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Gene polymorphisms of the Vascular Endothelial Growth Factor (VEGF) in Promotor Region -2578 C/A and -460 T/C as a Risk Factor of Diabetic Retinopathy in Egyptian Patients with Diabetes Mellitus

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ABSTRACT

Background: Promotor analysis is not a routine method in DNA diagnostics, despite the fact that the promoter mutations are known to impact gene expression in ways that are functionally evocative.

The most frequent microvascular consequence of diabetes mellitus (DM) is diabetic retinopathy (DR), which can cause blindness by damaging the retina to the point of vision impairment. Vascular endothelial growth factor (VEGF) is a signalling protein subfamily that is produced by a variety of cells and is involved in angiogenesis and vasculogenesis.

One of the outcomes linked to the VEGF gene polymorphisms is an increased risk of DR. The aim of this study was to assess the association between VEGF gene polymorphisms in the promotor region in a small sample of the Egyptian population. Three types of diabetic patients were recruited for this study: those with proliferative diabetic retinopathy (PDR), non-proliferative diabetic retinopathy (NPDR), and diabetics without retinopathy (DWR). Random blood sugar (RBS), glycosylated haemoglobin (HbA1c), and serum levels of VEGF were all examined.

Results: A significant increase in serum levels of RBS and HbA1c in the PDR group was observed compared to both DWR and NPDR. The serum level of VEGF in the PDR group showed a significant increase compared to the DWR group. The genotyping analysis revealed a significant difference in -2578 C/A polymorphism among diabetic groups. The risk factor escalation was considerably higher in the NPDR groups with the mutant heterozygous genotype (CA)

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than in the PDR groups. In the genotyping analysis, a significant difference was found. A significant rise in the mutant homozygous genotype (CC) was observed when comparing PDR patients to NPDR.

Conclusion: Two polymorphisms in the VEGF gene, -2578 C/A (rs699947) and -460 T/C (rs833061), have been linked to an increased risk of developing DR. Over time, their expression may contribute to this risk.

1. Background

Many organs, including the retina, may suffer damage to their capillary endothelium as a result of the excessive glucose accumulation in these cells [1]. Diabetic retinopathy is one of the microvascular complications of DM that affects the retina of the eyes by increased vascular permeability and endothelial dysfunction with marked circulating small extracellular vesicles [2], causing irreversible retinal damage, vision loss, and blindness at the end [3]. Hyperglycaemia is also associated with DM, a chronic metabolic condition that is complicated and has detrimental long-term consequences on key organs like the heart, brain, kidneys, and eyes and raises patient mortality [4,5].

Diabetes can cause two different types of retinopathies: PDR and NPDR [6]. The initial stage of DR, known as NPDR, is characterised by the dilatation of microvessels, or microaneurysms, in the retinal blood vessels [7].

The aetiology of DR is complex and multifactorial [8] and is influenced by environmental and genetic factors. Genetic variability may explain the occurrence and development of DR in individuals. Numerous potential genes, such as aldose reductase (ALR), endothelial nitric oxide synthase (ENOS), receptors for advanced glycation end products (RAGE), and VEGF, are connected to the pathophysiology of DR [9]. Strongly angiogenic, vascular endothelial growth factor was initially identified as a vital growth factor for vascular endothelial cells, contributing to the creation of blood vessels (vasculogenesis) in both normal and aberrant angiogenesis. The association of VEGF elevation and DR indicates that the genetic variability in the VEGF gene elevates its protein expression levels [10].

A single-base mutation is the most common and basic form of genetic diversity. It is referred to as single nucleotide polymorphism (SNP) and occurs when one nucleotide in DNA replaces another nucleotide [11]. SNPs are associated with many diseases and drug efficacy. The extra variants of SNPs are known as genetic polymorphisms [12].

An essential component in controlling gene expression is the promoter region, which is found upstream of the initiation site. The transcription factor binding sites may be altered by a variation in the promoter region, which would impact gene expression. A significant but mostly unstudied class of genetic diversity is represented by these functional polymorphisms. Observed variations in gene expression may occasionally be explained by a natural binding site that is created or removed by a regulatory SNP. In the complex networks of developmental gene expression, the core promoter region is a crucial regulatory module [13].

The human VEGF gene is made up of a 14 Kb coding region with 8 exons and 7 introns, and it is found on chromosome 6 (6p21.3). The promotor region of the highly polymorphic VEGF gene has been found to contain many SNPs [14,15]. Various studies have found association between VEGF -460 T/C (rs833061) and -2578 C/A (rs699947) polymorphisms and DR risk, while other studies have reported no association and risk of DR [16,17,18,19,20].

In this study, the possible relationship between the VEGF gene polymorphisms of -2578C/A (rs699947) and -460T/C (rs833061) alleles in the promotor region was tested in the samples of DR Egyptian patients and the increased DR risk.

2. Subjects and Methods

2.1 Subjects

This study was conducted on a sample of Egyptian patients with type 1 or type 2 diabetes mellitus who were seen in the Research Institute of Ophthalmology's (RIO, Giza, Egypt) outpatient clinic.

