

Blood Glucose, HbA1c Level, and its Correlation with VEGF-A (+405G/C) Polymorphism as Biomarker Predicts the Risk of Retinopathy and Nephropathy in Type 2 Diabetic Patients

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Abstract

Background: Diabetes-related vascular complications linked to increase in the expression of VEGF and its receptors. It helps to accelerate tissue damage inflicted by hyperglycemia, which is potential risk for diabetic complications. The study aimed to assess VEGF genetic polymorphism and its correlation with glucose and HbA1C level among Sudanese patients with diabetic retinopathy and nephropathy.

Methods: A case-control study was conducted among a total of 252 subjects and divided into four groups of 63 subjects each. Glucose and HbA1c were measured then the VEGF gene was amplified using polymerase chain reaction. The data were analyzed using SPSS.

Results: The HbA1c, and blood glucose levels had significantly (P value ≤ 0.00001) highest mean in the DR group, DN group followed by DM. There is a non-significant correlation between VEGF Genotypes and HbA1c, and blood glucose levels (P value $\leq 0.102, 0.173$) Patients with GC genotypes will be 74.6%, and 54% higher at risk to develop DR, and DN respectively and 40 % lower at risk to develop DM than those without GC genotype. While patients with CC genotypes will be 22.2% higher at risk of developing DM and 9.5%, 12.2% higher at risk of developing DR and DN respectively.

Conclusions: The VEGF +405G/C gene polymorphism is linked to diabetic retinopathy, and diabetic nephropathy in type 2 Sudanese diabetics, and the presence of the GC genotypes and G allele is a significant predictor for retinopathy. There is no significant relation between HbA1C serum levels, blood glucose, and the VEGF +405G/C gene polymorphism.

Keywords: Diabetic Nephropathy, Diabetic Retinopathy, Blood Glucose, HbA1c, Gene polymorphism, VEGF-A.

Introduction

Diabetes Mellitus (DM) is a metabolic disorder and a chronic hyperglycemia caused by inadequate insulin secretion, insufficient

insulin activity, or maybe both (1). Diabetes' chronic hyperglycemia is related to numerous of long-term microvascular complications targeting the eyes, kidneys, and nerves, and perhaps an enhanced risk of cardiovascular

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disease (CVD) (2). Diabetes is still a global challenge, with an estimated 424.9 million adults suffering from the disease, with that number expected to rise to 628.6 million patients (3). The WHO Eastern Mediterranean area has the world's highest burden of disease. Seven countries in this region have a high prevalence of diabetes, while another seven (including Sudan) have a moderate prevalence (9-12%) (4). Reportedly, the prevalence estimates rates of diabetes in Sudanese are approaching epidemic levels, leading to the emergence of a public health problem with significant socioeconomic consequences. The number of people with diabetes is 4 million, 20% of whom are children, and 8 million are expected to be infected. The northern states have the highest percentage (20%), followed by Khartoum (19%). Diabetes Mellitus (DM) in Sudan is associated with poor glycemic control, a high prevalence of complications, a low quality of life, and particularly with morbidity (5). Type 2 diabetes mellitus (T2DM) is the most common type of diabetes, accounting for approximate 90% of all cases. In 2017, the approximate incidence of diabetes in Africa was 3.3%, with Sudan among the countries with a prevalence of more than 12% (6). Patients with complications were older, had a relatively long duration, and had elevated serum cholesterol and triglyceride levels, as anticipated. The glycemic control was only acceptable (HbA1c <7.5%) in 12.5% of the patients.

The glycoprotein vascular endothelial growth factor (VEGF) is released into the bloodstream by the vascular endothelium, smooth muscle cells, and certain other cell types. Therefore, it is important to study *VEGF* promoter gene polymorphisms and its association with the disease which can help to identify subjects with diabetic Mellitus and to evaluate the diagnostic value of VEGF as a marker for diabetes mellitus complication, especially retinopathy and nephropathy. Vascular Endothelial Growth Factor (VEGF), formerly recognized as vascular permeability factor (VPF), (7) is a transmitter protein released by cells that activates blood vessel

