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Relationship between endometriosis and vaspin *RS2236242* gene polymorphism

Vztah mezi endometriózou a polymorfizmem genu pro vaspin *RS2236242*

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Summary: **Objective:** The aim of this study is to investigate the relationship between endometriosis and the vaspin *RS2236242* gene polymorphism. **Materials and methods:** This prospective cross-sectional case-control study included patients with grade 4 endometriosis and a healthy control group. Vaspin *RS2236242* gene polymorphism was evaluated in these study groups. **Results:** Thirty eight endometriosis individuals and 17 women from the control group in the study. The group of individuals with endometriosis exhibited similar characteristics to the control group in terms of sex, body mass index (BMI), and age (control mean age: 29.6 ± 4.62 years; BMI: 24.02 kg/m^2; endometriosis mean age: 30.4 ± 5.01 years; BMI: 23.63 kg/m^2). According to the statistical analysis, there was a significant difference in the genotype distribution of the vaspin *RS2236242* polymorphism between people with endometriosis and controls (P = 0.027). Also, the AT genotype was more likely to cause endometriosis than the OR: 2.474 (95% CI 0.668–9.169) genotypewhen we looked at the genotypes' relative risk ratio for endometriosis. Significant differences were observed in total AT and TT genotype frequencies between cases and controls (OR = 2.31; 95% CI 0.86–0.92; P = 0.03). AT and TT genotypes were associated with endometriosis risk. **Conclusion:** A significant association was observed between vaspin *RS2274907* A/T polymorphism and the probability of developing endometriosis.

Key words: endometriosis – vaspin *RS2274907* – SNP – DNA isolation

Souhrn: **Cíl:** Cílem této studie je prozkoumat vztah mezi endometriózou a polymorfizmem genu pro vaspin *RS2236242*. **Materiál a metody:** Tato prospektivní průřezová studie případů a kontrol zahrnovala pacientky s endometriózou 4. stupně a kontrolní skupinu zdravých žen. V těchto studijních skupinách byl hodnocen polymorfizmus genu pro vaspin *RS2236242*. **Výsledky:** Studie se zúčastnilo 38 jedinců s endometriózou a 17 žen z kontrolní skupiny. Skupina žen s endometriózou vykazovala podobné charakteristiky jako kontrolní skupina, pokud jde o pohlaví, index tělesné hmotnosti (BMI) a věk (průměrný věk kontroly: 29,6 ± 4,62 let; BMI: 24,02 kg/m^2; průměrný věk žen s endometriózou: 30,4 ± 5,01 let; BMI: 23,63 kg/m^2). Podle statistické analýzy byl významný rozdíl v distribuci genotypu polymorfizmu pro vaspin *RS2236242* mezi ženami s endometriózou a kontrolami (p = 0,027). Genotyp AT způsoboval endometriózu s vyšší pravděpodobností než genotyp TT (OR 2,474; 95% CI 0,668–9,169), když jsme sledovali poměr relativního rizika endometriózy genotypů. Mezi případy a kontrolami byly pozorovány signifikantní rozdíly v celkové četnosti genotypů AT a TT (OR 2,31; 95% CI 0,86–0,92; p = 0,03). Genotypy AT a TT byly spojeny s rizikem endometriózy. **Závěr:** Byla pozorována významná souvislost mezi polymorfizmem pro vaspin *RS2274907* A/T a pravděpodobností rozvoje endometriózy.

Klíčová slova: endometrióza – vaspin *RS2274907* – SNP – izolace DNA

Introduction

Endometriosis is a benign gynecological disease affecting approximately 6–10% of cases in women of reproductive age [1]. Endometriosis is a complex disease resulting from estrogen-dependent, chronic inflammatory conditions. Inflammation caused by the effects of estrogen through the estrogen receptor β is the underlying main process [2]. Although endometriosis affects primarily pelvic organs and ovaries, it is a systemic disease. The exact molecular and pathophysiological pathways of disease are not clearly determined. It was revealed that heredity has a 50% effect in twin studies, so the etiology of endometriosis may be related to genetic factors [3]. Genome-wide association studies (GWASs) have helped us learn more about the genetic and epigenetic aspects of

endometriosis. These studies found that single nucleotide polymorphisms (SNPs) were strongly linked to the risk of getting endometriosis [4,5].

