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An Association of Genetic Polymorphism in Endothelial Growth Factor and a Prevalence of Diabetic Retinopathy

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ABSTRACT

The objective of this study was determining the prevalence of diabetic retinopathy (DR) in patients with diabetes mellitus and its association with growth factor polymorphism rs2010963. A cross-sectional study looked at genetic and biochemical markers in Diabetic patients from Al-Hussein Teaching Hospital's Diabetes Unit. Whole blood samples were collected by venipuncture. He gave them a questionnaire to identify sociodemographic, clinical, and ophthalmological variables, then an ophthalmologist performed biomicroscopy, tonometry, and fundus assessment using indirect ophthalmoscopy under pupil dilation and a retinal fluorangiography study to classify diabetic retinopathy as no retinopathy, mild, moderate, or severe; severe and proliferative diabetic retinopathy (PDR). The identification of the polymorphism was carried out by polymerase chain reaction (PCR) through the identification of the PCR-RFLP Assays polymorphism; DNA extraction, integrity evaluation, and measurement from peripheral blood by using ARMS-PCR assay and statistical analysis, simple frequencies, bivariate analysis, allele frequencies, and Odd ratios were calculated. In the bivariate analysis, the category proliferative retinopathy associated with the presence of DR and the genetic polymorphism of growth factor polymorphism rs2010963 were recorded as Homozygous to the ancestor was 39.5%, Heterozygous was 42%, while that of Homozygous to the variant was 12.9%. In conclusion: Study group prevalence was 24.9% for indirect ophthalmoscopy and 61.7% for fluorangiography retina as well as according to our study the G/G gene variation increases non-proliferative diabetic retinopathy risk.

1. Introduction

The term diabetes encompasses a set of metabolic diseases characterized by the presence of high levels of glucose in the blood that may be caused by poor insulin secretion, resistance to its action, or a mixture of both (Barber and Franks, 2012). There are multiple systemic complications, which can be macro- and microangiopathies, with diabetic retinopathy (DR) is being the most frequent complication in up to 40% of all those affected by T2D, causing blindness 25 times among people aged 30-69 years (Bansal et al., 2013). Diabetic retinopathy (DR) is the main manifestation of ocular involvement, being a public health problem of great magnitude. The lesions are visible at the time of diagnosis in up to 30% of patients, which means that the disease has evolved for several years without being diagnosed. The DR generally has a progressive course, although in its early phases certain lesions may remit spontaneously (Sun and Jampol, 2019).

Some of the genes that have shown associations or are being explored in relation to diabetic retinopathy polymorphisms include “vascular endothelial growth factor (VEGF) gene polymorphism, it was found certain variations in the VEGF gene may influence the expression or activity of VEGF, potentially affecting the development and progression of diabetic retinopathy (Khan et al., 2020); Aldose reductase (ALR2) gene polymorphism is an enzyme involved in the metabolism of glucose. Polymorphisms in the ALR2 gene have been investigated as potential risk factors for diabetic retinopathy (Ereqat et al., 2023) and receptor for advanced glycation end products (RAGE) gene polymorphism which encodes a cell surface receptor involved in the pathogenesis of diabetic complications (Qayyum et al., 2021). There is also evidence that the Rho/Rho-kinase pathway may be involved in the regulation of the expression of cytokines such as growth factors (GF) in DR, with this same pathway also being activated by hypoxia. Growth factors are low-molecular-weight soluble proteins, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), platelet-derived growth factor (EGF), basic fibroblast (bFGF) and growth factor insulin type 1 (IGF-1) In diabetics, a series of anatomical changes are also observed, such as alterations in the capillary basement membrane and loss of pericytes with increased permeability and elasticity (Chegini et al., 1992). Numerous substances have also been described with angiogenic capacity,

such as the polypeptides and growth factors. VEGF is the factor with the most clinical and experimental evidence; it is synthesized by different retinal cells that include ganglion cells, pigment epithelium cells, pericytes, endothelial cells, astrocytes and Müller cells. Its expression is mediated by hypoxia and this increases the production of VEGF by retinal cells up to 30 times (Rattner et al., 2019). The effects of VEGF are mediated by binding to tyrosine kinase receptors on the plasma membrane of the vascular endothelium (Klettner et al., 2021).

