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RESEARCH ARTICLE

Vascular Endothelial Growth Factor +405 G/C Polymorphism and Diabetic Retinopathy

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Abstract

Diabetic retinopathy (DR) is the most common micro vascular complication of diabetes mellitus. Vascular endothelial growth factor (VEGF) is a major mediator of vascular permeability and angiogenesis and also an important mediator of retinal ischemia-associated intraocular neovascularization. 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.

A total of 117 subjects (healthy, diabetics without retinopathy and diabetics with DR) were studied. The relationship between DR and +405 G/C VEGF gene polymorphism and serum VEGF were determined.

This study reveals that diabetics with +405 G/C polymorphism would have 25 times risk to develop proliferative diabetic retinopathy (PDR), 2 times risk to develop non proliferative diabetic retinopathy (NPDR) and 3 times risk to develop DR in general than those without polymorphism (+405 G). Also C allele is significantly related to PDR group ($p < 0.001$).

Serum VEGF level in CC genotype diabetic patients (DNR plus DR) is significantly higher than level of GG genotype diabetic patients ($p=0.03$). Moreover serum VEGF level is highest in diabetic patients with PDR. Those results lead to conclude that +405 VEGF G/C polymorphism is an independent risk factor for the development of PDR.

Introduction

Diabetic retinopathy (DR) is one of the most common complications of diabetes mellitus (DM) affecting millions of working adults worldwide, in which the retina, a part of the eye becomes progressively damaged, leading to vision loss [1]. DR is characterized by gradually progressive alterations in the retinal microvasculature, leading to areas of retinal nonperfusion, increased vasopermeability, and pathologic intraocular proliferation of retinal vessels [2, 3]. DR is responsible for 4.8% of the 37 million cases of blindness throughout the world [4]. According to the American Diabetes Association (ADA) report, DR is the most frequent cause of blindness in American working-age populations (20–70 years); with 12,000 to 24,000 diabetics losing their sight each year [5]. DR-related blindness will become more common in the future, unless some breakthrough occurs in basic and clinical diabetic researches. Several recent studies have provided that even in the context of poor glycemic control, about 20% of the patients who go on to develop type 2 DM do not exhibit significant retinal changes of the type that can easily be observed in patients with non proliferative (NPDR) and proliferative DR (PDR) [6]. In contrast, others may develop PDR at a relatively early stage of diabetes despite good glycemic control [7, 8]. Therefore, DR might be a complex multifactorial disorder, resulting from an interaction of genetic as well as environmental etiologies. Useful clinical markers for genetic susceptibility to a disease are either familial aggregation or a variation in disease frequency, which are not explained by environmental, biochemical, or biological risk factors. DR displays these characteristics as clinical studies on human subjects with diabetes reveal substantial variation in the onset and severity of retinopathy that are not fully explained by the known risk factors.

The risk of severe DR in the siblings of affected individuals is substantially increased [9] moreover the Diabetes Control and Complications Trial has shown that retinopathy tends to cluster in families [10]. Furthermore, differences in the frequency of disease in ethnic populations [11] also suggest that genetic influences are operating in DR. One of candidate genes associated with the development and progression of DR is vascular endothelial growth factor (VEGF) gene.

The role of VEGF gene polymorphism in PDR is controversial. The VEGF gene is located on chromosome 6p21.3 and consists of 8 exons exhibiting alternate splicing to form a family of proteins [21, 22]. Polymorphism within the gene leads to differences in VEGF expression between individuals and could influence the etiology of a variety of pathologic conditions with which VEGF has been associated [22]. Several polymorphisms at the VEGF 5′ untranslated region also have been characterized and evaluated as risk alleles for the susceptibility to and/or progression of both DR and diabetic nephropathy through case-control studies [23-25]. To our knowledge, no information about the relation between DR and +405 G/C polymorphism of VEGF gene is available in the Iraqi population.

The aim is to evaluate the possible association of +405 G/C VEGF gene polymorphism with DR and assess its effect on serum level of VEGF.

Subjects and Methods

The study was conducted in Babil-Iraq, from December 2012 to May 2013. This case-control study enrolled 117 subjects which attended different medical centers including Al-Hilla Teaching General Hospital, and Marjan Medical City. Informed consent was obtained from all participants. The practical side of the study was performed at the laboratory of biochemistry department in College of Medicine /Babylon University, General Health Laboratory in Hilla, and Al-Nahrian Forensic DNA Training Centre in Baghdad.

Sixty four patients with diabetic retinopathy (DR) were recruited from the Ophthalmological Clinic, and had underwent complete ophthalmological examination, including best corrected visual acuity, and slit-lamp examination with high power condensing lens (78, 90 diopter) done after pupillary dilation by tropicamide 1% ophthalmic drops. The examination was performed by the same senior ophthalmologist.