Vaspin is a recently discovered adipokine that is released from visceral adipose tissue. It consists of 415 amino acids [6,7]. This substance belongs to the group of serine protease inhibitors, and its levels in the blood are linked to inflammation. Scientists have discovered that this adipokine has anti-inflammatory, anti-migratory, and anti-apoptotic properties on vascular endothelial and smooth muscle cells [8,9]. The vaspin gene is situated on chromosome 14q32.13 and is composed of 6 exons and 5 introns [10].

The vaspin gene contains two intronic polymorphisms, specifically intron 2 *RS77060950* G/T and intron 4 *RS2236242* A/T, which have been linked to various diseases, including polycystic ovary syndrome (PCOS) [11], type 2 diabetes mellitus metabolic syndrome, obesity [12,13], Coronary Artery Disease (CAD), and Nonalcoholic Fatty Liver Disease (NAFLD) [14].

Our objective was to examine the distribution of genotypes and high-risk alleles of vaspin in endometriosis. In addition, we also performed the correlation of genotype and stage of endometriosis with various parameters to understand the relationship between them.

Materıals and methods

Working population

The study we conducted was a cross-sectional case-control study, which included 43 individuals with endometriosis and 17 control cases. Since DNA isolation could not be performed in five cases, the study was continued with 17 controls and 38 endometriosis cases. The study was carried out in the gynecology and obstetrics clinic of Selcuk University Medical Faculty between November 20, 2019 and May 20, 2020. In this study, all procedures involving participants complied with the ethical standards of the Selcuk Medical Sciences University ethics committee, the Helsinki Declaration, and the ethical standards of 1964. This research was supported by Selcuk University Scientific Research Projects Coordination with the project number 19102061. All individual participants who took part in the study provided informed consent.

The stage of the disease in endometriosis cases was assessed using the new American Society for Reproductive Medicine categorization system (ASRM 1996). The study exclusively selected women diagnosed with stage IV endometrioma.

Blood from patients with endometriosis and control cases was collected in an EDTA anticoagulant tube and stored at –20 °C until genotyping.

Reagents used

DNA isolation

High Pure PCR Template Preparation Kit Cat. No. 11 796 828 001 (ROCHE brand) was used.

Vial/cap label contents/function is described below.

1. White: Tissue Lysis Buffer is a solution containing 20 mL of the following components: 4 M urea, 200 mM Tris, 20 mM NaCl, and 200 mM EDTA, with a pH of 7.4 at 25°C.
2. Green: Binding Buffer • The solution consists of 20 milliliters of a mixture containing 6 molar guanidine-HCl, 10 millimolar urea, 10 millimolar Tris-HCl, and 20% Triton X-100 (volume/volume), with a pH of 4.4 at 25 degrees Celsius.
3. Pink: Proteinase K. The product is a lyophilized recombinant PCR-grade substance that is used for sample lysis and the inactivation of endogenous DNase.

4a. Black: Inhibitor Removal Buffer • Combine 33 ml with an additional 20 mL of 100% ethanol. This will result in a final concentration of 5 M guanidine-HCl and 20 mM Tris-HCl at a pH 6.6 (25°C) when the ethanol is added.

4b. Blue: Wash Buffer • Add 20 ml of 100% ethanol, then 80 ml. • After the addition of ethanol, the final concentrations of NaCl and Tris-HCl will be 20 mM and 2 mM, respectively, with a pH of 7.5 at 25°C.

5. Colorless Elution Buffer • The solution contains 40 mL of Tris-HCl solution with a concentration of 10 millimolars and a pH of 8.5 at 25 degrees Celsius. Filter tubes with a high level of purity. There are two bags containing 50 polypropylene tubes, each of which has two layers of glass fiber fleece. These tubes may hold a sample volume of up to 700 µL. There are eight bags containing 50 polypropylene tubes, each with a volume of 2 mL.

