

## RESEARCH ARTICLE

# Contribution of VEGF polymorphisms to variation in VEGF serum levels in a healthy population

Heba H. Al-Habboubi, Mai S. Sater, Ahmad W. Almawi, Ghada M. Al-Khateeb, Wassim Y. Almawi

Department of Medical Biochemistry, Arabian Gulf University, Manama, Bahrain

**Correspondence:** W.Y. Almawi, Ph.D, Department of Medical Biochemistry, College of Medicine & Medical Sciences, Arabian Gulf University, PO Box 22979, Manama, Bahrain  
<wassim@agu.edu.bh>

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**ABSTRACT.** *Objective.* Vascular endothelial growth factor (VEGF) is a pro-angiogenic factor. Variability in VEGF expression, induced by specific VEGFA variants, are involved in angiogenesis-related disorders. This study examined the genotype distribution and functional role (VEGF expression) of rs699947, rs833061, rs1570360, rs2010963, rs833068, rs833070, rs3025020, and rs3025039 VEGFA variants and their haplotypes in 519 healthy Bahraini individuals of both genders. *Methods and results.* The distribution of the eight VEGFA polymorphisms screened was in Hardy-Weinberg equilibrium. The minor allele frequencies of rs699947 (0.42), rs833061 (0.32), rs1570360 (0.31), rs2010963 (0.33), rs833068 (0.37), rs833070 (0.42), rs3025020 (0.33), and rs3025039 (0.13) were generally compared to those established for Caucasians. Of the variants tested, rs3025020 was associated with increased VEGF serum levels ( $p=0.019$ ), while rs3025039 was associated with decreased levels ( $p=0.038$ ). Linkage analysis identified two VEGFA blocks, the first, spanning 16 kb, was not associated with altered VEGF levels, while the second, spanning 3 kb containing rs3025020 and rs3025039, was linked with higher VEGF expression, of which the  $-583T/+936T$  haplotype ( $p=0.008$ ) was linked with higher VEGF levels compared to the  $-583C/+936C$  (all wild-type) haplotype. *Conclusion.* These results support the association of rs3025020 and rs3025039, and specific VEGF haplotypes, with altered VEGF serum levels, although the exact functional mechanisms remain to be elucidated.

**Key words:** angiogenesis, haplotype, polymorphisms, vascular endothelial growth factor

VEGF is a potent, 45 kDa, vasculogenic and pro-angiogenic factor that acts by regulating the growth and development of healthy vasculature [1, 2]; it also plays an important role in inducing vascular permeability [1, 3]. VEGF also acts as a mitogen [4], and increases the adhesiveness of endothelial cell by upregulating the endothelial expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), and other adhesion molecules [2, 5]. The VEGF gene is located on chromosome 6, and comprises a 14-kb coding region with eight exons and seven introns [6]. Several polymorphisms in the VEGF have been reported, including the promoter variants -2578C/A (rs699947), -1154G/A (rs1570360), -634G/C (rs2010963) and -460T/C (rs833061), and the intron/exon variants +936C/T (rs3025039) [7, 8].

The production of VEGF is genetically determined [8], and select VEGFA variants, notably rs3025020 and rs3025039, contribute to the variation in VEGF serum levels [7, 8], and to altered susceptibility to diseases linked with altered angiogenesis, including recurrent spontaneous miscarriage [7], cancer [9], and microvascular [10-12] and macrovascular [13, 14] diabetic complications. Similar to other cytokines, heterogeneity in the distribution of VEGFA variants and protein expression has been reported among healthy individuals [8]. Ruggiero

investigated the genetic variation in VEGFA exons, intron-exon junctions, promoter and regulative regions in 1,957 individuals from three small, isolated villages in South Italy (Campora, Gioi and Cardile), and identified three common SNPs affecting VEGF serum levels in one population (Campora), and an additional variant affecting VEGF levels in another population (Cardile). This points to the contribution of distinct VEGFA variants, in different populations, to varying VEGF serum levels [8].