Written informed consent forms have been obtained from all subjects prior to study inclusion in accordance with the principles of the Declaration of Helsinki. Acceptance of Scientific Ethical Committee has also been obtained from RIO, Giza, Egypt, in 27/3/22. No. 2021120601.

Inclusion criteria: Before the 300 individuals were included in the study; at least five years had passed after their initial diagnosis.

Table 1: Clinical characters of 300 diabetic individuals, both those with and those without retinopathy

Characteristics	DWR group (n=100)	NPDR group (n=100)	PDR group (n=100)
Gender n (%)			
Male	52 (52%)	48 (48%)	67 (67%)
Female	48 (48%)	52 (52%)	33 (33%)
Age mean	40	56	55
<30	14 (14%)	0 (0%)	0 (0%)
30-40	12 (12%)	6 (6%)	2 (2%)
41-60	68 (68%)	73 (73%)	60 (60%)
>60	6 (6%)	27 (27%)	32 (32%)
Duration of diabetes			
=<10	58 (58%)	15 (15%)	6 (6%)
11-20	42 (42%)	63 (63%)	72 (72%)
>20	0 (0%)	22 (22%)	22 (22%)

Exclusion criteria: Patients suffering from severe medical illnesses like liver disease, congestive heart failure, cancer, inflammatory processes, or pregnancy, as well as those with local ocular ailments like uveitis, glaucoma, or cataracts.

Data of age, gender, and duration of diabetes from all patients are presented in Table 1.

Data are expressed as number, percentage and mean; DWR, diabetic without retinopathy, NPDR, Non-proliferative diabetic retinopathy; PDR, Proliferative diabetic retinopathy.

2.2 Methods

Sample collection

5 ml of whole peripheral venous blood samples have been collected from all subjects, under aseptic conditions, with EDTA for whole blood or without EDTA for separation of serum samples. Samples are stored at -20°C to measure the following: random blood glucose, glycosylated haemoglobin (HbA1c), serum VEGF levels, and genotyping tests (DNA extraction and SNP genotyping).

Biochemical Tests

Random blood glucose was determined by using glucose-liquizyme GOD-PAP single reagent (Spectrum, Egypt). Haemoglobin A1c (HbA1c) was determined by the interaction of antigen and antibody method by using the HbA1c turbidimetric immunoassay kit (Spectrum, Egypt). Vascular endothelial growth factor (VEGF) in serum was measured by using an ELISA kit (Bioassay Technology Laboratory, China).

Genotyping analysis

Genomic DNA has been extracted and purified from 100 µl of whole blood according to manufacture instructions using QIAamp DNA Blood Mini Kits (Qiagen GmbH, Hilden, Germany), and then the extracted DNAs were stored at -20°C till used. The selected SNPs' polymorphisms (-2578 C/A and -460 T/C) in VEGF have been performed by using restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) with an applied Bio-Systems 7500 system using the Taq PCR Master Mix Kit (QIAGEN, Germany) according to the manufacturer recommended protocol. The PCR reaction mixture (25 µl) contained the following: patient's DNA (3 µl), forward primer (2 µl), reverse primer (2 µl), PCR buffer (2.5 µl), dNTPase (2.5 µl), Taq master mix (0.5 µl), and sterile water (12.5 µl). The thermal cycler was programmed for both -2758 C/A (rs699947) SNP and -460 T/C (rs833061) according to the QIAGEN manufacturer's instructions [21]. The thermal cycler conditions were as follows: initial denaturation cycle for 5 min (94°C), denaturation cycle for 45 sec (94°C), annealing cycle for 45 sec (60°C), extension cycle for 45 sec (72°C), and final extension step for 7 min (72°C). The suggested sequences of PCR primers used for −2578 C/A (rs699947) polymorphism were 5′GGATGGGGCTGACTAGGTAAGC-3′ forward primer and 5′- AGCCCCCTTTTCCTCCAAC -3′ reverse primer, and the suggested sequences of PCR primers used for −460 T/C (rs833061) polymorphism were 5′-TGTGCGTGTGGGGTTGAGCG -3′ forward primer and 5′- TACGTGCGGACAGGGCCTGA -3′ reverse primer according to [21]. Then the PCR products were electrophoresed using a 2% agarose gel, stained with 0.5µg/mL ethidium bromide, and visualised under UV light.

Table 2: The restriction enzymes and PCR-RFLP products of VEGF polymorphisms

VEGF polymorphisms	Restriction enzymes	Product sizes
-2578 C/A (rs699947)	BgLII	CC: 324 bp CA: 324 bp, 202 bp and 122 bp AA:202 bp and 122 bp
-460 T/C (rs833061)	BSH12361	CC: 155 bp and 20 bp CT: 157 bp, 155 bp and 20 bp TT: 175 bp

Table 3: The number and percentage of gender of diabetic patients in relation to the existence of DR

Groups	Male		Female		*P* value
	No.	%	No.	%	
DWR	52	52%	48	48%	**0.366**
DR	115	57.5%	85	42.5%	
Total	167	55.7%	133	44.3%	

Data are expressed as number (NO.) and percentage (%). Chi-square test (χ^2) was performed to evaluate the statistical significance between groups and among groups. DWR, diabetic without retinopathy; DR, diabetic with retinopathy.