Before you start the experiment

The dry heat block is brought up to 70 °C.

The oven is brought up to 37 °C.

Proteinase K is put into 4.5 mL distilled water proteinase K bottle (pink cap). This solution is cut into small pieces and divided into Eppendorf tubes and stored at –15/–25 °C. It is dissolved by removing the appropriate amount.

Inhibitor removal buffer is put into a 20 mL pure ethanol inibitor removal buffer (black cap) bottle and mixed gently. Thus, the solution is ready.

Wash buffer is put in an 80 mL pure ethanol wash buffer bottle (blue cap) and mixed gently. Thus, the solution is ready.

Two elution buffers are put into 1.5 mL Eppendorf tubes and placed in a dry heat block at 70 °C for later use.

Necessary materials

1-İsopropanol.

Other materials required

96–100% ethanol (purity suitable for molecular biology use):

- sterile dH_2O;
- heater block or water bath;
- vortex device;

- microcentrifuge;
- microcentrifuge tubes (1.5 mL);
- micropipette set and filter micropipette tips.

Procedure

1. Sample + 200 µL. Binding Buffer (green cap) + 40 µL. Put in proteinase K and set it at 70 °C.
   Meanwhile, the number of patients in an Eppendorf tube was put in the X200 µL elution buffer (colorless c).
   1. Sample + 200 µL Binding Buffer (green cap) + 40 µL. Put in proteinase K and set it at 70 °C. Capped tube is heated to 70 °C.
2. For samples over 100 µL, add isopropanol and mix. Put the sample number on the bottom of the filtered tube; the lid of the filtered tube was closed, and the number was written on it.
3. Tubes containing this combination were placed in a centrifuge and subjected to a centrifugal force of 8,000 g for 1 min. The lower tube was discarded, and was replaced with a new tube.
4. Filter tube. The inhibitor removal buffer (contained in a tube with a black top) was placed upside down and then subjected to centrifugation with a force of 8,000 times the acceleration due to gravity for 1 min. The lower tube was discarded, and was replaced with a new tube.
5. 500 µL was put into the filter tube. Wash buffer (blue-capped tube) was placed and centrifuged at 8,000 g for 1 min by overturning. The bottom tube was discarded, and was replaced with a clean tube.
   Repeat steps 6–5, then pour the liquid into the bottom tube, place it under the repeating filter tube, and re-centrifuge at 13,000 Xg at maximum speed.
7. Normal 1.5 mL solution. The Eppendorf tube was taken, and labeled with a number written on the cap. The filtered tube was placed in this Eppendorf tube. Over 200 µL solution. The heated elution buffer (colorless cap tube) was placed at 70 °C and centrifuged at 8,000 × g for 1 min (this stage was 2 × 200).

Primer and probe design

All amplification primers are standard phosphoramide chemistry (MWG-Biotech) and all VIC/FAM-labeled probes are synthesized by Thermo Fisher and purified by reverse-phase HPLC. One set of primary probes was used for vaspine in amplification. Primary probes marked VIC/FAM were used. Nucleotide A was detected with the VIC probe, and the T nucleotide was detected with the FAM probe.

PCR conditions

Concentration and quantities of materials used for the *RS2236242* analysis are given in Tab. 1, while PCR conditions for polymorphism analysis are given in Tab. 2.

Genotype determination (end point analysis)

VIC and FAM hydrolysis probes were used in genotype determination, which was done by using the binding feature of the probes. One probe binds to nucleotide A, and the other binds to T nucleotide. End-point analysis makes use of this difference and distinguishes between natural and mutant types.

Five cases were excluded from the study because DNA isolation could not be performed.

Statistical analyses

Statistical analyses were performed using SPSS 18.0 software.

Hardy-Weinberg analysis was performed to compare the observed and expected genotype frequencies using the χ^2 test. We used odds ratios (ORs) and 95% confidence intervals (CIs) from a logistic regression analysis to figure out the link between vaspin *RS2236242* genotypes and the risk of endometriosis ($P < 0.05$ was considered statistically significant).

