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ASSOCIATION OF ENDOMETRIOSIS RISK AND GENETIC POLYMORPHISMS IN SLOVAK WOMEN

ABSTRACT: Endometriosis is a benign gynecological disease affecting approximately 10% of pre-menopausal women. Although, it is not a life-threatening condition, it may cause pain and infertility in women, significantly reducing quality of life. Results of epidemiological studies suggest that endometriosis is a genetic disorder with polygenic multifactorial inheritance. The aim of this study was to explore and clarify the connection between selected polymorphisms in ESR1 *and* PDCD6 *genes and endometriosis development risk in patients belonging to the majority population of Slovakia. Genomic DNA was extracted from buccal swabs. Genetic analysis of polymorphisms (rs2234693, rs9340799 and rs4957014) was performed by Real-Time PCR. The PCR amplification was performed on StepOne™ Real-Time PCR System. The findings of our study suggest that the allele C and genotype CC of rs2234693 polymorphism is significantly associated with an increased risk of endometriosis (*P = *0.044) in women from Slovakia. For rs9340799 and rs4957014 polymorphisms, no obvious association was found. Currently, surgical therapy is the preferred approach for diagnosis and treatment of endometriosis. In the future, polymorphism PvuII may serve as a non-surgical diagnostic genetic marker for predicting susceptibility to endometriosis.*

KEY WORDS: Endometriosis – Estrogen receptor-alpha gene – Single nucleotide polymorphism – Slovak population – Programmed cell death 6 gene

INTRODUCTION

Endometriosis is a chronic, estrogen dependent, benign, inflammatory disorder. It is defined as the presence of endometrial-like glandular epithelium and stroma present outside the uterus in ectopic locations (Vinnatier *et al.* 2001, Giudice, Kao 2004, Kennedy *et al.* 2005). Estimates of its frequency vary from 2 – 10% in women of reproductive age and approximately 30% in women with subfertility problems (Viganò 2004). Dysmenorrhea,

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dyspareunia, chronic pelvic pain, heavy menstrual flow, as well as infertility are associated with endometriosis. Making a diagnosis purely on presentable symptoms is difficult as there is considerable overlap with other diseases. This often results in a delay in diagnosis. This is important when one considers that endometriosis is a progressive disease that worsens with time (Barton-Smith *et al.* 2006). Surgery under general anesthesia, most commonly a laparoscopy, is required to make a definitive diagnosis but this is expensive and potentially associated with complications (Vercellini *et al.* 2009).

Despite its relatively high prevalence, pathogenesis and the molecular mechanism of the development of endometriosis have puzzled investigators for years and still remains an enigma. Endometriosis is a polygenic/multifactorial disease, which is related to the complex interactions between hormones, immunoinflammatory processes, genetic factors and the environment. However, the arguments for a genetic factor in the etiology of endometriosis are strong. Simpson *et al.* (1980) first suggested a genetic basis for endometriosis in 1980. The involvement of genetic factors is supported by concordance among monozygotic twins and higher rates of endometriosis found among relatives of endometriosis cases (Trabert *et al.* 2011).

As a disease affecting mostly women of reproductive age and regressing after menopause or ovariectomy, the development of endometriosis is clearly dependent on the metabolism of sex steroid hormones, specifically estrogen (Gurates, Bulun 2003). Estrogen is a steroid hormone, which plays a major role in female physiology and pathology. It regulates the normal physiological aspects of ovulation and menstruation, but its altered regulation promotes various benign and malignant uterine pathologies (Govindan *et al.* 2009). At present, molecular research has focused on steroid hormone receptors and hormone metabolism and their role in endometriosis. The estrogen receptor α gene (*ESR1*), which is located on chromosome 6q25.1, contains some gene polymorphisms, including intron 1 polymorphisms XbaI (rs9340799; A/G) and PvuII (rs2234693; T/C). Effects of these polymorphisms could be the result of a high linkage disequilibrium with functional variants that affect sensitivity to estrogen (Yaich *et al.* 1992).