formation. They are essential biochemical proteins that contribute in both vasculogenesis and angiogenesis. It is a component of the system that restores oxygen supply to tissues when blood circulation is insufficient, as in hypoxic conditions (8). Diabetes mellitus has an elevated level of plasma VEGF concentration (9). The normal function of the VEGF is to form new blood vessels throughout embryonic development, new blood vessels after injury, muscle after exercise, and new vessels, collateral circulation, to bypass blocked vessels. It can stimulate angiogenesis, improve collateral vessel formation, and increase microvasculature permeability (10,11). Diabetes-related microvascular changes in the retina induce hypoxia, that enhances VEGF production (12). Such a protein is intended to involve an important part in the development of diabetic retinopathy (DR) and diabetic nephropathy (DN) since it is implicated in the progression of diabetic nephropathy because anti-VEGF antibodies substantially lower hyperfiltration, albuminuria, and glomerular hypertrophy in experimental models. (13,14). So current study aimed to assess VEGF genetic polymorphism and its correlation with glucose and HbA1C level among Sudanese patients with diabetic retinopathy and nephropathy.

Materials and Methods

Study design and population

This is a descriptive case-control study design and a hospital-based study conducted among Sudanese who are clinically diagnosed with T2DM with diabetic retinopathy, diabetic nephropathy complication, diabetic patients without any complication, and health individual regarded as control. Samples were collected from Selma center of kidney disease for diabetic nephropathy, MAKAA HOSPITAL, and ZENAM hospital for diabetic retinopathy during the period from June 2018 to June 2020. Any subjects with Cancer, asthmatic, myocardial infarction (MI), chronic renal failure, severe illness, hemolytic anemia, mutable endocrine, and any other retinal disease not caused by

diabetes mellitus were excluded from the study. A pre-designed structural questionnaire was used for the collection of the demographic and clinical data concerning each participant was obtained from the registry database office.

Sampling technique and sample size

The sample was collected using the multistage sampling technique, where a total of 189 diabetic patients was enrolled. The sample size was calculated and obtained from statistical formula equation ($H = Z^2 P(1-P)/D^2$) which

Z = statistic for a level of confidence (1.96) P = expected prevalence (20%)

D = precision (0.05) $3.96 \times 0.16 / 0.0025 = 252$

Then patients were divided into 63 normal individuals, 63 diabetic retinopathy, 63 diabetic nephropathies, and 63 diabetic patients without any complication.

Blood sampling

A total of eight ml of venous blood was withdrawn from each volunteer in a disposable plastic syringe then poured in containers which are plain (2 ml) for VEGF level; fluoride oxalate container (2 ml) for glucose and EDTA container (4 ml) for HBA1c and molecular analysis. Blood samples which were collected in plain and fluoride oxalate were centrifuged at 1008 G to obtain serum and plasma respectively.

Biochemical measurement

Plasma level of glucose was using commercial reagent kits from Bio System Company (Spain) and HBA1c level too and measured by spectrophotometry.

DNA extraction from blood samples and PCR of VEGF-A gene

Genomic DNA was extracted using the guanidine chloride (prepared manually) method and stored at - 20 °C until use. The VEGF gene was amplified by PCR using these following primers:

DR Forward primer

5'-ATTTATTTTGGCTTGCCATT-3' and Reverse primer

5'-GTCTGTCTGTCTGTCCGTCA-3'

DN Forward primer: 5'-

GCTGAGAGTGGGGCTGACTA GGTA-3'

and Reverse primer: 5'-

GTTTCTGACCTGGCTATTTCCAGG-3'

The PCR was done in a final volume of 26 µl (3 µl Genomic DNA + 1.5 µl F-primer + 5 µl master mix + 0.5 enhancer + 16 DDW). Using the following conditions:

Denaturation at 94 °C for 4 min, followed by 35 cycles of (denaturation at 94 °C for 45 seconds, annealing at 58 °C for 1 min and extension at 72 °C for 1 min.), the final extension was at 72 °C for 5 minutes and then held at 4 °C indefinitely. The amplification products were then separated by electrophoresis through a 2% agarose gel stained with ethidium bromide. For the VEGF 405 + polymorphism, the PCR product was digested with the BsmFI restriction nuclease (Bio labs) (18). Best digestion conditions 9.3 ul of water were added to 2 ul of NEB buffer, 0.2 BSA, 8 ul of PCR product, and 0.5 ul BsmFI enzyme to make a final volume of 20 ul, which was mixed and incubated at 65 °C for 3 hours, (the uncut fragment was 300 base pairs (bp) (C allele) and G allele) digestion products were 200 bp and 100 bp approximately.