Results

Thirty eight endometriosis individuals and 17 control groups participated in the study. The group of individuals with endometriosis exhibited similar characteristics to the control group in terms

Tab. 1. Concentration and quantities of materials used in *RS2236242* polymorphism analysis.
Tab. 1. Koncentrace a množství materiálů použitých při analýze polymorfizmu *RS2236242*.

Content	Quantity
taqman assay master mix	10 µL
taqman assay primer probe	0.5 µL
DNAas RNAas free water	4.5 µL
sample DNA	5 µL
total reaction volume	20 µL

Tab. 2. PCR conditions for vaspin polymorphism analysis.
Tab. 2. Podmínky PCR pro analýzu vaspinového polymorfizmu.

PCR Steps		Target heat (°C)	Standby time	Heat transition rate (°C/second)	Fluorescent reading
Denaturation		95	10 minutes	4.4	no
Amplification (40 cycles)	denaturation	95	15 seconds	4.4	no
	elongation	60	60 seconds	2.2	single
	cooling	37	1 minute	2.2	no

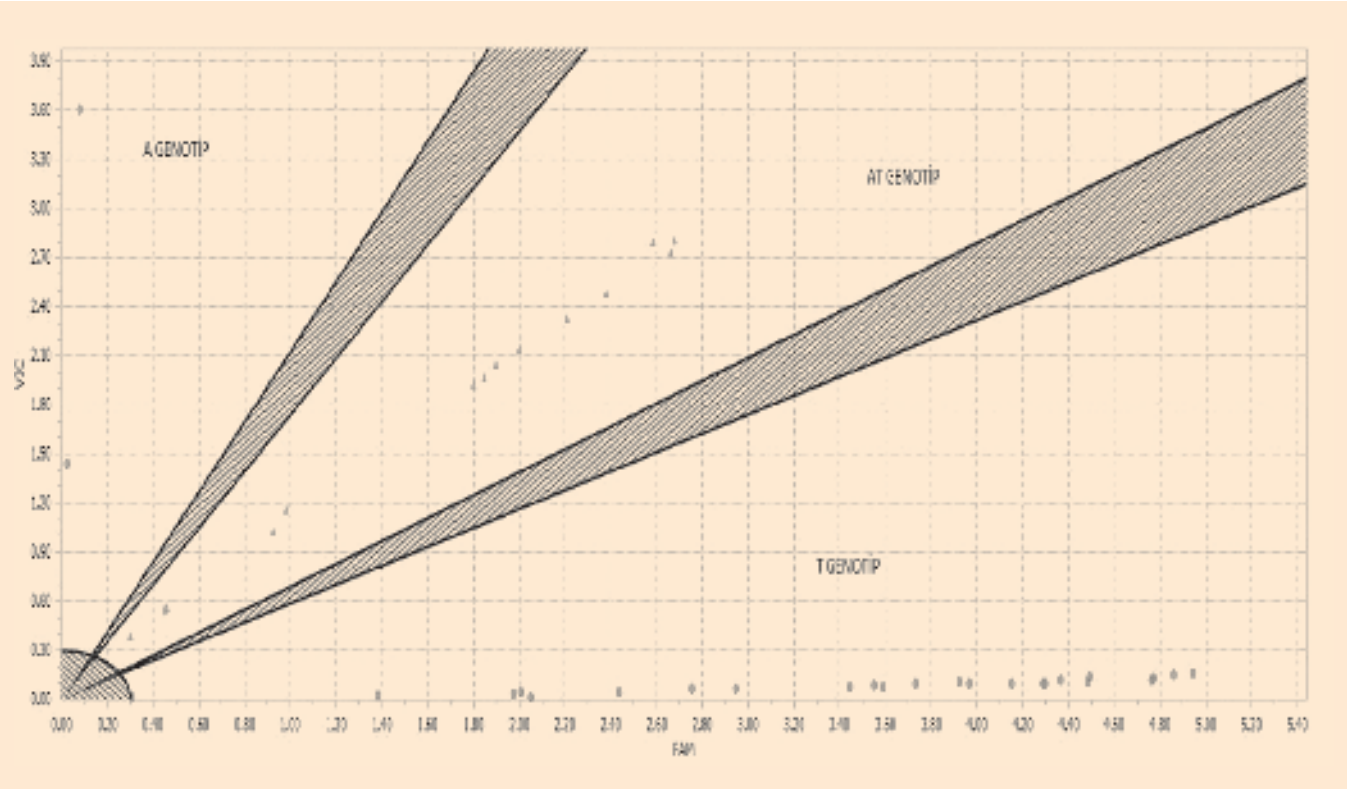

Fig. 1. Endpoint analysis of cases, genotype distributions.
Obr. 1. Analýza výsledků případů, distribuce genotypů.

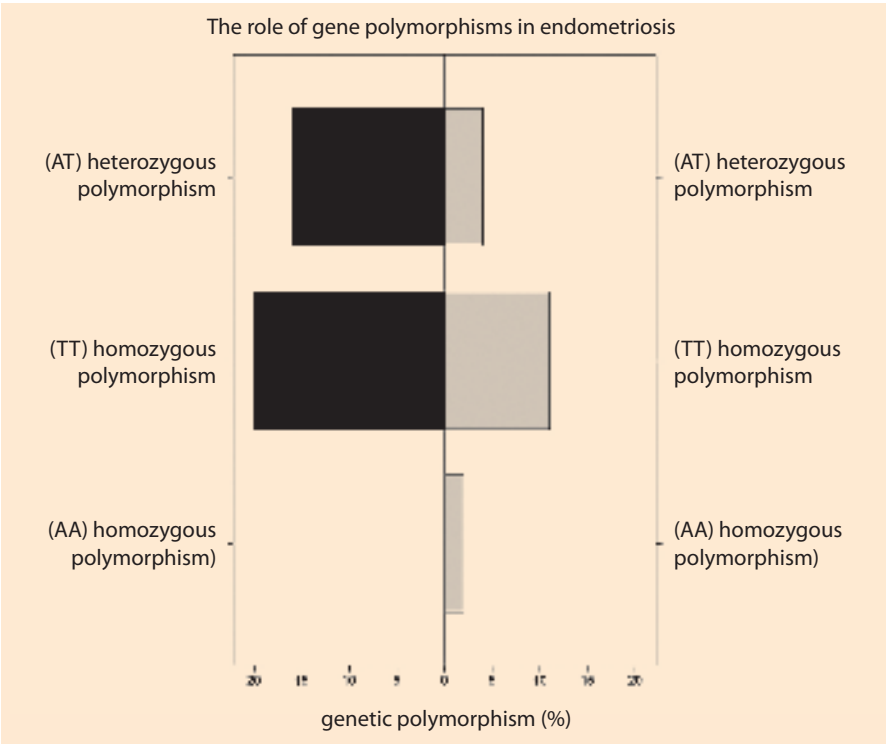

Fig. 2. Population pramid graph showing genotype difference of endometriosis cases and control group.
Obr. 2. Populační pramidový graf ukazující rozdíl v genotypech případů endometriózy a kontrolní skupiny.

of sex, body mass index (BMI), and age (control mean age: 29.6 ± 4.62 years; BMI: 24.02 kg/m^2; endometriosis mean age: 30.4 ± 5.01 years; BMI: 23.63kg/m^2).

DNA isolation of all cases was performed except for 5 cases, and the end point analysis of all cases is given in Fig. 1. According to the statistical analysis, there was a significant difference in the genotype distribution of the vaspin *RS2236242* polymorphism between people with endometriosis and controls (P = 0.027). Also, the AT genotype was more likely to cause endometriosis than the OR: 2.474 (95% CI 0.668–9.169) genotype when we looked at the genotypes' relative risk ratio for endometriosis (Tab. 2).

Significant differences were observed in total AT and TT genotype frequencies between cases and controls (OR = 2.31; 95% CI 0.86–0.92; P = 0.03). AT and TT genotypes were associated with endometriosis risk.

