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Original Research Article

Association of Vascular Endothelial Growth Factor +405G/C Polymorphism with Diabetic Retinopathy among Sudanese Patients

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Abstract

Background: Diabetic retinopathy (DR) is a major cause of new-onset blindness among diabetic adults and is characterized by increased vascular permeability, tissue ischemia, and neo-vascularization. Neovascularization of the retina carries a high risk of blindness as a result of vitreous hemorrhage and fibrosis. Vascular Endothelial Growth Factor (VEGF), originally known as vascular permeability factor (VPF), is a signal protein produced by cells that stimulates the formation of blood vessels. Polymorphisms within the VEGF gene lead to differences in VEGF expression between individuals and could influence the etiology of a variety of pathologic conditions with which VEGF has been associated. The aim of this study is to investigate +405G/C polymorphism of VEGF gene in Sudanese patients with type 2 diabetes mellitus (T2DM) and to evaluate its relationship with the development and improvment of diabetic retinopathy. ***Materials and Methods***: A total of 189individuals subjects divided to 3 groups (diabetics with DR, diabetics without DR and health individuals) were observed to determine the relationship between DR and +405 G/C VEGF gene polymorphism. ***Results:*** This study revealed that patients with GC genotypes are about 57.6 % at risk (95% CI 1.022-2.431) to develop DR, while the risk is about 40% (95% CI 0.404-0.893) in DM patients without DR than those without GC genotype. ***Conclusion:*** These results indicated that +405 VEGF G/C polymorphism could be used in the evaluation, development, and progression of DR.

Keywords: Diabetic retinopathy (DR), tissue ischemia, (VPF), Diabetic Retinopathy.

INTRODUCTION

Diabetes mellitus (DM), commonly known as diabetes, is a group of metabolic disorders characterized by a high blood sugar level over a prolonged period of time [1]. If left untreated, diabetes can cause many complications including diabetic ketoacidosis, hyperosmolar hyperglycemic state and ultimately may lead to death [2, 3]. Serious long-term complications include cardiovascular disease, stroke, chronic kidney disease, foot ulcers, damage to the nerves, damage to the eyes and cognitive impairment [2, 5]. These complications impose burdens on the health care systems worldwide. There are three main types of diabetes mellitus; type 1, type 2 and Gestational diabetes [2]. This categorization is according to the pathogenic processes involved which are ranged from autoimmune destruction of beta cells of the pancreas that leads to the insulin deficiency developing Type 1 diabetes Mellitus, aka insulin-dependent diabetes mellitus (IDDM), to a combination of resistance to insulin action and inadequate insulin secretion which led to the development of Type 2 diabetes mellitus [2-4]. Type 2 diabetes is also known non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes and it has become the common type among Sudanese patients and of major concern to diabetologists. The third type, gestational diabetes mellitus (GDM), occurs when pregnant women without a previous history of diabetes develop high blood sugar levels [2]. One of the most common long-term complications of diabetes is diabetic retinopathy (DR) which may cause vision impairment [6]. It often refers to retinal vascular disease, or damage to the retina caused by abnormal blood flow. Frequently, it's an ocular manifestation of systemic disease as seen in diabetes or hypertension [7]. The development of

retinopathy can be broken down into proliferative and non-proliferative types. Both types cause disease by altering the normal blood flow to the retina through different mechanisms [8].

Vascular Endothelial Growth Factor (VEGF), originally known as vascular permeability factor (VPF) [9], is a signal protein produced by cells that stimulates the formation of blood vessels. They are important signaling proteins involved in both vasculogenesis and angiogenesis. It is part of the system that restores the oxygen supply to tissues when blood circulation is inadequate such as in hypoxic conditions [10]. Serum concentration of VEGF is high in diabetes mellitus [11]. The VEGF's normal function is to create new blood vessels during embryonic development, new blood vessels after injury, muscle following exercise, and new vessels (collateral circulation) to by pass blocked vessels. It can stimulate angiogenesis, enhance collateral vessel formation, and increase the permeability of the microvasculature [12, 13]. Diabetic micro vascular changes in the retina lead to hypoxia, which stimulates production of VEGF [14]. This protein is believed to play a significant role in the development of DR by inducing hyper permeability of retinal vessels, breakdown of the blood–retinal barrier and neovascularization [15, 16]. These complications can arise as a result of abnormal barrier function of new vessels, leading to intraregional hemorrhage and exudation. Additionally, New blood vessels with increased fragility leading to sudden severe loss of vision due to vitreous hemorrhage.

