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ACE I/D polymorphism in Polish patients with endometriosis

Polimorfizm I/D genu ACE u polskich pacjentek z endometriozą

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Abstract

Objectives: *To analyse I/D polymorphism of ACE gene in women with diagnosed endometriosis and to determine the correlation of the identified genotypes with the stage of the disease and its clinical picture.*

Material and methods: *The analysis of ACE I/D gene polymorphism was performed in a group of 121 women with endometriosis and in the control group of 122 women. In the study group the stage of the disease, number of pathological foci, occurrence of chronic pain in pelvis minor and infertility were taken into consideration.*

Results: *In the study group the following distribution was found of ACE gene genotypes: II – 25.62%, ID – 47.93%, DD – 26.45%, whereas in the control group: II – 35.25%, ID – 41.80%, DD – 22.95%. The comparison of the frequency of analysed genotypes and alleles between the study and control groups did not demonstrate statistically significant differences (p>0.05). Similarly no correlation was found for these parameters when the four stages of the disease acc. to rAFS (p>0.05) were compared. In the study group the frequency was compared of the analysed ACE gene genotypes and alleles in women with infertility (n=59) and in fertile women (n=62). The comparison of these parameters did not demonstrate statistically significant differences between the analysed groups (p>0.05). The frequency of genotype II was 17.07%, ID – 46.34% and DD – 36.59% in women with endometriosis complaining of pain (n=41). In the group of women with endometriosis without pain the frequency of the investigated genotypes (II, ID, DD) was respectively: 26.53%, 59.18%, 14.29%. DD genotype and D allele were more frequent in patients with pain complaints (p<0.05).*

Conclusion: *In analysed population no association was found of ACE I/D polymorphism and the prevalence of endometriosis, its stages or the number of disease foci. However, the association of DD genotype and D allele with the occurrence of pain within pelvis minor in women with endometriosis was found.*

Key words: **endometriosis** / **angiotensin I converting enzyme** / **genetic polymorphism** / / **pelvic pain** /

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Streszczenie

Cel pracy: *Analiza polimorfizmu I/D genu ACE u kobiet ze zdiagnozowaną endometriozą oraz określenie korelacji zidentyfikowanych genotypów ze stopniem zaawansowania choroby i jej obrazem klinicznym.*

Materiał i metody: *Przeprowadzono analizę polimorfizmu I/D genu ACE w grupie 121 kobiet z endometriozą i 122 kobiet z grupy kontrolnej. W grupie badanej wzięto pod uwagę stopień zaawansowania i liczbę ognisk choroby, występowanie przewlekłego bólu miednicy mniejszej i bezpłodności.*

Wyniki: *W grupie badanej stwierdzono następujący rozkład genotypów genu ACE: II – 25,62%, ID – 47,93%, DD – 26,45%, a w grupie kontrolnej: II – 35,25%, ID – 41,80%, DD – 22,95%. Porównanie częstości występowania badanych genotypów i alleli pomiędzy grupą badaną i kontrolną nie wykazało różnic istotnych statystycznie (p>0,05). Podobnie brak korelacji dla tych parametrów zaobserwowano w przepadku porównania czterech stopni zaawansowania choroby wg. rAFS (p>0,05).*

W grupie badanej porównano częstość występowania analizowanych genotypów i alleli genu ACE u kobiet z niepłodnością (n=59) i płodnych kobiet (n=62). Analiza tych parametrów nie wykazała różnic statystycznie istotnych pomiędzy badanymi grupami (p>0,05). U kobiet z endometriozą, które uskarżały się na ból (n=41) genotyp II występował z częstością 17,07%, ID – 46,34%, DD – 36,59%. W grupie kobiet z endometriozą, u których ból nie występował, badane genotypy (II, ID, DD) występowały odpowiednio z częstością: 26,53%, 59,18%, 14,29%. Genotyp DD i allel D występował częściej u kobiet z dolegliwościami bólowymi (p<0,05).