In this study we investigated the distribution of eight common VEGFA polymorphisms, and their effect on VEGF serum levels, in the island-kingdom of Bahrain, which is located in the Arabian Gulf, and whose inhabitants derive their origin from three major roots: Jaafari Arabs, Sunni Arabs, and Iranians [15]. The present-day Bahraini population is characterized by a unique genealogy, combined with significant consanguinity and a small number of founders.

## PATIENTS AND METHODS

### Study subjects

Study subjects comprised 519 healthy, Bahraini individuals (266 males and 253 females; age:  $31.1 \pm 14.0$  years), recruited as blood bank donors, university employees, or

volunteers. Study subjects were from different zones of Bahrain, and were asked to sign a consent form agreeing to participate in the study: all institutional ethics requirements were met. None of the subjects recruited had any current or recent illness, which may have affected VEGF levels. EDTA-anti-coagulated blood and coagulated blood specimens were obtained from each participant by venipuncture, and were processed shortly thereafter.

### VEGF analysis

*VEGF* genotyping was investigated using the allelic (VIC- and FAM-labelled) discrimination method. TaqMan assays, as assay-on-demand, were ordered from Applied Biosystems (Applied Biosystems; Foster City, NJ, USA): C\_8311602\_10 (rs699947), C\_1647381\_10 (rs833061), C\_1647379\_10 (rs1570360), C\_8311614\_10 (rs2010963), C\_11400864\_10 (rs833068), C\_1647373\_10 (rs833070), C\_1647366\_10 (rs3025020), and C\_16198794\_10 (rs3025039). The reaction was performed in 6  $\mu$ L volumes on StepOne real-time PCR system, as recommended by the manufacturer (Applied Biosystems). Replicate, blinded, quality control samples were included to assess reproducibility of the genotyping procedure; concordance was >99%. Serum specimens were prepared by centrifugation of coagulated blood tubes at 2000 g for 10 min at room temperature, and were tested for VEGF by quantitative sandwich ELISA according to the manufacturer's instructions (R&D Systems Europe, Abingdon, UK). Results are given as pg/mL, with assay sensitivity being 5 pg/mL.

### Statistical analysis

Statistical analysis was performed on SPSS v. 17.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as a percentage of total (categorical variables) or mean  $\pm$  SD (continuous variables). Analysis of variance (ANOVA) was used in testing differences in serum VEGF means, and the Pearson  $\chi^2$  test was used in assessing inter-group significance. Allele frequencies were calculated by the gene-counting method; each polymorphism was tested for Hardy-Weinberg equilibrium using  $\chi^2$  goodness-of-fit test using HPlus 2.5 software (<http://qge.fhcrc.org/hplus>). All analyses were conducted assuming an additive genetic effect, as this is the most conservative mode. Linkage disequilibrium (LD) analysis and haplotype reconstruction was performed using Haplovie 4.1 (<http://www.broad.mit.edu/mpg/haplovie>). Logistic regression analysis was performed in order to determine the odds ratios (OR) and 95% confidence intervals (95%CI).

## RESULTS

### *VEGFA* alleles

The distribution of *VEGFA* rs699947 (p=0.224), rs833061 (p=0.516), rs1570360 (p=0.117), rs2010963 (p=0.131), rs833068 (p=0.452), rs833070 (p=0.476), rs3025020 (p=0.138), and rs3025039 (p=0.471) genotypes was in Hardy Weinberg equilibrium among the study subjects (table 1). The minor allele frequencies of the eight *VEGFA* variants tested ranged from 0.126 for rs3025039 to 0.419 for rs833070. These were compatible with

**Table 1**  
*VEGFA* SNPs analyzed.

Name	Position <sup>1</sup>	Gene Location	HWE p-value	MAF	Alleles
rs699947	43726139	Promoter	0.224	0.416	C:A
rs833061	43737236	Promoter	0.516	0.324	T:C
rs1570360	43737580	Promoter	0.117	0.31	G:A
rs2010963	43738100	5'-UTR	0.131	0.329	G:C
rs833068	43742277	Intron 2	0.452	0.372	G:A
rs833070	43742376	Intron 2	0.476	0.419	G:A
rs3025020	43748860	Intron 6	0.138	0.332	C:T
rs3025039	43752286	3'-UTR	0.471	0.126	C:T

MAF: Minor allele frequency; HWE: Hardy-Weinberg Equilibrium; HET: heterozygosity.