Table 4: The number and percentage of diabetic patients in different age categories according to the presence of DR

Groups	<30		30-40		41-60		>60		*P* value
	No.	%	No.	%	No.	%	No.	%	
DWR	14	14%	12	12%	68	68%	6	6%	**<0.001**
DR	0	0%	8	4%	133	66.5%	59	29.5%	
Total	14	4.6%	20	6.7%	201	67%	65	21.7%	
Mean ± SD	**52.2 ± 10.6**								

Data are expressed as number (No.) and percentage (%) and as mean ± SD. Chi-square test (χ^2) was performed to evaluate the statistical significance between groups and among groups. DWR, diabetic without retinopathy; DR, diabetic with retinopathy.

Table 5: The number and percentage of diabetic patients in different categories of duration of diabetes according to the presence of DR

Groups	=<10		11-20		>20		*P* value
	No.	%	No.	%	No.	%	
DWR	58	58%	42	42%	0	0%	**<0.001**
DR	21	10.5%	135	67.5%	44	22%	
Total	79	26.3%	177	59%	44	14.7%	

Data are presented as number (No.) and percentage (%). Chi-square test (χ^2) was performed to evaluate the statistical significance between groups and among groups. DWR, diabetic without retinopathy; DR, diabetic with retinopathy.

The PCR products of SNPs -2578C/A (rs699947) and -460 T/C (rs833061) were digested with the restriction enzymes FastDigest BgLII and FastDigest BSH12361 (Thermo Fisher Scientific, USA), respectively, according to the manufacturer's instruction. The digested RFLP-PCR products were electrophoresed on a 2% agarose gel stained with 0.5μg/mL ethidium bromide, and visualised under UV light to detect the genotypes according to the size of DNA fragments after digestion as shown in Table 2.

Statistical analysis

The statistical package for social studies program for Microsoft Windows (SPSS version 28) was used to analyse all of the data. Standard deviation (±SD) was used to express quantitative data. For every piece of qualitative data, including age and gender, frequencies and percentages were computed. The significance of variations in proportions was examined using the chi-square test (χ^2). When a P value was less than 0.05, differences were deemed significant. By using logistic regression, the odds ratio (OR) and 95% confidence interval (CI) were also determined.

3. Results

3.1 Clinical characteristics

300 diabetic patients were divided into 100 diabetic patients with DWR, 100 diabetic patients with NPDR, and 100 diabetic patients with PDR, as shown in Tables 3, 4 and 5. The patients' average age in this investigation was 52.2 ± 10.6 years, with 5 years as the minimum duration of diabetes and 30 years as the maximum.

There was no statistically significant difference between two groups of the study (DWR and DR) in terms of gender (P=0.366), while there was a significant variance in terms of age between DWR and DR subjects (P<0.001). Most diabetic patients who have DR were between 41 and 60 years (44.3%). The number of > 30 category was 14 patients in DWR, while it was 0 patients in DR. The number of patients in 30-40 category was 12 in DWR while there was 8 in DR. The number of 41-60 patients in DWR was 68, while there were 133 patients in the DR. The number of patients in the > 60 category was 6 patients in DWR, while there were 59 patients in DR. The findings showed that 10.5% of patients had retinopathy and had diabetes for less than ten years, 67.5% had diabetic retinopathy and had the disease for between eleven and twenty years, and 22% had diabetic retinopathy and had the disease for more than twenty years. On the other hand, there was a difference of statistical significance (P<0.001) in the duration of diabetes between DWR and DR.

3.2 Biochemical results

Biochemical results of diabetic patients are shown in Table 6. There was a noteworthy variation observed in RBS levels between the groups (P<0.001). When comparing NPDR to DWR, the RBS level showed no noticeable difference. On the other hand, the RBS level in PDR was noticeably greater than in DWR or NPDR (P<0.001). There is a notable variation in HbA1c values between groups (P<0.001). A substantial rise in HbA1c in correlation with the DR: individuals with PDR had a significantly higher HbA1c (9.2 ± 1.3) than individuals with non-proliferative retinopathy (7.6 ± 1.2) or those without retinopathy (6.7 ± 0.5). There is a strong link (P <0.05) between the VEGF enzymes and the development of DR. The VEGF enzyme levels in the PDR patients were significantly higher than those in the DWR patients (1361 ± 958.6 pg/mL) early 1.6-fold.

3.3 Genotyping for -2578 C/A VEGF gene polymorphism

The PCR products of VEGF-2578 C/A polymorphisms at the promoter region resulted in one type of band of DNA, the CC genotype, with a length of 324 bp. The PCR-RFLP products of VEGF-2578 C/A polymorphism at the promoter region after digestion by restriction enzyme (BgLII) are presented in Fig. 1. Three different kinds of bands were produced as a result of the restriction site that was introduced by the C to A transition at position -2578.: one fragment of the homozygous CC genotype at 324 bp, two fragments of the heterozygous CA genotype at 202 bp and 124 bp, and three fragments of the AA genotype at 324 bp, 202 bp, and 122 bp (Fig. 2).