Those were divided into 2 groups:

Group (1): 42 patients with non proliferative diabetic retinopathy **(NPDR)** and age mean 53.8 ± 8.7 years

Group (2): 22 patients with proliferative diabetic retinopathy **(PDR)** and age mean 51.8 ±10.6 years.

While the control include fifty three subjects which in turn also divided into two groups:

Group (1): 29 diabetic patients with no clinical signs of DR **(DNR)** with age mean 49.3 ±13.9 years.

Group (2): 24 healthy volunteers **(HC)** with no history of DM or any major clinical disorders and age mean 47.15 ±13.1 years.

Five to eight milliliters of blood were collected in 2 tubes, one plane tube without anticoagulants for measuring VEGF using ELISA kit (BIOO Scientific-USA) and other with EDTA tube which was used for fresh isolation of DNA by Genomic DNA Mini Kit (Geneaid-Italy), +405 G/C (rs 2010963) polymorphism in VEGF gene was detected by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Amplification was performed in a programmable thermal cycler gradient PCR system (Veriti 96 well applied Biosystem, Singapore).

The forward and reverse primers' sequences [26-28] were revealed in table (1):

Table (1): Sequences of primer used for PCR amplification of +405 G/C VEGF gene

primer	**Sequence**
Forward	5'- ATTTATTTTTGCTTGCCATT -3'
Reverse	5'- GTCTGTCTGTCTGTCCGTCA -3'

- **PCR conditions that give best result**:

Genomic DNA was amplified in a final volume of 26 µl (7 µl Genomic DNA+ 0.7 µl F-primer + 0.7 µl R-primer +12.5 µl Promega master mix with Green Taq DNA polymerase + 5.1 µl DDW) using the following conditions: Denaturation at 94 ^{o}C for 4 min. followed by 35 cycles of (denaturation at 94 ^{o}C for 45 seconds, annealing at 58 ^{o}C for 1 min. and extension at 72 ^{o}C for 1 min.) and a final extension was at 72 ^{o}C for 5 min. and then hold at 4 ^{o}C for indefinite time. Then the amplification products were separated by electrophoresis through 2% agarose gel stained with ethidium bromide, then for the VEGF +405 polymorphism the PCR product was digested with the BsmFI restriction nuclease (Biolab, USA) [28].

- **Digestion conditions that give best result**:

9.3 µl of water were added to 2 µl of NEB buffer, 0.2 BSA, 8 µl of PCR product and 0.5 µl BsmFI enzyme to a final volume of 20 µl, those were mixed and incubated at 65 °C for 3 hours.

The uncut fragment was 300 base pairs (bp) (C allele) and digestion products were 200 bp and 100 bp (G allele) approximately.

Statistical Analysis

The data were analyzed using SPSS statistical software (SPSS version 17). $P < 0.05$ was considered statistically significant. The distribution and comparison of alleles and genotypes frequency of VEGF (+405 G/C) gene polymorphism in each was made using the Chi-square test. Odds ratio (OR) with 95% confidence intervals (CI) were estimated for the effect of high risk alleles.

Results

The amplification PCR product was approximately 300 bp as figure (1) revealed [26, 27] containing the site needed.

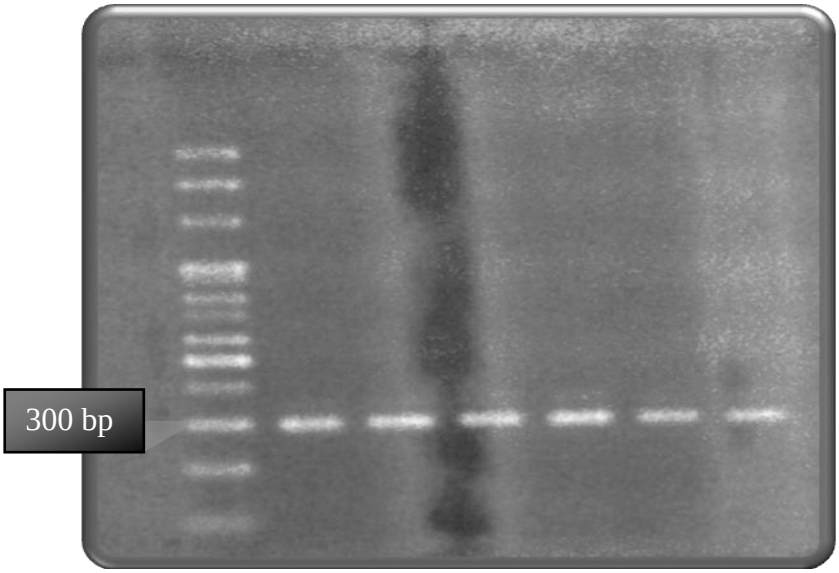

Figure (1): PCR product using Promega master mix on 2% agarose, 70V, and for 60 minute (10µl of DNA loaded in each well).

