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VASCULAR ENDOTHELIAL GROWTH FACTOR GENE POLYMORPHISM (-2578 T/A) (RS699947) IN DIABETIC FOOT ULCER AND ITS CORRELATION WITH OXIDATIVE STATUS

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ABSTRACT

Background: Diabetes mellitus is a metabolic disorder characterized by the presence of chronic hyperglycemia accompanied by greater or lesser impairment in the metabolism of carbohydrates, lipids and proteins. Diabetic foot ulcer (DFU) is complications of diabetes that affect the lower extremities are common, complex, and costly. The vascular endothelial growth factor is encoding VEGF that encompass 14 kb is located in chromosome region 6p21.3 and contains 8 exons and 7 introns. The current study deals with more frequent one (-2578 T/A) (rs699947). **Objective:** To investigate the molecular basis of *VEGF* (-2578 T/A) (rs699947) polymorphism and its correlation with biochemical parameters (HbA1c, GSH and MDA) in patients had DFU, in Kerbala province, Iraq. **Materials and Methods:** This study was a cross - sectional study. Sample size is 240 persons of both sexes randomly selected were conducted in the study divided into two groups, 120 had DFU and 120 had T2DM as control groups. Genotyping of *VEGF* was performed by amplification refractory mutation system Polymerase Chain Reaction (ARMS-PCR) of DNA extracted from peripheral blood mononuclear cells of 120 unrelated patients and 120 unrelated control donors. The most important SNP that affected on *VEGF* gene were involved in current study (-2578 T/A) (rs699947). **Results:** The significant results (p value ≤ 0.01) that showed in biochemical parameters (HbA1c, GSH and MDA) between DFU patients and T2DM as control group. The *VEGF* (-2578 T/A) (rs699947) polymorphism has a significant association with DFU. The AT genotype significantly raised the risk of DFU (p value ≤ 0.01). While the T allele significantly raised the risk of DFU (P value ≤ 0.01). The correlation of genotypes of *VEGF* (-2578 T/A) (rs699947) polymorphism with parameters (HbA1c, GSH and MDA) between DFU patients and T2DM as control groups, the correlation of AA genotype with parameters between DFU patients and T2DM as control group were significant (P value ≤ 0.01), but the correlation of AT genotype with parameters between DFU patients and T2DM as control group were significant (P value ≤ 0.01) while the correlation of TT genotype with parameters between DFU patients and T2DM as control group were significant (P value ≤ 0.01). **Conclusion:** The significant correlations were observed between (HbA1c, GSH and MDA) in DFU patients and T2DM as control group. The significant association between *VEGF* (-2578 T/A) in (rs699947) gene polymorphism and DFU patients as compared with T2DM as control group.

KEYWORDS: DFU, VEGF gene, (-2578 T/A) SNP, (HbA1c,GSH and MDA), Kerbala, Iraq.

INTRODUCTION

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The fact that increased cellular resistance to insulin may be the causative factor to develop DM is considerable. The disease is associated with different types of complication that is attributed to morbidity and mortality.The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels.[1,2]

These range from autoimmune destruction of the b-cells of the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action.[3] The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is deficient action of insulin on target tissues. Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the

complex pathways of hormone action.[4] Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often unclear which abnormality, if either alone, is the primary cause of the hyperglycemia.[5] Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss, sometimes with polyphagia, and blurred vision.[6]

Diabetic foot ulcer (DFU) Complications of diabetes that affect the lower extremities are common, complex, and costly. Foot ulceration is the most frequently recognized complication.[7,8]

DFU has developed; there is an increased risk of ulcer progression that may ultimately lead to amputation. Overall, the rate of lower limb amputation in patients with DM is 15 times higher than patients without diabetes. It is estimated that approximately 50%-70% of all lower limb amputations are due to DFU.[9] Ulcer healing with appropriate therapy - surgical debridement, off-loading of pressure, attention to infection, and if necessary, vascular reconstruction - foot ulcers heal in many patients, and the need for amputation is averted.[10,11]

Vascular endothelial growth factor (*VEGF*) which is also named as vasculotropine or vascular permeability factor (VPF) is produced by vascular endothelium, pericytes, retinal pigment epithelium, macrophages, T cells and some other cells.[12]

The *VEGF* receptors (*VEGFR-1 and 2*) are exclusively found on the endothelial cells. *VEGFR-1* (FL T) seems to mediate vascular hyper-permeability and *VEGFR-2* (FLK-1) appears to be involved in angiogenesis.[12] The gene encoding *VEGF* encompass 14 kb located in chromosome region 6p21.3 and contains 8 exons and 7 introns.[12,13]

There are many different SNPs for *VEGF* gene but the more common SNPs that affected on DFU was *VEGF* (-2578 T/A).

