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A study of the genotyping and vascular endothelial growth factor polymorphism differences in diabetic and diabetic retinopathy patients

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Abstract

Background: Retinopathy is one of the major causes of visual impairment which is the most severe microvascular complication of diabetes mellitus (DM). The aim of this study was to evaluate the association between diabetic retinopathy (DR) and two SNPs (– 152G > A and – 165C > T) located in the promoter region of the vascular endothelial growth factor (VEGF) gene in a small sample from Egyptian population. One hundred diabetic patients without retinopathy (DWR) and two hundred diabetic patients with retinopathy were included in this study. Genotype analysis for the two SNPs (– 152G > A and – 165C > T) was assessed by using the PCR–RFLP technique. In addition, the serum protein level of VEGF was measured by ELISA assay.

Results: The results showed a significant relationship between – 152G > A (rs13207351) polymorphism and both proliferative and non-proliferative retinopathy in genotypes (GG, GA, AA). The risk factor increment in the mutant heterozygous genotype (GA) was significantly increased in NPDR compared to PDR (OR = 16.3, 95%CI = 0.80–331.7); (OR = 20.4, 95%CI = 1.08–385.3), respectively. There was no significance between VEGF – 165C > T (rs79469752) gene polymorphism and retinopathy. Moreover, the serum protein level of VEGF showed a highly significant increase ($P = 0.0001$) in PDR (Mean \pm SD = 3691 \pm 124.9) when compared to both DWR (Mean \pm SD = 497.3 \pm 18.51) and NPDR (Mean \pm SD = 1674.5 \pm 771.7). These results were supported by the increased level of VEGF in serum protein which is positively correlated with the severity of retinopathy. Measuring VEGF protein level in DR patients would help as a biomarker in early diagnosis.

Conclusion: The increase in the mutant heterogeneous GA genotype in VEGF – 152G > A SNP could be a risk factor for the progression of severe retinopathy in diabetic patients.

Keywords: VEGF polymorphism, Diabetes Mellitus, Egyptian and RFLP

Background

Diabetic retinopathy (DR) is an ocular complication of diabetes mellitus (DM) causing a microvascular complications of the retina of the eye; causing vascular severe complications in the eye and represents a major cause

of blindness around the world [1–3]. Without advanced treatment, DR evolved from mild-NPDR, to moderate and then to severe NPDR, finally to PDR [4, 5]. DR is known as a stage of proliferation that characterized by the formation of new blood vessels, anterior retinal hemorrhage, leading to vision loss [1].

Genetic factors may play a role in the pathogenesis and prevalence of DR in different ethnic populations [6]. Consequently, it is important to identify molecular markers that may help in the diagnosis of DR in various

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populations [7]. The human vascular endothelial growth factor (VEGF) is one of these risky genetic factors that plays a critical role in angiogenesis [8] observed in several ophthalmic diseases including PDR [9]. VEGF gene is located on chromosome 6 (6p21.3) and consists of 8 exons and 7 introns. It is a polymorphic gene with several single nucleotide polymorphisms (SNPs) in the 5'-untranslated region (UTR), 3'-UTR, and promoter regions [10].

Many literatures have studied (-152G>A and -165C>T) SNPs in the promoter region of VEGF gene in numerous diseases, included DR [11, 12]. Yet, the two SNPs were poorly studied in Egyptian patients. Hence the aim of this study was to establish the genetic risk factor between two SNPs (-152G>A and -165C>T) in relation to diabetic retinopathy among Egyptian patients.

Patients and methods

A total of 300 of DM patients who attend diabetes polyclinic of Research Institute of Ophthalmology (RIO) Hospital, Giza, Egypt, between April 2018 and December 2019; all were willing to participate in this study upon written consent form in accordance with the principles of the Declaration of Helsinki (WMA Declaration of Helsinki 2013), Also the protocol was approved by the RIO Ethical Committee on 4/7/2021. The 300 DM patients were classified into 100 diabetic patients without retinopathy (DWR) and 200 diabetic retinopathy patients were later classified into 100 diabetic patients with proliferative retinopathy (PDR) and 100 diabetic patients with non-proliferative retinopathy (NPDR), all the DM patients have a fundoscopic examination in the ophthalmology clinic using fundus biomicroscopic using a 90-diopter lens by slit lamp examination.