2. Methodology

Ethical approval

The study was adapted to the scientific and ethical principles that justify medical research, especially with regard to its possible contribution to the solution of health problems ; it was carried out by health professionals in medical institutions that act under the supervision of the competent health authorities.

Sample size preparation

To calculate the minimum sample size, the following formula was used to according to the following formula with using an online website (https://statulator.com/SampleSize/ss1P.html):

$$n = \frac{z_{\alpha/2}^{\ 2}\, pq}{d^2}$$

N= 385

Sample collection

Two to four milliliters of venous blood were taken from each participant using 5 mL sterile disposable plastic syringes after washing the aspiration site with 70% ethyl alcohol from Al-Hussein Teaching Hospital's Diabetes Unit. After aspiration, blood was put into 10-ml disposable plastic tubes. Blood tubes were bench-centrifuged at 3000-4000 RPM for 5 minutes to separate sera in the lab. Aspirated serum was put into 1.5 micro-centrifuge tubes for measurement blood parameters and 200 µl of blood were used DNA extraction kits (G-spin™ Total DNA Extraction Mini Kit/ Korea) and investigation the genetic FEVG polymorphism.

The identification of the polymorphism was carried out with the separation of the plasma from the buffycoat, and the DNA was extracted, which was quantified and its purity and concentration measured by spectrophotometry at 260/280 nm as well as electrophoresis, Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis was utilized to conduct genotyping of the rs2010963 polymorphism in a group of 240 women and 145 men with diabetic retinopathy. The polymerase chain reaction (PCR) was conducted in a total volume of 30 µL. The reaction mixture included approximately 100 ng of genomic DNA, 1× PCR buffer, 1 mM $MgCl_2$ (25 mM stock), 2 µM of dNTP, 20 pmol of each forward and reverse primer (F: 5′-CCG ACG GCT TGG GGA GAT TG-3′; R: 5′-CGG CGG TCA CCC CCA AAA G-3′), 1 unit of Taq DNA polymerase, and 5% Dimethyl sulfoxide. The amplification program consisted of an initial denaturation step at 94°C for 10 minutes, followed by 40 cycles of denaturation at 94°C for 45 seconds, annealing at 62°C for 45 seconds, and extension at 72°C for 30 seconds. A final extension step was performed at 72°C for 10 minutes. The PCR products obtained were subjected to digestion with 0.2 U of the *BsmFI* restriction enzyme. The resulting digested fragments were then analyzed using 10% polyacrylamide gel electrophoresis. Following the treatment with the restriction enzyme, the CC genotype was identified by the presence of a single 197 bp fragment. The CG genotype was characterized by the presence of three fragments measuring 197, 167, and 30bp. Lastly, the GG genotype was identified by the presence of two fragments measuring 167 and 30bp.

Subsequently, an ophthalmological evaluation was performed evaluating visual acuity with Snellen charts at a distance established at 20 feet, followed by a pinhole test or the ability visual if necessary. The assessment of the anterior segment was through Biomicroscopy with 10x and 15x magnification lenses to rule out the presence of rubeosis irisis (vascularization on the iris), The patients in this study were categorized into three groups based on their blood cholesterol levels using the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) guidelines (Parhofer and Laufs, 2019) according to these guidelines, hypertriglyceridemia is classified as follows: Mild hypertriglyceridemia: This category includes individuals with triglyceride (TG) levels ranging from 150-199 mg/dL. High hypertriglyceridemia: This group encompasses individuals with TG levels between 200-499 mg/dL and very high hypertriglyceridemia: This category comprises individuals with TG levels exceeding 500 mg/dL.

Statistical analysis

A descriptive analysis was carried out with simple frequencies, trend measures and dispersion. Chi-Square Test (x^2) will be used for the comparison of proportions for the comparison of means and medians, t-student or Mann Whitney U tests respectively according to the distribution of the variables (Gharban *et al*., 2022). The prevalence of diabetic retinopathy will be calculated, classifying it into its different degrees of severity and the allele frequencies of the variants of the *rs2010963* gene.