DM is a large problem worldwide. A total of 424.9 million adults have been estimated to have had DM, and this is estimated to rise to 628.6 million patients [15]. The WHO eastern Mediterranean region has the highest prevalence of DM in the world. Seven countries in this region have a high prevalence of DM and a further seven countries (including Sudan) have a medium prevalence (9–12%) of DM [16]. Type 2 diabetes mellitus (T2DM) is the major type of DM, accounting for approximately 90% of all cases. The estimated prevalence of DM in Africa in 2017 was 3.3%, and Sudan was among the countries that had a prevalence of DM of more than 12% [15].

SUBJECTS AND METHODS

The Research Ethical Committee of the Ministry of Health and of Al-Neelain University have approved the present study and a verbal informed consent has been taken from all patients, or their legal representatives in case of disability. This case-control study was carried out in Khartoum-Sudan from August 2018 to August 2020 and were included a total of 189 diabetes patients which were categorized into three groups. The first group are patients diagnosed with DR and had submit complete ophthalmological examination enrolled from the Ophthalmological Clinic, and attended from MAKAA HOSPITAL. The second group were patients diagnosed with DM without DR which attended from ZENAM hospital and the third group were healthy individuals.

The practical side of the study was performed at the laboratory of biochemistry department-Faulty of medical laboratory- Al-Neelain University, and at the National University Biomedical Research Institute. Full medical examination was done which include, full personal, family, and medical history including a standardized questionnaire for any chronic diseases, gender, age, age of onset of diabetes, duration of diabetes and Medical examination which include (ophthalmological examination, fasting glucose level, HBA1c) and genetic study.

Methods Laboratory investigations

Sample Collection

Six milliliters of blood were collected in 2 tubes, Sterile fluoride vacationer tube for fasting blood sugar (2 ml). Sterile ethylene diamine-tetra-acetate "EDTA" vacationer (4 ml) tubes used for HbA1c estimation by using ichroma^{TM} and analysis of VEGF +405G/C genotype by using sequences of primer used for PCR amplification of +405G/C VEGF gene [17, 18].

Primer Sequence

Forward5'-ATTTATTTTTGCTTGCCATT-3'
Reverse5'-GTCTGTCTGTCTGTCCGTCA-3'

PCR test

Genomic DNA was extracted using quinidine chloride method and the targeted region was amplified in a final volume of 26µl (3µl Genomic DNA + 1.5µlF-primer + 5µl master mix + 0.5 enhancer + 16 DDW). The polymerase chain reaction was set for denaturation at 94°C for 4min followed by 35cycles of denaturation at 94°C for 45seconds, annealing at 58°C for 1min and extension at 72°C for 1min and define extension was at 72°C for 5min and then hold at 4°C for in definite time. Then the amplification products were separated by electrophoresis through 2% agarose gel stained with ethidium bromide ,then for the VEGF405+ polymorphism the PCR product was digested with the Bsm FI restriction nuclease (Bio labs) [18]. Digestion conditions that give best result 9.3 µl of water were added to 2µl of NEB buffer, 0.2BSA, 8 µl of PCR product and 0.5µl Bsm FI enzyme to a final volume of 20µl, those were mixed and incubated at 65°C for 3hours .The uncut fragment was 300 base pairs (bp) (Callele) and G allele) digestion products were 200bp and 100bp approximately

STATISTICAL ANALYSIS

Data was examined using the statistical package of social science (IBMSPSS version 20.0) for windows software package. A *P* value of ≤ 0.05 was interpreted as statistically significant. Categorical variables, alleles and genotypes frequency were

analyzed using a Pearson's Chi square test or Fisher's Exact Test. The strength of significant was done by calculating the contribution to chi square of each cell using adjusted residuals P values (adjusted P value = 0.05/number of new adjusted residuals or cells). Comparison of groups was done by Kruskal Wallis test and the post hoc was done using Independent samples Kruskal Wallis.

