

The -2549 insertion/deletion polymorphism in the promoter region of *VEGF* is associated with the risk of recurrent spontaneous abortion

MOHAMMAD HASHEMI^{1,2}, HIVA DANESH², FATEMEH BIZHANI², MOJGAN MOKHTARI³, GHOLAMREZA BAHARI², FARHAD TABASI⁴ and MOHSEN TAHERI⁵

¹Cellular and Molecular Research Center, Zahedan University of Medical Sciences; Departments of

²Clinical Biochemistry and ³Obstetrics and Gynecology, School of Medicine, Zahedan University of Medical Sciences;

⁴Student Research Committee, Zahedan University of Medical Sciences; ⁵Department of Genetics, School of Medicine, Zahedan University of Medical Sciences, Zahedan 98167-43181, Iran

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Abstract. Recurrent spontaneous abortion (RSA) is a common health problem affecting women of reproductive age. Altered expression of vascular endothelial growth factor (*VEGF*) has been associated with spontaneous abortion. The present case-control study aimed to evaluate the impact of the 18-bp insertion/deletion (ins/del) polymorphism (rs35569394) in the promoter region of the *VEGF* gene on idiopathic RSA. Genomic DNA from 93 patients with RSA and 93 healthy fertile women of southeastern Iran was isolated using the salting-out method. Genotyping of the rs35569394 variant was performed by a polymerase chain reaction (PCR) method. The findings indicated that the *VEGF* 18-bp ins/del variant significantly increased the risk of RSA under codominant (ins/ins vs. del/del; OR=2.85, 95% CI=1.31-6.22, P=0.019), dominant (del/ins+ins/ins vs. del/del; OR=2.19, 95% CI=1.20-4.01, P=0.015) and allelic (ins vs. del; OR=1.90, 95% CI=1.25-2.88, P=0.003) inheritance models. In summary, the findings propose a significant association between the *VEGF* 18-bp ins/del polymorphism and risk of RSA in a sample of the southeast Iranian population. Further studies on larger sample sizes and different ethnicities are required to validate the present findings.

Introduction

Recurrent spontaneous abortion (RSA), one of the most common complications of pregnancy, refers to the occurrence

of at least two consecutive unexplained pregnancy losses before the 20th week of gestation (1). It is reported that approximately 2% of women experience RSA (2). Although the pathogenic mechanism of RSA remains to be fully elucidated, increasing data proposes that RSA may occur as a result of certain fetal and maternal factors, including genetic factors, endocrine and metabolic disorders, and autoimmune abnormalities (3,4). However, the definitive cause of RSA is undetermined in approximately 50% of cases (5). Genetic variations have been suggested as an influential factor for RSA and as of 2012, approximately 100 candidate genes had been inspected (6).

The human vascular endothelial growth factor (*VEGF*) gene (OMIM: 192240) is mapped to chromosome 6 (6p12-p21) and consists of 8 exons separated by 7 introns, the alternative splicing of which produces a family of proteins (7). *VEGF*, also known as VEGFA, is a key regulator of physiological vasculogenesis and angiogenesis during pregnancy (8). It has been proposed that altered expression of the *VEGF* gene may serve a role in the pathogenesis of RSA (9-13). Numerous studies have investigated the *VEGF* genetic polymorphisms and RSA risk in diverse ethnic groups (14-19); these led to inconsistent results, indicating the varying degree of association between *VEGF* polymorphism and RSA risk among different ethnicities.

Polymorphisms in the promoter, introns, exons and untranslated regions (3'-and 5'-UTRs) of a gene may affect the manufacture or function of the corresponding protein. The *VEGF* gene is highly polymorphic (20) and functional polymorphisms of the *VEGF* gene modulate *VEGF* protein expression (13,21,22). A functional 18-bp insertion/deletion (ins/del) polymorphism, located at position -2549 in the promoter region of *VEGF* (21) affects gene expression, whereby the del allele leads to a 1.95-fold increase in transcriptional activity compared to the ins allele (22).

Due to the important roles of *VEGF* during pregnancy, the dysregulated expression of the *VEGF* gene in RSA, and the potential divergence in genetic risk among various populations, the current study was designed to investigate the impact of the 18-bp ins/del polymorphism (rs35569394) in *VEGF* on RSA risk in a southeast Iranian population.