3. Results and Discussion

The population characteristics were as follows: 37.7% (n=145) malesand 62.3% (n=240) female. The percentage of patient retinal hemorrhages was 6.90% and 5.83% in male and female respectively; percentage of patient non- proliferative retinopathy 42.76% and 32.92% in male and female, respectively, as well as percentage of patient with proliferative retinopathy 50.34% and 61.25% in male and female, respectively (Figure 1). Complications of diabetes mellitus was divided according to whether the patient suffered from neuropathy and/or nephropathy where 13.79% of the study population was positive for this in male and 6.25% in female, 84.82 % and 24.58% of patients suffer from hypertension in male and female respectively, on the other hand 61.37% and 15.8% presents positive smoking in male and female respectively (Figure 2). The age range of most patients was 15 to 60 years old, patients who had more prevalence was 45-54 years of diabetes mellitus was 37.93% in male while more prevalence in 35-45 years of diabetes mellitus was 32.51% in female (Table 1).

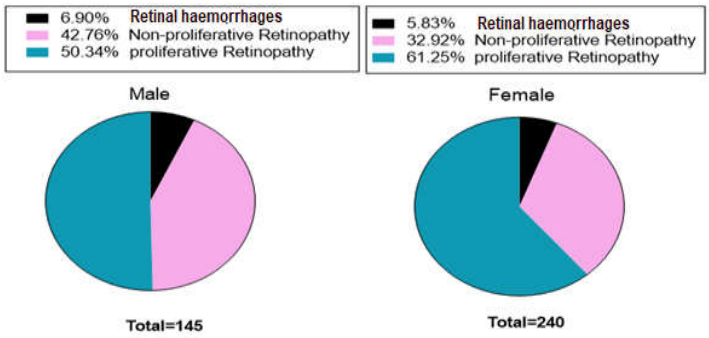


Figure 1 Percentage of diabetic retinopathy according to gender

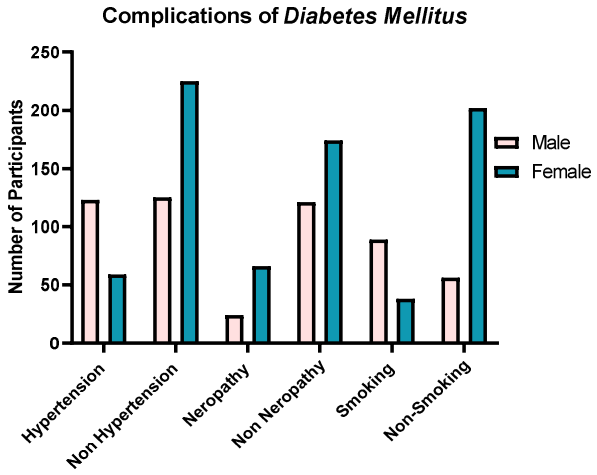


Figure 2 Complication of diabetes retinopathy according to gender

Table 1 Distribution of diabetic retinopathy according to age

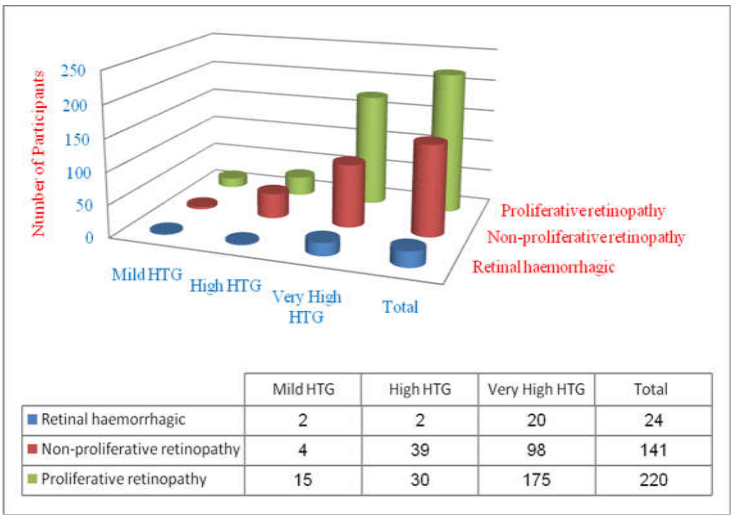
Variables	Study groups male (N=145) No.%				Study groups female (N=240) No.%			
Age (Years) Number	15-24	25-34	35-44	45-54	15-24	25-34	35-44	45-54
	11 (7.59)	36 (24.82)	43 (29.66)	55 (37.93)	38 (15.83)	50 (20.83)	78 (32.51)	74 (30.83)
Retinal hemorrhages	1	2	4	3 (5.45)	4 (10.53)	3	3	4