A history of all patients determined the duration of the disease and age followed by blood samples were withdrawn from all patients for testing random blood sugar, (RBS) and glycosylated haemoglobin (HbA1c) to confirm DM all are presented in Table 1. All the diabetic patients were on diabetic medication of insulin and oral tablets. VEGF protein levels was measured by ELISA and VEGF gene polymorphisms for -152G>A and -165C>T SNPs using the Polymerase Chain Reaction-Restriction Fragment Length Polymorphisms (PCR-RFLP).

Inclusion criteria

Participants diagnosed to have DM with and without DR were also included in this study.

Exclusion criteria

Participants with severe medical conditions congestive heart failure, liver disease, malignancy, inflammatory process, pregnancy and, with local eye diseases cataract and glaucoma or uveitis. Participants with diabetic duration less than 5 years. Finally, when declined to sign the consent form.

Laboratory investigations for biochemical and genotyping

Five milliliter of venous blood was obtained from patients with DM then divided into two portions, 2 ml added to EDTA tube for HbA1c test and for the genotyping assay, and the remaining of the 3 ml was saved for the routine laboratory investigations. HbA1c was assessed according to the method of, using glycosylated hemoglobin (GHb) Kit of Spectrum Diagnostics Co., (Egypt). Plasma glucose was measured according to the method of, using glucose—liquizyme GOD-PAP (Single Reagent) Kit of Spectrum Diagnostics Co., (Germany). Serum VEGF was also, measured by using ELISA (Enzyme-Linked

Table 1 Clinical and biochemical characteristics of the diabetic patients with and without retinopathy

Characteristics	DWR group (n = 100)	NPDR group (n = 100)	PDR group (n = 100)	P-value	
				NPDR versus DWR	PDR versus DWR
Age (years)	45.6 ± 11.3	56.4 ± 8.3	54.4 ± 10.2	.00	.00
Gender, n (%)					
Male	53% (53)	55% (55)	66% (66)	.777	.061
Female	47% (47)	45% (45)	34% (34)		
Duration of diabetes (years)	11.20 ± 4.26	17.04 ± 5.37	18.6 ± 5.8	.00	.00
HbA1C	6.7 ± 1.06	8.92 ± 1.43	9.03 ± 1.5	.00	.00
RBS	177.5 ± 49.7	202.6 ± 60.8	240.2 ± 83.4	0.002	.00
VEGF	497.3 ± 18.51	1674.5 ± 771.7	3691 ± 124.9	0.00	0.00

Data are expressed as number and percent and Mean ± SD. *t*-test was performed to evaluate the statistical significance between the two groups at $p < 0.05$. DWR, diabetic without retinopathy, NPDR, non-proliferative diabetic retinopathy, PDR, proliferative diabetic retinopathy, RBS, Random blood sugar; HbA1c, glycated haemoglobin A1c

Immunosorbent Assay) kit, according to the manufacturer's recommendation with commercially available kit (Bioassay Technology Laboratory Yangu Dist. Shanghai. China).

DNA extraction

Peripheral blood samples were collected from all participants (2 ml blood from each) on EDTA-rinsed vacutainer tubes. Genomic DNA was extracted by spin-column based kit, Qiagen (QIAamp Whole Blood Genomic DNA Purification Mini Kit), blood DNA extraction kit according to the protocol provided by the manufacturer (QIAamp DNA Mini Kit). The final samples were kept at -30°C until use.

PCR conditions

The PCR reaction included the enzyme Taq DNA polymerase, dNTPs, and PCR buffer. Where each 25 μl of the PCR reaction contained: Genomic DNA 3 μl , PCR buffer 2.5 μl , Forward primer 2 μl , Reverse primer 2 μl , Taq polymerase 0.5 μl , dNTPs 2.5 μl and nuclease free H₂O 12.5 μl . PCR was carried out on a Biometra™ Thermal Cycler, (Model: TProfessional Basic).

Determination of the VEGF genotypes

Both VEGF polymorphisms SNPs were detected using primers containing a single base-pair mismatch adjacent to the polymorphic site in order to introduce a restriction site into the wild-type nucleotide sequences after amplification.

$-152\text{G}>\text{A}$ polymorphism was determined using a PCR-RFLP technique based protocol as described by [12] We use forward primer 5'-TCCTGCTCCCTCCTCGCCAATG-3' and reverse primer 5'-GGCGGGGACAGGCGAGCCTC-3'. The reaction was amplified on a thermal cycler (Biometra™ thermalcycler), where the cycles was programmed as follows: 10 min at 95°C followed by 35 cycles for 1 min at 94°C , 35 cycles for 1 min at 62°C , 35 cycles for 1 min at 72°C , and 5 min at 72°C .

