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ORIGINAL ARTICLE

VEGF Gene Polymorphisms as a Genetic Biomarkers for Diabetic Retinopathy

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ABSTRACT:

Background: Diabetic retinopathy (DR) is one of the potential etiologies of blindness globally. It is featured by elevated vascular permeability, hemostatic abnormalities, retinal ischemia and neo angiogenesis. Theis study aim is to give better understanding for the genetic causes of DR and predict the impact of the polymorphism vascular endothelial growth factor(VEGF) gene and DR occurrence.

Methods: This study is a case-control study, included 70 cases diagnosed as T2DM, the cases were allocated into 3 groups: Group (1): included 20 healthy participants as a control group. Group (2): included 20 type 2 diabetic (T2DM) cases without DR (NDR). Group (3): included 30 T2DM cases with DR. All cases of the studied groups were subjected to: full history taking, complete physical and clinical assessment, laboratory tests, and fundus examination. rs2010963 VEGF single nucleotide gene polymorphism was detected.

Results: AA genotype, A allele was significantly dominant by (14.08 fold), (4.8 fold) in DR group as compared with control group (P = 0.001 and P = 0.0004 respectively). AA genotype, A allele was significantly dominant by (5.9 fold), (2.9 fold) in DR group as compared with the NDR group (P = 0.015 and P = 0.01 respectively).GG genotype, was significantly dominant by (8 fold) in males compared with females in DR group (P = 0.009).

Conclusions: We concluded that VEGF gene polymorphisms had a role in developing diabetic retinopathy and they can be used as a genetic predictor for diabetic retinopathy.

Keywords: VEGF Gene; Diabetes; Retinopathy; Polymorphisms.

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease of the metabolism of carbohydrates that is typified by a decreased capacity of the body to either create or react to insulin. type 1 (T1DM) and type 2 (T2DM) are the types of DM. Insulin shortage causes T1DM, while insulin resistance (IR), decreased insulin secretion, and elevated levels of glucose cause T2DM [1] . The two types of DM raise the chance of developing long-term issues that take years to manifest. Blood vessel injury is one of the main long-term consequences. The threat of cardiovascular disorders is doubled by DM. Stroke and peripheral artery disease are two more macrovascular disorders [2].

Microvascular complications that affect small blood vessels, cause serious pathogenic disabilities in diabetic patients. The most precious organs include the nerves, kidneys, and eyes [3].

The diabetic patient eyes should be followed up continuously in long term diabetic patient, as the diabetic retinopathy (DR) that impacts retinal arterioles, venules and capillaries that can lead to their damage. As a result, eventual blindness and gradual vision loss will occur. In addition, DM elevates the risk of having cataracts, glaucoma, and other ocular disorders [4].

Diabetic retinopathy is one of the potential blindness causes by incidence of 51% in western world. It is featured by elevated vascular permeability, haemostatic abnormalities, retinal ischemia and neo angiogenesis. Lesion varies from microaneurysm, hemorrhage, exudate in early stages but late neovascularization occur with retinal detachment and blindness. Without treatment, diabetic retinopathy develops mild non proliferative DR (NPDR), then moderate and then to severe NPDR, finally it may develop proliferative diabetic retinopathy (PDR) [5].The progression of DR depends on DM duration, blood pressure and glucose levels although these factors alone do not explain cause of DR. Many previous studies tried to explain the pathogenesis of DR, yet the exact pathogenesis is still obscure [6].

The genetic role of DM pathogenesis was proved in several investigations accordingly, many reports suggested that DR has genetic tendency. Growth factors are associated in DM pathogenesis as they regulate angiogenesis [7].

Vascular endothelial growth factor (VEGF) is a crucial growth factor family member. VEGF is a potential proangiogenic factor. VEGF is used as a prognostic marker for many diseases that their pathogenesis includes angiogenesis such as diabetic angiopathies remarkably diabetic retinopathy [8].VEGF increases vascular permeability manages vascular endothelial proliferation and promote angiogenesis in RD which cause retinal detachment with visual impairment. Several studies predict that VEGF expression is elevated in cases with DR. The genetic variance that involves in the gene promotor area can be associated with increase VEGF expression that is recorded in many vascular diseases [9].