	(9.09)	(5.56)	(9.3)			(6)	(3.85)	(5.41)
Non-proliferative diabetic retinopathy	6 (54.55)	14 (38.89)	17 (39.53)	25 (45.45)	11 (28.95)	15 (30)	26 (33.33)	27 (36.49)
Proliferative diabetic retinopathy	4 (36.36)	20 (55.56)	22 (51.16)	27 (49.09)	23 (60.53)	32 (64)	49 (62.82)	43 (58.11)

The mean levels blood HbA1c and serum glucose and insulin in proliferative retinopathy cases were significantly higher than in non-proliferative retinopathy and retinal hemorrhage patients, the concentration of blood HbA1c and serum glucose and insulin were 10.5± 1.6 mg/dl ,401± 18.9 mlU/ml, respectively, with significant differences P=0.039), (Table 2).

Table 2 Hyperglycemic parameters associated with diabetic retinopathy

Parameters	Retinal Hemorrhages	Non-Proliferative retinopathy	Proliferative retinopathy	P value
HbA1c (%)* Mean± SD (min-max)	8.3± 1.8	9.6± 2.2	10.5± 1.6	0.047
glucose (mg/dl) Mean± SD (min-max)	398± 24.9	355± 20.3	401± 18.9	0.062
Insulin (mlU/ml) Mean± SD (min-max)	11.7± 7.3	16.9± 16.3	21.8± 15.5	0.039



	Mild HTG	High HTG	Very High HTG	Total
Retinal haemorrhagic	2	2	20	24
Non-proliferative retinopathy	4	39	98	141
Proliferative retinopathy	15	30	175	220

Figure 3 Parameters of hypertriglyceridemia associated with diabetic retinopathy

Figure 3 showed that there is a high significant relationship between patients with diabetic retinopathy and an increase in the level of cholesterol in their blood, it was found that out of 24 patients with retinal hemorrhages, there were 20 patients (83.3%) with very high hypertriglyceridemia, and out of 141 patients with non-proliferative retinopathy, there were 98 patients (69.5%) with very high hypertriglyceridemia. Finally, out of 220 patients with proliferative retinopathy, there were 175 patients (79.5%) with very high hypertriglyceridemia.

A total of 385 subjects were genotyped for the VEGF +405G/C (rs2010963) SNP using the PCR-RFLP approach (Figure 4). The bands for the GG genotype (wild) were visualized at 167 and 30 bps, the CC genotype (mutant) at 197 bp, and the GC genotype (hetero) at 197, 167, and 30 bps.