Tissue homeostasis is a delicate balance between cell proliferation, differentiation, and cell death. Alterations in this balance are often observed in a variety of human diseases (Krebs, Klemenz 2000). Programmed cell death 6 (*PDCD6*) gene, also named as apoptosis-linked gene 2 (*ALG-2*) is located on chromosome 5p15.33. PDCD6 protein is an apoptosis-linked calcium-binding protein with five EF-hand motifs and required for programmed cell death in response to various apoptotic agents (Shi *et al.* 2013). We selected one SNP in this gene (rs4957014) which is located in the intron region of the *PDCD6* gene.

The purpose of the present case-control study was to investigate whether the two polymorphisms in the *ESR1* gene and one polymorphism in *PDCD6* gene were related with endometriosis. Herein, evaluated the distributions of rs2234693, rs9340799 and rs4957014 polymorphisms in Slovak women with endometriosis. We hypothesized that single nucleotide polymorphisms (SNPs) in these genes may be associated with endometriosis. The identification of the related genes is essential for genetic diagnosis and gene therapy for genetic associated disease. Genetic polymorphisms that predispose women to increased risk of developing endometriosis may serve as useful genetic biomarkers for the disease.

MATERIALS AND METHODS

Subjects

All women who participated in this association study belonged to the majority population of Slovakia. Women were divided into two groups. Forty-eight women with endometriosis (patients) ranged in age from 20 to 44 years (mean age: 32.33 ± 6.53 years) and 122 women without endometriosis (control individuals) ranged in age from 18 to 41 years (mean age: 28.47 ± 5.38 years). Diagnosis of endometriosis was confirmed by ultrasound scan, laparoscopy, or laparotomy. The normal controls were recruited during annual health examination and whether these women had any clinical signs of endometriosis or other symptoms relating to endometriosis.

This study was approved by the ethical committee of University hospital with health center of J. A. Reimana in Prešov. Informed consent was obtained from all the participants of this study.

The stage of endometriosis was assigned by the gynecologist according to the revised American Society for Reproductive Medicine (rASRM) scoring system (Haas *et al.* 2013). For our purposes, stages I (minimal) and II (mild) were grouped as light while stages III (moderate) and IV (severe) were grouped as severe; or the stages I, II and III were grouped as rASRM < 4 and stage IV were as rASRM = 4.

DNA isolation and genotyping

Genomic DNA was extracted from buccal swabs using ReliaPrep™ gDNA Tissue Miniprep System kit

(Promega, Madison, USA) according to the manufacturer's instructions. This system is designed for obtaining intact gDNA without the use of ethanol washes or precipitations. DNA concentration was measured with NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, USA).

Genetic analysis of polymorphisms was performed by Real-Time PCR. TaqMan allelic discrimination analyses were performed according to Applied Biosystems standard protocols (Applied Biosystems, Waltham, USA). The analyzed SNPs were as follows: *ESR1* rs9340799 also known as XbaI polymorphism (C__3163591_10); *ESR1* rs2234693 also known as PvuII polymorphism (C__3163590_10) and PDCD6 rs4957014 (C__11855391_10) (Applied Biosystems). Genotypes for XbaI and PvuII polymorphisms were termed AA/AG/GG and TT/TC/CC, respectively. Genotypes for *PDCD6* rs4957014 were termed TT/GT/GG. The PCR amplification was performed on StepOne™ Real-Time PCR System (Applied Biosystems). Real-Time PCR conditions for all polymorphisms are shown in *Table 1*.

Statistical Analysis

Allele and genotype frequencies for all polymorphisms were obtained by directed counting. Differences in allele distribution were assessed by chi-square analysis, which was also used to test for Hardy-Weinberg equilibrium, by online software SNPs. Genotypic association tests in a case-control pattern assuming codominant, dominant, recessive, overdominant, or log-additive genetic models were performed using SNPstats (Solé *et al.* 2006). Association between haplotypes and endometriosis risk was calculated by using the $\chi 2$ test. Odds ratio test with 95% confidence intervals (CIs) was used to assess the strength of association of genotype and allele frequency and risk of disease occurrence. A *P*-value < 0.05 was considered statistically significant.