The present work aim was to give better understanding for the genetic causes of DR and predict the impact of the polymorphism VEGF gene and diabetic retinopathy occurrence

METHODS

This case-control study was carried out at Medical Biochemistry Department and Outpatient Clinics of Ophthalmology department, Faculty of Medicine, Zagazig University. Seventy patients diagnosed as T2DM, who attend Outpatient Clinics at Ophthalmology Department, Zagazig University Hospitals for regular care were included. The cases were allocated into 3 groups: Group (1): 20 normal participants as a control. Group (2): 20 T2DM cases without DR. Group (3): included 30 T2DM cases with DR.

The study was conducted after obtaining approval from Institutional Review Board (IRB#10572-14-3-1023) and written informed consent from all cases. The research was conducted under the World Medical Association's Code of Ethics (Helsinki Declaration) for human research.

Cases with the following criteria were included; T2DM patients were diagnosed regarding American Diabetes Association (ADA) criteria: Fasting blood glucose (FBG) >126 mg/dl. (2-h) postprandial blood glucose level > 200 mg/dl. HbA1c > 6.5 % in cases with the classic symptoms of hyperglycemia. Random blood glucose > 200 mg/dl. Patients with abnormal oral glucose tolerance test.

Cases with the following characteristics were excluded; any local eye diseases cataract and glaucoma or uveitis. Any medical disorder. Participants with diabetic duration less than 5-year. Patients with T1DM. Participant lacks of cooperation or refuses to give consent

All patients were subjected to: Full history taking including: gender, age, the disease duration and onset, family history, smoking, diet, and physical activity. Complete physical

and clinical examination, laboratory tests (Hb0A1c), lipid profile, insulin, fasting blood glucose), and fundus examination.

FBG, HbA1c, HDL-cholesterol, LDL-cholesterol, and Total cholesterol, Triglycerides levels were obtained from patients' sheets and were auto analyzed by (cobas® 8000 modular analyzer series).

For determination of IR: calculation of the homeostasis model assessment (HOMA) score according to Mari A et al. [10].

DNA extraction

Employing the commercially supplied Blood Genomic DNA Extraction Kit (Solarbio-China-Cat#D1800), genomic DNA was isolated from whole blood. DNA extracted according to the manufacturer instructions.

Quantification and purity of DNA

To measure the amount of DNA and assess its purity, 20 µl of each DNA specimen was mixed with 1 ml of deionized water in a quartz cuvette. The Milton Roy Spectronic 3000 Array was used to detect absorbance at 260 and 280 nm wavelengths. Since the resonance structures of the purine and pyrimidine bases are what cause the absorbance, DNA has its highest absorbance at 260 nm. At 260 nm, an absorbance of 1.0 indicates a 50 µg/ml DNA content. Tyrosine, phenylalanine, and tryptophan cause proteins to absorb most at 280 nm. [11].

PCR Protocol:

Utilizing a DNA thermal cycler 480, PERKIN ELMER (Norwalk, CT 06856, USA, Serial No. P16462), the amplification was performed regarding the following protocol: five minutes of initial denaturation at 94°C, 30 cycles of denaturation at 94°C for five seconds, annealing for 45 sec. at 61°C, extension for 45 sec. at 72°C, and final extension at 72°C for five minutes. Ethidium bromide (EtBr) staining, 2% agarose gel electrophoresis, and UV transilluminator visualization were performed on the PCR products in order to identify the stained 210-bp [15].

