DOI: 10.4149/BLL_2016_088

Bratisl Med J 2016; 117 (8)
452 – 455

CLINICAL STUDY

Association of two selected polymorphisms with developed endometriosis in women from Slovakia

Pitonak J[1], Galova J[2], Bernasovska J[3]

Gyneacology and Maternity Ward II, University Hospital and Health Centre of JA Reiman, Presov, Slovakia.
dr.pitonak@gmail.com

ABSTRACT
OBJECTIVES: To clarify the connection between two selected mononucleotide polymorphisms (rs4957014 and rs3756712) in programmed cell death gene 6 (PDCD6) and endometriosis development risk in patients belonging to the majority population of Slovakia.
METHODS: From all women involved in the research a buccal DNA sample was taken. A genetic analysis of selected polymorphisms was implemented using Real-time PCR method. Variance in allelic and genotype frequencies was statistically evaluated between the controlgroup and the group of patients.
RESULTS: The analysed group consisted of 52 women suffering from endometriosis and the control group of 63 women. Variant G allele frequency in the group of patients in case of polymorphism rs3756712 had a value of 0.42 and in the control group 0.29; that represents its statistically and significantly higher occurrence in the group of patients suffering from endometriosis (p = 0.029 and OR = 1.833). Presence of G allele is related to almost 1.9 times higher risk of endometriosis development.
CONCLUSION: Achieved results show that polymorphism rs3756712 is significantly associated with the risk of endometriosis development in Slovak women. Polymorphism rs4957014 did not show any connection with development of endometriosis *(Tab. 5, Ref. 10)*. Text in PDF *www.elis.sk.*
KEY WORDS: apoptosis, endometriosis, programmed cell death gene 6, mononucleotide polymorphisms.

Introduction

Nowadays, endometriosis is a relatively common chronic benign inflammatory disease. It is not life-threatening, but might cause pain and infertility that strongly affects quality of life. It is characterized by the presence of implants of abnormally placed tissue similar to endometrium, including glands and stroma, outside the uterine cavity (Vinatier et al, 2001; Giudice and Kao, 2004; Kennedy et al, 2005).

Apoptosis or programmed cell death is one of the basic processes responsible for homeostasis in the organisms. There is a lot of evidence showing apoptosis helps to keep homeostasis during menstruation of healthy women removing senescent cells from the functional layer of endometrium at the end of the secretory and menstrual phase of a menstrual cycle (Dmowski et al, 2001). However, in women suffering from endometriosis the percentage of endometrial cells undergoing apoptosis is significantly decreased and on the contrary the number of surviving cells is increased and they still show physiological activity. Endometriosis is typical of ectopically placed endometrial cells that show dysregulation between proliferation and apoptosis when influenced by particular stimuli. Eutopic endometrium of women suffering from endometriosis increases the expression of anti-apoptotic factors and decreases the expression of pro-apoptotic factors in comparison with endometrium of healthy women. All these factors contribute to survival of endometrial cells returning to the peritoneal cavity by retrograde menstruation and consequent development of endometriosis (Taniguchi et al, 2011).

Resistance of endometrium to apoptosis in women suffering from endometriosis might be caused by various factors, including an inappropriate transduction of a signal for apoptosis or an inability of endometrium to produce induction or inhibition proteins in patients suffering from endometriosis. Calcium binding protein ALG-2 is encoded by programmed cell death gene 6 (PDCD6) also known as apoptosis linked gene 2 (ALG2). Mahul-Mellier et al, (2008) confirmed that this protein plays an important role in many processes of apoptotic pathways caused by endoplasmic reticulum stress, that induce cell death with Ca^{2+} ions involved. Inappropriate expression of a gene encoding this protein leads to excessive survival of endometrial cells. Therefore, we focused on two selected mononucleotide polymorphisms found in this gene and tried to confirm/disapprove its role in the development and progression of endometriosis.

[1]Gyneacology and Maternity Ward II, University Hospital and Health Centre of JA Reiman, Presov, Slovakia, [2]Biology Department, Faculty of Humanities, University of Presov, Slovakia, and [3]Biology Department, Faculty of Humanities, University of Presov, Slovakia

Address for correspondence: J. Pitonak, MD, PhD, Gyneacology and Maternity Ward II, University Hospital and Health Centre of JA Reiman, Jana Holleho 14, SK-081 81 Presov, Slovakia.
Phone: +421.907973754

Material and methods

Analysed groups

This associational research dealt only with women from the majority population of eastern Slovakia. Researched women were divided into two groups. The first group consisted of 52 women suffering from endometriosis (patients) at the age of 20–44. The diagnosis of these women was confirmed by ultrasonography, laparoscopy or laparotomy. The second group consisted of 63 healthy women (check-ups) at the age of 18–41. These women, who did not suffer from any symptoms of the researched disease, were selected accidentally when coming for a routine check-up.