Table 6: Biochemical results of 300 diabetic patients

Groups	DWR (No.=100)	NPDR (No.=100)	PDR (No.=100)	*P* value
RBS (mg/dL)	181.5 ± 38.5	186.3 ± 18.3	214.9 ± 46.3 [a b]	**<0.001**
HbA1c % ± SD	6.7 ± 0.5	7.6 ± 1.2 [a]	9.2 ± 1.3 [a b]	
VEGF (pg/mL)	822.2 ± 918	1217 ± 814.3 [a]	1361 ± 958.6 [a]	

Data are expressed as mean ± SD; Chi-square test (χ^2) was performed to evaluate the statistical significance between groups and among groups. a: significant difference at ($p < 0.05$) compared to DWR; b: significant difference at ($p < 0.05$) compared to NPDR.

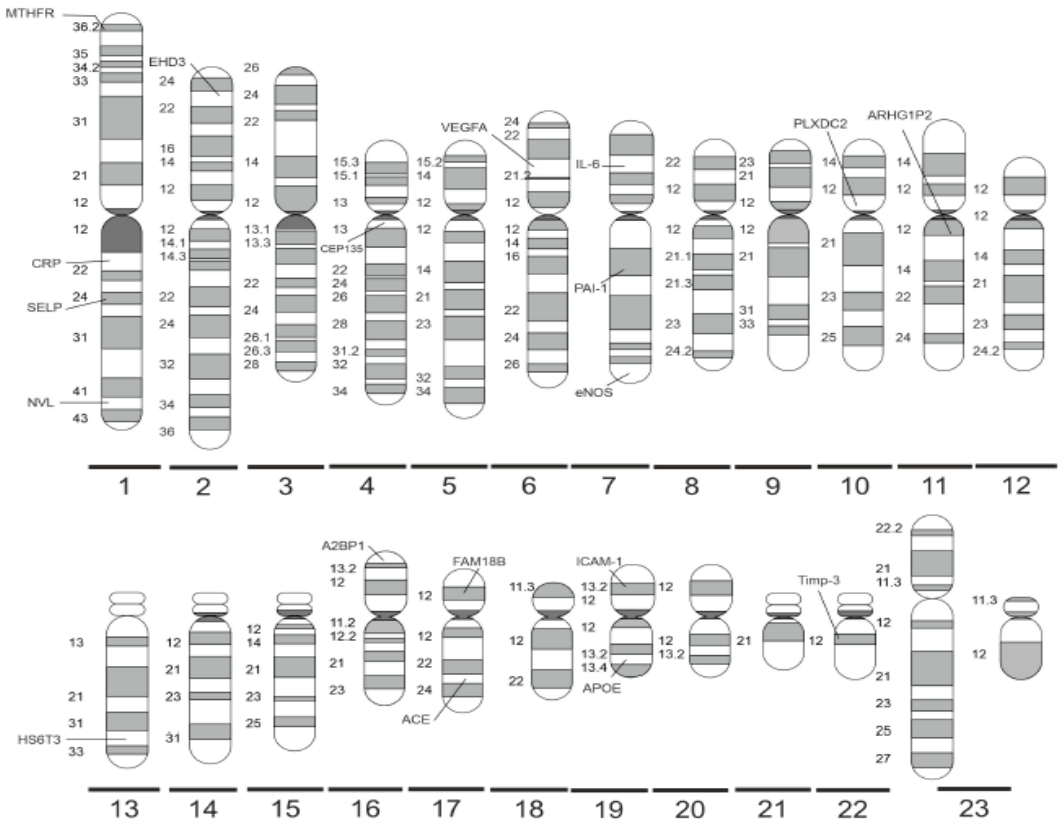

Figure 1: Important DR-related genes located on particular chromosomes in humans [43]

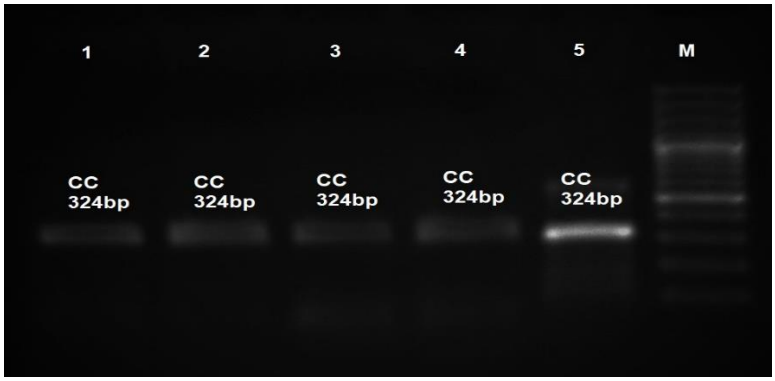

Fig. 2: -2578 C/A VEGF gene polymorphism at a promoter region was determined by PCR-RFLP detection. Samples were run through a gel documentation system, stained with ethidium bromide, and electrophoresed on a 2% agarose gel. Lane M is the DNA ladder (100 bp); lanes 1, 2, 3, 4, and 5 are the CC genotype (one band at 324 bp).