The PCR product was then digested with 1 μl of the restriction enzyme Fast Digest HpyF3I (Ddel) (Thermo Fisher Scientific) 30 min at 37°C .

The $-165\text{C}>\text{T}$ polymorphism was determined by using the same technique with the same conditions as mentioned above.

The PCR product was then digested with 1 μl of the restriction enzyme Fast Digest MvaI (BstNI) (Thermo Fisher Scientific) 30 min at 37°C .

Statistical analysis

Statistical analyses of data were done by using Statistical Package for Social Science Version 22 (SPSS, Inc., Chicago, IL). Qualitative variables expressed as number

and percentage. Quantitative variables are expressed as mean \pm standard deviation. Statistical significance was defined as $P \text{ value} \leq 0.05$. For quantitative data, a student t-test was used to compare between two groups. Chi-square test and Fisher's exact test were used for qualitative data.

Odds ratio and 95% confidence interval used to assessed the associations between the VEGF polymorphisms and DR susceptibility, [7].

Results

The distributions of demographic and clinical characteristics of diabetic patients in all groups are represented in Table 1. The DWR group had a shorter duration of DM (11.21 ± 4.26) and were younger (45.6 ± 11.3) than patient with NPDR (56.4 ± 8.3) and PDR (54.4 ± 10.2).

There was a significant increase ($P < 0.05$) in the plasma level of HbA1c in retinopathy groups (NPDR = 8.92 ± 1.43 ; PDR = 9.03 ± 1.5) when compared to the DWR group (6.7 ± 1.06). Also, the serum level of VEGF increased significantly ($P < 0.05$) in both NPDR (1674.5 ± 771.7) and PDR (3691 ± 124.9) when compared with the DWR group (497.3 ± 18.51). However, RBS showed a significant increase ($P < 0.05$) in PDR rather than NPDR when compared to DWR which represented in Table 1.

VEGF gene rs13207351 ($-152\text{G}>\text{A}$) polymorphism

There was a highly significant increase in GA genotype distribution in both retinopathy groups versus DWR (NPDR: OR = 16.3, CI = 0.80–331.7 and PDR: OR = 20.43, CI = 1.08–385.3). While, the AA genotype distribution showed a significant decrease in retinopathy groups (NPDR: OR = 0.17, CI = 0.04–0.65; PDR: OR = 0.12, CI = 0.03–0.42) versus DWR. The GG genotype distribution was non-significant in NPDR ($P = 0.14$) when compared with DWR. But, it was on the edge of significance in PDR OR = 3.90, CI = 1.05–14.48 versus DWR. Haplotype analysis revealed a strong evidence of an association for $-152\text{G}>\text{A}$ SNP with DR. The risk factor for the progression of retinopathy increased significantly with G allele in NPDR OR (CI) = 4.29 (1.62–11.37); PDR OR (CI) = 18.06 (7.32–44.52). The frequency of haplotype G was 64% in PDR group $P = 0.004$ and 29% in NPDR $P = 0.0001$. Meanwhile, A allele showed a significant decrease in NPDR OR (CI) = 0.23 (0.09–0.61) and PDR 0.05 (0.02–0.14) when compared with DWR (Tables 2 and 3).

The presence of a G to A transition at position-152 introduced a restriction site that, the PCR product band at 204 bp, after digestion, three types of bands were observed—a complete HpyF3I(Ddel) cut representing homozygous VEGF ($-152\text{G}/\text{G}$), resulting in

Table 2 VEGF gene polymorphisms in NPDR patients and DWR

Polymorphisms	DWR group (n = 100)	NPDR group (n = 100)	OR (95% CI)	P-value
rs13207351 (-152G>A)				
<i>Genotype</i>				
GG	9 (9%)	22 (22%)	3.01 (0.72–12.62)	0.141
GA	0 (0%)	14 (14%)	16.3 (0.80–331.7)	0.0074
AA	91 (91%)	64 (64%)	0.17 (0.04–0.65)	0.01
<i>Allele</i>				
G	18 (9%)	58 (29%)	4.29 (1.62–11.37)	0.004
A	182 (91%)	142 (71%)	0.23 (0.09–0.61)	
rs79469752 (-165C>T)				
<i>Genotype</i>				
CC	89 (89%)	100 (100%)	0.16 (0.0086–3.099)	0.16
CT	9 (9%)	0 (0%)	0.27 (0.013–5.46)	0.54
TT	2 (2%)	0 (0%)	0.39 (0.018–8.41)	1
<i>Allele</i>				
C	187 (93.5%)	200 (100%)	0.17 (0.009–3.23)	0.17
T	13 (6.5%)	0 (0%)	0.17 (0.009–3.23)	0.17