DNA samples of women included in the research were taken in 2014 and 2015 and everything was performed afteran approval of the Ethics Committee of Faculty Hospital and Health Centre of J. A. Reiman in Prešov. All women participating in the research were informed about its nature and importance in advance and signed informed consent and agreed voluntarily to be involved in the research.

The stage of endometriosis of patients was determined by their gynaecologist in accordance with data established by the American Fertility Society (1985). To simplify the statistical processing of the data, theoriginal four-stage classification including stage I – minimal, stage II – mild, stage III – moderate and stage IV – severe was reduced to two stages. Stages I and II were united to the mild stage and stages III and IV to the severe stage of endometriosis.

DNA isolation and a genetic analysis

Samples for the genetic analysis were taken in the form of a buccal smear. To isolate DNA we used commercially available kit ReliaPrep™ gDNA Tissue Miniprep System (Promega), designed to isolate human DNA from buccal smears. Isolation of samples was done in accordance with appropriate protocol. Concentration and cleanness of isolated DNA samples were determined by measures of NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific).

Detection of quantitative PCR products was performed by specific dually labelled hydrolysis probes TaqMan®. We analysed two selected mononucleotide polymorphisms in gene PDCD6: rs4957014 (C__11855391_10) and rs3756712 (C__1578218_10) (Applied Biosystems). Both polymorphisms represent mononucleotide substitution of thymine for guanine in a nucleotide chain. Genotyping was performed using StepOne™ Real-Time PCR System (Applied Biosystems). A selected thermal programme consisted of initial denaturation at 95 ºC for 15 minutes and subsequent 45 cycles of denaturation at 95 ºC for 15 seconds and anelation and polymerisation at 60 ºC for 1 minute. A total volume of PCR reaction mixture for one sample was 15 µl in both polymorphisms. Genotypes of a particular polymorphism of individual samples were read on the basis of a dot chart or amplification curves.

Statistical analysis

Each polymorphism was given genotype and allelic frequencies. Hardy–Weinberg equilibrium and differences among allelic multiplicity in analysed groups were evaluated by Pearson´s chi-square test (χ^2 test) using online SNPs software. The analysis of polymorphisms in particular genetic models was implemented by means of online SNPStats application suggested by Solé et al (2006). Power of association between endometriosis occurrence and relevant polymorphism was assessed by OR (Odds ratio) with values corresponding with 95% of CI (confidence interval). All statistical tests were assessed in accordance with a confidence interval $p < 0.05$.

Results

The average age of the women with diagnosed endometriosis (32.39 ± 6.31 years) was higher in comparison with average age of women in the control group (28.62 ± 5.12 years) ($p < 0.05$).

Considering the diagnosed stage of endometriosis, the group of patients consisted of 5 women (9.62 %) withstage I, 19 women (36.54 %) withstage II, 21 women (40.38 %) withstage III and 7 women (13.46 %) with stage IV.

A frequency in individual alleles and genotypes in the patients and the check group are summarised in Tables 1 and 2 for both studied polymorphisms. We did not detect any statistically significant deviations from Hardy–Weinberg equilibrium. It means our genotype frequencies were identical with theoretically calculated frequencies, as a value of Pearson´s test shows.

In polymorphism rs4957014, the most frequent allele occurring in both groups was allele G with a frequency of 0.65 in the patients and 0.73 in the controls. Allele T occurred in the group of patients with a frequency of 0.35 and 0.27 in the group of control. We did not succeed in finding significant variance in allele frequencies of polymorphism in the analysed groups.

In polymorphism rs3756712, the most frequent allele occurring in both groups was allele T with a frequency of 0.58 in the patients and 0.71 in the controls. The frequency of variant allele G had a value of 0.42 in the patients and 0.29 in the controls; which represents its statistically and significantly higher occurrence in the women with diagnosed endometriosis ($p = 0.029$ and OR = 1.833). OR value indicates that presence of allele G is connected with almost 1.9 times higher risk of endometriosis progression (Tabs 1, 2).