RESULTS

This study included 189 subjects divided into three groups of 63 subjects each. Regarding the gender in the present study there was 55.6%, 41.3% and 54% males, and 44.4%, 58.7% and 46% females in healthy, DM without DR and DM with DR groups, respectively. The healthy group had age mean of 62.24±1.74, the DM group had age mean of 62.08±1.54 and the DR group had age mean of 67.92±1.13.The HBA1c levels had the highest mean (9.94±0.22%) in the DR group followed by DM without DR (8.58±0.19%) compared with healthy control group (4.89±0.11%). Similarly, the glucose levels in the DR group had the highest mean (190.10±8.04) followed by the DM group (144.92±2.33) and the NC group (4.89±0.11.

Table-1: Descriptive analysis of study population

Variables	Descriptive showed as	All groups	NC	DM	DR
Gender	Males %	95	55.6%	41.3%	54%
	Females %	94	44.4%	58.7%	46%
AGE	Mean ± SE.	64.08±.88	62.24±1.74	62.08±1.54	67.92±1.13
HBA1c level	Mean ± SE.	7.80±.19	4.89±0.11	8.58±.19	9.94±.22
Glucose level	Mean ± SE.	142.04±4.07	91.10±1.10	144.92±2.33	190.10±8.04
Total number	-	189	63	63	63

Genotypes and Alleles Frequency

Comparison of Genotypes frequency between the three Groups

The present results indicated that all groups were significantly different form each other (Chi square P.value = 0.00001) (Table-5).

Likewise, the GC genotype percentage (74.6%) in the DR group was the highest compared to the normal and DM without DR groups (34.3% and 19.6% respectively). Also, the CC genotype percentage (60.9%) in DM group was the highest compared to the normal and DR groups (13% and 26.1% respectively), and the GC genotype was significantly (adjusted p value = 0.00279) less than expected. In addition, the DR group showed the less prevalence of the GG genotype compared to the other groups (these results were considered as the most significant results after measuring the contribution of cells to chi square with an adjusted p values) (Table-2).

Table-2: The frequency of Genotypes between the Normal, DM and DR groups

Groups		Genotypes			Total
		GC	GG	CC	
Normal	Count	35	25	3	63
	Expected Count	34.0	21.3	7.7	63.0
	Within group %	55.6%	39.7%	4.8%	100%
	Within genotype %	34.3%	39.1%	13.0%	33.3%
DM	Count	20**a	29	14**d	63
	Expected Count	34.0	21.3	7.7	63.0
	Within group %	31.7%	46.0%	22.2%	100%
	Within genotype %	19.6%	45.3%	60.9%	33.3%
DR	Count	47**b	10**c	6	63
	Expected Count	34.0	21.3	7.7	63.0
	Within group %	74.6%	15.9%	9.5%	100%
	Within genotype %	46.1%	15.6%	26.1%	33.3%
Total	Count	102	64	23	189
	Expected Count	102.0	64.0	23.0	189.0
	% within Groups	54.0%	33.9%	12.2%	100.0%
	% within genotype	100.0%	100.0%	100.0%	100.0%

** Mostly contributed to chi square (adjusted P value 0.005) (p values a= 0.00001, b= 0.00006, c= 0.00022, d= 0.00279)
0DM (diabetes mellitus) DR (diabetic retinopathy)

Alleles Frequency in all three groups

The current study observed that there is a significant difference (Chi square, P value = 0.027) in allele frequency among all groups. The G allele was dominant (64.5%) over the C allele (35.5%).

The C allele has 24.2% in NC group, 38.4% in DM group and 37.4% in DR group. While the G allele had 73.9%in NC group, 64.2% in DM group and 54.3% in DR group (Table-3).