Data are expressed as number and percent, OR, odds ratio; CI, confidence interval, Fisher's exact test was performed to evaluate the statistical significant ($P < 0.05$), DWR, diabetic without retinopathy, NPDR, non-proliferative diabetic retinopathy, PDR, proliferative diabetic retinopathy

Table 3 VEGF gene polymorphisms in PDR patients and DWR

Polymorphisms	DWR group (n = 100)	PDR group (n = 100)	OR (95%CI)	P-value
rs13207351 (-152G>A)				
<i>Genotype</i>				
GG	9 (9%)	28 (28%)	3.90 (1.05–14.48)	0.051
GA	0 (0%)	17 (17%)	20.43 (1.08–385.3)	0.0
AA	91 (91%)	55 (55%)	0.12 (0.03–0.42)	0.0005
<i>Allele</i>				
G	18 (9%)	127 (63.5%)	18.06 (7.32–44.52)	0.001
A	182 (91%)	73 (36.5%)	0.05 (0.02–0.14)	
rs79469752 (-165C>T)				
<i>Genotype</i>				
CC	89 (89%)	100 (100%)	0.12 (0.007–2.348)	0.14
CT	9 (9%)	0 (0%)	0.205 (0.01–4.14)	0.27
TT	2 (2%)	0 (0%)	0.29 (0.014–6.37)	0.51
<i>Allele</i>				
C	187 (93.5%)	200 (100%)	0.133 (0.007–2.45)	0.16
T	13 (6.5%)	0 (0%)	0.133 (0.007–2.45)	0.16

Data are expressed as number and percent, OR, odds ratio; CI, confidence interval, Fisher's exact test was performed to evaluate the statistical significant ($P < 0.05$), DWR, diabetic without retinopathy, PDR, proliferative diabetic retinopathy

two fragments of 143 and 61 bp (GG); a partial cut representing heterozygous VEGF (-152G/A), resulting in three fragments of 204, 143 and 61 bp (GA); and an uncut 204 bp (AA) fragment representing homozygous VEGF (-152A/A) were visualized on a 2% agarose gel (Figs. 1, 2 and 3).

VEGF gene rs79469752 (-165C>T) polymorphism

There was no significant difference in genotype distribution and allele frequency in the DR groups (NPDR and PDR) when compared to DWR group (Tables 2 and 3).

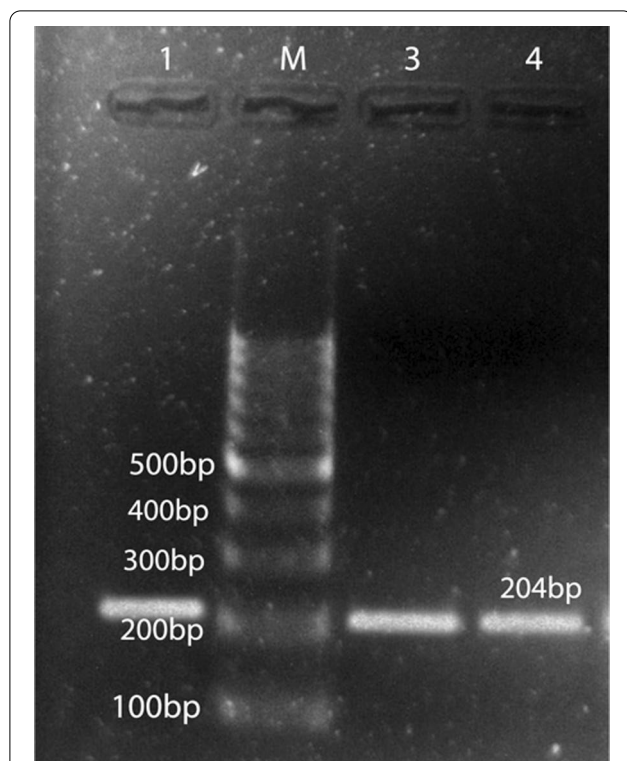


Fig. 1 The product of the PCR polymerase chain reaction amplification of $-152G > A$ and $-165C > T$ VEGF polymorphisms represented in lane 1, 3, 4. Lane M marker represents ladder 100 bp