RESULTS

Genotype distribution of polymorphisms in our cases and control subjects were not deviated from Hardy-Weinberg equilibrium. *Table 2* presents allele frequencies of polymorphisms included in the study. Proportions of PvuII genotypes TT/TC/CC in patients were 10.42/54.16/35.42% and in controls were 24.60/50.00/25.40%, respectively. Frequencies of T and C alleles were in patients 0.37/0.62 and in controls 0.49/0.50, respectively. Significantly increased endometriosis risk was found to be associated with C allele of PvuII polymorphism ($P = 0.044$; OR = 1.640). Results indicate a significant association of CC genotype with endometriosis. Statistical analysis carried out with different genotype models (*Table 3*) shows that significantly increased endometriosis risk was found to be associated with the CC-TC genotypes in a dominant model, compared with TT genotypes ($P = 0.04$; OR = 2.80; 95% CI = 1.02 - 7.73). Significantly elevated endometriosis risks were found to be associated with PvuII polymorphism by using log-additive analyses. Proportions of XbaI genotypes AA/AG/GG in patients were 43.75/54.17/2.08% and in controls were 40.98/45.08/13.94%, respectively. Frequencies of A and G alleles were in patients 0.71/0.29 and in controls 0.64/0.36, respectively. For XbaI polymorphism no association was observed between endometriosis risk and genotypes, even in different genetic models. Proportions of rs4957014 genotypes TT/GT/GG in patients were 16.67/35.42/47.91% and in controls were 7.38/36.88/55.74%, respectively. Frequencies of T and G alleles were in patients 0.34/0.66 and in controls 0.26/0.74, respectively. For rs4957014 polymorphism also no association was observed between endometriosis risk and genotypes, even in different genetic models.

The rASRM classification of the endometriosis collective resulted in 5 (10.42%) patients with stage I,

TABLE 1: Real-Time PCR conditions for *ESR1* and *PDCD6* gene polymorphisms.

Gene	Polymorphism	Allelic variants	Initial denaturation	Denaturation	Annealing	Extension
ESR1	rs9340799	A (reference) G (alternative)	95 °C	95 °C	60 °C	60 °C
	rs2234693	T (reference) C (alternative)	15 min	15 s	1 min	1 min
PDCD6	rs4957014	T (reference) G (alternative)				

18 (37.50%) patients with stage II, 20 (41.66%) patients with stage III and 5 (10.42%) patients with stage IV. The patient group was stratified by stage of endometriosis for a light and severe stage and for rASRM < 4 and rASRM = 4 (*Table 4*). There was a difference in the distribution of the PvuII and XbaI genotypes between the groups with rASRM < 4 and rASRM = 4. Allele C in PvuII polymorphisms was statistically more frequent in patients with rASRM = 4 than in patients with rASRM < 4. In patients with rASRM = 4 the CC genotype was only observed. For rs4957014 polymorphisms, there was no difference in the distribution of genotypes between the groups of light and severe stages, or between the groups with rASRM < 4 and rASRM = 4.

Haplotype CA/CA was more frequently observed and over-represented in the patients (*Figure 1*). Haplotype CG/CG was not observed in any group. Haplotype TG/TG was over-represented in control group, with a significant difference in frequency between the case and the control group ($P = 0.0036$) (*Table 5*).

These findings indicate that PvuII alternative genotype and allele are strongly associated with higher susceptibility of endometriosis.

DISCUSSION

The diversity of the clinical manifestations of endometriosis complicates the exact identification of

TABLE 2: Allele frequencies of selected SNPs in *ESR1* and *PDCD6* gene among endometriosis patients and control group. n corresponds to the number of individuals; OR (odds ratio); statistically significant results are shown in bold.

Polymorphisms	Allele	Patients (n = 48)	Controls (n = 122)	OR	*P* value
rs2234693	T C	36 (37.5 %) 60 (62.5 %)	121 (49.6 %) 123 (50.4 %)	1.640	**0.044**
rs9340799	A G	68 (70.8 %) 28 (29.2 %)	155 (63.5 %) 89 (36.5 %)	0.717	0.202
rs4957014	T G	33 (34.4 %) 63 (65.6 %)	63 (25.8 %) 181 (74.2 %)	0.664	0.115

