)	No	11	26.2	26	61.9	5	11.9	0.457
	A	4	26.7	10	66.7	1	6.7	
	B	6	20.0	18	60.0	6	20.0	
	C	8	25.8	14	45.2	9	29.0	

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(Continued)

Kit	Source
Vascular endothelial growth factor (VEGF)	VEGF Human Elisa Kit supplied by Thermo-Fisher Scientific US
DNA extraction kits	DNA isolation kit QIA amp Mini Spin,50 tests supplied by QIAGEN
Universal MasterMIX	Thermo-fisher Scientific US
TaqmanProbs	Applied Biosynthesis–Analysis Biotechnology Company, Cairo, Egypt

Ethics approval and consent to participate
Not applicable.

Consent for publication
I am and all authors have approved the manuscript for submission to your journal hoping that it is going to be accepted for publication.

Competing interests
The authors declare that they have no competing interests.

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