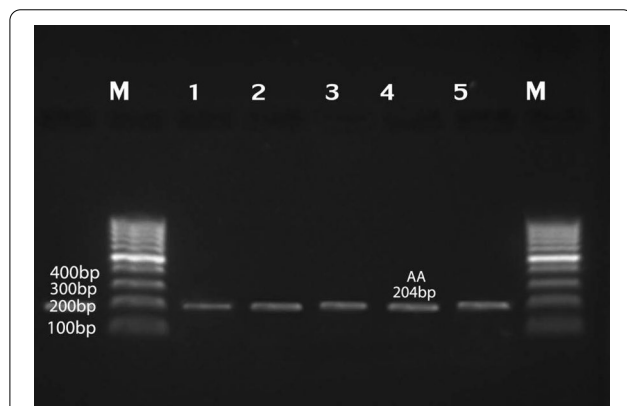


Fig. 2 Digestion products for $-152 G > A$ VEGF polymorphism by DdeI restriction enzyme, samples was electrophoresed on a 2% agarose gel. Lane M represents ladder 100 bp. Lanes 1, 2, 3, 4, 5 represent AA mutant genotype (one band at 204 bp)

The presence of a C to T transition at position-165 introduced a restriction site that, the PCR product band at 204 bp, after digestion, three types of bands were observed a complete MvaI (BstNI) cut representing homozygous VEGF ($-165C/C$), resulting in two

fragments of 130 and 74 bp (CC); a partial cut representing heterozygous VEGF ($-165C/T$), resulting in three fragments of 204, 130 and 74 bp (CT); and an uncut 204 bp (TT) fragment representing homozygous VEGF ($-165 T/T$) were visualized on a 2% agarose gel (Figs. 1 and 4).

Discussion

Diabetic retinopathy ruins the most collective reason of visual impairment in controlled – aged individuals. Hyper glycaemia is the main critical factor that causes a severe ocular complications (retinopathy) associated with diabetes [13]. The progression of diabetic retinopathy is influenced by both genetic and environmental agents [2, 14, 15].

In the present study, an elevated HbA1c level, for longer duration of diabetes and an increment in mean age were detected in NPDR and PDR. These observations were in agreement with [16–18] who reported that these changes may be considered as indicators of poor glycemic control and significant risk factors for development and progression of DR. Additionally, Wat et al. [15] indicated that a prolonged exposure to hyperglycemia increases the chance to develop retinopathy. An impaired adaptive responses to hypoxia could contributed to the pathophysiology of diabetes and its complication [19, 20]. Emerging evidence indicates that the conditions of diabetes cause different tissues to be hypoxic [21–25], including the retina [26].

Moreover, it was established that oxidative DNA damage occurs as a consequence of hypoxic exposure in different species including human subjects [27–30]. This oxidative damage could lead to genomic instability, perhaps related to acquisition of mutations [31]. Hypoxia is a major regulator of VEGF expression via hypoxia inducible factor (HIF) and other hypoxia-regulated genes which coordinate with VEGF expression and, in turn, VEGF-driven signaling [32, 33].

There are two families of polypeptides with opposed features; proangiogenic, and antiangiogenic, that produced from the splicing and proteolytic processing of VEGF transcripts [34, 35]. The disparity of balance between pro- and antiangiogenic VEGF further crucially give an explanation of the anatomic variation of the diabetic eye that may result in vision deterioration [36]. The present study has confirmed the participation of VEGF in the development of DR as suggested by previous studies [16, 37–41]. Moreover, the present data indicated increased levels of VEGF protein with 3.6 folds in the NPDR patients and 7.4-folds in the PDR patients compared to DWR patients. The higher level of VEGF in PDR than NPDR is in concordance with Zhou et al. [42].

