Original Article

Association of Vascular Endothelial Growth Factor (VEGF) +405 G>C Polymorphism with Endometriosis in an Iranian Population

Memariani, Toktam (B.Sc.)[1]; Salimi Nejad, Kioomars (M.Sc.)[1]; Kamali, Kourosh (M.D., M.P.H.)[1, 5]; Shervin, Adel (M.D.)[2]; Mohajer-Maghari, Behrokh (M.Sc.)[1]; Akhondi, Mohhamad Mehdi (Ph.D.)[1, 3]; Khorram Khorshid, Hamid Reza (M.D., M.P.H., Ph.D.)[1, 4*]

1- Reproductive Biotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran
2- Gynecological Surgery Ward, Tehran Clinic Hospital, Tehran, Iran
3- Avicenna Infertility Clinic, Avicenna Research Institute, ACECR, Tehran, Iran
4- Genetic Research Center, Social Welfare and Rehabilitation Sciences University, Tehran, Iran
5- Department of Epidemiology and Biostatistics, Faculty of Public Health, Tehran University of Medical Sciences, Tehran, Iran

Abstract

Introduction: Angiogenesis, growth of new blood vessels from pre-existing vessels, is a crucial physiological process for tissue regeneration. This state is also seen in pathological processes such as malignancies and endometriosis. Vascular endothelial growth factor (VEGF) is a major mediator of angiogenesis and vascular permeability which is known to play an important role in the development of endometriosis. The aim of this study was to investigate the relationship between +405 G>C VEGF polymorphism and endometriosis in an Iranian population.

Materials and Methods: The study population was comprised of 105 women with and 150 women without laparoscopic evidence of endometriosis. Genomic DNA from blood cells was extracted using salting out method. Genotype and allele frequency of +405 G>C polymorphism was compared between women with endometriosis and the controls using PCR-RFLP. Statistical analysis was performed using SPSS 13.0 software. Chi-squared test and odds ratio plus 95% confidence interval were determined. A p-value less than 0.05 was considered statistically significant.

Results: While the +405 VEGF genotype frequencies in the case group were 41.3% G/G, 46.2% C/G and %12.5 C/C, they were 32% GG, %53.3 GC and 14.7% CC in the control group. The distribution of three genotypes and allele frequencies of +405 G>C VEGF polymorphism between the case and control groups did not demonstrate any significant difference.

Conclusion: In contrast to previous studies, no significant correlation was found between +405 G>C VEGF polymorphism and endometriosis. Since this was the first study in an Iranian population, further investigation with bigger sample sizes may be indicated to be able to generalize the findings.

* *Corresponding Author:* Dr. Hamid Reza Khorram Khorshid, Reproductive Biotechnology Research Center, Avicenna Research Institute (ARI), ACECR, Shahid Beheshti University, Velenjak, P.O. Box: 19615-1177, Tehran, Iran.
E-mail: h.korramkhorshid@avicenna.ac.ir

Received: Dec. 19, 2009
Accepted: Feb. 3, 2010

Keywords: Angiogenesis, Endometriosis, Polymorphism, Vascular endothelial growth factor.
To cite this article: Memariani T, Salimi Nejad K, Kamali K, Shervin A, Mohajer-Maghari B, Akhondi MA, et al. Association of Vascular Endothelial Growth Factor (VEGF) +405 C>G Polymorphism with Endometriosis in an Iranian Population. J Reprod Infertil. 2010;11(1): 33-37.

Downloaded from http://www.jri.ir

Introduction

Endometriosis is defined as the growth of endometrial tissue including endometrial glands and stroma outside the uterine cavity, causing diverse diseases and signs such as infertility, pelvic pain, and dysmenorrhea (1, 2). Depending on the diagnostic method, the prevalence of this disease during reproductive period is reported to be 5 - 10% in the general population. The frequency of this disease among women who visit gynecologists for fallopian tube obstruction varies between 1 - 7% and in infertile women it varies between 9 - 50% (3, 4).

Although, this disease has been described and diagnosed for more than 100 years now, the

mechanisms underlying the development of the disease are still unclear. While the transplantation of endometrial tissue into the pelvic peritoneum via retrograde menstruation is considered as one of the most widely accepted explanation for the pathogenesis of the disease, this physiological process does not lead to the onset of the disease in the majority of cases. Therefore, diverse factors are thought to be involved in the development of the disease, such as environmental, immunological, endocrine and genetic predispositions (5).