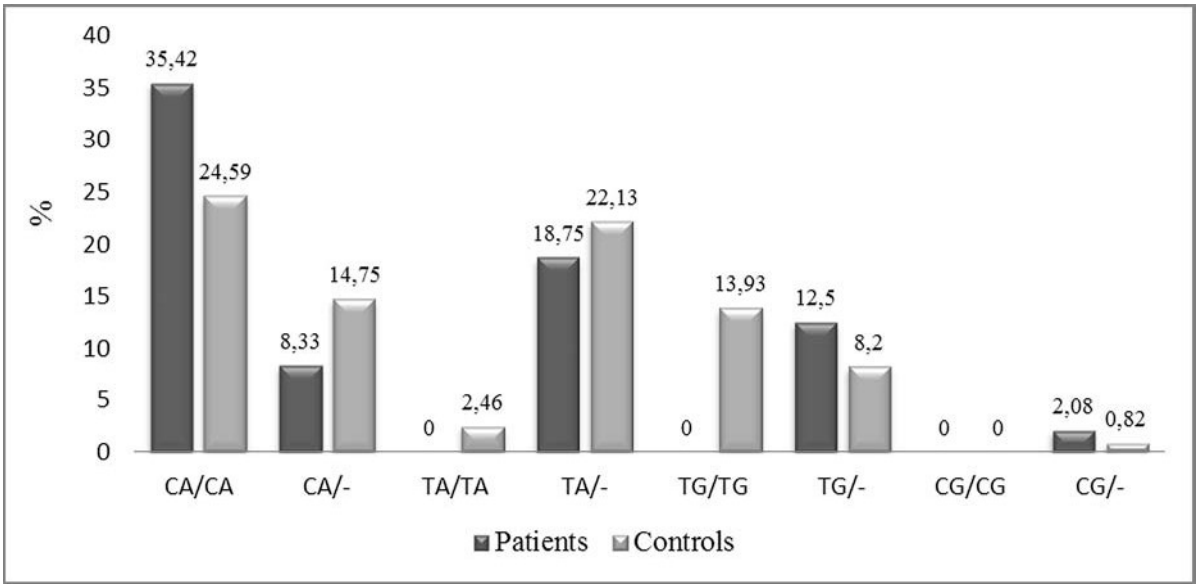

FIGURE 1: Distribution of haplotypes.

TABLE 3: Genotype frequencies of selected SNPs in *ESR1* and *PDCD6* gene among endometriosis patients and control group in different genetic models. n corresponds to the number of individuals; OR (odds ratio); CI (confidence interval); statistically significant results are shown in bold.

Genetic model	Genotype	Patients n = 48 (%)	Controls n = 122 (%)	OR (95 % CI)	*P* value
rs2234693 T/C					
Codominant	TT	5 (10.42)	30 (24.60)	1.00 (reference)	
	TC	26 (54.16)	61 (50.00)	2.56 (0.89 – 7.32)	0.07
	CC	17 (35.42)	31 (25.40)	3.29 (1.07 – 10.05)	**0.03**
Dominant	TT	5 (10.42)	30 (24.60)	1.00 (reference)	
	CC - TC	43 (89.58)	92 (75.40)	2.80 (1.02 – 7.73)	**0.04**
Recessive	TT - TC	31 (64.58)	91 (74.60)	1.00 (reference)	
	CC	17 (35.42)	31 (25.40)	0.62 (0.30 – 1.27)	0.19
Overdominant	TT - CC	22 (45.84)	61 (50.00)	1.00 (reference)	
	TC	26 (54.16)	61 (50.00)	1.18 (0.61 – 2.31)	0.62
Log-additive				0.60 (0.36 – 0.98)	**0.04**
rs9340799 A/G					
Codominant	AA	21 (43.75)	50 (40.98)	1.00 (reference)	
	AG	26 (54.17)	55 (45.08)	1.13 (0.56 – 2.25)	0.74
	GG	1 (2.08)	17 (13.94)	0.14 (0.02 – 1.12)	0.04
Dominant	AA	21 (43.75)	50 (40.98)	1.00 (reference)	
	GG - AG	27 (56.25)	72 (59.02)	0.89 (0.45 – 1.75)	0.74
Recessive	AG - AA	47 (97.92)	105 (86.06)	1.00 (reference)	
	GG	1 (2.08)	17 (13.94)	0.13 (0.02 – 1.02)	0.01
Overdominant	AA - GG	22 (45.83)	67 (54.92)	1.00 (reference)	
	AG	26 (54.17)	55 (45.08)	1.44 (0.74 – 2.81)	0.29
Log-additive				0.70 (0.41 – 1.19)	0.18
rs4957014 G/T					
Codominant	GG	23 (47.91)	68 (55.74)	1.00 (reference)	
	GT	17 (35.42)	45 (36.88)	1.12 (0.54 – 2.32)	0.21
	TT	8 (16.67)	9 (7.38)	2.63 (0.91 – 7.61)	0.07
Dominant	GG	23 (47.91)	68 (55.74)	1.00 (reference)	
	GT – TT	25 (52.09)	54 (44.26)	1.37 (0.70 – 2.67)	0.36
Recessive	GG - GT	40 (83.33)	113 (92.62)	1.00 (reference)	
	TT	8 (16.67)	9 (7.38)	2.51 (0.91 – 6.95)	0.08
Overdominant	TT - GG	31 (64.58)	77 (63.12)	1.00 (reference)	
	GT	17 (35.42)	45 (36.88)	0.94 (0.47 – 1.88)	0.86
Log-additive				1.45 (0.89 – 2.37)	0.14