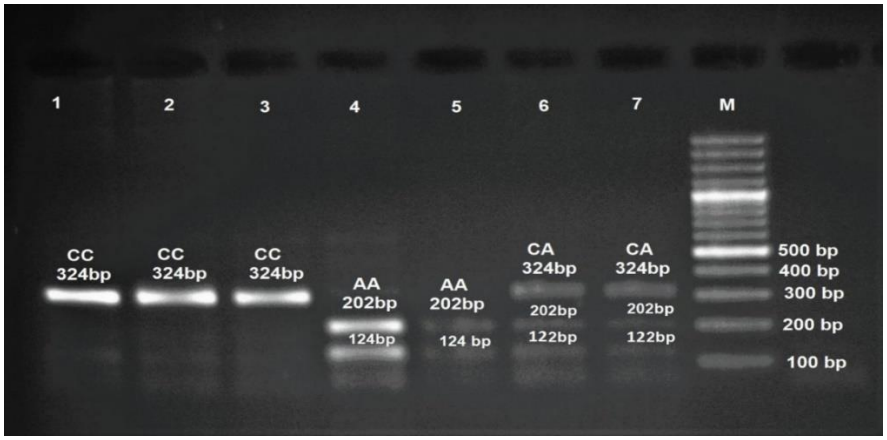

Fig. 3: -2578 C/A VEGF polymorphism at a promoter region digested by restriction enzyme (BgLII) and determined by PCR-RFLP detection. Samples were electrophoresed on 2% agarose gel electrophoresis, stained with ethidium bromide, and viewed under the gel documentation system. Lane M represents the (100 bp) DNA ladder; lanes 1,2, and 3 represents the CC genotype (one band at 324 bp); lanes 4,5 represents CA genotype (two bands at 202 bp and 124 bp); and lanes 6,7 represents AA genotype (three bands at 324 bp, 202 bp, and 122 bp).

Table 7: Genotype frequency of -2578 C/A VEGF polymorphism in patients with and without diabetic retinopathy

		DWR	NPDR	PDR	Test value	P-value
		No. = 100	No. = 100	No. = 100		
Genotyping C	**C/C**	68 (68%)	33 (33%)	33 (33%)	45.259	**<0.001**
	C/A	14 (14%)	55 (55%)	45 (45%)		
	A/A	18 (18%)	12 (12%)	22 (22%)		
Genotyping C allele	**C**	150 (75%)	121 (60.5%)	111 (55.5%)	17.739	**<0.001**
	A	50 (25%)	79 (39.5%)	89 (44.5%)		

Data are presented as no. and percentage. Significant difference is calculated by Chi-square (χ^2). a: significant change at ($p < 0.05$) compared to DWR; b: significant change at ($p < 0.05$) compared to NPDR.

Table 7 represents the genotypes of VEGF-2578 C/A. Between the three groups of type 2 diabetic patients, there was a substantial variation in the genotype distribution (C/C, C/A, and A/A) of the VEGF-2578 C/A polymorphism expression levels ($\chi 2$ (df 4) = 45.25, P < 0.001). The highest frequency of homozygous C/C genotype was 68% in both genders of DWR patients; the frequency of C/C genotyping decreased in both NPDR and PDR patients by 33%. The frequency of heterozygous C/A genotype was 14% in DWR patients. However, it increased in both NPDR and PDR patients to 55% and 45%, respectively. The frequency of homozygous A/A mutant genotype was 18% in DWR patients, and it was 12% in NPDR patients, while in PDR patients it was 22%. The frequency of the haplotype A allele in PDR was 44.5% and in NPDR was 39.5%, while it was 25% in DWR patients. The frequency of the haplotype C allele in PDR was 55.5% and in NPDR was 60.5%, while it was 75% in DWR patients.

Table 8 represents the odds ratio (OR) and risk factor of the presence of -2578 C/A in diabetic patients. It meant patients without retinopathy who have heterozygous C/A mutants were 8 times more likely to develop non-proliferative retinopathy (OR = 8.095, 95% CI = 3.94-16.61) and patients without retinopathy and have heterozygous C/A mutants were 7 times more likely to develop proliferative retinopathy (OR = 6.62, 95% CI = 3.19-13.74). While patients without retinopathy with homozygous A/A mutants were 1.5 times more susceptible to developing non-proliferative retinopathy (OR = 1.374, 95% CI=0.59-3.18) and patients without

retinopathy who have homozygous mutant A/A were 2.5 times more susceptible to developing proliferative retinopathy (OR = 2.51, 95% CI = 1.19-5.32). The C allele in both NPDR and PDR markedly raised the risk factor for the advancement of retinopathy (OR = 2.4 95% CI = 1.5-3.6).