MATERIALS AND METHODS

This study was designed as a cross-sectional study. Total 240 subjects were recruited, including 120 patients with DFU as a case group, and 120 patients have T2DM as a control group. The patients were diagnosed by a specialist physician at Al-Hassan Medical Center for Endocrinology, in the Al-Hussein Medical City/Kerbala, Iraq. It should be noted that Kerbala is a holy city and one of the biggest cities in Iraq, where thousands of Iraqis and foreigners visit every week, also the diabetic center is an important center in Iraq and recruits patients from all cities of Iraq, therefore our study population could be representatives of the Iraqi population. The study protocol was approved by the Medical Ethics Committee of College of Medicine/Kerbala University and the Kerbala Health Directorate.

Written informed consent was obtained from the patients who participated in this study. The patients' privacy was protected and the entire process was performed with a prior written consent.

The glycated hemoglobin (HbA1c) level was assessed, using the COBAS HbA1c kit (Roche, Germany), by COBAS INTEGRA® 400 (Roche, Germany); the turbidity measurement was made with a spectrophotometer to determine the light absorbance. malondialdehyde (MDA) assayed by using thiobarbituric acid reactive substance method, glutathione (GSH) measured based on 5,5-Dithiobis (2-nitrobenzoic acid) DTNB. The amount of light blocked by particle suspension depends not only on concentration, but also on the particle size, because particles tend to aggregate and settle out of suspension, consequently sample handling becomes critical.

Blood samples were collected from DFU patients and the control group in EDTA tubes. Genomic DNA was extracted from the peripheral blood sample (1 ml), using the kit ReliaPrep™ Blood gDNA Miniprep System obtained from (Promega USA). The protocol was followed according to the manufacturers' recommendations. DNA concentration and purity were measured, using a BioDrop (UK). Amplification refractory mutation system Polymerase Chain Reaction (ARMS-PCR) technique (Table 1) was used to determine the *VEGF* (-2578 T/A) genotypes**.** The primer sequences are shown in Table 2. The PCR products were resolved on agarose gel (Promega, USA), and visualized by staining with ethidium bromide.

Table 1: The program of ARMS-PCR for SNPs of VEGF gene.

Type of Cycle	Temperature °C	Time	No. of Cycles
Initial denaturation	95	5 min.	1 cycle
Denaturation	95	30 sec.	35 cycles
Annealing	60	30 sec.	
Extension	72	1 min.	
Final extension	72	5 min.	1 cycle
Hold	4	10 sec.	
Total time: 2 hours and 2 minutes			

The exon 2 of *VEGF* genes (-2578 T/A) (rs699947) was amplified by means of specific primers to study the SNP.

Our result was used primer oligonucleotide designing by using Homo sapiens vascular endothelial growth factor gene, complete CDs, reference sequence (accession number AH001553), depending on https://www.ncbi.nlm.nih.gov/ websites.

Table 2: Primer sequence for *VEGF* (-2578 T/A) SNP, number of base pairs (bp) of primers.

SNPs of *VEGF* gene	Alleles	Sequences of Primers	Number of bp in primers	Product Size	%GC
-2578 (C/A) (rs699947)	A allele (wild)	Inner reverse: **5**'TCAGTCTGATTATCCACCCAGATCT **3**'	25 bp	295 bp	44 %
	T allele	Inner reverse: **5**'TCAGTCTGATTATCCACCCAGATCA **3**'	25bp		44 %
	Common primer	Outer forward: **5**'CTAGTGCACGAATGATGGAAAGG **3**'	23 bp		48%

The data were expressed as mean±SD. The student t-test and ANOVA were used for calculating the probability. The PAST version 3.09, 2004 was used for calculating the probability value (P value), Chi- square (χ^2), odds ratio (OR) and confidence interval 95% (CI 95%), to express the significance in polymorphisms, biochemical parameters and demographic characteristics between the study groups. In all statistical analysis the significant p value is $P < 0.05$.

RESULTS AND DISCUSSION

The results of the present study that shown in table (3) there was a significant difference between GSH in DFU patients group (2.986 ± 0.56) and T2DM as control group (5.24 ± 0.85) (P value ≤ 0.01). The significant result (P value ≤ 0.01) that shown in differentiation in MDA between DFU patients group (77.55 ± 8.97) and T2DM as control group (36.69 ± 2.25). The result of HbA1c indicate a significantly higher levels in DFU patients (10.59 ± 2.03) than that found in T2DM as control group (8.4 ± 1.97) (P value ≤ 0.01).

Table 3: Biochemical parameters in DFU patients as compared with T2DM as control groups.

