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Investigation of *VEGF* gene polymorphism rs35569394 in endometriosis

Investigação do polimorfismo rs35569394 do gene VEGF *em endometriose*

Lysandra T. Bruno; Marco Fábio Prata-Lima; Mariangela T. Ruiz-Cintra; Alessandra Bernadete Trovó de Marqui

Universidade Federal do Triângulo Mineiro (UFTM), Minas Gerais, Brazil.

ABSTRACT

Introduction: Endometriosis is a chronic gynecological condition characterized by the presence of endometrial tissue in locations outside the uterus. Its symptoms include dysmenorrhea, dyspareunia, chronic pelvic pain, infertility, dysuria, and dyschezia. Several studies have related the vascular endothelial growth factor (*VEGF*) gene polymorphisms as a factor for the development of endometriosis. **Objectives:** The aim of this study was to compare the frequency of the *VEGF* gene polymorphism rs35569394 in women with endometriosis and a control group, as well as to investigate the association of this polymorphism with the risk of endometriosis. **Methods:** This case-control study included 45 endometriosis patients (cases) and 78 controls. The molecular analysis was performed by polymerase chain reaction (PCR). The chi-square test was used to compare the genotype and allele frequencies, and the Hardy-Weinberg equilibrium was also tested using the chi-square test, considering the significance of $p < 0.05$. **Results:** The frequency of the II, ID and DD genotype was 24.4% versus 26.9%; 35.6% versus 37.2%; and 40% versus 35.9%, in patients and the control group, respectively. The allele frequencies were I: 0.42 (cases) and 0.45 (control); D: 0.58 (cases) and 0.55 (control). The allele and genotype frequency of *VEGF* -2549I/D polymorphism was not different between women with endometriosis and the control. **Conclusion:** In the present study, the frequency of the *VEGF* gene polymorphism rs35569394 is similar between cases and control. This genetic variant is not associated with endometriosis in the investigated population.

Key words: endometriosis; genetic polymorphism; polymerase chain reaction.

INTRODUCTION

Endometriosis is a chronic gynecological condition characterized by the presence of endometrial tissue in ectopic locations. Its symptoms include dysmenorrhea, dyspareunia, chronic pelvic pain, infertility, dysuria and dyschezia[1]. Due to the symptomatology, patients usually exhibit high levels of depression, anxiety[2] and sexual dysfunction[3].

Endometriosis presents characteristics similar to neoplasia, since neovascularization is needed for the implantation and development of endometrial tissue[4] and therefore, angiogenesis regulating genes, such as vascular endothelial growth factor (*VEGF*), may be related to the development of the disease. A recent review[5] presents an overview of the basic mechanisms of vascularization in endometriosis, with particular emphasis on its future clinical implications in the diagnosis and therapy of the disease. The authors conclude that the establishment of approaches based on vascularization in the management of endometriosis is still a great challenge. For diagnostic purposes, reliable angiogenic and vasculogenic biomarkers with high sensitivity and specificity must be identified[5].

The *VEGF* gene is highly polymorphic, and several studies have related the polymorphisms in this gene as a factor for the development of endometriosis[4]. An insertion/deletion (I/D) polymorphism was identified at position -2549 of the promoter region of the *VEGF* gene (rs35569394) and is characterized by an 18 base pairs (bp) deletion at that region (D allele). This genetic variant has been studied and related to several conditions such as Behçet's disease[6, 7], renal disease[8], breast cancer[9], diabetic retinopathy[10], diabetic nephropathy[11], recurrent spontaneous abortion[12], preeclampsia[13] and uterine fibroids (leiomyomas)[14]. However, this polymorphism was not associated with hepatocellular carcinoma[15], recurrent miscarriage[16],

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essential hypertension[17] and systemic lupus erythematosus[18]. However, we are not aware that studies on the relationship of this polymorphism with endometriosis have been carried out.

It is worth mentioning that a recent review has shown that there is a growing and significant scientific production on the role of genetic polymorphisms or single nucleotide polymorphisms (SNPs) in the etiology of endometriosis, stating their participation in the development of this condition[19]. Considering the assumption and the numerous scientific evidence of the contribution of the *VEGF* gene to the pathogenesis of endometriosis, the present research is justified.

OBJECTIVES

The aim of this study was to compare the frequency of the *VEGF* gene polymorphism rs35569394 in women with and without endometriosis, as well as to verify its association with this condition.

METHODS

This project was approved by the Research Ethics Committee [Comitê de Ética em Pesquisa (CEP)] of the Universidade Federal do Triângulo Mineiro (UFTM) – protocol no. 1628. Forty-five samples of women with endometriosis (age ranged from 24 to 58 years) and 78 samples of women without endometriosis (age ranged from 22 to 70 years) from the Gynecology and Obstetrics Outpatient Clinic of the UFTM were evaluated. The inclusion criteria were that the woman must has performed laparoscopy or laparotomy that allowed the confirmation of the presence of endometriosis (patients) or their absence (control), and to voluntarily participate in the study by signing the informed consent form. Women who did not meet the criteria previously described were excluded. The period for data collection was 18 months.