According to the restriction digestion pattern that obtains in this study after digestion of PCR product with restriction enzyme; figure (2), 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 (300 bp) is variant homozygote (CC); presence of polymorphism.

3- Three bands (300 bp, 200 bp, and 100 bp) is variant and wild type heterozygote (GC); presence of polymorphism.

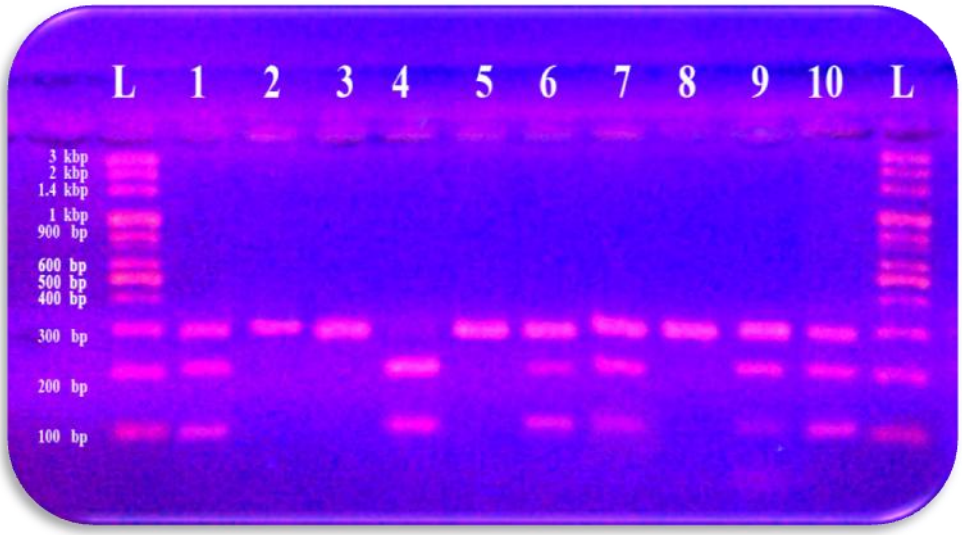

Figure (2): Restriction digestion of PCR products demonstrating the patterns of digestion in different genotypes of VEGF +405 G>C polymorphism on 2% agarose, 70V, and for 75 minute (8µl of DNA loaded in each well).
Lane L: 100 bp plus ladder
Lane 1, 6, 7, 9 and10 show 3 bands (100 bp, 200 bp, and 300 bp); GC genotype
Lane 2, 3, 5, and 8 show 1 band (300 bp); CC genotype
Lane 4 shows 2 bands (100 bp, and 200 bp); GG genotype

❖ **Association of VEGF +405 G/C Polymorphism with DR**

This study reveals that diabetic patients with +405 G/C polymorphism would have 25 times risk (95% CI 3.631 – 181.437) to develop PDR, 2 times risk to develop NPDR and 3 times risk to develop DR in general than those without polymorphism (+405 G), and C allele significantly related to PDR group ($p < 0.001$).

Also, the current study shows a significant difference in allele frequency among all groups (p value < 0.001); (C-allele has 32.6% in NPDR, 87.5% in PDR, 21.4% in DNR and 25% in HC while G-allele has 67.4% in NPDR, 12.5% in PDR, 78.6% in DNR and 75% in HC) as in table (2). Moreover, there is a significant increase in the frequency of the C allele in the diabetic retinopathy patients (NPDR plus PDR) compared with control (DNR plus HC) (p value < 0.001).

Table (2): Alleles' frequency in studied groups.

Allele		Groups				Total
		NPDR	PDR	DNR	HC	
C	% within allele	44.1%	41.2%	8.8%	5.9%	100.0%
	% within group	32.6%	87.5%	21.4%	25%	44.0%
G	% within allele	62.0%	4.0%	22.0%	12.0%	100.0%
	% within group	67.4%	12.5%	78.6%	75%	56.0%

The relative frequency for GG, GC, and CC genotypes of the +405 VEGF gene was respectively 12.5%, 62.5%, and 25% in the PDR group; and 26.1%, 60.9%, and 13% in the NPDR group as table (3) illustrated.