Detection of rs2010963 (-634 C/G) polymorphism of VEGF gene using amplification refractory mutation system (ARMS)technique:

Genomic DNA was isolated and kept at -20°C in Babylon University's biotechnology lab. Tetra primers and the ARMS-PCR technique were used to genotype the SNPs in the study. The rs2010963 SNPs' outside primers produce the confirmative amplicon for the promoter region and 5' UTR, where the SNPs are located. The 3' end of the primer was mismatched to create the inner reverse primers for the mutant allele. For enhance the specificity, the third nucleotide of the 3' end was also mismatched. The following were the PCR steps: To attain the lowest annealing for 40 sec at 58°C and 72°C for extension and final extension for 40 s and 7 min, respectively, 40 cycles of denaturation for 35 sec at 95°C and annealing at 65°C steadily decreased each cycle. Initial denaturation at 95°C for 2 minutes is done to activate the PCR cycles [16]. Following 2% agarose gel electrophoresis, EtBr staining, and UV transilluminator visualization, the PCR products were examined.

Total of four primers are used:
F0:CGACGGCTTGGGGAGATTGCTCTAC,
F1:GCGTGCGAGCAGCGAATGC,
R0:GGCGGTGTCTGTCTGTCTGTCCGT,
R1:CAGGTCACTCACTTTGCCCCTGACC

Statistical Methods

All data were analyzed utilizing SPSS 26.0 for windows. Quantitative data were expressed as the mean ± SD & range and qualitative data were presented as number and percentage. F: Anova test was employed to compare between more than two groups of normally distributed variables. If anova test was significant, Scheffé's test is a method for testing pairwise Sheffe's test to find out which pairs of means are significant. Percent of categorical variables were compared employing Chi-square test or Fischer exact test. An odds ratio is a assess of relationship between an exposure and an outcome. Confidence interval (CI) is an interval which is expected to 95% confident that the true value falls within that range. Data with P<0.05 considered significant.

RESULTS

There was significantly abnormal laboratory finding in DR, NDR patients, in HOMA, HbA1c, and T.cholesterol ($p<0.05$) (Table 1).

Percent of CC, GC, and GG for VEGF Gene were 10, 33.3, and 57.7% in DR group.35, 45, and 20% in NDR group. and 50, 40, and 10% in control group. GG genotype, was significantly in DR group compared with the NDR group and control group (P = 0.018, 0.007 respectively). G allele was significantly dominant in DR group compared with the NDR group, healthy control group (P = 0.002, 0.0001 respectively). (Table 2)

Regarding VEGF, GG genotype, G allele was significantly dominant by (24.8 fold), (6.4 fold) in DR group as compared with the control group (P = 0.0004 and P = 0.0001 respectively). CC genotype, C allele was significantly dominant by (9.1 fold), (3.72 fold) in DR group as compared with the NDR group (P = 0.018 and P = 0. 001 respectively). (Table 3)

Regarding DR group, Percent of CC, GC, and GG for VEGF gene were 6.7, 13.3, and 80% in males compared to 13.3, 53.3, and 33.3% in females. C, G allele, were 13.3 and 86.7% in males compared 40%/60% in females. GG genotype, G allele were significantly dominant by (8.7 fold), (4.3 fold) in males compared with females in DR group (P = 0.03 and P = 0. 02 respectively).GG genotype , was significantly dominant by (8 fold) in males compared with females in DR group (P = 0.009). (Table 4)

Concerning NDR group, Percent of CC, GC, and GG for VEGF Gene were 30, 40, and 30% in males compared to 40, 50, and 10% in females. C, G allele, were 1:1 in males compared 65 and 35% in females. There was no remarkable variance between males and females, in NDR group regard VEGF Gene frequency or allele ($p>0.05$). (Table 5)

Table (1): Demographic characters of studied groups:

Demographic data	**Control group n.20**	**NDR group n.20**	**DR group n.30**	χ^2 /f-test	p-value
Gender n(%) females males	10(50.0%) 10(50.0%)	10(50.0%) 10(50.0%)	15(50.0%) 15(50.0%)	0.143c	0.931
Age per years Mean ±SD (range)	56.73±6.79 49-77	61.45±8.32 45-75	56.73±6.79 49-77	2.82	0.067
HOMA(insulin resistance)(mg/dl) mean ± SD range	1.39±0.27 (1-1.9)	4.06±0.41 (3.1-4.7)	4.14±0.42 (3.5-4.9)	392.6	0.0001*,^
"HbA1c(%)" mean ± SD range	4.91±0.34 (4.4-5.5)	8.45±1.3 (6.5-11.4)	7.92±0.82 (6.5-9.5)	95.6	0.0001*,^,$
"T.cholesterol (mg/dl)" mean ± SD range	129.4±9.79 (110-147)	170.45±26.75 (126-200)	175.1±26.63 (133-220)	25.8	0.0001*,^
TG(mg/dl) mean ± SD range	113.65±13.32 (94-134)	154.25±15.63 (130-178)	163.9±12.99 (143-188)	82.6	0.0001*,^,$

χ^2 Chisquare test (c), f: Anova, Data are expressed as mean ± standard deviation (SD), Range. (* significant Compare DR group &NDR group), ^, significant Compare DR group &control group), ($significant Compare NDR group &control group)

Table (2): Frequency distribution of VEGF gene in DR, NDR and control group:

	Control group n.20 n(%)	NDR groupn.20 n(%)	DR group n.30 n(%)	X2	p-value	Compare between groups
VEGF Gene						
CC	10(50.0)	7(35.0)	3(10.0)	16.92	0.002*	P1=0.018* P2=0.007* P3=0.53
GC	8(40.0)	9(45.0)	10(33.3)			
GG	2(10.0)	4(20.0)	17(57.7)			
VEGF Allele						
C	28(70.0)	23(57.5)	16(26.7)	21.05	0.002*	P1=0.002* P2=0.0001* P3=0.25
G	12(30.0)	17(42.5)	44(73.3)			

χ 2 Chi square test of significant, *p<0.05 significant p>0.05 no significant, (P1, Compare DR group &NDR group), p2 Compare DR group &control group), p3 Compare NDR group &control group)

Table 3: Frequency distribution of VEGF Gene in DR , NDR, and control groups:

GENETIC	DR group n.30	control group n.20	χ 2	p	Odd (95%CI)DR
VEGF Gene Domain model n(%)					
CC pulse GC	13(43.3)	18(90.0)	Ref		
GG	17(56.7)	2(10.0)	11.09	0.0008*	11.8(2.3-60)
VEGF Gene recessive mode n(%)					
CC	3(10.0)	10(50.0)	Ref		
GG pulse GC	27(90.0)	10(50.0)	9.9	0.001*	9(2.05-39.5)
GENETIC	**NDR group n.20**	**control group n.20**	**χ 2**	**p**	**Odd (95%CI)**
VEGF Gene Domain model n(%)					
CC pulse GC	16(80.0)	18(90.0)	Ref		
GG	4(20.0)	2(10.0)	f	0.66	2.2 (0.27-27.4)
VEGF Gene recessive mode n(%)					
CC	7(35.0)	10(50.0)	Ref		
GG pulse GC	13(65.0)	10(50.0)	0.92	0.34	1.86(0.52-6.61)
GENETIC	DR group n.30	NDR group n.20	χ 2	p	Odd (95%CI) DR
VEGF Gene Domain model n(%)					
CC pulse GC	13(43.3)	16(80.0)		Ref	
GG	17(56.7)	4(20.0)	6.62	0.01*	5.2(1.4-19.4)
VEGF Gene recessive mode n(%)					
CC	3(10.0)	7(35.0)	f	Ref	
GG pulse GC	27(90.0)	13(65.0)		0.073	4.8(0.89-32.7)

χ 2 Chi square test of significant,f: Fisher exact test, ref(reference gene or allele). 95%CI:95% confidence interval,*p<0.05 significant p>0.05 no significant

Table 4: Frequency distribution of VEGF Gene in DR and NDR groups according gender:

GENETIC	DR group n.30		χ 2	p	Odd (95%CI)
	Males n.15	Females n.15			
VEGF Gene frequency n(%)			**6.82**	**0. 032***	
CC	1(6.7)	2(13.3)	Ref		
GC	2(13.3)	8(53.3)	**f**	**0.03***	8.7(1.2-113)b
GG	12(80.0)	5(33.3)	f	0.54	4.4(0.2-306)a
VEGF Gene Domain model n(%)					
CC pulse GC	3(20.0)	10(66.7)	Ref		
GG	12(80.0)	5(33.3)	6.65	**0.009***	8(1.5-42)
VEGF Gene recessive mode n(%)					
CC	1(6.7)	2(13.3)	Ref		
GG pulse GC	14(93.3)	13(86.7)	f	0.99	2.1(0.1 -66.7)
VEGF Allele frequency n(%)					
C	4(13.3)	12(40.0)	Ref		
G	26(86.7)	18(60.0)	5.4	**0.02***	4.3(1.2-15.6)

χ 2 Chi square test of significant,f: Fisher exact test, ref (reference gene or allele). 95%CI:95% confidence interval,*p<0.05 significant p>0.05 no significant, (a, Compare GG&CC), (b, Compare GG&GC)

Table 5: Frequency distribution of VEGF Gene in NDR group according gender:

Genetic	NDR group n.20		χ 2	p	Odd (95%CI)
	Males n.10	Females n.10			
VEGF Gene frequency n(%)			1.25	0.53	
CC	3(30.0)	4(40.0)	Ref		
GC	4(40.0)	5(50.0)	f	0.68	3.4(0.18-235)b
GG	3(30.0)	1(10.0)	f	0.69	3.5(0.2-261)a
VEGF Gene Domain model n(%)					
CC pulse GC	7(70.0)	9(90.0)	Ref		
GG	3(30.0)	1(10.0)	f	0.58	3.6(0. 23-224)
VEGF Gene recessive mode n(%)					
CC	3(30.0)	4(40.0)	Ref		
GG pulse GC	7(70.0)	6(60.0)	f	0.99	1.5(0.17-15)
VEGF Allele frequency n(%)					
C	10(50.0)	13(65.0)	Ref		
G	10(50.0)	7(35.0)	0.92	0.34	1.8(0.5-6.6)

χ 2 Chi square test of significant, f: Fisher exact test, ref (reference gene or allele). 95%CI:95% confidence interval, *p<0.05 significant p>0.05 no significant, (a, Compare GG&CC), (b, Compare GG&GC)

DISCUSSION

DM is a metabolic disease of the metabolism of carbohydrates that is typified by a decreased capacity of the body to either secrete or react to insulin. Insulin shortage causes T1DM, while IR, decreased insulin production, and elevated glucose intake cause T2DM [17].

Each of the types of diabetes raises the chance of developing long-term issues that take years to manifest. Blood vessel injury is one of the potential long-term consequences. The risk of cardiovascular disorder is doubled by diabetes. Peripheral artery disease and stroke are two more macrovascular conditions [18].

Our study was aiming to give better understanding for the genetic causes of DR and predict the impact of the polymorphism of VEGF gene and DR occurrence.

In this study, the age of all selected groups range from 45-77 years old and there was no variation in studied group regard age, (p>0.05). The selection of our patients from exact age group was done according to the scientific finding of Wyczalkowska-Tomasik el al, [19] who found that inflammatory markers change with age. This was supported with another study that estimated the insufficiency of VEGF signaling that happened with age [20].

In our study we select the gender of our groups with no difference regard gender, p>0.05. According to Malamitsi-Puchner A et al., [21] study they found that serum VEGF values are generally elevated in females than in males, with variations across different life stage. Also, another study highlights the influence of sex hormones on the expression and function of PPARs [22].

In this study, there was significantly abnormal laboratory finding in DR, NDR patients, in HOMA (IR) (mg/dl), "HbA1c (%)", "T.cholesterol (mg/dl)" TG (mg/dl) (p<0.05)

We enforced our data by clinical information and estimation Of HbA1c (%) that assure the results of IR. And this result was agreed with Zhai et. al. [23].