Wnioski: *W badanej populacji kobiet nie wykazano istnienia związku między polimorfizmem genotypu I/D genu ACE, a występowaniem endometriozy, stopniem jej zaawansowania oraz liczbą ognisk choroby. Natomiast wykazano związek genotypu DD i allelu D z występowaniem u kobiet z endometriozą objawów bólowych w obrębie miednicy mniejszej.*

Słowa kluczowe: **endometrioza** / **enzym konwertujący angiotensynę** / / **genetyczny polimorfizm** / **ból miednicy mniejszej** /

Introduction

Endometriosis is a benign estrogen-dependent disease of women, mainly during their reproductive years, when endometrial cells grow outside of the uterus which evokes chronic inflammatory reaction. Pelvis minor organs and peritoneal surface are the locations most frequently affected [1]. Endometriosis is most often divided into three categories: peritoneal endometriosis, ovarian endometrial cysts and rectouterine pouch endometriosis [2]. Occasionally endometriosis can also develop in other parts of the body e.g. in lungs, appendix. It has been estimated that it occurs in about 10% of women in their reproductive years but it increases even to 35-50% of women complaining of pelvic pain or infertility [3, 4].

Aetiology of endometriosis has not been explained although there exist several theories competing with each other. Retrograde menstruation is thought to be one of the causes of the disease. During a woman's menstrual flow some of the menstrual effluent goes in reverse into the peritoneal cavity (implantation theory). There comes to ectopic implantation of endometrial cells in the peritoneum and to establishment of the disease foci [5]. Other frequently mentioned and already classical theories explaining the development of endometriosis are the theory of metaplasia and the theory of induction [1]. Furthermore, short menstrual cycle, prolonged menstrual bleeding and small number of labours are factors increasing the risk of endometriosis development [1, 4, 5]. In recent years, the share of the angiogenesis in the development and persistence of endometriosis foci in peritoneal cavity has been indicated [4-6].

Angiopoetins, increasing the exposure of endothelial cells to the activity of angiogenic factors, matrix metalloproteinases (MMP) degrading the components of extracellular matrix and facilitating penetration of newly formed blood vessels, vascular-endothelial growth factor (VEGF) and interleukins (IL-8 and IL-6) are the factors playing an important role in the process of neovascularisation in endometriosis [4-7].

Genetic factors also play a role in the development of endometriosis [3, 8-10]. In the case of severe endometriosis the occurrence of hereditary predispositions of multigene inheritance is observed [11]. The contribution of a genetic factor in the development of endometriosis was also confirmed by studies on monozygotic twins [9]. Up till now, multiple candidate genes have been analysed as their involvement in the pathogenesis of endometriosis seems to be of major importance, among others: genes encoding cytokines and pro-inflammatory factors, adhesive proteins and extracellular matrix enzymes, growth factors, cell cycle and apoptosis regulatory proteins, receptors for hormones [reviewed 9, 10]. Renin-angiotensin system (RAS) was suggested to be another factor contributing to the development of endometriosis. It acts as hormonal system [12].

The RAS gene system comprises the renin, angiotensinogen (AGT), angiotensin I converting enzyme (ACE) and angiotensin II receptor types 1 and 2 (AT_1, AT_2) genes. [13]. Renin is synthesized in kidneys and released from granular juxtaglomerular cells. This enzyme demonstrates aspargine protease activity and takes part in angiotensinogen (synthesized in liver) to angiotensin I conversion. Then, angiotensin I is converted to angiotensin II – a key compound of RAS system which transmits the signal into the cells through membrane receptors of type 1 and 2 [13]. Besides angiotensin systemic activity its local (tissue) potential is emphasized. Deletion of 287-base pair fragment in intron 16 of *ACE* gene results in increased level of angiotensin I convertase in plasma [14].

Objectives

The aim of the study was the analysis of insertion/deletion polymorphism in the angiotensin I converting enzyme gene (*ACE*) in women with diagnosed endometriosis and to determine the correlation of the identified genotypes with the stage of the disease and its clinical picture.