markers that may preferably be used to verify or refute the diagnosis of endometriosis. Ballard *et al.* (2006) state that the diagnosis of endometriosis may be delayed by 7–12 years, contributing significantly to the deterioration of the quality of life of women with this disease. Varga *et al.* (2012), based on the results of several studies, indicate that up to 65% of women with endometriosis are initially diagnosed with other illnesses. Non-invasive methods such as ultrasound or blood tests have so far not been successful in diagnosing this disease. Although the increased levels of glycoprotein of high molecular weight CA-125 were confirmed in serum and peritoneal fluid in women with endometriosis, which pointed out the potential diagnostic marker, its significance at the disease detection has not been confirmed. There are many other causes, pathological and physiological, which may lead to increased levels of CA-125 in the body. For these reasons, surgical intervention is required to confirm a definitive diagnosis of endometriosis. However, invasive surgery poses a potential risk of developing other health problems. Delay in diagnosis, the cost of surgery and persistent pain associated with endometriosis could be reduced by using non-invasive procedures and methods of molecular genetics.

In the present study we have tried to confirm/exclude the connection of selected single nucleotide polymorphisms with the emergence and development of endometriosis. Based on the results, evaluate whether these polymorphisms could serve as genetic SNP markers for the identification of the disease. This approach could help reduce the number of women undergoing surgery and in selecting women who are required to undergo surgical examination.

Endometriosis leads to estrogen-dependent growth of ectopic endometriotic tissue. Due to this fact, we

TABLE 4: Association of endometriosis patients with different rASRM stages and genotype. n corresponds to the number of individuals; statistically significant results are shown in bold.

	rs2234693 genotype			
	TT n (%)	**TC n (%)**	**CC n (%)**	***P* value**
Light (n = 23)	4 (17.39)	12 (52.17)	7 (30.44)	0.246
Severe (n = 25)	1 (4.00)	14 (56.00)	10 (40.00)	
rASRM < 4 (n = 43)	5 (11.62)	26 (60.47)	12 (27.91)	**0.013**
rASRM = 4 (n = 5)	0 (0.00)	0 (0.00)	5 (100)	
	rs9340799 genotype			
	AA n (%)	**AG n (%)**	**GG n (%)**	***P* value**
Light (n = 23)	8 (34.78)	15 (65.22)	0 (0.00)	0.477
Severe (n = 25)	13 (52.00)	11 (44.00)	1 (4.00)	
rASRM < 4 (n = 43)	16 (37.20)	26 (60.47)	1 (2.33)	**0.037**
rASRM = 4 (n = 5)	5 (100)	0 (0.00)	0 (0.00)	
	rs4957014 genotype			
	TT n (%)	**GT n (%)**	**GG n (%)**	***P* value**
Light (n = 23)	3 (13.04)	8 (34.78)	12 (52.18)	0.436
Severe (n = 25)	5 (20.00)	9 (36.00)	11 (44.00)	
rASRM < 4 (n = 43)	6 (13.95)	17 (39.54)	20 (46.51)	0.734
rASRM = 4 (n = 5)	2 (40.00)	0 (0.00)	3 (60.00)	

TABLE 5: Haplotype frequencies of PvuII and XbaI polymorphisms in control and study group. Statistically significant results are shown in bold.