Our study revealed that there was significantly abnormal laboratory finding in DR, NDR patients, (T cholesterol and TG) (p<0.05).

These results came with Li Z et al., [24] study who published that Elevated LDL cholesterol values were correlated with elevated risk of DR. LDL cholesterol can conduct to atherosclerotic plaques formation, which can block blood flow to the retina. Moreover, investigations reported a positive relationship between high triglyceride values and the development of DR. High triglycerides can contribute to oxidative stress and inflammation, which can harm the blood vessels in the retina.

To clarify VEGF polymorphism role in DR, in this study we have selected rs2010963 (-634 C/G) polymorphisms of the VEGF polymorphism.

The rs2010963 SNP is located in the promoter region of the VEGF, 634 base pairs upstream of the transcription start site. This SNP involves a single nucleotide change that can affect the binding of transcription factors to the promoter region, which can in turn affect the level of VEGF gene expression.

Our results showed that a polymorphism in the VEGF Gene was correlated with DR presence. Allele (G) for VEGF -634 C/G gene which was significantly dominant by (6.4 fold) in the DR group compared with control group (p=0.0001). The VEGF -634 gene G homozygote and G allele are related to a higher risk of DR progression 57.7%, 73.3 % in the DR group when compared with control group, respectively (p=0.0001).

Our results demonstrated that the percent of CC, GC, and GG for VEGF Gene were 10%, 33.3% and 57.7% in DR group respectively while they were 35%, 45% and 20% respectively in NDR group however they were 50%, 40% and 10% in the control group

respectively. The homozygote C allele are related to a declined risk of DR progression.

In agreement with us Awata et al., [29] study. They assumed that C-634G was correlated with DR presence. they found The VEGF -634 gene G homozygote and G allele are related to a higher risk of DR formation and they detected that the relationship was stronger in diabetic macular edema cases.

Another study agreed to our results and they failed to find any association between VEGF -634 Gene Polymorphisms and NDR, but not in Kamal et al., [30] had announced that, Regarding the VEGF -634 G/C polymorphism, there was no substantial variance between DM cases and controls. Additionally, they hypothesize that this polymorphism primarily works in concert with diabetic circumstances to create retinopathy.

In contrary to us, Churchill et al, [22] have shown that C allele presence at this position is correlated with an elevated risk of developing DR. Specifically, individuals with the CC genotype have been found to have a higher susceptibility to DR compared to those with the GG genotype.

Additionally, the severity of DR can be influenced by specific genetic variations, highlighting the complex genetic basis of this complication. Understanding these genetic differences is crucial for identifying individuals at higher risk for DR, developing targeted prevention strategies, and potentially personalizing treatment approaches for DM with DR.

The investigated populations' varied genetic backgrounds could be the reason for this discrepancy. For example, this discrepancy might suggest that various populations have distinct associations with particular genetic variations.

Finding the genes linked to this illness is crucial for both gene therapy and genetic diagnosis. Due to the uncertainty of a polygenic feature, genetic investigations of multifactorial disorders like DR are challenging. Elevated mitogenic activity and upregulation of growth factors could result from the gene's biallelic expression [32].

CONCLUSIONS

Early accurate diagnosis of diabetic retinopathy must be the major goal of the physician while facing these cases from the first second to avoid the probable dangerous sequences. VEGF gene polymorphisms had a role in developing diabetic retinopathy and they can be used as a genetic predictor for diabetic retinopathy**.**

Ethics declarations

This study was conducted following the ethical principles of the Declaration of Helsinki (Edinburgh 2000) and the approval of the Institutional Review Board. The Institutional Review Board granted an exemption for informed consent.

All participants provided informed written consent.

This study was approved by the Institutional Research Board (IRB) at Zagazig University

Conflict of interest: The authors declare that they have no competing interest.

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