Material and methods

Subjects

A cohort of 121 women diagnosed upon laparoscopic examination with different stages (I – minimal, II – mild, III – moderate, IV - severe) of endometriosis using the revised American Fertility Society (rAFS) classification system [15] were recruited at the Department of Surgical and Endoscopic Gyneacology, Polish Mother's Memorial Hospital, Lodz, Poland. Twenty-nine patients were classified as stage I of the disease (mean age 32.1±4.7, range 23-42), 22 as stage II (mean age 33.8±5.8, range 25-51), 35 as stage III (mean age 33.2±6.8, range 21-49) and 35 patients were stage IV (mean age 31.2±5.6, range 20-56).

A population of 122 women without endometriosis from the Department of Surgical and Endoscopic Gyneacology were studied as a control group (mean age 40.8±10.4, range 22-67).

The protocol had been previously approved by the Bioethical Committee of the Medical University of Lodz (RNN/47/09/KB).

Molecular analysis

Genomic DNA was isolated from the peripheral blood with the Blood Mini kit (DNA-Gdańsk, Poland) according to the manufacturers procedure. Analysis of *ACE* genotypes was performed using polymerase chain reaction (PCR).

The primer sequences were as follows: sense primer - 5'CTGGAGACCACTCCCATCCTTTCT3' and antisense primer: 5'GATGTGGCCATCACATTCGTCAGAT3', as described by Hsieh and coworkers [16].

PCR reaction was performed in a final volume of 50µl containing 150ng/sample of primers, 0.1 mM/sample of dNTPs, DNA buffer 1X/sample, 1.5 U/sample of *Taq* polymerase and 50ng of genomic DNA. The DNA was amplified for initial denaturation at 94°C for 5 min. followed by 35 cycles of 94°C for 30 sec, 62°C for 30 sec and 72°C for 45 sec following a final extension of 72°C for 10 min. (Genius, Techne).

The amplification products were analyzed by electrophoresis of 5 µl samples on 2% agarose gels and visualizing with ethidium bromide staining. The products were of the size about 190 bp and 490 bp for *I* and *D* allele respectively. (Figure 1).

Statistical analysis

Statistical analysis was performed using the Statistica 8.0 pl. Allele and genotype frequencies were compared between groups using the χ^2 test (or Fisher's exact test when necessary). Non-parametric variables were analyzed with the U-Mann-Whitney test. $P < 0.05$ was considered statistically significant.

Results

In the study group (n=121) the following distribution was found of *ACE* gene genotypes: *II* – 25.62% *ID* – 47.93%, *DD* – 26.45%, whereas in the control group (n=122): *II* – 35.25%, *ID* – 41.80%, *DD* – 22.95%. (Table I).

I and *D* allele frequencies were: *I* – 49.59%, 56.15%; *D* – 50.41%, 49.85% respectively for the study and control groups. (Table II).

The comparison of the frequency of *II, ID* and *DD* genotypes and *I* and *D* alleles between the study and control groups did not demonstrate statistically significant differences (respectively: p=0.265; p=0.147). No correlation was found as regards the frequency of the investigated genotypes (Table I) and alleles (Table II) when the four stages of the disease acc. to rAFS were compared (respectively: p=0.266; p=0.624).