Data analysis

Data were analyzed using Microsoft office excel 2007 and statistical package for social sciences (SPSS) version 21 software programs to estimate the frequencies of clinical data. Cross tabulation and Chi-square tests were used for biochemical and immunological Results with the p-value of <0.05 considered as statistically significant.

Ethical consideration

Permission of this study was obtained from the ethical committee of the institutional review board, ALNEELAIN University. Verbal informed consent was obtained from participants.

Results

The present study included 189 individuals, 69 patients with diabetic retinopathy (DR) as a test group, and 669 diabetic patients without

any complication (DM) all these were compared with 69 normal health individuals (NC) as a control group. All data were summarized in Table 1.

Table 1. Baseline data of the study participants.

Variables		All groups n=189 (%)	NC n=63 (%)	DM n=63 (%)	DR n=63 (%)
Gender	Males %	95 (50.3%)	35 (55.6%)	26 (41.3%)	34 (54%)
	Females %	94 (49.7%)	28 (44.4%)	37 (58.7%)	29 (46%)
AGE (Years)	Mean \pm SD	64.08 \pm .88	62.24 \pm 1.74	62.08 \pm 1.54	67.92 \pm 1.13
HBA1c level (%)	Mean \pm SD	7.80 \pm .19	4.89 \pm 0.11	8.58 \pm .19	9.94 \pm .22
Glucose level mg/dl	Mean \pm SD	142.04 \pm 4.07	91.10 \pm 1.10	144.92 \pm 2.33	190.10 \pm 8.04
Total number		189 (100%)	63 (100%)	63 (100%)	63 (100%)
P-value		0.00001			

Frequency of HBA1c and glucose levels between all groups

The results have shown that there is a significant difference within all groups (Independent samples Kruskal-Wallis test, P-Value = 0.001). Also, a significant difference

was observed between groups (DM-DR, P-value \leq 0.009) (DM-No, P-value \leq 0.001) (No-DR, P-value \leq 0.001). The DR group showed the highest HBA1c levels followed by the DM group and the Normal group respectively, findings illustrated in Table 2.

Table 2. The frequency of HBA1c and glucose levels in all groups (Normal, DM, DN, and DR).

Parameters	Mean \pm SD	P. value
HBA1c Level	Mean \pm SD	0.0001
Normal	4.86% \pm 0.9%	No-DR 0.001
DM	8.58% \pm 1.5%	DM-No 0.001
DR	9.94% \pm 1.74%	DM-DR 0.009
Glucose Level	Mean \pm SD	0.001
Normal	91.1 \pm 8.7	No-DR 0.001
DM	144.9 \pm 18.5	DM-No 0.001
DR	190.1 \pm 63.8	DM-DR 0.022

Molecular results

The present results indicated that all groups were significantly different from each other (Chi-square P-value = 0.00001), (Fig. 1). Likewise, the GC genotype percentage (74.6%) in the DR group was the highest

compared to the normal and DM groups (34.3% and 19.6%, respectively). Also, the CC genotype percentage (60.9%) in the 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 \leq 0.00279) less than expected. there is a significant difference (Chi-square, P-value \leq 0.022) in allele frequency among all groups The G allele was significantly dominant (64.9%) over the C allele (35.1%).

VEGF Genotype and HbA1c levels

There was no significant difference between the Genotypes and HbA1c levels in DR, and DM group (P. value =0.970, and 0.107), respectively (Table 3).

Table 3. Comparison of Genotypes and HbA1c levels in DR group.

Genotypes	HbA1c % (Mean \pm SD)		
	DR	DN	NC
GC	9.94% \pm 0.26%	8.41% \pm 0.26%	5.03% \pm 0.16%
GG	9.96% \pm 0.48%	8.37% \pm 0.30%	4.70% \pm 0.17%
CC	9.90% \pm 0.89%	9.27% \pm 0.42%	4.80% \pm 0.46%
P value	0.970	0.107	0.365

Comparison of Genotypes and HbA1c levels in diabetic groups (DM+DR)

There was no significant difference (Kruskal

Wallis, P-value =0.102) between the Genotypes and HbA1c levels in diabetic groups (DM+DR) (Table 4 and Fig. 2).