3.4 Genotyping for -460T/C VEGF gene polymorphism

The PCR products of the VEGF-460 T/C polymorphism at the promoter region before digestion resulted in one band of DNA; TT genotype, with a length of 175 bp. Fig. 3 shows the PCR-RFLP products of VEGF-460 T/C polymorphism at the promoter region after digestion by restriction enzyme (BSH12361). Two distinct band types were produced by the restriction site that was introduced by the T to C transition at position -460: one fragment of homozygous T/T genotype at 175 bp and two fragments of heterozygous C/C genotype at 155 bp and 20 bp (Fig. 4).

Table 9 indicates a significant difference in the genotype of the -460 T/C polymorphism expression levels between the three groups of diabetic patients ($\chi 2$ (df 2) = 38.75, $P < 0.001$). The highest frequency of homozygous T/T genotype was in DWR patients (82%), then the frequency of T/T genotype decreased in both NPDR and PDR patients (61%) and (39%), respectively. The frequency of homozygous C/C mutant genotype was 18 (18%) in DWR patients and then increased in NPDR patients (39%) and increased more in PDR patients (61%). The frequency of the haplotype T allele in PDR was 39% and in NPDR was 61%, while it was 82% in DWR patients. The frequency of the haplotype C allele in PDR was (61%) and in NPDR was 39%, while it was 18% in DWR patients.

Table 10 shows the odds ratio and risk factor of the presence of -460 T/C in diabetic patients. It means that patients without retinopathy who have the homozygous allele C/C mutant were 3 times more susceptible to developing non-proliferative retinopathy (OR = 2.91, 95% CI = 1.52-5.76) while patients without retinopathy with the homozygous allele C/C mutant were suspected to be 7 times more likely to develop proliferative retinopathy (OR = 7.12, 95% CI = 4.5-11.27). The C allele in PDR considerably raised the risk factor for the development of retinopathy (OR=7.12, 95% CI= 4.5-11.27), while in NPDR (OR=2.91, 95% CI= 1.84 - 4.61).

4. Discussion

Two polymorphisms were examined in this study: the promotor region -2578 C/A (rs699947) and -460 T/C (rs833061) alleles. Variations in the rs699947 regions alleles can alter how the VEGF gene is expressed. This is the result of unique individual differences. Several studies have reported both significant and negligible differences in the connection between the VEGF SNP at position -2578 (rs699947) and variations in alleles C and A, which have been found to be risk factors in a number of individuals [16,22,23].

The most prevalent microvascular consequence of DM that results in permanent blindness is DR [24]. Diabetic retinopathy is categorised into two types: proliferative diabetic retinopathy (PDR) and non-proliferative diabetic retinopathy (NPDR). The more refined and progressive version of NPDR is called PDR [25]. In T2DM patients, there was no statistically significant correlation between gender and the development of DR (P = 0.366) [14,26]. However, DR was observed to be more prevalent in male in India [27] and in the UAE [28].

Table 8: Odds ratio and risk factor of -2578 C/A polymorphism of VEGF gene

		DWR	NPDR	Odds ratio (95% CI)	P-value	PDR	Odds ratio (OR)	P-value
		No. = 100	**No. = 100**			**No. = 100**		
Genotyping C	**C/C**	68 (68%)	33 (33%)	*Ref.*	*Ref.*	33 (33%)	*Ref.*	*Ref.*
	C/A	14 (14%)	55 (55%)	8.095 (3.944 – 16.617)	**<0.001**	45 (45%)	6.623 (3.192 – 13.742)	**<0.001**
	A/A	18 (18%)	12 (12%)	1.374 (0.593 – 3.184)	**0.459**	22 (22%)	2.519 (1.191 – 5.326)	**0.016**
Genotyping C allele	**C**	150 (75%)	121 (60.5%)	2.405 (1.585 – 3.649)	**<0.001**	111 (55.5%)	2.405 (1.573 – 3.678)	**<0.001**
	A	50 (25%)	79 (39.5%)			89 (44.5%)		

OR= Odds Ratio; and 95% (CI) = 95% Confidence Interval (lowest range – highest range) are calculated by logistic regression.

Table 9: Genotype frequency of -460 T/C VEGF polymorphism in patients with and without diabetic retinopathy

		DWR	NPDR	PDR	Test value	P-value
		No. = 100	**No. = 100**	**No. = 100**		
Genotyping T	**T/T**	82 (82%)	61 (61%)	39 (39%)	38.750	**<0.001**
	C/C	18 (18%)	39 (39%)	61 (61%)		
Genotyping T allele	**T**	164 (82%)	122 (61%)	78 (39%)	77.500	**<0.001**
	C	36 (18%)	78 (39%)	122 (61%)		

Data are presented as number and percentage. Significant difference is calculated by Chi-square ($\chi 2$). a: significant change at ($p < 0.05$) compared to DWR; b: significant change at ($p < 0.05$) compared to NPDR.