Table (3): Genotype distribution of VEGF +405 G/C polymorphism in all groups

GROUPS		GENOTYPES			Total
		CC	GC	GG	
NPDR					
	% within group	13.0%	60.9%	26.1%	100.0%
	% within genotype	42.9%	60.9%	50.0%	54.8%
PDR					
	% within group	25.0%	62.5%	12.5%	100.0%
	% within genotype	28.6%	21.7%	8.3%	19.0%
DNR					
	% within group	28.5%	28.6%	42.9%	100.0%
	% within genotype	28.6%	8.7%	25.0%	16.7%
HC					
	% within group	0.0%	50.0%	50.0%	100.0%
	% within genotype	0.0%	8.7%	16.7%	9.5%
Total					
	% within group	16.7%	54.8%	28.6%	100.0%
	% within genotype	100.0%	100.0%	100.0%	100.0%

There were no significant differences in genotype distribution between all groups and also study revealed non significant association between +405 genotypes (GG, GC, CC) and studied groups (HC, DNR, NPDR, PDR) p value > 0.05. Moreover GC genotype percentage in DR groups was higher than control groups and the expected number for GC genotype in this study is 39 while the observed is 64 with chi-square equal to 26.205 and highly significant p value < 0.001 as demonstrated in table (4).

Table (4): Observed and expected number forVEGF+405 genotypes

Genotypes	Observed	Expected	Chi-Square	P-value
GG	33	39.0	26.205	0.000
GC	64	39.0		
CC	20	39.0		
Total	117			

Control subjects (both diabetic without retinopathy and healthy) with GC genotype would have six times risk (95% CI 0.504 - 77.494) to develop PDR and three times risk (95% CI 0.574 -14.824) to develop NPDR and three times risk (95% CI 0.703 -16.385) to develop DR in general than those with GG genotype. At the same time, those with CC genotype would have five times risk (95% CI 0.273 - 91.518) to develop PDR and two times risk (95% CI 0.241-13.215) to develop DR than those with GG genotype. In addition to that, diabetic patients with CC genotype would have three times risk to develop PDR than those with GG genotype (95% CI 0.150 -59.890).

❖ **Association of VEGF Polymorphism with Plasma Levels**

To assess the functional relevance of the +405 G/C polymorphism on serum VEGF levels, VEGF concentration in serum of all participants were measured.

Study finds that serum VEGF level in CC genotype diabetic patients (DNR plus DR) is significantly higher than level of GG genotypes diabetic patients (p=0.03) and at the same time higher than level of GC genotype diabetic patients but not significantly, as figure (3) demonstrates.

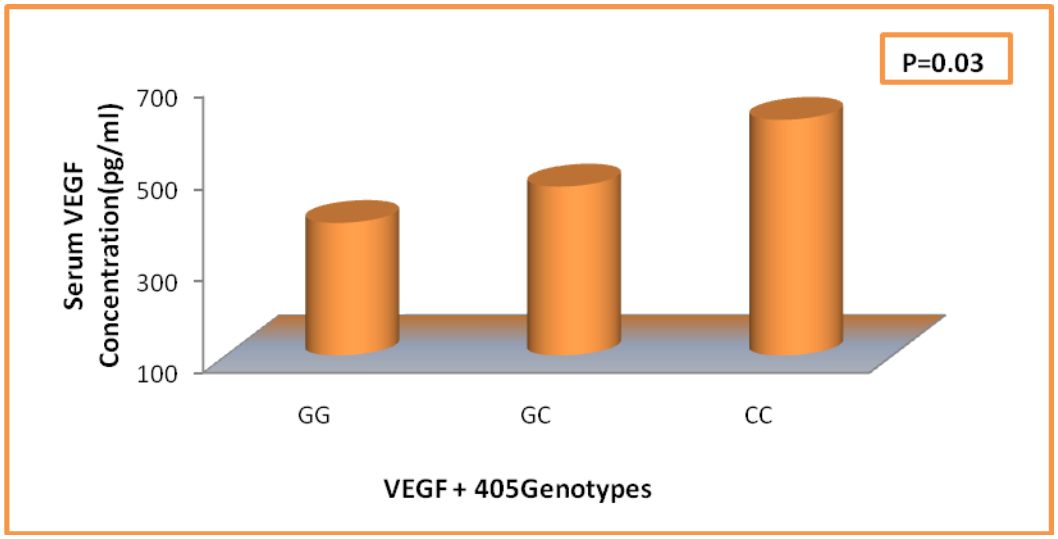

Figure (3): Serum level of VEGF according to +405 genotypes.

❖ **Association between VEGF Plasma Levels and diabetic Retinopathy**

There is significant difference in serum VEGF level between studied groups (HC, DNR, NPDR, PDR) (p=0.004) and the highest level is in PDR group as figure (4) reveals.