Table-3: Alleles Frequency in studied groups

Allele		Groups			Total
		NC	DM	DR	
C	Within allele %	24.2%	38.4%	37.4%	100.0%
	Within group %	26.1%	35.8%	45.7%	35.5%
G	Within allele %	37.8%	37.8%	24.4%	100.0%
	Within group %	73.9%	64.2%	54.3%	64.5%

Comparison of HBA1c levels between all groups

The results have shown that there is a significant difference within all three groups (Independent samples Kruskal Wallis test, P Value = 0.001). Also, a significant difference was observed between groups (DM-DR, P value 0.009) (DM-No, P value 0.001) (No-DR, P value 0.001). The DR group showed the highest HBA1c levels followed by the DM group and the Normal group respectively (Fig-1).

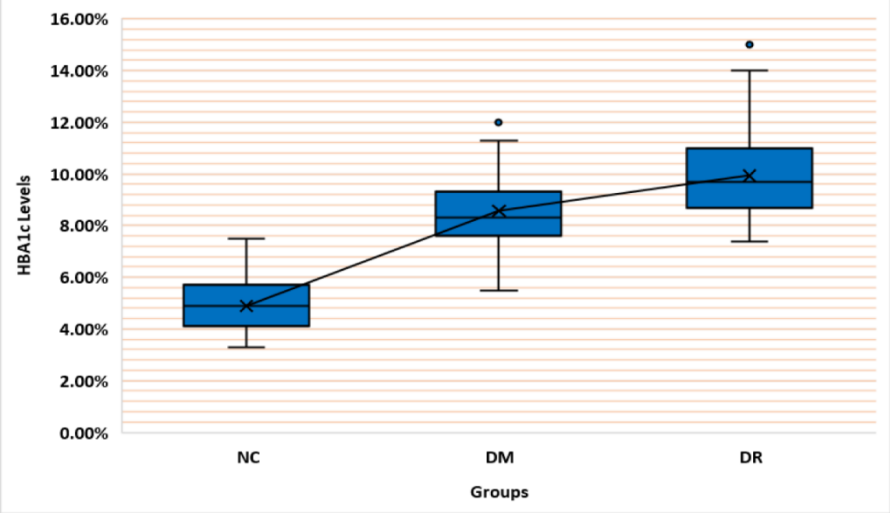

Fig-1: The frequency of HBA1c levels in all three groups (Normal, DM and DR)

Comparison of glucose levels between all groups

The study has demonstrated that there is a significant difference within all three groups (Independent samples Kruskal Wallis test, P Value = 0.001). Also, a significant difference was observed between groups (DM-DR P value 0.022) (DM-No P value 0.001) (No-DR P value

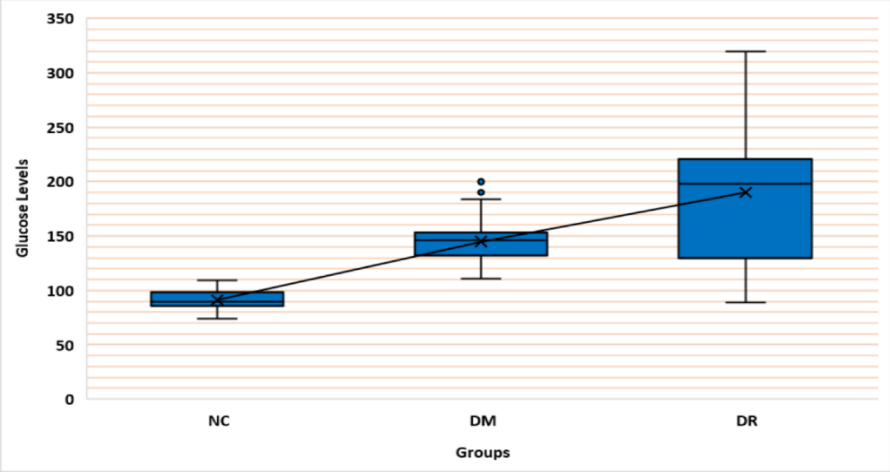

Fig-2: The frequency of Glucose levels in all groups (Normal, DM and DR)

The amplification PCR product was approximately 300bp as Figure-**3** revealed [17, 19] containing the targeted site.