<sup>1</sup> Location on chromosome based on dbSNP build 125.

frequencies established elsewhere for populations of Caucasian descent.

### *VEGFA* genotype distribution and association with VEGF levels

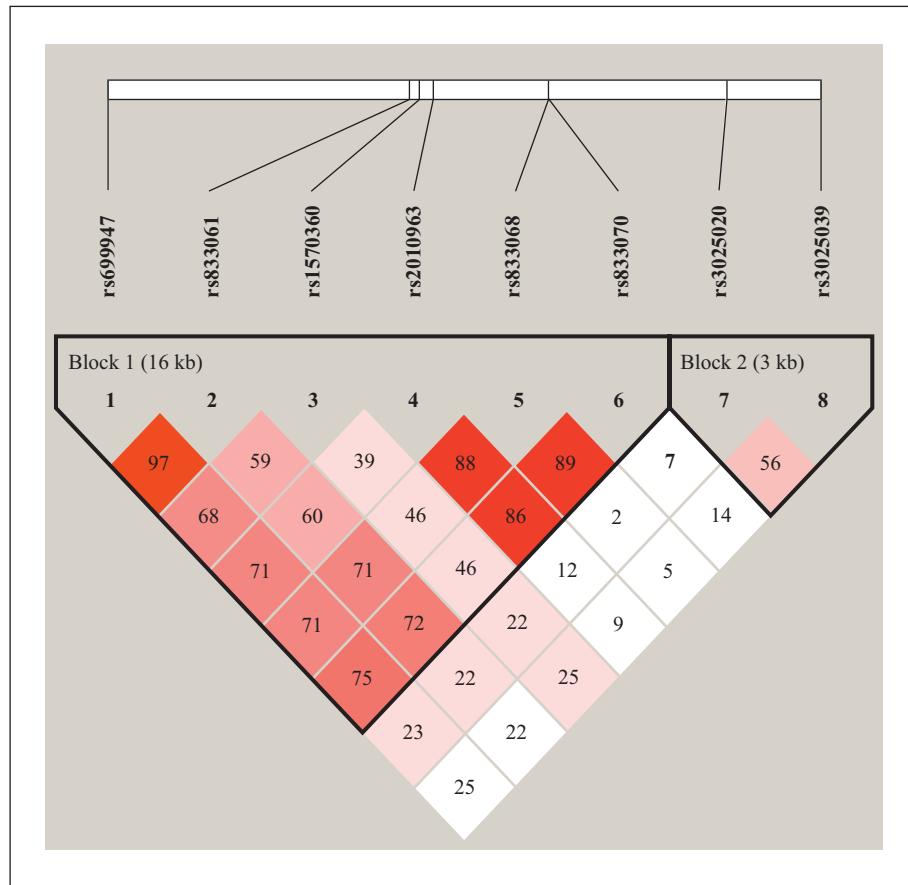
Similar to their allelic distribution, the genotype distribution of the eight *VEGFA* variants tested was similar to their distribution among Caucasians and Europeans, but markedly different from Asians (Chinese, Japanese, Koreans), and Africans (African-Americans and Sub-Saharan Africans). *VEGFA* SNPs were then tested for association with VEGF serum levels. Of the eight *VEGFA* variants analyzed, only rs3025020 (p=0.019) and rs3025039 (p=0.038) were associated with altered VEGF levels. The T/T genotype of rs3025039 was associated with lower VEGF levels ( $295.2 \pm 215.4$  pg/mL) compared to the C/C genotype ( $475.9 \pm 288.3$  pg/mL), while the T/T genotype of rs3025020 variants was associated with higher VEGF serum levels ( $548.1 \pm 208.6$  pg/mL) compared to the C/C ( $365.3 \pm 254.9$  pg/mL) genotype (table 1). Limited linkage disequilibrium was observed among rs3025020-rs3025039 ( $D' = 0.56$ , LOD=2.37) (figure 1).