Tab. 1. Genotype and allelic frequencies of polymorphism rs4957014 in gene *PDCD6* in the patients and the check group.

rs4957014	Patients (n=52)	Check-ups (n=63)
Genotype TT	8 (15.39 %)	6 (9.52 %)
Genotype GT	20 (38.46 %)	22 (34.92 %)
Genotype GG	24 (46.15 %)	35 (55.56 %)
f (T)	0.35	0.27
f (G)	0.65	0.73
p (Pearson) for HWE	0.278	0.366
χ^2	1.57	
OR	0.698	
Statistical significance	p = 0.211	

TT – homozygous genotype; GT – heterozygous genotype; GG – homozygous genotype; f (T) – standard allele frequency; f (G) – variant allele frequency; n – total; HWE – Hardy-Weinberg equilibrium; χ^2 – chi-square test; OR – odds ratio

Tab. 2. Genotype and allelic frequencies of polymorphism rs3756712 in gene PDCD6 in the patients and the check group.

rs3756712	Patients (n=52)	Check-ups (n=63)
Genotype TT	20 (38.46 %)	33 (52.38 %)
Genotype GT	20 (38.46 %)	24 (38.10 %)
Genotype GG	12 (23.08 %)	6 (9.52 %)
f (T)	0.58	0.71
f (G)	0.42	0.29
p (Pearson) for HWE	0.126	0.597
χ^2	4.74	
OR	1.833	
Statistical significance	p=0.029	

TT – homozygous genotype; GT – heterozygous genotype; GG – homozygous genotype; f (T) – standard allele frequency; f (G) – variant allele frequency; n – total; HWE – Hardy-Weinberg equilibrium; χ^2 – chi-square test; OR – odds ratio

The statistical analysis implemented in five various genetic models showed that significantly increased susceptibility to endometriosis development appears in polymorphisms rs3756712 (Tab. 4) connected with presence of homozygous genotype GG that is shown in statistical significance found in the co-dominant genetic model (p = 0.03; OR = 3.30; 95% CI = 1.07–10.18). Each variant allele G modifies risk in an additive way, that is clear from the confidence interval found in the log-additive genetic model (p = 0.04). The statistical analysis implemented in polymorphisms rs4957014 (Tab. 3) did not confirm any obvious association of this polymorphism with endometriosis development in neither observed models (Tab. 4).

Stratifying the group of patients on the basis of the stage of endometriosis development, mild and severe, we did not manage to confirm any statistically significant differences in distribution of alleles and genotypes in the studied groups in neither of the studied polymorphisms (Tab. 5).

Discussion

Endometriosis is a multifactorial disease with polygenic heredity. Oestrogen level increase aids proliferation and cell growth and that leads to endometriosis development. Subsequently, genes regulating the vascular endothelial growth factor (growth and reproduction of endometrial cells) and genes aiding survival of cells and eliminating apoptosis are activated. This is the reason why, in connection with endometriosis, we focused on the analysis of two selected mononucleotide polymorphisms (rs4957014 and rs3756712) in programmed cell death gene 6 (PDCD6) in a group of women from eastern Slovakia and decided to contribute to clarification and better understanding of association of these

Tab. 3. Genotype frequencies of polymorphism rs4957014 in the patients and the check group and their association with endometriosis development risk in particular genetic models.

Genetic model	Genotype	Patients n=52 (%)	Check-ups n=63 (%)	OR (95 % CI)	p
rs4957014 G/T					
Co-dominant	GG	24 (46.15)	35 (55.56)	1.00 (reference)	
	GT	20 (38.46)	22 (34.92)	1.33 (0.60–2.94)	0.48
	TT	8 (15.39)	6 (9.52)	1.94 (0.60–6.32)	0.26
Dominant	GG	24 (46.15)	35 (55.56)	1.00 (reference)	
	GT – TT	28 (53.85)	28 (44.44)	1.46 (0.70–3.05)	0.32
Recessive	GG – GT	44 (84.61)	57 (90.48)	1.00 (reference)	
	TT	8 (15.39)	6 (9.52)	1.73 (0.56–5.34)	0.34
Over-dominant	TT – GG	32 (61.54)	41 (65.08)	1.00 (reference)	
	GT	20 (38.46)	22 (34.92)	1.16 (0.54–2.50)	0.69
Log-additive				1.37 (0.81–2.34)	0.24

TT – homozygous genotype; GT – heterozygous genotype; GG – homozygous genotype; n – total; OR – odds ratio; CI – confidence interval

Tab. 4. Genotype frequencies of polymorphism rs3756712 in the patients and the check group and their association with endometriosis development risk in particular genetic models.

Genetic model	Genotype	Patients n=52 (%)	Check-ups n=63 (%)	OR (95 % CI)	p
rs3756712 G/T					
Co-dominant	TT	20 (38.46)	33 (52.38)	1.00 (reference)	
	GT	20 (38.46)	24 (38.10)	1.37 (0.61–3.10)	0.10
	GG	12 (23.08)	6 (9.52)	3.30 (1.07–10.18)	0.03
Dominant	TT	20 (38.46)	33 (52.38)	1.00 (reference)	
	GT – GG	32 (61.54)	30 (47.62)	1.76 (0.83–3.71)	0.14
Recessive	TT – GT	40 (76.92)	57 (90.48)	1.00 (reference)	
	GG	12 (23.08)	6 (9.52)	2.85 (0.99–8.23)	0.05
Over-dominant	TT – GG	32 (61.54)	39 (61.90)	1.00 (reference)	
	GT	20 (38.46)	24 (38.10)	1.02 (0.48–2.16)	0.97
Log-additive				1.70 (1.01–2.87)	0.04

TT – homozygous genotype; GT – heterozygous genotype; GG – homozygous genotype; n – total; OR – odds ratio; CI – confidence interval

Tab. 5. Distribution of genotypes among various stages of endometriosis in the group of patients.