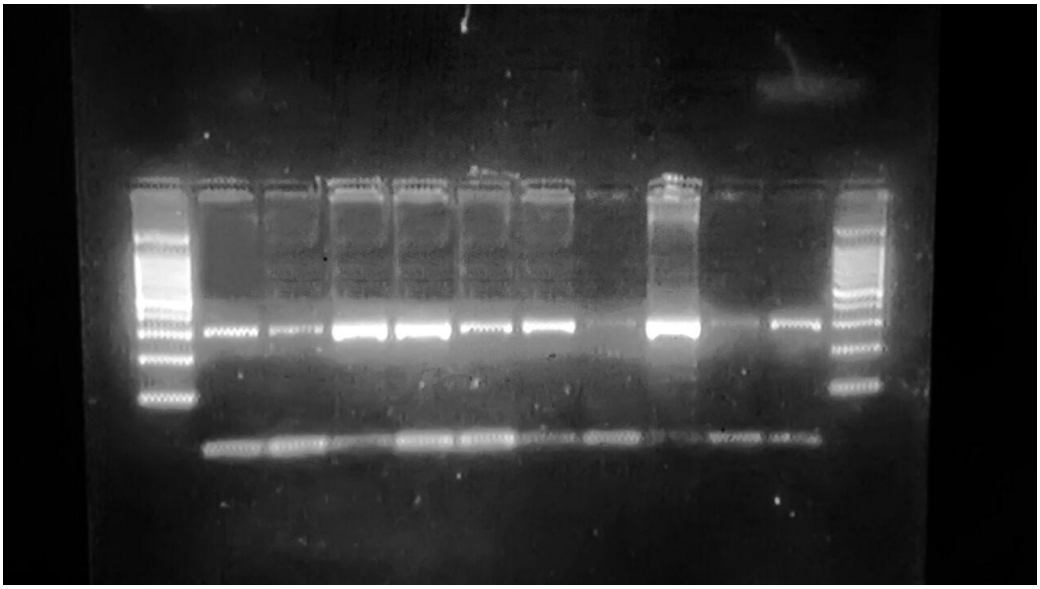

Fig-3: PCR product, 2% agarose and for 40 minute (7µl of DNA loaded in each well)

Figure-4 shows the restriction digestion pattern that obtained in this study after digestion of PCR product with restriction enzyme. The revealed genotypes of the subjects were divided into 3 groups based on the presence or absence of polymorphism:

1. Tow bands (200 bp and 100 bp) is wild-type homozygote (GG); absence of polymorphism.
2. One band; the uncut fragment (300bp) is variant homozygote (CC); presence of polymorphism.
3. Three bands (300bp, 200bp, and 100 bp) is variant and wild type heterozygote (GC); presence of polymorphism