Table 10: Odds ratio and risk factor of -460T/C polymorphism

		DWR	NPDR	Odds ratio (95% CI)	P-value	PDR	Odds ratio (OR)	P-value
		No. = 100	**No. = 100**			**No. = 100**		
Genotyping T	**T/T**	82 (82%)	61 (61%)	*Ref.*	*Ref.*	39 (39%)	*Ref.*	*Ref.*
	C/C	18 (18%)	39 (39%)	2.913 (1.521 – 5.576)	**<0.001**	61 (61%)	7.125 (3.722 – 13.641)	**<0.001**
Genotyping T allele	**T**	164 (82%)	122 (61%)	2.913 (1.840 – 4.610)	**<0.001**	78 (39%)	7.125 (4.502 – 11.278)	**<0.001**
	C	36 (18%)	78 (39%)			122 (61%)		

OR= Odds Ratio; and 95% (CI) = 95% Confidence Interval (lowest range – highest range) are calculated by binary logistic regression).

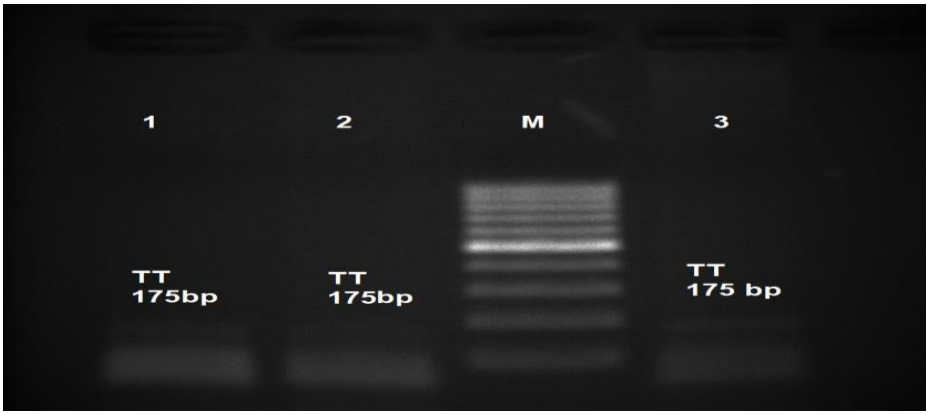

Fig. 4: -460 T/C polymorphism at a promoter region was determined by PCR-RFLP detection. Samples were seen using a gel documentation system after being electrophoresed on a 2% agarose gel and stained with ethidium bromide. Lane M represents the (100 bp) DNA ladder; lanes 1, 2, and 3 represented the TT genotype (one band at 175 bp).

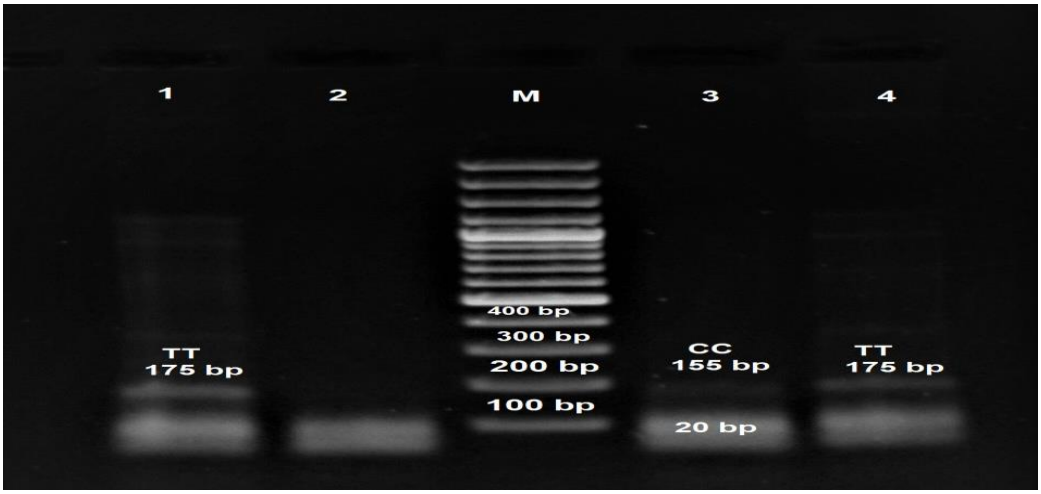

Fig. 5: -460 T/C VEGF polymorphism at a promoter region digested by restriction enzyme (BSH12361) and determined by PCR-RFLP detection. Using a 2% agarose gel electrophoresis, samples were examined under a gel documentation system after being stained with ethidium bromide. Lane M represents (100 bp) DNA ladder; lanes 1, 4 represented TT genotype (one band at 175 bp); lanes 3 represented CC genotype (two bands at 155 bp and 20 bp).

In the present study, there was a significant (P <0.05) correlation found between age and the development of DR. Retinopathy was most common in patients over 40 (61–60 years = 66.5% and over 60 years = 29.5%), indicating that age is a risk factor for the development of diabetic retinopathy. Previous research from a variety of populations found a link between ageing and developing DR [26,29-32].

The duration of diabetes can induce progression of DR [25]. Our findings showed a strong association (P <0.05) between the duration of diabetes and the onset of DR, which is consistent with previous research. [14,26,29]. Similarly, research conducted on Saudi and Japanese populations [23,33,34]. However, a study in the Mexican population by [35] indicated that the difference in the severity of diabetes between the NPDR and PDR groups was not statistically significant.

