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

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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.

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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'-AAGATCTGGGTGGATAATC AGACT-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 μl genomic DNA (~100 ng/μl), 1 μl of each primer (10 μM), 10 μl 2X Prime Taq Premix and 7 μl ddH_2O 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 μg/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 ± 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±4.98 years and the control group consisted of 93 unrelated healthy women with a mean age of 30.01±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 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.

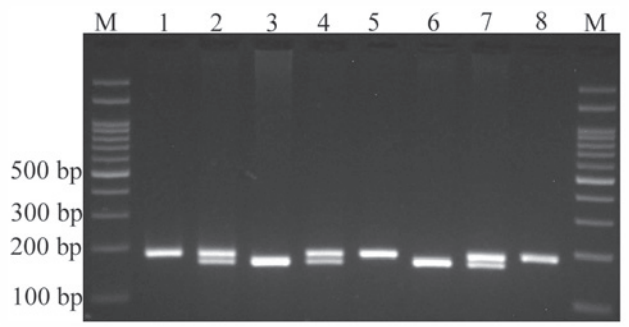

Figure 1. Agarose electrophoresis pattern of polymerase chain reaction amplification products of the vascular endothelial growth factor -2549 ins/del polymorphism. M: DNA marker; lanes 1, 5, 8: Homozygote for ins allele (ins/ins); lanes 2, 4, 7: Heterozygote (ins/del); lanes 3, 6: Homozygote for del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

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. Pereza *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.

VEGF 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.