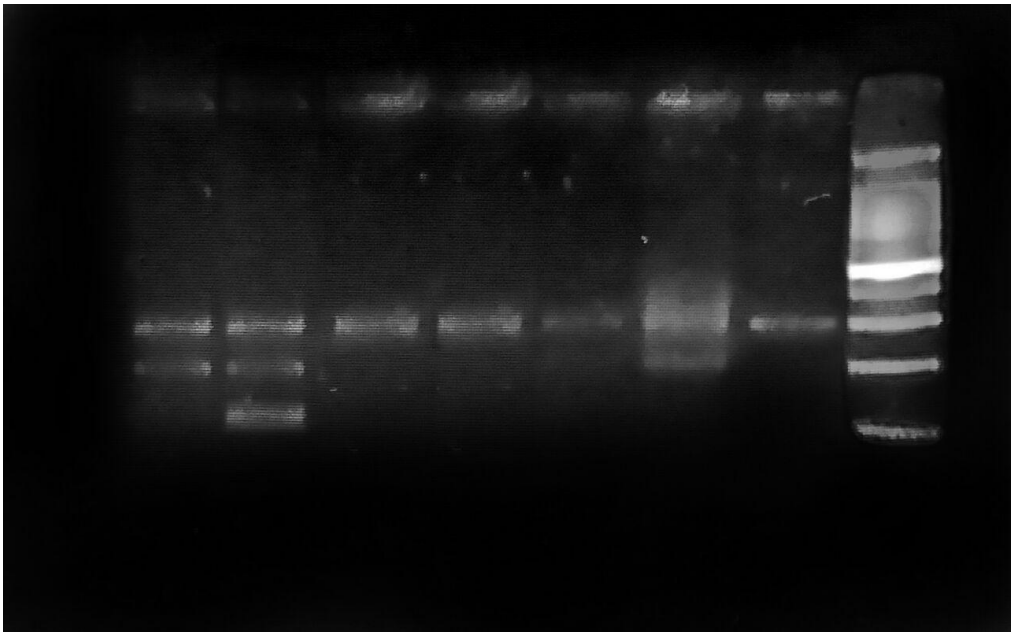

Fig-4: Restriction digestion of PCR products demonstrating the patterns of digestion in different genotypes of VEGF +405 G>C polymorphism on 2% agarose and for 50 minutes (8µl of DNA loaded in each well)

DISCUSSION

VEGF is believed to play a significant role in the development of DR by inducing hyper permeability of retinal vessels, breakdown of the blood–retinal barrier and neovascularization [20, 21]. Complications can arise as a result of abnormal barrier function of new vessels, leading to intra retinal hemorrhage and exudation. New blood vessels have increased fragility leading to sudden severe loss of vision due to vitreous hemorrhage. The VEGF polymorphisms might be a

useful predictive marker for the development and progression of diabetic retinopathy at an earlier stage [22].

This study revealed that patients with GC genotypes will be 57.6 % higher at risk (95% CI 1.022-2.431) to develop DR and 40% lower at risk (95% CI 0.404-0.893) to develop DM than those without GC genotype. While, patients with CC genotypes will be 83.2 % higher at risk (95% CI 1.354-2.479) to develop DM and 36.8% higher at risk (95% CI .0.832-2.252) to develop DR than those without CC genotype. In addition, patients with GG genotypes will be 13.7 % higher at risk (95% CI 0.803-1.610) to develop DM and 50.9 % Lower at risk (95% CI .282 – .852) to develop DR than those without GG genotype.

This is in agreement with the results of Ray *et al*., [23]; it was observed that the presence of the G allele is a risk marker for developing retinopathy as well as PAD, suggesting a potential association between this allele and an increase in activity in the promoter region of the gene. These data differ from those and our study which described by in Szaflik *et al*., [24] which the C allele of +405 genes was associated with an increased risk of DR.

On the other hand, Watson *et al*., [25] Found is in a coincedance with our study in which the G allele at position +405 affects the transcriptional activity and increases VEGF production in peripheral blood mononuclear cells in response to lipopolysaccharide. They also showed a dose-dependent effect of the G allele.

This study observed that the GC genotype and the G allele of +405 G/C polymorphism of VEGF gene are increased related to the severity of DR (PDR and DME) in Sudanese type 2 diabetic patients suffering from DR than those without the disease. This is in accordance with other results where the SNP +405 G/C (rs: 2010963) has previously been associated with DR in other populations [26-28]. However, to our knowledge, there are no studies regarding the behavior of this SNP in the Sudanese diabetic population suffering from DR.

Some SNPs of VEGF gene such as +405 G/C polymorphism impact VEGF protein expression and has functional significance on VEGF protein production (Watson et al. [25]). These data have no suspicion call for a possibility that +405 C/C VEGF in diabetics could be engaged in DR development and severity through increasing VEGF expression and hence production.

Emerging data show that anti-VEGF therapy, which is less destructive than laser, can reverse the diabetic retinopathy. This supports the suggestion of the role of this SNP in the pathogenesis of DR [29, 30]. Moreover, the American Academy of Ophthalmology (AAO) preferred practice pattern committee now stated that there is sufficient evidence for the treatment of DR with anti-VEGF treatment (American Academy of ophthalmology [31].

CONCLUSION

We showed for the first time in Sudan diabetic population that the VEGF +405G/C poly morphism is associated with DR as a genetic marker for predicting DR in type 2 Sudanese diabetics.

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