The main surgical indications were chronic pelvic pain, dysmenorrhea and dyspareunia. According to the criteria established by the American Society of Reproductive Medicine[20], 47% of women exhibited stages III-IV (moderate-severe endometriosis) and for 38% of patients, this information was not available in the medical record. Only 15% of the patients exhibited minimal-to-mild endometriosis (stages I-II). In the present study, laparoscopy combined with histological confirmation was the standard method for diagnosis of endometriosis.

Participants' deoxyribonucleic acid (DNA) was extracted from peripheral blood according to the technique described by Miller *et al*. (1988)[21], quantified in spectrophotometer and amplified by polymerase chain reaction (PCR).

The polymorphism rs35569394 in the promoter region of the *VEGF* gene was amplified using the following primer sequence: sense primer: 5' GCT GAG AGT GGG GCT GAC TAG GTA 3' and antisense primer: 5' GTT TCT GAC CTG GCT ATT TCC AGG 3'. PCR conditions were 1× buffer, 2 mM MgCl2, 0.2 uM of each deoxyribonucleotide triphosphate (dNTP), 20 pmols of each *primer*, 1 U of Taq DNA polymerase and approximately 100 ng of DNA. Amplification occurred with initial denaturation at 95°C for 10 minutes, followed by 35 cycles with denaturation at 95°C for 45 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 45 seconds, and finally extension at 72°C for 10 minutes. The deletion of 18 bp allele produces a 211 bp fragment, and the insertion, a 229 bp band, that is, three genotypes will be possible: DD (211 bp band), II (229 bp band) and ID (two bands, one of 211 bp and one of 229 bp).

For the determination of the genotypes, electrophoresis was performed on 5% polyacrylamide gel stained with silver nitrate. The staining steps were: 1. fixation solution (83.3 ml of ethanol; 3.33 ml of acetic acid, distilled water sufficient for 500 ml qsp for 10 minutes; 2. silver solution (1 g silver nitrate; distilled water 500 ml qsp) for 10 minutes; 3. two quick washes with distilled water; 4. developer solution (15 g NaOH; 3.33 ml formaldehyde; distilled water 500 ml qsp) for 5 minutes; and 5. storage in fixation solution.

Statistical analysis used the chi-square test for comparison of genotype and allele frequencies; the Hardy-Weinberg equilibrium (HWE) was also tested using the chi-square test, considering significance of $p < 0.05$. The statistical power obtained was 85%, using the GPower program.

RESULTS

The distribution of genotypes and allele frequency in patients and controls is shown in the **Table**.

Statistically, there was no significant difference between the two groups regarding the distribution of genotype ($\chi^2 = 0.216$; $p = 0.90$) and allele ($\chi^2 = 0.325$; $p = 0.57$) frequencies of the *VEGF* gene polymorphism -2549 I/D in patients and control, respectively. The distribution of the genotype was in the Hardy-Weinberg equilibrium in patients ($\chi^2 = 3.31$; $p = 0.07$), but not in the control group ($\chi^2 = 4.89$; $p = 0.03$).

TABLE – Genotype and allele frequency of study and control groups

	Endometriosis *n* (%)	Control *n* (%)
	Genotype frequency	
II	11 (24.4)	21 (26.9)
ID	16 (35.6)	29 (37.2)
DD	18 (40)	28 (35.9)
Total	45	78
	Allele frequency	
I	0.42	0.45
D	0.58	0.55

DISCUSSION

The *VEGF* gene is of great importance for the development of endometriosis, and some polymorphisms of this gene have been associated with this disease, such as *VEGF* +405 G>C, *VEGF* +936 C/T, *VEGF* -1154G>A and *VEGF* -2578C>A[22-24]. On the other hand, the *VEGF* gene polymorphism rs35569394 was not investigated in patients with endometriosis, but in several biological conditions[6-18], with controversial results. The present study is the first one to investigate it in endometriosis, however, no association was found between this polymorphism and the disease. According to the literature, most of the published studies positively associated this polymorphism with the conditions investigated[6-14], some of them with emphasis on II genotype and/or I allele[6, 7, 9, 14] and others for the genotype and allele DD and D, respectively[8, 10, 11-13].

The **Board** compares the genotype and allele frequencies of our study with those published in the literature that did not associate the polymorphism with the conditions investigated.

According to the literature, there were no deviations of the HWE among the studied groups[9, 10, 12, 13, 15], disagreeing with the present study, which showed deviations in the control group. HWE is used to check if frequencies remain unchanged over generations. HWE deviations suggest that there is, for example, some sort of selection acting in the population, or that there is migration, or inbreeding, or these phenomena associated[25].