Parameters	Mean ± SD		*P. value*
	Patient N=120	Control N=120	
GSH, µM/ g	2.986 ± 0.56	5.24 ± 0.85	≤ 0.01
MDA, nM/g	77.55 ± 8.97	36.69 ± 2.25	≤ 0.01
HbA1c	10.59 ± 2.03	8.4 ± 1.97	≤ 0.01

Table (3) there was a significant difference between GSH in DFU patients group and T2DM as control group, DFU is associated with oxidative stress (OS), which arises in cells and tissues from excessive generation of free radicals in the presence of a decreased antioxidant defense system.[15]

Depressed GSH level is thought to be responsible for some of the metabolic disturbances seen in diabetic patients, and subsequent to the onset of diabetic complication.[16]

Soroush and Johansen has been demonstrated that hyperglycemia induces production of ROS, which finally leads to increased OS in diabetic patients. OS leads to increased lipid peroxidation and elevated production of MDA which is the end product of Lipid peroxidation assay (PUFA).[17,18]

Oxidative stress is considered to instigate the development of insulin resistance, β cell dysfunction and impaired tolerance to glucose in T2DM patients.[19]

Malondialdehyde levels are indicative of the extent of lipid peroxidation as a result of oxidative degeneration of polyunsaturated fatty acids due to increased generation of free radicals and impaired antioxidant defenses. Increased lipid peroxidation can be destructive to various body tissues.[20] It has been shown in some studies that patients with diabetes and foot ulcer have higher level of serum MDA and lymphocyte MDA in comparison to patients without ulcer or healthy controls.[21]

HbA1c were significantly higher in DFU participants due to poor glycemic control has been hypothesized to stimulate the increased production of lipid peroxidation.[22,23]

The current study were classified the (-2578 T/A) polymorphisms into three genotypes, one homozygous for the A allele (AA) wild type, one heterozygous (AT) and the last one was homozygous for the allele T (TT). Table (4) were revealed the genotyping of study subjects according to (-2578 T/A) polymorphism of the *VEGF* gene.

Various genetic disorders have been investigated in Iraqi with type 2 diabetic patients with and without diabetic foot ulcers.[24,25] The results of the current study of genotype distribution of the *VEGF* (-2578 T/A) (rs699947) SNP exhibited a significant associations (P ≤ 0.01) were noticed between AT and TT genotype and incidence of DFU patients when compared with those of the T2DM as control group. The results of calculating the odds ratios for AT and TT were (0.21 and 0.32) respectively.

Table 4: Genotype of *VEGF* (-2578 T/A) polymorphism.

VEGF genotype (-2578 T/A)	Patients N (%)	Control N (%)	Odds ratio	CI 95%	*P value*
AA (Ref)	31 (25.83%)	72 (60%)	-	-	-
AT	73 (60.83%)	36 (30%)	0.21	0.12 – 0.38	≤ 0.01
TT	16 (13.34%)	12 (10%)	0.32	0.14 – 0.76	≤ 0.01

The results of the current study of genotype distribution of the *VEGF* (-2578 T/A) (rs699947) SNP were showed a significant associations (P ≤ 0.01) between AT and TT genotype and incidence of DFU patients when compared with patients have T2DM as control group. In table (4) the classification of genotype into AA, AT and TT, the more frequent genotype is heterozygous AT.

The works of several teams have already proved the connection of other polymorphic loci of the *VEGFA* gene with the development of DFS. Xiaolei demonstrated the protective role of rs699947-locus of the *VEGFA* gene regarding the DFS development in Chinese patients.[26] The lower frequency of A allele might lead to the increase of angiogenesis, further reduce the morbidity of DFU but when high frequency of A allele might lead to decrease of angiogenesis so increase complication of DFU.[14]

The results in current study were showed in table (5) the correlation between *VEGF* (-2578 T/A) SNP and biochemical parameters (GSH, MDA and HbA1c).

Table 5: Correlation between *VEGF* (-2578 T/A) SNP and biochemical parameters in DFU patients as compared with control group.