Table 4. Comparison of Genotypes and HbA1c levels in diabetic groups (DM+DR).

Genotypes	HbA1c % (mean \pm SD)	P. value	Glucose level mg/dl (mean \pm SD)	P-value
GC	9.48% \pm 0.21%	0.102	147.16 \pm 6.13	0.173
GG	9.78% \pm 0.28%		130.09 \pm 5.67	
CC	9.46% \pm 0.39%		152.57 \pm 10.54	



Fig. 1. The gel electrophoresis of VEGF +405G/C Polymorphism. The size of amplification PCR product was approximately 300 bp. Lane 1 DNA ladder (100 bp) and lanes 2-11 show the 300 bp amplicon.

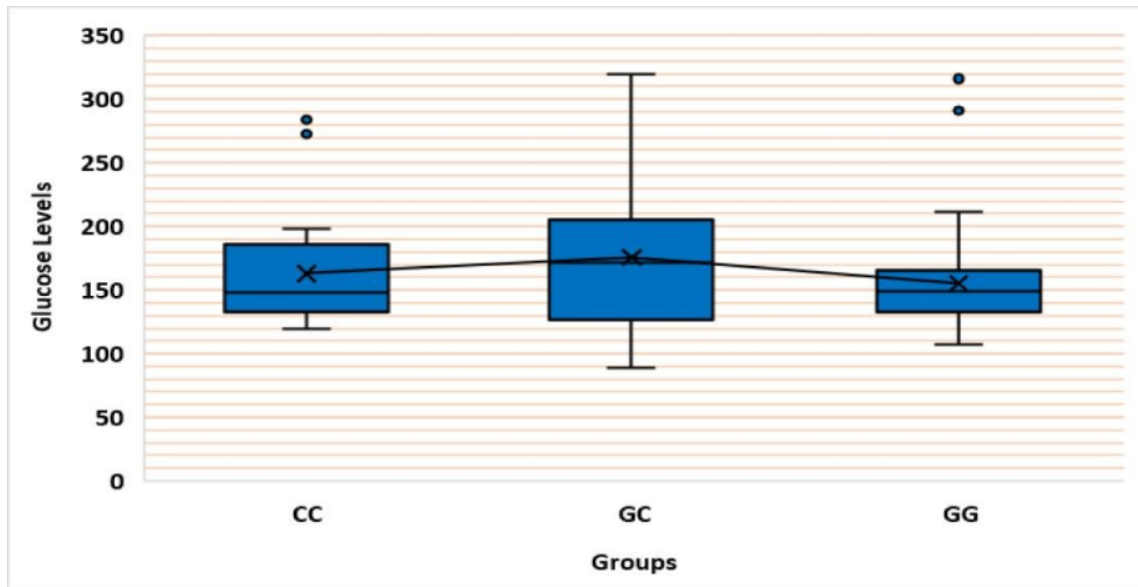


Fig. 2. Comparison of Genotypes and Glucose levels in diabetic groups (DM+DR).

Discussion

The present study aimed to assess such correlation of Genetic VEGF-A gene polymorphisms (+405 G/C (rs2010963) among Sudanese diabetic patients with retinopathy and nephropathy. The study among diabetic retinopathy patients revealed that patients with GC genotypes were 57.6 % higher at risk to develop DR than those without GC genotype. While patients with CC genotypes were 83.2 % higher at risk to develop DM and about 36.8 % higher at risk to develop DR than those without CC genotype, this is in agreement with the results of Ray et al (15); who was encountered that the existence of the G allele is a predisposing factor retinopathy, implying a possible link between this allele and an increase in activity in the gene's promoter region. The DR group showed less prevalence of the GG genotype compared to the other groups which is inconsistent with findings of Szaflik et al. (16) that was previously reported that the C allele of +405 genes were linked to a potential risk of DR.

The current study observed that there is a significant difference in allele frequency among all groups. The DR group showed the highest HbA1c levels followed by the DM group and the Normal group respectively and there is a significant difference in allele

frequency among all groups. Interestingly Watson et al (17) finding is consistent with our study, which observed that the G allele at position +405 influences transcriptional enhances VEGF production in peripheral blood mononuclear cells in responding to lipopolysaccharide. They also noted that the G allele has a dose-dependent impact.