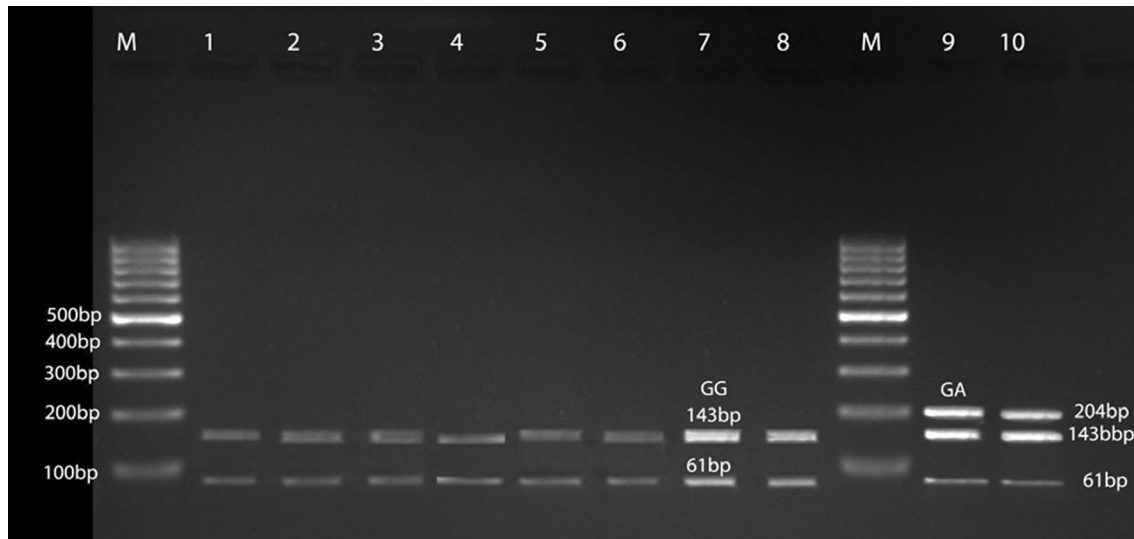


Fig. 3 Digestion products for – 152G>A VEGF polymorphism by DdeI restriction enzyme, samples were electrophoresed on a 2% agarose gel. Lane M represents ladder 100 bp. The G allele was cut into two fragments of 61 and 143 bp, whereas the A allele remained uncut (204 bp). Lane 9 and 10 represent GA genotype (three bands at 61, 143 and 204 bp). Lanes 1, 2, 3, 4, 5, 6, 7, 8 represent GG genotype (two bands at 61 and 143 bp)

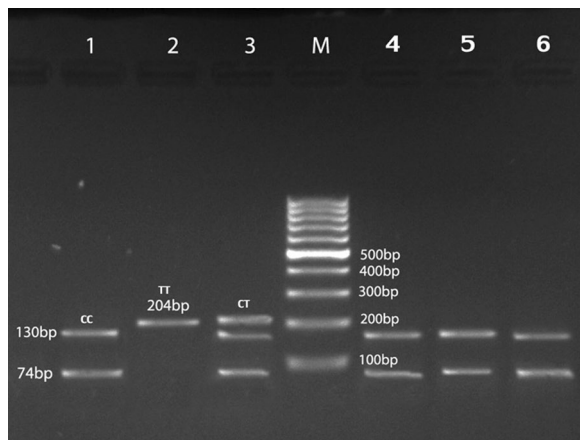


Fig. 4 Digestion products for – 165C>T VEGF polymorphism by BstNI restriction enzyme, samples were electrophoresed on a 2% agarose gel. Lane M represents ladder 100 bp. The C allele was cut into two fragments of 74 and 130 bp, whereas the T allele remained uncut (204 bp). Lane 3 represents CT genotype (three bands at 74, 130 and 204 bp). Lanes 1, 4, 5, 6 represent CC genotype (two bands at 74 and 130 bp). Lane 2 represent TT mutant genotype (one band at 204 bp)

Many studies investigated specific polymorphisms in the promoter and 5 untranslated region (UTR) of VEGF gene [43] which found to be associated with the risk of angiogenesis in DR [44]. In this current study a significant association of VEGF rs13207351 gene polymorphism with retinopathy in diabetic patients

was noted when compared to DWR group. Therefore, VEGF – 152G>A polymorphism could be a genetic marker for sever DR in Egyptian diabetic patients. This is in agreement with previous studies by Churchill et al. [45–48] who indicated the effect of VEGF genetic variants on the severity of retinopathy through increasing VEGF expression.