References

1. Practice Committee of American Society for Reproductive Medicine: Definitions of infertility and recurrent pregnancy loss: A committee opinion. Fertil Steril 99: 63, 2013.
2. Ford HB and Schust DJ: Recurrent pregnancy loss: Etiology, diagnosis, and therapy. Rev Obstet Gynecol 2: 76-83, 2009.
3. Brown S: Miscarriage and its associations. Semin Reprod Med 26: 391-400, 2008.
4. Toth B, Jeschke U, Rogenhofer N, Scholz C, Würfel W, Thaler CJ and Makrigiannakis A: Recurrent miscarriage: Current concepts in diagnosis and treatment. J Reprod Immunol 85: 25-32, 2010.
5. McNamee K, Dawood F and Farquharson R: Recurrent miscarriage and thrombophilia: An update. Curr Opin Obstet Gynecol 24: 229-234, 2012.
6. Rull K, Nagirnaja L and Laan M: Genetics of recurrent miscarriage: Challenges, current knowledge, future directions. Front Genet 3: 34, 2012.
7. Vincenti V, Cassano C, Rocchi M and Persico G: Assignment of the vascular endothelial growth factor gene to human chromosome 6p21.3. Circulation 93: 1493-1495, 1996.
8. Burton GJ, Charnock-Jones DS and Jauniaux E: Regulation of vascular growth and function in the human placenta. Reproduction 138: 895-902, 2009.
9. Amirchaghmaghi E, Rezaei A, Moini A, Roghaei MA, Hafezi M and Aflatoonian R: Gene expression analysis of VEGF and its receptors and assessment of its serum level in unexplained recurrent spontaneous abortion. Cell J 16: 538-545, 2015.
10. Bagheri A, Kumar P, Kamath A and Rao P: Association of angiogenic cytokines (VEGF-A and VEGF-C) and clinical characteristic in women with unexplained recurrent miscarriage. Bratisl Lek Listy 118: 258-264, 2017.
11. Pang LH, Li MJ, Li MQ, Yang DM and Shi L: Vascular endothelial growth factor (VEGF) and the VEGF soluble receptor-1 (sFlt-1) in chorionic villus tissue from Chinese women with early recurrent spontaneous abortion. J Int Med Res 39: 830-837, 2011.
12. Pang L, Wei Z, Li O, Huang R, Qin J, Chen H, Fan X and Chen ZJ: An increase in vascular endothelial growth factor (VEGF) and VEGF soluble receptor-1 (sFlt-1) are associated with early recurrent spontaneous abortion. PLoS One 8: e75759, 2013.
13. Al-Khateeb GM, Mustafa FE, Sater MS and Almawi WY: Effect of the functional VEGFA-583C/T variant on vascular endothelial growth factor levels and the risk of recurrent spontaneous miscarriage. Fertil Steril 95: 2471-2473, 2011.
14. Ghasemi N, Dehghani Firouzabadi R and Ahmadi S: Association of -460C/T and +405 G/C polymorphisms of vascular endothelial growth factor gene and susceptibility to ovarian hyperstimulation syndrome. Int J Reprod Biomed (Yazd) 15: 87-92, 2017.
15. Aggarwal S, Parveen F, Faridi RM, Phadke S, Borkar M and Agrawal S: Vascular endothelial growth factor gene polymorphisms in north Indian patients with recurrent miscarriages. Reprod Biomed Online 22: 59-64, 2011.
16. Pereza N, Ostojić S, Smirčić A, Hodžić A, Kapović M and Peterlin B: The -2549 insertion/deletion polymorphism in the promoter region of the VEGFA gene in couples with idiopathic recurrent spontaneous abortion. J Assist Reprod Genet 32: 1789-1794, 2015.
17. Su MT, Lin SH and Chen YC: Genetic association studies of angiogenesis- and vasoconstriction-related genes in women with recurrent pregnancy loss: A systematic review and meta-analysis. Hum Reprod Update 17: 803-812, 2011.
18. Zhang B, Dai B, Zhang X and Wang Z: Vascular endothelial growth factor and recurrent spontaneous abortion: A meta-analysis. Gene 507: 1-8, 2012.
19. Xu X, Du C, Li H, Du J, Yan X, Peng L, Li G and Chen ZJ: Association of VEGF genetic polymorphisms with recurrent spontaneous abortion risk: A systematic review and meta-analysis. PLoS One 10: e0123696, 2015.
20. Rogers MS and D'Amato RJ: The effect of genetic diversity on angiogenesis. Exp Cell Res 312: 561-574, 2006.
21. Brogan IJ, Khan N, Isaac K, Hutchinson JA, Pravica V and Hutchinson IV: Novel polymorphisms in the promoter and 5' UTR regions of the human vascular endothelial growth factor gene. Hum Immunol 60: 1245-1249, 1999.
22. Yang B, Cross DF, Ollerenshaw M, Millward BA and Demaine AG: Polymorphisms of the vascular endothelial growth factor and susceptibility to diabetic microvascular complications in patients with type 1 diabetes mellitus. J Diabetes Complications 17: 1-6, 2003.
23. Hashemi M, Mokhtari M, Khazaeian S, Bahari G, Rezaei M, Nakhaee A and Taheri M: Evaluation of HLA-G 14-bp ins/del and +3142G>C polymorphisms with susceptibility to recurrent spontaneous abortion. Taiwan J Obstet Gynecol 56: 276-280, 2017.
24. Hashemi M and Mokhtari M: Evaluation of trancobalamin II rs1801198 and transcobalamin II receptor rs2336573 gene polymorphisms in recurrent spontaneous abortion. J Obstet Gynaecol, 2017.
25. Hashemi M, Hanafi Bojd H, Eskandari Nasab E, Bahari A, Hashemzehi NA, Shafieipour S, Narouie B, Taheri M and Ghavami S: Association of adiponectin rs1501299 and rs266729 gene polymorphisms with nonalcoholic fatty liver disease. Hepat Mon 13: e9527, 2013.
26. Rezaei M, Hashemi M, Sanaei S, Mashhadi MA and Taheri M: Association between vascular endothelial growth factor gene polymorphisms with breast cancer risk in an Iranian population. Breast Cancer (Auckl) 10: 85-91, 2016.
27. Barišić A, Pereza N, Hodžić A, Ostojić S and Peterlin B: A single nucleotide polymorphism of DNA methyltransferase 3B gene is a risk factor for recurrent spontaneous abortion. Am J Reprod Immunol 78, e12765, 2017.
28. Zhang Y, Wu YY, Qiao FY and Zeng WJ: Association between p53 polymorphism at codon 72 and recurrent spontaneous abortion. J Huazhong Univ Sci Technolog Med Sci 36: 402-405, 2016.
29. Wang G and Sun J: Interactive effects of Snps located within CD28/B7pathway and environment on susceptibility to recurrent spontaneous abortion. Cell Physiol Biochem 43: 2185-2199, 2017.
30. Rah H, Chung KW, Ko KH, Kim ES, Kim JO, Sakong JH, Kim JH, Lee WS and Kim NK: miR-27a and miR-449b polymorphisms associated with a risk of idiopathic recurrent pregnancy loss. PLoS One 12: e0177160, 2017.
31. Yalcintepe SA, Silan F, Hacivelioglu SO, Uludag A, Cosar E and Ozdemir O: Fetal Vegf genotype is more important for abortion risk than mother genotype. Int J Mol Cell Med 3: 88-94, 2014.
32. Almawi WY, Saldanha FL, Mahmood NA, Al-Zaman I, Sater MS and Mustafa FE: Relationship between VEGFA polymorphisms and serum VEGF protein levels and recurrent spontaneous miscarriage. Hum Reprod 28: 2628-2635, 2013.
33. Watson CJ, Webb NJ, Bottomley MJ and Brenchley PE: Identification of polymorphisms within the vascular endothelial growth factor (VEGF) gene: Correlation with variation in VEGF protein production. Cytokine 12: 1232-1235, 2000.
34. Awata T, Inoue K, Kurihara S, Ohkubo T, Watanabe M, Inukai K, Inoue I and Katayama S: A common polymorphism in the 5'-untranslated region of the VEGF gene is associated with diabetic retinopathy in type 2 diabetes. Diabetes 51: 1635-1639, 2002.
35. Mohammadi M, Bazrafshani MR, Day PJ and Ollier WE: Vascular endothelial growth factor production is regulated by gene polymorphisms. Iran J Immunol 6: 119-129, 2009.
36. Papazoglou D, Galazios G, Papatheodorou K, Liberis V, Papanas N, Maltezos E and Maroulis GB: Vascular endothelial growth factor gene polymorphisms and idiopathic recurrent pregnancy loss. Fertil Steril 83: 959-963, 2005.
37. Saboori S, Noormohammadi Z and Zare-Karizi S: Genetic variation in vascular endothelial growth factor gene and its association with recurrent spontaneous abortion. Bratisl Lek Listy 117: 80-86, 2016.
38. Vagnini LD, Nascimento AM, Canas MC, Renzi A, Oliveira-Pelegrin GR, Petersen CG, Mauri AL, Oliveira JB, Baruffi RL, Cavagna M and Franco JG Jr: The relationship between vascular endothelial growth factor 1154G/A polymorphism and recurrent implantation failure. Med Princ Pract 24: 533-537, 2015.
39. Samli H, Demir BC, Ozgöz A, Atalay MA and Uncu G: Vascular endothelial growth factor gene 1154 G/A, 2578 C/A, 460 C/T, 936 C/T polymorphisms and association with recurrent pregnancy losses. Genet Mol Res 11: 4739-4745, 2012.
40. Shim SH, Kim JO, Jeon YJ, An HJ, Lee HA, Kim JH, Ahn EH, Lee WS and Kim NK: Association between vascular endothelial growth factor promoter polymorphisms and the risk of recurrent implantation failure. Exp Ther Med 15: 2109-2119, 2018.