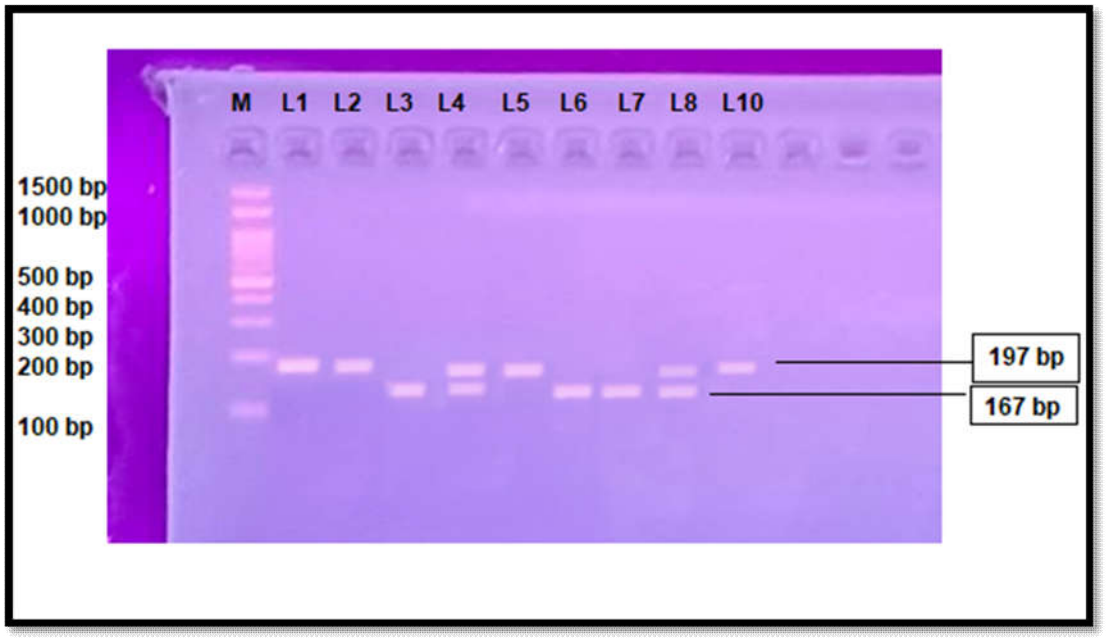


Figure 4 Agarose gel electrophoresis pattern of the VEGF +405G/C (rs2010963) gene SNP using PCR-RFLP; M1: 100 bp molecular weight marker; the homozygous mutant genotype was observed in lanes L1, L2, L5 and L10, which showed a single band at 197 bp; The heterozygote genotype was observed in lanes L4, and L8, which displayed three bands (197 bp, 167 bp and 30 not appear) and wild type allele which display two band (167 bp and 30 bp not appear)

In the univariate analysis, no statistically significant differences were observed between the three categories for polymorphisms rs2010963 in retinal hemorrhages, non-proliferative retinopathy except Proliferative retinopathy ($\chi 2$=2.201, p=0.33; $\chi 2$=1.91 and $\chi 2$=25.43, p=0.001, respectively). The allele and genotype frequencies of VEGF SNP were (Table 3). The genotypic distribution of these SNP was found to be in Hardy–Weinberg equilibrium except in Proliferative retinopathy groups. The frequency of minor CC genotypes was increased in Proliferative retinopathy groups (38.2%), followed by retinal hemorrhages (27.37%), compared to non-proliferative retinopathy groups (19.2%).

Table 3 Genotype and allele frequencies of the VEGF +405G/C (rs2010963) single nucleotide polymorphism (SNP) among the study subjects

Genotype frequency	Retinal hemorrhagic	Frequency	Non-proliferative retinopathy	Frequency	Proliferative retinopathy	Frequency
GG	2 (9%)	0.1674	54 (38.3%)	0.3549	64 (29%)	0.2066
GC	14 (63.63%)	0.4835	60 (42.5%)	0.4817	72 (32.8%)	0.4959
CC	6 (27.37%)	0.3492	27 (19.2%)	0.1634	84 (38.2%)	0.2975
Allelic frequency	No. (%)	Frequency	No. (%)	Frequency	No. (%)	Frequency
G	9 (41%)	0.41	84 (60%)	0.60	99 (45%)	0.45
C	13 (59%)	0.59	57 (40%)	0.40	121 (55%)	0.55