Correspondence to: Professor Mohammad Hashemi, Department of Clinical Biochemistry, School of Medicine, Zahedan University of Medical Sciences, Khalij Fars Boulevard, Zahedan 98167-43181, Iran

E-mail: mhd.hashemi@gmail.com

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Patients and methods

Patients. A total of 186 subjects including 93 RSA cases and 93 controls were enrolled in the present case-control study. This cohort was used in previous studies by our group on gene polymorphisms and RSA risk, as detailed elsewhere (23,24). The participants were selected between January 2015 and February 2016 from individuals attending the obstetrics and gynecology clinic at the Ali ibn Abi Talib Hospital affiliated to Zahedan University of Medical Sciences (Zahedan, Iran). RSA was defined as two or more consecutive pregnancy losses before 20 weeks of gestation. All of the patients were without anatomical, microbial, viral, hormonal or genetic disease. The control group consisted of healthy fertile women without any history of miscarriage. The local research Ethics Committee of Zahedan University of Medical Sciences approved the project and informed consent was obtained from all participants. The salting-out method was used for genomic DNA extraction from peripheral blood samples as described previously (25).

Genotyping. Genotyping of *VEGF* variants was performed using a polymerase chain reaction (PCR) method as described previously (26). The forward and reverse primers used for detection of polymorphism were 5'-AAGATCTGGGTGGATAATCAGACT-3' and 5'-AACTCTCCACATCTTCCCTAAGTG-3', respectively. These primers were produced by Macrogen, Inc. (Seoul, Korea). PCR was performed using commercially available Prime Taq Premix (Genet Bio, Inc., Daejeon, Korea) according to the manufacturer's protocol. Briefly, into 0.20-ml PCR reaction tubes, 1 μ l genomic DNA (~100 ng/ μ l), 1 μ l of each primer (10 μ M), 10 μ l 2X Prime Taq Premix and 7 μ l ddH₂O were added. The PCR cycling conditions were 5 min at 95°C, followed by 30 cycles of 30 sec at 95°C, 30 sec at 61°C and 30 sec at 72°C, with a final step at 72°C for 5 min. The PCR products were resolved on 2.5% agarose gel electrophoresis containing 0.5 μ g/ml ethidium bromide and visualized with an ultraviolet transilluminator (Fig. 1). The product sizes for the ins and del alleles were taken to be 188 and 170 bp, respectively (26).

Statistical analysis. Statistical analysis was performed using SPSS 20.0 software (IBM Corp., Armonk, NY, USA). Categorical data (represented by the mean \pm standard deviation) and continuous data (represented by frequency) were analyzed by χ^2 test and independent sample t-test, respectively. The potential associations between *VEGF* variants and RSA risk were evaluated by computing the odds ratio (OR) and 95% confidence intervals (95% CI) from unconditional logistic regression analysis. $P < 0.05$ was considered to indicate statistical significance.

Results and Discussion

As reported previously (23,24), the RSA group consisted of 93 women with a mean age of 28.88 \pm 4.98 years and the control group consisted of 93 unrelated healthy women with a mean age of 30.01 \pm 4.77 years. There was no significant difference between the groups regarding age ($P = 0.116$).

With regard to the current experiment, the genotype and allele frequencies of the *VEGF* 18-bp ins/del polymorphism

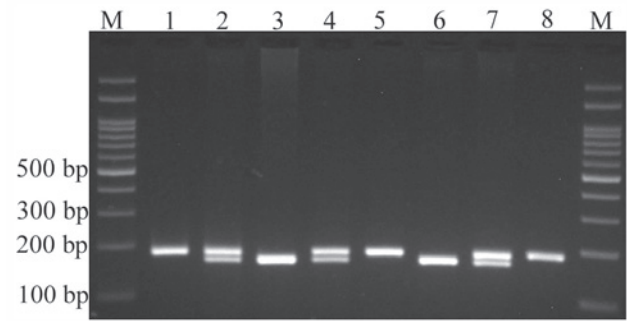


Figure 1. Agarose electrophoresis pattern of polymerase chain reaction amplification products of the vascular endothelial growth factor -2549 ins/del polymorphism. M: DNA marker; lanes 1, 5, 8: Homozygote for ins allele (ins/ins); lanes 2, 4, 7: Heterozygote (ins/del); lanes 3, 6: Homozygote for del allele (del/del). ins, insertion; del, deletion.

in the cases and controls are presented in Table I. The results indicated that the 18-bp ins/del polymorphism significantly increased the risk of RSA under codominant (ins/ins vs. del/del; OR=2.85, 95% CI=1.31-6.22, $P = 0.019$), dominant (del/ins+ins/ins vs. del/del; OR=2.19, 95% CI=1.20-4.01, $P = 0.015$) and allelic (ins vs. del; OR=1.90, 95% CI=1.25-2.88, $P = 0.003$) inheritance models. There were no significant differences in the rates of RSA between the case and control groups under the recessive and overdominant inheritance models.

RSA is a multifactorial disorder caused by various genetic and non-genetic factors. A number of studies have demonstrated an association between genetic variants and the risk of RSA (27-30). *VEGF* is among the established regulators of angiogenesis during pregnancy and has been associated with RSA (31,32). Increasing data indicates that polymorphisms of the *VEGF* gene, including -1154 G/A (rs1570360), -2578 A/C (rs699947), +936 C/T (rs3025039) and -2549 ins/del (rs35569394) are associated with *VEGF* expression level (33-36).