	CA/CA	CA/-	TA/TA	TA/-	TG/TG	TG/-	CG/CG	CG/-
Patients	0.3542	0.0833	0.0000	0.1875	0.0000	0.1250	0.0000	0.0208
Controls	0.2459	0.1475	0.0246	0.2213	0.1393	0.0820	0.0000	0.0082
***P* value**	0.1834	0.3187	0.5596	0.6823	**0.0036**	0.3918	1.0000	0.4862

focused primarily on the analysis of two polymorphisms located in the gene for the estrogen receptor α (*ESR1*), in particular the XbaI (rs9340799) and PvuII (rs2234693) polymorphism and their connection with the establishment and development of endometriosis. Polymorphism PvuII was analyzed in a number of studies, and it was associated with a number of pathologies in women of reproductive age (Surekha *et al.* 2007, Ivanova *et al.* 2007), indicating that this polymorphism is very important and greatly influences the function of estrogen receptors. The connection between this polymorphism and endometriosis was initially observed by Georgiou *et al.* (1999). In their study of Greek women, they reported a significant difference in the frequency of the mutated allele C polymorphism between the patients and the control group (0.72 versus 0.49, respectively). These results are comparable to the results obtained in our study. C allele frequency was 0.62 in patients, and 0.51 in controls, which statistically has a significantly higher incidence in women diagnosed with endometriosis ($P = 0.044$). The value of the odds ratio (OR) indicates that the C allele is associated with a 1.6-fold higher risk of developing endometriosis. We achieved statistical significance in a dominant genetic model ($P = 0.04$), via confirming a significant predisposition in individuals carrying the C allele for endometriosis. Accordingly, it can be argued that even the presence of one allele C is sufficient for the modification of endometriosis risk.

The results of existing studies, which focused on XbaI polymorphism were widely divergent. It is noted that the standard allele A is associated with the physiological state of the organism, which corresponds to the optimum effect of estrogen and, therefore, its presence reduces the risk of endometriosis, while the presence of allele G increases it. In our study group, we compared the frequency of allele G in patients (0.29) with the frequency of this allele in controls (0.36), without observing any significant difference ($P = 0.202$). We conclude that this polymorphism itself is unlikely to have any effect on the development of endometriosis in Slovak women.

Both studied polymorphisms are located in intron regions of *ESR1* gene of the chromosome 6q25. These two polymorphisms which are separated by just 46 bp, are in strong binding disequilibrium. We attempted to ascertain whether there was a link between the occurrence of different haplotypes, observed in our group which originated from a combination of different alleles and genotypes of these two polymorphisms, and the development of endometriosis. We found significance only in the prevalence of haplotype TG/TG ($P = 0.0036$), which was more frequent in the control group of women compared with patients. Based on these results, the risk of developing endometriosis could not be linked with the presence of a haplotype.

The occurrence of the risk allele C in the PvuII polymorphism is likely to result in higher sensitivity of the estrogen receptor and increased levels of estrogen. It also promotes proliferation and growth of cells and hence the development of endometriosis. Subsequently, genes regulating vascular endothelial growth factor (growth and proliferation of endometriotic cells) and genes promoting cell survival and inhibiting apoptosis are activated. In connection with endometriosis, we focused on a gene of programmed cell death 6 (*PDCD6*), which is involved in the regulation of apoptotic pathways. We specifically examined the rs4957014 polymorphism located in this gene. In our study group, we were not able to confirm any connection between this polymorphism and development of endometriosis. No statistical analysis conducted at five different genetic models (codominant, dominant, recessive, overdominant and log-additive) confirmed any apparent association among women from Slovakia.

CONCLUSIONS

It appears that the polymorphism in the gene for the estrogen receptor α (PvuII, rs2234693) is associated with the development of endometriosis in women

belonging to the majority Slovakia population. Although, the pathogenesis of endometriosis is still not fully understood, our results showed that estrogen and its receptors play an important role. This polymorphism involved in the biosynthesis and signaling pathways of steroid hormones could be a useful genetic biomarker for predicting susceptibility to endometriosis. Thus, it would allow for early therapeutic intervention for women at high risk for developing this disease. To our knowledge, this is the first study concerned with the connection between polymorphisms rs2234693 and rs9340799 in *ESR1* gene and rs4957014 polymorphism in the gene *PDCD6* and endometriosis in women in a Slovakian population.

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