Diabetic retinopathy is an eye complication caused by diabetes that affects the retina specifically; uncontrolled diabetes can lead to vision problems and blindness if the blood vessels in the retina are damaged. Regular eye exams are an essential part of diabetes management (Mansour *et al*., 2020). This study analyzed 385 patients, 123 and 59 were diagnosed with hypertension in male and female respectively. By presenting the results previously, the study found that there is a significant increase in retinal hemorrhagic more than in women, and it is also associated with high blood pressure. Males who develop retinal hemorrhages may suffer from several causes and risk factors, such as hypertension, which can damage the retinal blood vessels, resulting in hemorrhages. Males, in general, may have a higher prevalence of hypertension than females, which could contribute to an increased risk of retinal hemorrhage (Thiagarajah *et al*., 2021). In addition, males may also be more likely to engage in activities that could damage the retina, such as smoking or drinking alcohol (Yang *et al*., 2022). On the other hand,

there were different results between the sexes when compared with neuropathy; the results found women more suffering from neuropathy than in males. Diabetic retinopathy and neuropathy are two common complications of diabetes (Saini *et al*., 2021). Although neuropathy and retinopathy are distinct complications of diabetes, both are caused by long-term damage to blood vessels and nerves, their development may be influenced by chronic hyperglycemia (high blood sugar), oxidative stress, inflammation, and other metabolic abnormalities associated with diabetes (Rasheed *et al*., 2021). The results of the current study showed that smoking played an important role in the occurrence of diabetic retinopathy. Smoking is associated with an increased risk of diabetic retinopathy. Studies have shown that non-smokers are less likely than smokers with diabetes to develop and progress to advanced stages of retinopathy (Ansari *et al*., 2022).

The number of patients with class three Proliferative retinopathy (PDR), which is one of the types of diabetic retinopathy, increased with increasing age, as it was recorded at a number of patients of 49.09% in males, and 58.11 % in female at the age of 40-45 years old. In diabetic retinopathy, Proliferative retinopathy is the most advanced stage. An insufficient blood supply triggers the retina to release growth factors that stimulate new blood vessels. However, these blood vessels are abnormal and fragile, which can cause complications. If not treated promptly, the newly formed vessels can lead to bleeding, retinal detachment, and scar tissue formation, which can result in severe vision loss or blindness (Crabtree and Chang, 2021). Diabetic Retinopathy, including PDR, is more likely to occur in elderly individuals who have lived with diabetes for a longer period of time. When blood sugar levels are not controlled properly, abnormal blood vessels form in the retina, resulting in Proliferative retinopathy (Fabrikantov *et al*., 2023). Proliferative retinopathy patients had significantly higher HbA1c levels, serum glucose levels, and insulin levels than those with non-proliferative retinopathy and retinal hemorrhages (Kim *et al*., 2021). It is well known that high triglycerides are associated with cardiovascular disease, including conditions that can affect the eyes, such as diabetic retinopathy (Xu e*t al*., 2020). The results of the study showed that there is an occupational difference in the levels of cholesterol among patients with diabetic retinopathy, according to the three types. The study found that the third category patients (proliferative retinopathy) had high levels of cholesterol as Very high hypertriglyceridemia, especially when combined with high cholesterol levels or low high-density lipoprotein cholesterol levels, can lead to systemic vascular dysfunction, and it is possible that vascular dysfunction may affect blood vessels throughout the body, including those in the retina, which may increase the risk of retinopathy (Bryl *et al*., 2022). Minor CC genotypes were more common in proliferative retinopathy groups (38%), followed by retinal hemorrhages (27.37%) compared to non-proliferative retinopathy groups (19.2%). In conclusion the present study explored the association of VEGF +405 G/C (rs2010963) in subjects with DN which may provide a chance to measure the impact of a candidate gene on the progression of DN in the future. In this study, it was found that the *rs2010963* genotype increases the risk of renal hemorrhagic and proliferative retinopathy in the Iraqi population, there is a significant correlation between this susceptibility and the age at diagnosis and the severity stage of the retinopathy.

4. Conclusion

This study concluded that the category proliferative retinopathy was associated with the presence of DR and the genetic polymorphism of growth factor polymorphism rs2010963 and the prevalence was 24.9% for indirect ophthalmoscopy and 61.7% for fluorangiography retina as well as according to our study the G/G gene variation increases non-proliferative diabetic retinopathy risk.

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