The present study was presented as a limitation to the casuistry. It is worth noting that the gold standard for the diagnosis of endometriosis is laparoscopy, a surgical procedure performed in view of strong clinical suspicion. This factor interferes significantly in obtaining a larger sample size. However, there are recent studies published in the literature on genetic polymorphisms in endometriosis that investigated 52[26, 27] and 54[28] patients versus 42[26], 46[28] and 63[27] control. In our study, 123 women (45 patients and 78 control) were investigated, and the statistical power obtained guarantees the reliability/robustness of the results found.

Further studies of this SNP in other populations are suggested to clarify the real contribution of the *VEGF* gene polymorphism rs35569394 to the etiology of endometriosis. However, it is noteworthy that this scientific study is pioneering on this polymorphism in endometriosis.

CONCLUSION

In the present study, the frequency of the *VEGF* gene polymorphism rs35569394 is similar between cases and control. This genetic variant is not associated with endometriosis in the investigated population.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

BOARD – Genotype and allele frequencies from different studies that did not associate the *VEGF* -2549 I/D polymorphism to investigated diseases

Study	Sample	Genotype frequency	Allele frequency
Present study	45 cases of patients with endometriosis	II (24.4%) ID (35.6%) DD (40%)	I (0.42) D (0.58)
	78 control	II (26.9%) ID (37.2%) DD (35.9%)	I (0.45) D (0.55)
He *et al*. (2010)[15]	206 patients with hepatocellular carcinoma	II (7.3%) ID (35.4%) DD (57.3%)	I (0.25) D (0.75)
	302 control	II (6.6%) ID (37.7%) DD (55.6%)	I (0.25) D (0.75)
Aggarwal *et al*. (2011)[16]	200 women with recurrent miscarriage	II (30%) ID (47.5%) DD (22.5%)	I (0.54) D (0.46)
	200 control	II (28%) ID (49%) DD (23%)	I (0.53) D (0.48)
Timasheva *et al*. (2017)[17]	216 men with essential hypertension	II (27.7%) ID (51%) DD (21.3%)	Information not available
	314 healthy individuals	II (19.2%) ID (53%) DD (27.8%)	
Cassemiro *et al*. (2017)[18]	61 patients with systemic lupus erythematosus	II (15.7%) ID (40%) DD (44.3%)	I (0.49) D (0.51)
	33 control (healthy individuals)	II (27.3%) ID (48.5%) DD (24.2%)	I (0.52) D (0.48)

VEGF: vascular endothelial growth factor.

RESUMO

Introdução: *A endometriose é uma condição ginecológica crônica caracterizada pela presença de tecido endometrial em sítios extrauterinos. Seus sintomas incluem dismenorreia, dispareunia, dor pélvica crônica, infertilidade, disúria e disquezia. Diversos estudos têm relacionado os polimorfismos no gene* vascular endothelial growth factor (VEGF) *como um fator para o desenvolvimento da endometriose.* ***Objetivos:*** *O objetivo deste estudo foi comparar a frequência do polimorfismo rs35569394 do gene* VEGF *em mulheres com endometriose e controle, bem como investigar a associação deste polimorfismo ao risco de endometriose.* ***Métodos:*** *Este estudo caso-controle incluiu 45 pacientes com endometriose (casos) e 78 controle. A análise molecular foi realizada por reação em cadeia da polimerase (PCR). O teste do qui-quadrado foi empregado para comparar as frequências genotípicas e alélicas, e o equilíbrio de Hardy-Weinberg foi testado também por meio do teste do qui-quadrado, considerando a significância de* $p < 0,05$. ***Resultados:*** *A frequência do genótipo II, ID e DD foi de 24,4%* versus *26,9%; 35,6%* versus *37,2%; e 40%* versus *35,9%, em pacientes e controle, respectivamente. As frequências alélicas foram I: 0,42 (casos) e 0,45 (controles) e D: 0,58 (casos) e 0,55 (controle). A frequência de alelos e genótipos do polimorfismo* VEGF *-2549I/D não foi diferente entre as mulheres com endometriose e as controle.* ***Conclusão:*** *Na casuística analisada, a frequência do polimorfismo rs35569394 do gene* VEGF *é semelhante entre casos e controle. Essa variante genética não está associada à endometriose na população investigada.*

Unitermos: *endometriose; polimorfismo genético; reação em cadeia da polimerase.*

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CORRESPONDING AUTHOR

Alessandra Bernadete Trovó de Marqui

Instituto de Ciências Biológicas e Naturais; Departamento de Patologia, Genética e Evolução; Campus I; Praça Manoel Terra, 3330; CEP: 38015-050; Uberaba-MG, Brasil; Phone: +55 (34) 3700-6434; Fax: +55 (34) 3700-6466; e-mail: alessandratrovo@hotmail.com.