VEGF genotype			GSH		*P value*	MDA		*P value*	HbA1c		*P value*
			DFU N= 120 Mean ± SD	Control N= 120 Mean ± SD		DFU N= 120 Mean ± SD	Control N= 120 Mean ± SD		DFU N= 120 Mean ± SD	Control N= 120 Mean ± SD	
VEGF (-2578 T/A)	AA	DFU N= 120	2.79 ± 0.72	4.96 ± 0.81	≤ 0.01	76.42 ± 9.59	36.06 ± 2.36	≤ 0.01	10.63 ± 1.53	8.303 ± 1.85	≤ 0.01
		Control N= 120	3.005 ± 0.58	5.17 ± 1.39	≤ 0.01	77.56 ± 9.27	36.68 ± 2.26	≤ 0.01	10.62 ± 2.007	8.29 ± 1.99	≤ 0.01
	AT	DFU N= 120	2.99 ± 0.56	5.24 ± 1.14	≤ 0.01	77.69 ± 8.9	36.66 ± 2.23	≤ 0.01	10.6 ± 2.05	8.44 ± 1.97	≤ 0.01
		Control N= 120	2.99 ± 0.56	5.24 ± 0.85	≤ 0.01	77.55 ± 8.97	36.69 ± 2.25	≤ 0.01	10.59 ± 2.03	8.414 ± 1.97	≤ 0.01
	TT	DFU N= 120	3.005 ± 0.57	5.204 ± 1.39	≤ 0.01	77.98 ± 9.11	36.86 ± 2.213	≤ 0.01	10.5 ± 2.04	8.444 ± 1.99	≤ 0.01
		Control N= 120	3.008 ± 0.55	5.23 ± 1.37	≤ 0.01	77.82 ± 9.05	36.7 ± 4.36	≤ 0.01	10.69 ± 2.023	8.405 ± 1.99	≤ 0.01

The significant result (P value ≤ 0.01) were appeared between (AA) for *VEGF* genotype and biochemical parameters (GSH, MDA and HbA1c). The correlation between (AT) SNP for *VEGF* genotype and biochemical parameters (GSH, MDA and HbA1c) is significant (P value ≤ 0.01). The significant result (P value ≤ 0.01) were showed between (TT) SNP for *VEGF* genotype and biochemical parameters (GSH, MDA and HbA1c).

The results were showed in table (5) appeared the correlation between SNP of *VEGF* gene and biochemical parameters (GSH, MDA and HbA1c). Oxidative stress is considered to instigate the development of insulin resistance, β cell dysfunction and impaired tolerance to glucose in T2DM patients, oxidative stress is also associated with the long term complications of DM such as microvascular, macrovascular complications and DFU.[27,28] Free radicals overproduction is the starting point for a cascade of events that eventually leads to diabetic complications including lower extremity ulcerations.[29]

Oxidative stress increase in cells when exposed to a hyperglycemic environment. Oxidative stress occurs as a consequence to an increased oxidant load and decreased antioxidant status (glutathione). Oxidative stress causes cells damage by forming lipid peroxides (malonylaldehyde) and protein carbonyl.[30]

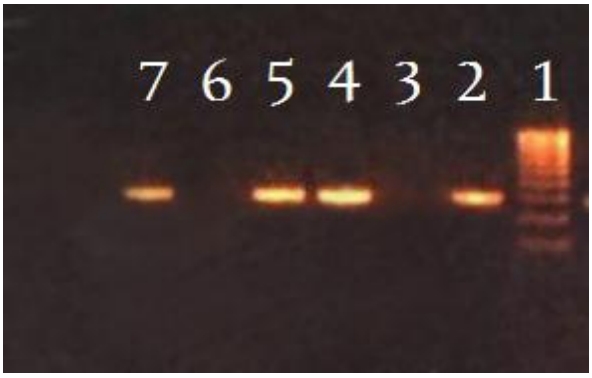

Figure 1: ARMS-PCR product (295 bp) of the *VEGF* polymorphism (rs699947) visualized on 1.5% agarose gel. Lane 1is a DNA marker of 100–1000 bp. Lane 2 and 3 are the 295 bp product of the *VEGF* polymorphism region for A allele (wild type). Lane 4 and 5 are the 295 bp product of the *VEGF* polymorphism region for AC alleles. Lane 6 and 7 are the 295 bp product of the *VEGF* polymorphism region for recessive C allele.

In current study the defect in SNPs of *VEGF* gene in DFU patients lead to increase oxidative stress (MDA) and decrease in antioxidant (GSH). HbA1c were significantly higher in DFU participants due to poor glycemic control has been hypothesized to stimulate the increased production of lipid peroxidation.[23,31] The amplification of *VEGF* polymorphism (-2578 T/A) region is giving rise to 295 bp product , as shown in Figure 1.

CONCLUSION

- The significant results were appeared in biochemical parameters (HbA1c, GSH and MDA) between DFU patients and T2DM as control group.
- The significant results were showed in *VEGF* (-2578 C/A) (rs699947) between DFU patients and T2DM as control groups.
- The significant results in the correlation of genotypes of *VEGF* (-2578 C/A) (rs699947) polymorphism with parameters (HbA1c, GSH and MDA) between DFU patients and T2DM as control groups.

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