Angiogenesis is known to play a key role in the implantation and growth of the endometrial tissue displaced by retrograde menstruation (6, 7). Endometriotic lesions with high proliferative activity have a higher microvessel density compared to lesions with low proliferative activity (8).

Vascular Endothelial Growth Factor (VEGF) is a heparin-binding glycoprotein which is known as one of the strongest factors with angiogenesis properties (6). This glycoprotein plays a fundamental role in regulating angiogenesis and vascular permeability in both physiological and pathological states (9). There are many reasons indicating the role of VEGF in the development of endometriosis, such as expression of VEGF in stromal and epithelial cells and its regulation by estrogen (10, 11). Comparison of endometrial epithelial cells in patients suffering from endometriosis with healthy subjects shows higher levels of VEGF, specifically at the end of the secretory phase of menstrual cycle, and these cells are found more in red than black endometriotic lesions (12, 13). Furthermore, peritoneal cavity fluid in patients with endometriosis shows significantly elevated levels of VEGF compared to that of controls (6, 14, 15). VEGF is also considered as a diagnostic tool and candidate gene for the diagnosis of endometriosis. Based on genetic association studies, a few mutations and single nucleotide polymorphisms (SNPs) found in VEGF gene are regarded functionally important. For example, +405 G>C polymorphism in 5' untranslated region affects the level of VEGF protein expression (16, 17).

Numerous studies have reported that VEGF gene polymorphisms are associated with prostate cancer, development of proliferative diabetic retinopathy and decreased risk of breast cancer (18 - 20).

Recently, the association of -460 C>T VEGF gene polymorphism with endometriosis was demonstrated (21). Furthermore, three research groups which have investigated the existence of any association between endometriosis and +405 G>C polymorphism in Chinese (21), South Indians (5) and Korean women (2), have reported positive findings, but to date, these results have not been confirmed in other populations.

In the present study, the correlation of VEGF gene +405 G>C polymorphism with endometriosis in an Iranian population was assessed.

Materials and Methods

Patients: To assess +405 G>C VEGF gene polymorphism, blood samples were collected from 20 – 50-year old women, who had been referred to Avicenna Infertility Clinic and Tehran Clinic Hospital in Tehran, Iran. One-hundred and five patients with endometriosis and 150 women with no gynecological problems were selected as cases and controls, respectively. The diagnosis of endometriosis was confirmed by laparoscopy.

Women having rheumatoid arthritis, giant cell arthritis, diabetic retinopathy, psoriasis and Behçet's disease were excluded from the study. The protocol of the study was also approved by the Ethics Committee of Avicenna Research Institute. A written informed consent was obtained from all the participants.

Genomic DNA Analysis: Five milliliters of peripheral blood samples were collected in tubes containing 200 μl of 0.5 *M* EDTA. Genomic DNA was extracted from the obtained blood samples using the salting-out method.

Genotyping of the 405 G>C polymorphisms in VEGF gene was determined by employing PCR / RFLP method. One pair of primers (Forward: 5′ CCACTTGAATCGGGCCGACG 3′, Reverse: 5′ GTCTGTCTGTCTGTCCGTCA 3′) was used to amplify the relevant fragment and analyze the +405 C>G variation**.** Each PCR reaction with a total volume of 25 μl, contained 50 *ng* of genomic DNA, 5 *pmol* of each primer, 1.5 *mM* of $MgCl_2$ (Roche, Germany) and one unit of DNA polymerase (Roche, Germany). Amplification was performed in a programmable thermal cycler gradient PCR system (Eppendorf, Germany). DNA was denatured at 94° *C* for 5 minutes. The PCR amplification was carried out for 30 cycles

Downloaded from http://www.jri.ir

(denaturation at 94° *C* for 30 sec, annealing at 61° *C* for 30 sec, extension at 72° *C* for 30 sec and final extension for 7 min at 72° *C*).

The PCR products, harboring the polymorphism at 5' UTR of VEGF gene were analyzed using 1.5% agarose gel electrophoresis followed by ethidium bromide staining and ultraviolet visualization. The PCR products were digested with restriction enzyme BsmFI (New England Biolabs, UK) at 65° *C* overnight for +405 C>G polymorphism, separated by polyacrylamide gel electrophoresis, and identified using silver nitrate staining. The +405 G allele was cut into two fragments of 101 and 181 bp, while the +405 C allele remained uncut (282 bp) (Figure 1).