### *VEGFA* haplotypes

Haplovie analysis identified two blocks: the first spanning 16 kb, and associated with no marked changes in serum VEGF levels, while the second spanning 3 kb and containing rs3025020 and rs3025039, was associated with altered VEGF expression (figure 1). Table 1 shows the two-locus [rs3025020 (-583C/T) and rs3025039 (+963C/T)] *VEGFA* haplotypes constructed based on their MAF prevalence and the LD between them. Compared to the  $-583C/+936C$  haplotype, the double-mutant  $-583T/+936T$  haplotype was associated with significantly higher serum VEGF levels (p=0.008). The single-mutant  $-583T/+936C$  and  $-583C/+936T$  haplotypes were also associated with increased serum VEGF levels, but these increases were not statistically significant.

## DISCUSSION

The human *VEGFA* gene is highly polymorphic, with some of the polymorphisms occurring at high frequency, while others are not as common. An ethnic/racial contribution to the distribution of common *VEGFA* variants, and



**Figure 1**

Haplotype graph of the 8 *VEGFA* SNPs analyzed. Light red/pink block,  $D'$  (normalized linkage disequilibrium measure or  $D$ )  $< 1.0$ , with logarithm of odds (LOD) score  $> 2.0$ ; white blocks,  $D' < 1.0$  with LOD  $< 2.0$ ; numbers in blocks denote  $D'$  value. The genomic organization is depicted above the LD plot. LOD being defined as  $\log_{10}(L1/L0)$ , where  $L1$ =likelihood of the data under linkage disequilibrium, and  $L0$ =likelihood of the data under linkage equilibrium.  $D'$  is calculated as per:  $D' = (D)$  divided by the theoretical maximum for the observed allele frequencies.

significant associations between *VEGFA* genotype and both VEGF secretion and disease states have been reported [7, 16]. Marked heritability of VEGF serum levels according to *VEGFA* genotypes has been reported among a healthy Bahraini population, in which VEGF variability was captured by two SNPs: rs3025020 and rs3025039. Haplotype analysis was consistent with this finding, and strong linkage was noted between these two variants in the *VEGFA* region. It was of interest to note that strong linkage was seen between the remaining six SNPs, thereby defining two blocks: one block (rs3025020 and rs3025039) linked with altered VEGF secretion, and another block (rs699947, rs833061, rs1570360, rs2010963, rs833068, rs833070) not linked with marked changes in VEGF levels.

The MAF of *VEGFA* SNPs seen in the Bahraini population were generally comparable to those established for Caucasian populations (<http://www.ncbi.nlm.nih.gov/snp>), and an ethnic contribution to *VEGFA* SNP distribution was seen, highlighted by the comparable MAF of rs699947, rs833061, rs833071, and rs3025039 in Bahraini and Europeans, which were higher than the rates established for Asians, Sub-Saharan Africans, and African Americans. In contrast, the MAF of rs2010963 was generally comparable between Bahraini (34.3%) and other populations of Asian, African, and European descent (29.0%-37.0%), while the MAF of rs833068 was higher among Asians, Sub-Saharan Africans, and African Americans (43.9-50.9%), compared with Bahraini (33.1%) or Caucasians (37.2%).