However, the TT genotype was found to be two times more common in people

with endometriosis (N = 20) than in people without endometriosis (N = 11) (52, 63, etc., 64.70% rates within their group). There was also a tendency for a more frequent prevalence of the AT genotype in individuals with endometriosis (N = 18) compared to the control group (N = 4) (47, 36, etc., 23.52% rates within their group). The AA genotype was only found in the control group (N = 2) (Fig. 2).

Tab. 3. Distribution of the vaspin *RS2236242* gene polymorphisms in endometriosis and controls groups.
Tab. 3. Distribuce polymorfizmů genu pro vaspin *RS2236242* v endometrióze a kontrolních skupinách.

***RS2236242* polymorphism**	**Endometriosis (%)**	**Control**	**OR allele (95% CI); P-value**
Genotype			
TT	20 (52.63)	11 (64.70%)	1
TA	18 (47.36)	4 (23.52%)	2.475 (0.668–9.169)
			P = 0.007
AA	0	2 (100%)	1
Total	38	17	

Discussion

In the present study, the relationship between the vaspin gene *RS2236242* polymorphism and endometriosis was investigated. In our study, which is the first research on this subject in the literature, a significant relationship was found between the vaspin gene *RS2236242* polymorphism and endometriosis.

Endometriosis is a benign gynecological disease affecting approximately 6–10% of cases in women of reproductive age [1]. It is a systemic disease that occurs with ectopic endometrial cell growth outside the uterus [15,16]. Endometriosis causes inflammation, resulting in pain, alteration of nearby tissues, fibrosis, the creation of adhesions, and infertility. Although it is believed that the immune system and inflammation are the causes of the disease, the precise molecular and pathophysiological mechanisms are still unknown [17,18].

Genome-wide association studies (GWASs) have helped us learn more about the genetic and epigenetic aspects of endometriosis. These studies found that single nucleotide polymorphisms (SNPs) were strongly linked to a person's likelihood of getting endometriosis [4,5]. In previous GWAS studies, ten genome-wide SNPs were detected in cases of advanced endometriosis in the Caucasian race [19–25]. Many of these genes were associated with breast and ovarian cancer in addition to endometriosis [26,27].

Adipokines generally function in cases such as inflamation, cell division, cell differentiation, tumor development, and metastasis [28]. Vaspin is an adipokine secreted by visceral adipose tissue, and has 415 amino acids [6,7]. This substance belongs to the group of serine protease inhibitors, and its levels in the blood are linked to inflammation. There isn't a lot of information about what vaspin does, but this adipokine has been shown to help vascular endothelial and smooth muscle cells by reducing inflammation, stopping migration, and stopping cell death [8,9]. Studies have shown that in isolated adipocytes, vaspine attenuates the inflammatory cytokine response triggered by IL-1 by inhibiting the NFkB pathway [29].

In our study, we found a significant difference in the frequency of the intronic polymorphism *RS2236242* A/T distribution between women with endometriosis and control groups. There were notable disparities in TA genotype frequencies between individuals with the condition and those without it (OR = 0.59; 95% CI 0.37–0.59; P= 0.03). The current study found that having the A allele was linked to a lower risk of getting endometriosis (OR = 0.67; 95% CI 0.46–0.96; P = 0.03), stopping the disease from getting worse. The difference between AT + AA and TT genotypes is that individuals with AT + TT genotypes had a greater risk of developing endometriosis (OR = 0.58; 95% CI 0.37–0.92; P = 0.02). SNP changes in the vaspin gene detected up until now may have much more value than is known in the disease development [10,30].

Therefore, our study opens up a new area to understand the role of vaspin in endometriosis. This study is the first to establish a connection between the vaspin *RS2236242* A/T polymorphism and endometriosis. However, similar to endometriosis, comprehensive research involving a substantial number of patients is necessary to investigate the vaspin gene and other chronic inflammatory conditions.

Conclusion

Our findings suggest a significant correlation between the vaspin *RS2274907* A/T polymorphism and the probability of developing endometriosis.

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