Statistical analysis: According to the restriction digestion pattern and gel staining analysis, genotypes of the participants were divided into three groups based on the presence or absence of polymorphism: wild-type homozygote, variant homozygote and heterozygote DNAs.

Statistical analysis was performed using SPSS statistical package (V. 13.0). Allele ratios and genotype distributions in the cases and controls were analyzed by logistic regression. GG genotype and G alleles were assumed as reference group in the analysis using Pearson's Chi-square exact test. Odds ratios were calculated with 95% confidence intervals (CIs). P-values less than 0.05 were considered statistically significant.

Results

The +405 VEGF genotype frequencies in the case group were as follows: 41.3% G/G, 46.2% C/G and %12.5 C/C representing 43, 48 and 13 patients respectively. The genotype frequencies in the control group were 32% GG, %53.3 GC and 14.7% CC in 48, 80 and 22 patients, respectively. There were no statistically significant differences between the cases and controls in terms of 405 G/C genotype distributions and allele frequencies (p = 0.267), Table 1.

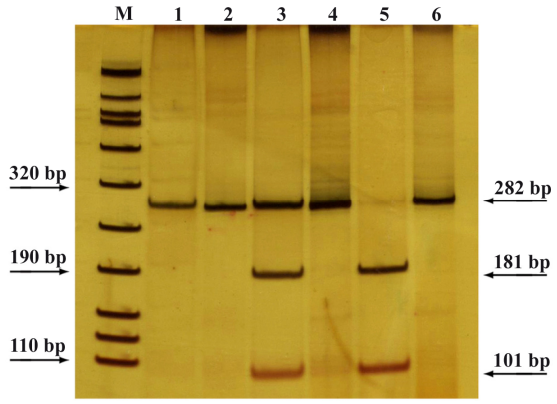


Figure 1. Restriction digestion of PCR products demonstrating the patterns of digestion in different genotypes of VEGF +405 G>C Polymorphism. M: Marker VIII (Roche), 1: Homozygote (variant), 3: Heterozygote (variant and wild type), 5: Homozygote (wild type), 2, 4 and 6: PCR product

Table 1. The distribution of genotype and allele frequencies in the studied groups

Genotype/ Allele	Study Group		P-value	OR
	Patients (*Number*)	Controls (*Number*)		
Genotype				
GG	48	83	Reference Group	
GC	80	48	0.150	0.67 (0.39 - 1.15)
CC	22	13	0.308	0.66 (0.3 - 1.47)
Allele				
G	133	176	Reference Group	
C	75	124	0.267	0.8 (0.55 - 1.17)

Discussion

A key mediator in angiogenesis is vascular endothelial growth factor, as it stimulates endothelial cell proliferation and migration and increases vascular permeability. Recent studies have shown the association of VEGF gene polymorphisms with the development of diseases in which angiogenesis plays an important role. In the present study, the possible correlation between endometriosis and +405 G>C VEGF gene polymorphism was studied in an Iranian population.

Awata et al. showed that +405 C/C VEGF gene polymorphism versus G/G genotype in type II diabetes mellitus increases the possibility of retinopathy of about three folds (22). Patients with psoriasis showed a significant increase in the frequency of C allele and +405 C/C genotype of VEGF gene compared to healthy controls (23).

VEGF gene is located on chromosome 6p21.3. This gene consists of 8 exons and the gene transcript undergoes alternative splicing processes to produce a protein family (24). There are a few transcription factor binding sites in 5' untranslated region in VEGF gene (25). Hence, polymorphisms in this region result in different levels of gene

Downloaded from http://www.jri.ir

expression in people, causing various ranges of diseases.

Three separate research groups carried out case-control studies on various populations to determine the association between endometriosis and +405 G>C VEGF gene polymorphism. Watson et al showed that the G allele at +405 possibly resided within a potential myeloid zinc finger protein (MZF1) binding site and had direct effects on the level of gene transcription and ultimately on LPS-stimulated VEGF protein expression in peripheral blood mononuclear cells (PBMCs) (17).