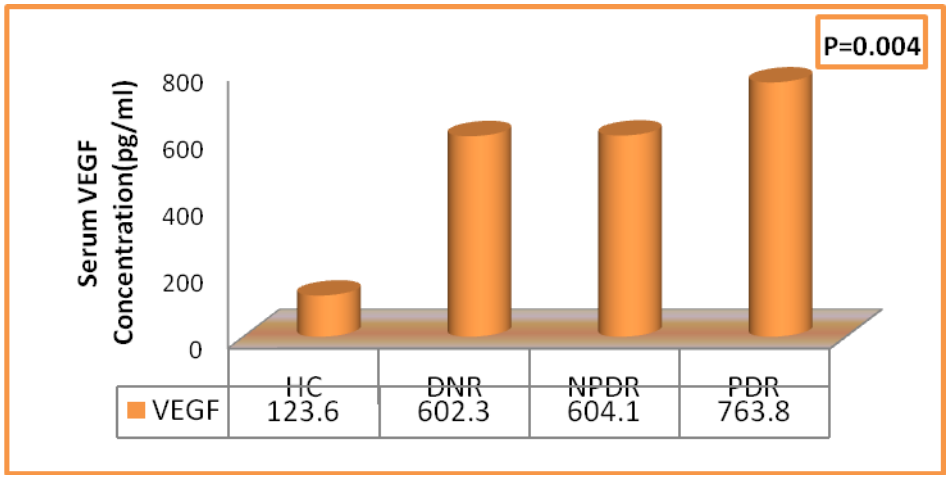

Figure (4): Mean of serum VEGF concentration in studied groups.

Discussion

VEGF plays an important role in the pathogenesis of diabetic microvascular complications. As VEGF is involved in the process of new blood vessel formation, it seems to be a potential candidate gene for DR [29-31].

The relationship between the VEGF gene polymorphism and proliferative diabetic retinopathy has been the subject of recent studies. VEGF polymorphisms might be a useful predictive marker for the development and progression of diabetic retinopathy at an earlier stage [32].

This study reveals that diabetic patients with +405 G/C polymorphism would have 25 times risk (95% CI 3.631 – 181.437) to develop PDR than those without polymorphism (+405 G), and C allele is significantly related to PDR group ($p < 0.001$) which agree with the results of Szaflik *et al*.[33] in which the C allele of +405 VEGF gene was associated with an increased risk of DR and where +405 polymorphism was strongly associated with DR in Japanese subjects and differ from those described by Ray *et al*. and Liinamaa *et al.* [22,34] whom did not find an association between +405 C allele and PDR.

Study also revealed a significant difference in allele frequency between all groups, and there was a significant increase in the frequency of the C allele in the DR patients (NPDR plus PDR) compared to control (DNR plus HC) which is similar to finding of Awata T. *et al*. [35] and in contrast to finding of Xiufen Yang *et al*.[36] which revealed no statistically significant difference in the allele frequencies.

Those different findings could be due to differences in the genetic background or environment of each population under examination; alternatively, these differences may be related to small sample size.

In addition to that there were no significant differences in genotype distribution between all groups. Also study revealed non significant association between +405 genotypes (GG, GC, CC) and studied groups (HC, DNR, NPDR, PDR) which is similar to finding of Buraczynska M. *et al.* and Watson *et al.* study [27, 23] that found no association between the +405 genotype and DR in Caucasian samples. Meanwhile study find that serum VEGF level in CC genotype diabetic patients (DNR plus DR) is significantly higher than level of GG genotypes diabetic patients which is tempting to speculate that the association between +405 VEGF-C allele and PDR risk is the result of increased VEGF production.

Awata *et al.* [35] measured the highest VEGF serum levels in patients who are homozygous for VEGF +405 C alleles however Watson *et al.* [23] stated a different result (demonstrating the highest VEGF levels in individuals with +405 GG genotype). This apparent inconsistency between studies may be caused in part to different methods and experimental models that were used.

The most likely mechanism underlying study's findings is that polymorphisms of the 5′ untranslated region of the VEGF gene appear to increase its basal VEGF gene promoter activity [22, 23, 35] and induce the expression of VEGF [37, 38], which leads to the stimulation of angiogenesis and subsequent development of PDR.

Conclusion

We showed for the first time in an Iraqi diabetic population that the VEGF +405 G/C polymorphism is associated with DR as independent factor. Hence, the potential value of identifying diabetic patients with a VEGF +405 polymorphism G/C in the advantage of early therapeutic intervention and reduced progression of DR and subsequent vision loss.

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