A previous study by Amoah et al. [12, 49] using the same SNP – 152G>A (rs13207351) gene polymorphism in the promoter of VEGF on breast and colon cancer patients revealed the same results as in our present study. However, there was no studies concerning analyzing the behavior of the mentioned SNP in the Egyptian diabetic patients suffering from DR, and the severity of DR in the present study, GA genotype showed an increase by 1.2-fold, while G allele showed an increase by 4.5 fold in PDR compared to NPDR. The association of wild G allele with the higher risk to develop retinopathy may be explained by the shifting of gene from antiangiogenic properties to angiogenic properties Bates et al. [50, 51]. The results of this study are in concordance with Marsh et al. [52] who revealed that G allele may exhibit higher expression of VEGF mRNA.

Furthermore, the AA genotype in the current work showed low risk factor for the severity of DR by 1.4-fold (OR=0.12, CI=0.03–0.42). On contrary, Churchill et al. [45–47, 53] demonstrated that AA genotype was a potential risk marker of diabetic retinopathy (OR=3.5, CI=1.5–7.7) in Britain and in Chinese populations (OR=3.7, CI=1.2–11.7).

In the present study, VEGF gene – 165C>T polymorphism have no obvious association with DR severity. This is in agreement with Amoah et al. [12, 49] who did not detect an association with breast cancer and coronary collateral vessels. We also observed non statistical difference between the three genotypes of – 165C>T polymorphism in the two diabetic groups. Nevertheless, these results were contradictory with Churchill et al. [45] where a single haplotype – 165C was significantly associated with PDR patients. This contradiction might be due to different ethnicity of the subjects or may be inter-related to other risk factors among those populations, which may need further investigation.

Conclusions

The present study attentive on new therapeutic strategy needed hand to hand with the health awareness to reduce the affliction associated with the common problems of diabetes in Egyptian population. The VEGF rs13207351 SNP polymorphism may be considered a reliable genetic marker for predicting DR severity. The mutant heterozygous form of GA polymorphism and Gallele at – 152 of VEGF gene might show a higher frequency of susceptibility to retinopathy as an ocular diabetic complication. However, there is no association with VEGF – 165C>T gene polymorphism. Serum VEGF are considered a biomarker for evaluating the development and progression of DR. VEGF level was strongly correlated with the NPDR and PDR, and its concentration was also found to be statistically correlated with some biochemical (HbA1c) and demographic findings (age and duration).

Further studies are needed to better our understanding of VEGF SNPs and DR susceptibility and to confirm these relationships in our societies.

Abbreviations

DM: Diabetes mellitus; DWR: Diabetic without retinopathy; NPDR: Non-proliferative diabetic retinopathy; PDR: Proliferative diabetic retinopathy; VEGF: Vascular endothelial growth factor; PCR: Polymerase chain reaction; RFLP: Restriction fragment length polymorphism; SNPs: Single nucleotide polymorphisms; RIO: Research Institute Ophthalmology; RBS: Random blood sugar; HbA1c: Hemoglobin blood A1c.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s43042-022-00277-x>.

Additional file 1. Raw data of Diabetic without retinopathy patients.

Additional file 2. Raw data of Non-proliferative diabetic retinopathy patients.

Additional file 3. Raw data of Proliferative diabetic retinopathy patients.

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Authors' contributions

SW performed in collecting the literature review, carried out the molecular and the biochemical techniques, analyzed and interpreted the patient data, Writing and revising the manuscript. MMH participated in revising the manuscript. KAR support in collecting medical samples from outpatients clinic, helping in revising the manuscript. AAAA provided the medical samples of patients who participated in the design of the study and contributed to revising the manuscript. OHE suggested the point of the study, participated in the design of the study, writing and revising the whole manuscript. The authors read and approved.

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Availability of data and materials

Data are presented in excel sheet as row data. They are also, included in this paper and should not be used by other authors without previous consent of the present authors (us). With our best regards.

Declarations

Ethics approval and consent to participate

All participated in this study upon written consent form in accordance with the principles of the Declaration of Helsinki (WMA Declaration of Helsinki 2013), also the protocol was approved by the RIO Ethical Committee on 4/7/2021.

Consent for publication

A consent form was signed by all the 300 participants to take part in this study. The participants were informed about the purpose of the study. Only blood samples were used in this research study. A specific questionnaire was filled with all included and excluded criteria. I have saved copies from these consent forms, if you want me to upload them as a Additional files 1, 2 and 3, I will be glad to send.

Competing interests

All authors hereby declare that there is no conflict of interest associated with this publication.

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