Bhanoori et al. evaluated 215 women with endometriosis and 210 with no evidence of the disease and reported significant difference between the prevalence of endometriosis and +405 G>C polymorphism ($p = 0.002$) in a way that the G allele frequency was 81.7% in rAFS stage III – IV endometriosis while it was 72.7% in the controls (5). In contrast, Kim et al. selected 215 women with an advanced stage of endometriosis as the case group, 219 women without endometriosis and 70 fertile women as the control group and showed that the frequency of +405 C/C genotype in patients with severe endometriosis was more than that of the control group (2). Similarly, Gentilini et al. examined 203 Italian women affected by endometriosis and 140 women without laparoscopic evidence of the disease and confirmed the possibility of endometriosis to be almost two times higher in patients carrying the C allele. Moreover, they showed that the existence of this allele could be a risk factor for the implantation of endometrial fragments which are refluxed into the peritoneal cavity (26). On the other hand, Awata et al. showed a high serum VEGF levels in healthy Japanese population with +405 C/C genotype distribution.

Our findings were not in lines with previously stated studies perhaps due to difference in geographical location or the genetic basis of the studied population, as the possibility of a disease occurrence depends on both its prevalence in the population and environmental factors (27). Race difference and genetic backgrounds are important factors in genetic association studies. Therefore selection of different control groups might lead to dissimilar results (27). For example, in the present study, the control group was comprised of women without the disease as confirmed by laparoscopy, while in a study on the Japanese population female neonates were selected as the control group (9). On the other hand, in a study carried out on the Korean population, the control group was made up of women who had either benign ovarian cysts, infertility, pelvic pain or dysmenorrhea (2).

Conclusion

In the present study, the genotype and allele distribution of the +405 C>G VEGF gene polymorphism was not significantly different between the cases and the controls. However, further studies on larger Iranian populations may be necessary to confirm these observations.

Acknowledgement

The authors would like to thank Avicenna Research Institute for supporting this study, Mrs. Jamshidi and other operating room personnel of the Gynecological Surgery Ward at Tehran Clinic Hospital and also Mrs. Elham Savad Shirazi, Mrs. Bahareh Beik at Avicenna Infertility Clinic for help in collecting the study subjects.

References

1. Olive DL, Schwartz LB. Endometriosis. N Engl J Med. 1993;328(24):1759-69.
2. Kim SH, Choi YM, Choung SH, Jun JK, Kim JG, Moon SY. Vascular endothelial growth factor gene +405 C/G polymorphism is associated with susceptibility to advanced stage endometriosis. Hum Reprod. 2005;20(10):2904-8.
3. Duignan NM, Jordan JA, Coughlan BM, Logan-Edwards R. One thousand consecutive cases of diagnostic laparoscopy. J Obstet Gynaecol Br Commonw. 1972;79(11):1016-24.
4. Williams TJ, Pratt JH. Endometriosis in 1,000 consecutive celiotomies: incidence and management. Am J Obstet Gynecol. 1977;129 (3):245-50.
5. Bhanoori M, Arvind Babu K, Pavankumar Reddy NG, Lakshmi Rao K, Zondervan K, Deenadayal M, et al. The vascular endothelial growth factor (VEGF) +405 G>C 5'-untranslated region polymorphism and increased risk of endometriosis in South Indian women: a case control study. Hum Reprod. 2005;20(7):1844-9.
6. Taylor RN, Lebovic DI, Mueller MD. Angiogenic factors in endometriosis. Ann N Y Acad Sci. 2002; 955:89-100.