Since patients are unable to lower the glucose transfer rate when they are in a chronic state of hyperglycaemia, this can cause damage to microvascular endothelial cells, which line the interior surface of blood and lymphatic arteries. So, T1DM and T2DM may lead to DR [5,7]. The longer the time period of diabetes happens without controlling blood sugar levels, the more incidence of DR will increase and accelerate its progression [36]. In this study, comparing the reported random blood sugar (RBS) data to those without DR, a significant increase in the PDR patients was reported (P <0.05) at 214 ± 46.3 mg/dL (1.2-fold).

When comparing NPDR to DWR, there was a substantial difference in the HbA1c level (P<0.001). Furthermore, a noteworthy variation in the HbA1c level was observed in PDR in contrast to DWR and NPDR (P<0.001). The development of diabetic retinopathy was influenced by HbA1c, as evidenced by a significant difference between groups (P <0.05). However, [29] discovered that among Egyptians with varying stages of diabetes, there was no statistically significant variance in HbA1c. Other studies on other groups did not find any statistically significant changes between HbA1c and DR patients [30,35,37].

The VEGF levels varied significantly (P<0.001) between the groups. A statistically substantial (P<0.001) rise in the VEGF level in NPDR relative to DWR. Furthermore, there was a noteworthy rise in VEGF levels in PDR relative to DWR (P<0.001). The difference in the VEGF level between PDR and NPDR was not statistically significant. Because of this, VEGF is thought to be a helpful biomarker for evaluating the duration and efficacy of DR treatment [14,20,38]. These results are consistent with [14]. Contrary, [35] reported that increased levels of VEGF may play a role in the progress of DR in the Mexican population; the same was reported by [33] in Saudi patients.

Since the VEGF gene is the main regulator of vascular expansion in both healthy and pathological conditions, it has been suggested that VEGF polymorphisms play a major part in the development of DR. Vascular endothelial growth factor is one type of cytokine produced by vascular endothelial cells that

is involved in angiogenesis, the process of blood vessel formation. In diabetic retinal disease, VEGF can lead to the development of new blood vessels, the destruction of the blood retinal barrier, and an increase in the permeability of the retinal vascular bed because of hypoxia and hyperglycaemia. Clinical studies showed a significant increase in circulating and vitreous VEGF levels in serum or plasma, as well as a strong positive correlation between these parameters in PDR patients.

SNP VEGF-2578 (rs699947) showed a considerable variation in allele frequency; in both the DR and NDR groups, the C allele was the one with the greatest prevalent allele variation. This study, which examined the connection between DR on T2DM and the polymorphism of VEGF rs699947, is similar to several others conducted in different nations [16,23,39,40]. Additionally, differences in the A and T alleles were determined in this study for the DR and NPDR groups, respectively. The frequency of haplotype A allele in PDR was 89 (44.5%) and in NPDR was 79 (39.5%), while it was 50 (25%) in DWR patients. The frequency of haplotype T allele in PDR was 78 (39%) and in NPDR was 122 (61%) while it was 164 (82%) in DWR patients.

Variants of VEGF-2578C/A or -2578C/A transposable genes in patients with type-2 diabetes mellitus in Bali, Indonesia, rs699947 are risk factors for diabetic retinopathy [41].

Diabetic retinopathies have also been linked to many SNPs. The prevalence of problems related to diabetes varies by ethnic group. Because variant allele frequencies change throughout ethnic regions, they may also have differing susceptibilities to various diseases. These polymorphic loci exhibit varying variant allele and haplotype frequencies among many super populations, including Africans, Europeans, admixed Americans, and South and East Asians [42]. Another study on Iraqi patients reported that the category proliferative retinopathy was associated with the presence of DR and the genetic polymorphism of growth factor polymorphism rs2010963 [22].

Gene frequency, or allele frequency, is the relative frequency of an allele at a certain locus in a population expressed as a fraction or percentage. That allele's specific percentage of all chromosomes in the population that carry it across the whole population or sample size.

In this study, the link between the two DR types and the two types of VEGF polymorphisms was assessed. Research indicates that DR is a complex illness resulting from a combination of hereditary and environmental variables [39]. Also, ethnicity plays an important role in the development and progression of DR [20].

Focusing on the impact of a single nucleotide change in the VEGF gene that can increase the chance of contributing to diabetic retinopathy (DR) risk in diabetic patients. The association between the -2578 /A (rs699947) and -460T/C (rs833061) VEGF polymorphisms and DR susceptibility was statistically significant, which in turn affects the VEGF protein level.

Conclusion

According to genotyping study, DR is strongly ($p<0.05$) correlated with the serum level of the VEGF gene -460 (T/C) polymorphism rather than the -2578 C/A gene polymorphism. Additionally, VEGF gene expression was higher in the mutant homozygous genotype (CC). It is still need to conduct more research with a larger sample size.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Research Institute of Ophthalmology, Giza, Egypt in 27/3/22. No. 2021120601

Consent to participate

Informed consent was obtained from all patients included in the study.

Consent for publication

The author confirms that t Consent to publish has been received from all participants.

Availability of data and material

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

The author confirms that there are no conflict of interests.

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