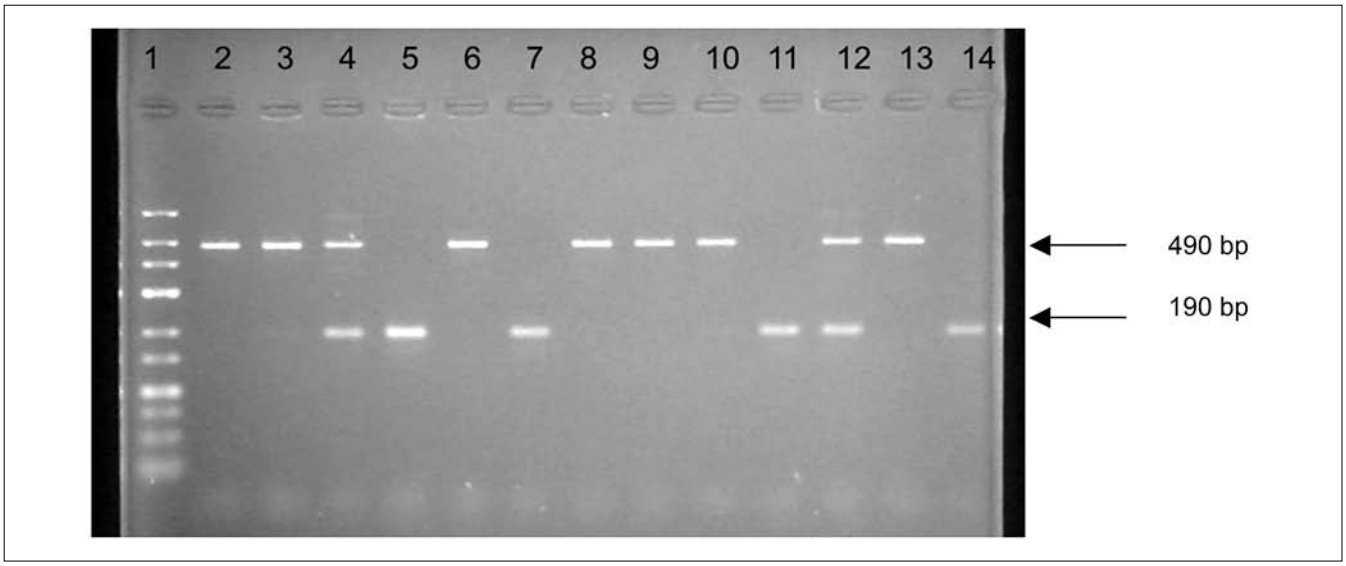

Figure 1. Gel electrophoresis of the *ACE I/D* polymorphism. 1: DNA size marker (Low Range); *II:* 2, 3, 6, 8-10, 13; *ID:* 4, 12; *DD:* 5, 7, 11, 14.

Table I. Genotype frequencies for *ACE* I/D gene polymorphism in individuals with and without endometriosis.

Disease stage (rAFS)*	*II* N (%)	*ID* N (%)	*DD* N (%)	Total N (%)
I	6 (20.69)	19 (65.52)	4 (13.87)	29 (100)
II	7 (31.82)	7 (31.82)	8 (36.36)	22 (100)
III	8 (22.86)	15 (42.86)	12 (34.29)	35 (100)
IV	10 (28.57)	17 (48.57)	8 (22.86)	35 (100)
Total	31 (25.62)	58 (47.93)	32 (26.45)	121 (100)
Control	43 (35.25)	51 (41.80)	28 (22.95)	122 (100)

* revised American Fertility Society

Table II. Alleles frequencies for *ACE* I/D gene polymorphism in individuals with and without endometriosis.

Disease stage (rAFS)*	*I* N (%)	*D* N (%)
I	32 (54.24)	27 (45.76)
II	20 (46.51)	23 (53.49)
III	31 (44.29)	39 (55.71)
IV	37 (52.86)	33 (47.14)
Total	120 (49.59)	122 (50.41)
Control	137 (56.15)	107 (43.85)

* revised American Fertility Society

Among patients with endometriosis (n=121) in 55 of them a single disease focus was observed, in 41-two and in 19 endometrial lesions comprised three localizations, in 4 patients – four, in 2 – five localizations. Table III demonstrates the frequency of individual genotypes of I/D polymorphism in *ACE* gene. The comparison of the investigated genotypes and alleles between these groups with the use of nonparametric Mann-Whitney test did not show statistically significant differences (respectively: p=0.594; p=0.947).

The frequency of the analysed *ACE* gene genotypes and alleles was compared in the study group in women suffering from infertility (n=59) and in fertile women (n=62). The following distribution of genotypes was observed in individual investigated groups: patients with infertility: *II* – 22.03%, *ID* – 52.54%, *DD* – 25.42%; patients without fertility disorders: *II* – 29.03, *ID* – 43.55%, *DD* – 27.42%. The frequency of *I* and *D* alleles was: *I* – 48.31%, 50.81%; *D* – 51.69%, 49.19% respectively for the patients with infertility and in those with normal fertility. (Table IV). The comparison of the frequency of genotypes and alleles of *ACE* gene did not demonstrate statistically significant differences between both analysed groups (respectively: p=0.567; p=0.697).