The GC genotype and G allele of the +405 G/C polymorphism of the VEGF gene have been identified to be more linked to the occurrence of DR (PDR and DME) in Sudanese type 2 diabetic patients with DR than those without the disease in this study. This is consistent with previous findings in which the SNP +405 G/C (rs: 2010963) has been linked to DR in other communities. (Buracznska et al. (18). Badre et al. (19); Fan et al. (20). To the best of our knowledge, neither study has been undertaken on the behavior of this SNP in the Sudanese diabetic population suffering from DR.

The study has demonstrated that there is, a significant difference was observed between groups. Several VEGF gene SNPs, such as the +405 G/C polymorphism, influence VEGF overexpression and have functional implications for VEGF synthesis of protein (19). Such findings suggest the risk that +405 C/C VEGF in diabetics may play a role in DR

progress and consequence by continuing to increase VEGF expression and thus generation. New evidence suggests that anti-VEGF therapy, which is less invasive than laser, can reverse diabetic retinopathy. This lends credence to the idea that this SNP plays a role in the pathophysiology of DR. (Gupta et al. (21); Wyckoff et al. (22). Furthermore, the American Academy of Ophthalmology's (AAO) favored training pattern committee has already noted that there is conclusive evidence for curing DR with anti-VEGF treatment (American Academy of ophthalmology) (23).

Chronic hyperglycemia has been shown to increase VEGF-A production and secretion. It sets off a chain reaction that leads to VEGF-A formation and, ultimately, DM microvascular complications (24). Cellular hypoxia and hyperglycemia are the primary physiological stimuli for VEGF production. Hyperglycemia, by enhancing oxidative stress, can play a role as a toxin to the endothelium. A high blood glucose concentration stimulates the production of vasoconstrictor substances, particularly endothelin-1 (25). Throughout this analysis, we revealed previous findings of higher plasma VEGF levels in diabetic patients, as well as higher plasma VEGF levels in patients with retinopathy and nephropathy. In DR may progress through several stages, including early non-proliferative diabetic retinopathy (NPDR), moderate NPDR, severe NPDR, and finally advanced proliferative, DR (PDR) (26-28). PDR is distinguished by increased vascular permeability, tissue ischemia, and neovascularization, which result in fibrovascular changes, vitreoretinal traction, and retinal detachment, ultimately leading to blindness (29-31). VEGF is generated by a variety of cell types in the retina. The retinal pigment epithelium (RPE), pericytes, endothelial, glial, Muller, and ganglion cells are all included (32,33). Müller cells and RPE are thought to be the primary sources of VEGF in the retina, while endothelial cells are thought to be the main focus of VEGF

(34,35). Numerous pathophysiological mechanisms have been suggested in DN to demonstrate the dysfunction of the glomerular filtration barrier, which eventually result in diabetic microalbuminuria and, subsequently, proteinuria. The synergistic effects of hyperglycemia and elevated VEGF-A in diabetic glomerulopathy may be clarified by the innovative hypothesis of "uncoupling of VEGF-A with nitric oxide (NO)" (36,37). Ordinarily, VEGF-A stimulates endothelial NO release, and NO is required for VEGF-A actions on endothelial cells. When hyperglycemia hinders regular endothelial function and decrease NO production, increased levels of glomerular VEGF- A found in diabetes may have a negative effect on endothelial cells, resulting to diabetic glomerulopathy. It's obvious that the VEGF +405G/C polymorphism is linked with DR, so it can be used as a genetic marker for predicting DR in type 2 Sudanese diabetics. GC genotype and G allele in the VEGF gene is associated with increased susceptibility to diabetic retinopathy in type 2 Sudanese diabetics, so genotyping analysis is important for DM patients. Estimation of VEGF level is recommended for DM patients.

The study concluded that the VEGF +405G/C gene polymorphism is associated with diabetic retinopathy among type 2 Sudanese diabetics, and the existence of the GC genotypes and G allele is a predisposing factor retinopathy. There is non-significant correlation between Serum level of HbA1C, as well as glucose level and VEGF +405G/C gene polymorphism.

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