Downloaded from http://www.jri.ir

7. McLaren J. Vascular endothelial growth factor and endometriotic angiogenesis. Hum Reprod Update. 2000;6(1):45-55.
8. Bourlev V, Volkov N, Pavlovitch S, Lets N, Larsson A, Olovsson M. The relationship between microvessel density, proliferative activity and expression of vascular endothelial growth factor-A and its receptors in eutopic endometrium and endometriotic lesions. Reproduction. 2006;132(3):501-9.
9. Ikuhashi Y, Yoshida S, Kennedy S, Zondervan K, Takemura N, Deguchi M, et al. Vascular endothelial growth factor +936 C/T polymorphism is associated with an increased risk of endometriosis in a Japanese population. Acta Obstet Gynecol Scand. 2007;86(11):1352-8.
10. Hyder SM, Nawaz Z, Chiappetta C, Stancel GM. Identification of functional estrogen response elements in the gene coding for the potent angiogenic factor vascular endothelial growth factor. Cancer Res. 2000;60(12):3183-90.
11. Mueller MD, Vigne JL, Minchenko A, Lebovic DI, Leitman DC, Taylor RN. Regulation of vascular endothelial growth factor (VEGF) gene transcriptt-tion by estrogen receptors alpha and beta. Proc Natl Acad Sci U S A. 2000;97(20):10972-7.
12. Donnez J, Smoes P, Gillerot S, Casanas-Roux F, Nisolle M. Vascular endothelial growth factor (VEGF) in endometriosis. Hum Reprod. 1998;13 (6):1686-90.
13. Tan XJ, Lang JH, Liu DY, Shen K, Leng JH, Zhu L. Expression of vascular endothelial growth factor and thrombospondin-1 mRNA in patients with endometriosis. Fertil Steril. 2002;78(1):148-53.
14. McLaren J, Prentice A, Charnock-Jones DS, Millican SA, Muller KH, Sharkey AM, et al. Vascular endothelial growth factor is produced by peritoneal fluid macrophages in endometriosis and is regulated by ovarian steroids. J Clin Invest. 1996;98(2):482-9.
15. Matalliotakis IM, Goumenou AG, Koumantakis GE, Neonaki MA, Koumantakis EE, Dionyssopoulou E, et al. Serum concentrations of growth factors in women with and without endometriosis: the action of anti-endometriosis medicines. Int Immunopharmacol. 2003;3(1):81-9.
16. Brogan IJ, Khan N, Isaac K, Hutchinson JA, Pravica V, Hutchinson IV. Novel polymorphisms in the promoter and 5' UTR regions of the human vascular endothelial growth factor gene. Hum Immunol. 1999;60(12):1245-9.
17. Watson CJ, Webb NJ, Bottomley MJ, Brenchley PE. Identification of polymorphisms within the vascular endothelial growth factor (VEGF) gene: correlation with variation in VEGF protein production. Cytokine. 2000;12(8):1232-5.
18. Ray D, Mishra M, Ralph S, Read I, Davies R, Brenchley P. Association of the VEGF gene with proliferative diabetic retinopathy but not proteinuria in diabetes. Diabetes. 2004;53(3):861-4.
19. Lin CC, Wu HC, Tsai FJ, Chen HY, Chen WC. Vascular endothelial growth factor gene-460 C/T polymorphism is a biomarker for prostate cancer. Urology. 2003;62(2):374-7.
20. Krippl P, Langsenlehner U, Renner W, Yazdani-Biuki B, Wolf G, Wascher TC, et al. A common 936 C/T gene polymorphism of vascular endothelial growth factor is associated with decreased breast cancer risk. Int J Cancer. 2003;106(4):468-71.
21. Hsieh YY, Chang CC, Tsai FJ, Yeh LS, Lin CC, Peng CT. T allele for VEGF gene-460 polymorphism at the 5'-untranslated region: association with a higher susceptibility to endometriosis. J Reprod Med. 2004;49(6):468-72.
22. Awata T, Inoue K, Kurihara S, Ohkubo T, Watanabe M, Inukai K, et al. A common polymorphism in the 5'-untranslated region of the VEGF gene is associated with diabetic retinopathy in type 2 diabetes. Diabetes. 2002;51(5):1635-9.
23. Young HS, Summers AM, Bhushan M, Brenchley PE, Griffiths CE. Single-nucleotide polymorphisms of vascular endothelial growth factor in psoriasis of early onset. J Invest Dermatol. 2004;122(1):209-15.
24. Vincenti V, Cassano C, Rocchi M, Persico G. Assignment of the vascular endothelial growth factor gene to human chromosome 6p21.3. Circulation. 1996;93(8):1493-5.
25. Akiri G, Nahari D, Finkelstein Y, Le SY, Elroy-Stein O, Levi BZ. Regulation of vascular endothelial growth factor (VEGF) expression is mediated by internal initiation of translation and alternative initiation of transcription. Oncogene. 1998;17 (2):227-36.
26. Gentilini D, Somigliana E, Vigano P, Vignali M, Busacca M, Di Blasio AM. The vascular endothelial growth factor +405 G>C polymorphism in endometriosis. Hum Reprod. 2008;23(1):211-5.
27. Zondervan KT, Cardon LR, Kennedy SH. What makes a good case-control study? Design issues for complex traits such as endometriosis. Hum Reprod. 2002;17(6):1415-23.

Downloaded from http://www.jri.ir