In the present study, the possible association between the 18-bp ins/del variant in the promoter region of *VEGF* and the risk of RSA was inspected in a sample of the southeast Iranian population. The results demonstrated that the *VEGF* 18-bp ins/del variant significantly increased the risk of RSA under codominant, dominant and allelic inheritance models. Previous studies have evaluated the impact of *VEGF* polymorphisms on the risk of RSA (16,37). Saboori *et al* (37) examined the possible association between +936 C/T, -1154 G/A, *VEGF* intron 5 C/T (rs3025010) and +5092 A/C (rs2146323) polymorphisms of the *VEGF* gene and the risk of RSA. They identified a significant association between the -1154 G/A and *VEGF* intron 5 C/T variants and the risk of RSA. Perez *et al* (16) reported that the *VEGF* 18-bp ins/del polymorphism in men may be associated with RSA. Furthermore, Vagnini *et al* (38) observed an association between the *VEGF* -1154 G/A variant and recurrent implantation failure (RIF). A meta-analysis performed by Xu *et al* (19) revealed that -1154 G/A, +936 C/T, -634 G/C (rs2010963) and -583 T/C (rs3025020) polymorphisms in the *VEGF* gene were associated with increased RSA risk. In particular, the -1154 G/A variant was significantly associated with the risk of RSA among non-Asian populations, while the +936 C/T variant was significantly associated with RSA risk among Asian populations (19). In addition, the

Table I. Genetic and allele frequencies of *VEGF* 18-bp ins/del polymorphism in recurrent spontaneous abortion cases and controls.

<i>VEGF</i> 18-bp ins/del polymorphism	Cases, n (%)	Controls, n (%)	OR (95% CI)	P-value
Codominant				
del/del	27 (29.0)	44 (47.3)	1.00	-
del/ins	38 (40.9)	33 (35.5)	1.88 (0.94-3.66)	0.092
ins/ins	28 (30.1)	16 (17.2)	2.85 (1.31-6.22)	0.019
Dominant				
del/del	27 (29.0)	44 (47.3)	1.00	-
del/ins+ins/ins	66 (71.0)	49 (52.7)	2.19 (1.20-4.01)	0.015
Recessive				
del/del+del/ins	65 (69.9)	77 (82.8)	1.00	-
ins/ins	28 (30.1)	16 (17.2)	2.07 (0.99-3.98)	0.057
Overdominant				
del/del+ins/ins	55 (59.1)	60 (64.5)	1.00	-
del/ins	38 (40.9)	33 (35.5)	1.26 (0.69-2.27)	0.546
Allele				
del	92 (49.5)	121 (65.1)	1.00	-
ins	94 (50.5)	65 (34.9)	1.90 (1.25-2.88)	0.003

VEGF, vascular endothelial growth factor; ins, insertion; del, deletion; OR, odds ratio; 95% CI, 95% confidence interval.

findings of Almawi *et al* (32) indicated an association of *VEGF* -460 T/C (rs833061), +398 G/A (rs833068), -583 T/C variants with the risk of RSA. On the contrary, Samli *et al* (39) demonstrated that the -2578 C/A (rs699947), -460 T/C and +936 C/T polymorphisms of the *VEGF* gene were not associated with the risk of RSA; while the -1154 G/A variant was related to the risk (39).

More recently, Shim *et al* (40) investigated the association between *VEGF* promoter polymorphisms -2578 C/A, -1154 G/A, -634 C/G and +936 C/T and RIF. Their findings revealed that the -2578 AA genotype was associated with an increased prevalence of RIF (≥ 4 implantation failures) compared with the CC genotype, whereas the *VEGF* -634 CG+GG genotype was associated with an increased incidence of total RIF and ≥ 4 RIFs compared with the CC genotype.

There are certain limitations to the current study. First, a relatively small sample size was used; second, only one polymorphism of *VEGF* was evaluated, and thus other polymorphisms of this gene should be assessed in equivalent populations; and third, the serum levels of VEGF were not determined to evaluate the association between the genotypes and serum levels of VEGF, which warrants further study.

In conclusion, the present findings support an association between *VEGF* 18-bp ins/del polymorphism and increased risk of RSA. Further association studies on larger sample sizes and different ethnicities are now required to verify the current findings.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article or available from the corresponding author on reasonable request.

Authors' contributions

MH designed the study, analyzed and interpreted data and drafted the manuscript. HD, FB, FT and MT performed experiments and data analysis and gave final approval of the manuscript. MM and GB collected, analyzed and interpreted data, and gave final approval of the manuscript.

Ethics approval and consent to participate

The local research Ethics Committee of Zahedan University of Medical Sciences (Zahedan, Iran) approved the project and informed consent was obtained from all participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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