	TT n (%)	GT n (%)	GG n (%)	p
rs4957014				
Mild stage (n=24)	3 (12.50)	9 (37.50)	12 (50.00)	0.504
Severe stage (n=28)	5 (17.86)	11 (39.29)	12 (42.85)	
rs3756712				
Mild stage (n=24)	8 (33.33)	11 (45.83)	5 (20.84)	0.782
Severe stage (n=28)	12 (42.86)	9 (32.14)	7 (25.00)	

TT – homozygous genotype; GT – heterozygous genotype; GG – homozygous genotype; n – total

two polymorphisms with development of such a complex disease as endometriosis is. Gene PDCD6 located on a short arm of the fifth chromosome encodes a protein that plays an important role in many processes of apoptotic pathways. Rho et al (2012) proved this gene is important for the angiogenesis process. Angiogenesis represents production of new blood vessels and bloodstream and already existing vessels and it is an essential step following implantation and proliferation of reflux endometrial tissue. Disrupting an optimum activity of PDCD6, there is no removal of endometrial cells by apoptosis and abnormal growth of endometrial tissue is increased, that might potentially lead to endometriosis development.

Potential association of these two polymorphisms in PDCD6 with endometriosis development was studied by Shi et al (2013) in a study implemented in women living in Han, Sichuan province, south-western China. Regarding polymorphism rs4957014, they did not detect significantly increased endometriosis risk in connection with allele G (OR = 1.31; p = 0.029), while regarding polymorphism rs3756712, they did not detect significant connection between alleles of this polymorphism and disease development (OR = 1.27; p = 0.066). They detected significantly increased risk of endometriosis development linked to genotype GG and GT in polymorphism rs3756712 in the dominant (p = 0.010), co-dominant (p = 0.017) and over-dominant (p = 0.005) genetic model.

In our studied group we detected a link to the disease only in polymorphism rs3756712, where allele G was connected with almost 1.9 times higher risk of endometriosis development in women from the majority population of eastern Slovakia. Polymorphism rs4957014 in the researched groups did not show any statistically significant differences in allelic frequencies. On the basis of achieved results it is possible to state that polymorphism rs3756712 plays an important role in the development and progression of endometriosis; polymorphism rs4957014 evidently does not influence endometriosis origin and development in Slovak women. Our results are in contrary with the results achieved by Shi et al. (2013), that shows the fact that the factor of ethnicity might have played an important role.

As far as we know, it was the first research dealing with link between these two polymorphisms in programmed cell death gene 6 and endometriosis in women of the majority population in Slovakia.

References

1. Vinatier D et al. Theories of endometriosis. Eur J Obstet Gynecol Reprod Biol 2001; 96 (1): 21–34.

2. Giudice LC, Kao LC. Endometriosis. Lancet 2004; 364 (9447): 1789–1799.

3. Kennedy S et al. ESHRE guideline for the diagnosis and treatment of endometriosis. Hum Reprod 2005; 20 (10): 2698–2704.

4. Dmowski WP et al. Apoptosis in endometrial glandular and stromal cells in women with and without endometriosis. Hum Reprod 2001; 16 (9): 1802–1808.

5. Taniguchi F et al. Apoptosis and endometriosis. Front Biosci 2011; 3: 648–662.

6. Mahul-Mellier AL et al. Alix and Alg-2 are involved in tumor necrosis factor receptor 1-induced cell death. J Biol Chem 2008; 283 (50): 34954–34965.

7. American Fertility Society. Revised American Fertility Society classification of endometriosis. Fertil Steril 1985; 43 (3): 351–352.

8. Solé X et al. SNPStats: a web tool for the analysis of association studies. Bioinformatics 2006; 22 (15): 1928–1929.

9. Rho SB et al. Programmed cell death 6 (PDCD6) inhibits angiogenesis through PI3K/mTOR/p70S6K pathway by interacting of VEGFR-2. Cell Signal 2012; 24 (1): 131–139.

10. Shi S et al. Association between two single nucleotide polymorphisms of PDCD6 gene and increased endometriosis risk. Hum Immunol 2013; 74 (2): 215–218.

Received March 4, 2016.
Accepted March 22, 2016.