Data on the prevalence of *VEGFA* variants among Arab populations are scarce. The MAF of rs1570360 in Bahrainis (0.329) was generally comparable to the rates established for central Tunisians (0.367) [17], while the MAF of rs699947 recorded for Bahrainis (0.329) was markedly lower than that established for (North) Tunisians (0.496) [18]. Interestingly, the MAF of rs699947 reported for Bahrainis (0.416) was in accordance with the results reported for (Central) Tunisians (0.416) according to one study [17], and higher (MAF=0.343) according to another study performed in the same area (Central Tunisia) [19]. The likely explanation for these apparently conflicting findings is attributed to the relatively low number of subjects screened in the studies of Smach [17] ( $n=113$ ) and Ben Nasr [19] ( $n=169$ ), as compared to the markedly larger sample used here ( $n=519$ ).

Several polymorphisms in the *VEGFA* gene have been identified, however, only few were associated with altered VEGF production. Of the eight *VEGFA* variants screened here, rs3025020 was associated with increased VEGF secretion, in agreement with our recent finding [7], and that of Ruggiero [8]. In contrast, rs3025039 was associated with reduced VEGF secretion, which was also in agreement with the study involving healthy Southern Italian subjects [8]. In contrast to previously studies on renal transplant recipients [20] and women with or without pregnancy complications [21], where the promoter variant rs699947 (-2578C/A) was linked with higher

**Table 2**  
Distribution of VEGFA genotypes.

SNP	Genotype	Number	Percent	Serum VEGF (pg/mL)	P <sup>1</sup>
rs699947	C/C	184	35.5	324.9±263.4	0.688
	C/A	238	45.9	365.1±329.1	
	A/A	97	18.7	463.5±309.9	
rs833061	T/T	241	46.4	284.4±204.3	0.712
	T/C	220	42.4	443.1±324.9	
	C/C	58	11.2	452.1±393.0	
rs1570360	G/G	255	49.1	364.8±186.5	0.493
	G/A	206	39.7	368.6±162.2	
	A/A	58	11.2	418.6±225.0	
rs2010963	G/G	215	41.4	358.5±180.0	0.472
	G/C	213	41.0	366.5±194.7	
	C/C	64	12.3	349.5±148.8	
rs833068	G/G	215	41.4	382.2±262.3	0.811
	G/A	229	44.1	373.3±239.0	
	A/A	75	14.5	368.9±289.4	
rs833070	G/G	180	34.7	329.9±193.6	0.593
	G/A	243	46.8	368.2±196.9	
	A/A	96	18.5	374.8±216.6	
rs3025020	C/C	239	46.1	365.3±254.9	0.019
	C/T	215	41.4	452.8±217.9	
	T/T	65	12.5	548.1±208.6	
rs3025039	C/C	400	77.1	475.9±288.3	0.038
	C/T	109	21.0	384.5±228.7	
	T/T	10	1.9	295.2±215.4	

<sup>1</sup> 2-way ANOVA.

levels of VEGF, we and others [8, 22] showed that VEGF levels was not affected by the rs699947 variant among healthy individuals. Furthermore, while the rs1570360 (-1154G/A) polymorphism has been previously shown to affect VEGF serum levels marginally [22], the results reported here and elsewhere [8, 23] do not support an influence of rs1570360 on VEGF expression.

The highly polymorphic nature of the VEGFA gene has hampered the identification of “common” haplotypes. Haplovew analysis identified two blocks with significant linkage disequilibrium among the SNPs within each of them. It is of interest that haplotype A, containing rs3025020 and rs3025039, showed markedly altered VEGF serum levels [8], while haplotype B, containing the remaining six SNPs, was not associated with changes in VEGF levels. It will be of interest to analyze comprehensively the presence of other SNPs across each block. This

would of help in gene linkage analysis, and in association studies of human disease linked with angiogenesis.

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**Table 3**  
Effects of VEGFA haplotypes on serum VEGF levels.

Haplotype	Haplotype frequency	Serum VEGF (pg/mL)	P <sup>1</sup>
-583C/+936C	0.562±0.022	323.9±218.7	Reference
-583T/+936C	0.313±0.054	582.6±308.4	0.008
-583C/+936T	0.106±0.052	281.8±200.6	0.602
-583T/+936T	0.019±0.127	452.8±177.5	0.090

<sup>1</sup> 2-way ANOVA.

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