Information was obtained from 90 patients with diagnosed endometriosis concerning the occurrence of chronic pelvic pain. In patients who complained of pain (n=41) the frequency of *II* genotype was 17.07%, of *ID* genotype – 46.34% and of *DD* genotype – 36.59%. The frequency of these genotypes in the group of patients with no pain was respectively: 26.53%, 59.18%, 14.29%. (Table V).

DD genotype was more frequent in patients with pelvic pain and the difference was statistically significant (χ^2=6.13, p=0.046). Similarly, the frequency of *D* allele in this group was significantly higher compared to the group of patients with no pain (χ^2=4.5, p=0.034).

Discussion

Endometriosis affects even 10% of all reproductive-aged women. Its aetiology still remains unrecognized. Endometrium is one of few mature tissues which is distinguished by regular periods of rapid growth and abruption. Thus, angiogenesis is an important element of this tissue functioning. That is why recently it has been suggested that the mechanism of the development and persistence of this disease foci may be engaged in the angiogenesis [4-7]. The fact that endometriotic implants are often surrounded by a network of blood vessels can be a proof. Neovascularization around and inside endometriotic lesions was also confirmed by microscopic analysis [6]. Inan et al. observed increased expression of von Willebrand factor and CD34 (antigens specific for vascular endothelium) in endometrial cysts in relation to the tissue of normal ovary [17]. In the authors' opinion this proves the significant role of neoangiogenesis in the development of endometriosis. Ria et al., who compared the structure of blood vessels in normal endometrium and in endometrial cysts came to the same conclusion [18]. Immaturity of newly formed vessels and high proliferative activity of endothelial cells in active inflammatory lesions were also observed in peritoneal endometriosis [5, 19, 20].

Owing to reports proving coexistence of endometriosis and neovascularization, there was undertaken the analysis of polymorphism of genes engaged in the development and functioning of blood vessels including also the genes of renin-angiotensin system [10].

RAS system regulates, among others, blood pressure by influencing vessel tension, renal haemodynamics and water and electrolyte balance [21]. The studies revealed that angiotensin I convertase is present on the surface of vessel epithelial cells within the whole circulatory system. Also renin released from kidneys can be captured by epithelial cells. This enables local production of angiotensin II which demonstrates autocrine/paracrine activity [13]. Furthermore, angiotensin II produced in tissues stimulates smooth muscle vessel proliferation. The contribution of RAS is also suggested as one of the factors in the initiation of the menstrual cycle [22].

Table III. Genotype and allele frequencies for *ACE* I/D gene polymorphism in individuals with different number of *endometriosis foci.*

	Number of *endometriosis foci*				
	1	**2**	**3**	**4**	**5**
II	15	7	6	3	0
ID	24	23	9	1	1
DD	16	11	4	0	1
Total	55	41	19	4	2

Table IV. Genotype and allele frequencies for *ACE* I/D gene polymorphism in individuals with endometriosis with and without infertility.

Infertility	*II* N (%)	*ID* N (%)	*DD* N (%)	Total N (%)	*I* N (%)	*D* N (%)
Yes	13 (22.03)	31 (52.54)	15 (25.42)	59 (100)	57 (48.31)	61 (51.69)
No	18 (29.03)	27 (43.55)	17 (27.42)	62 (100)	63 (50.81)	61 (49.19)
Total	31 (25.62)	58 (47.93)	32 (26.45)	121 (100)	120 (49.59)	122 (50.41)

Table V. Genotype and allele frequencies for *ACE* I/D gene polymorphism in individuals with endometriosis with and without pain.

Pain	*II* N (%)	*ID* N (%)	*DD* N (%)	Total N (%)	*I* N (%)	*D* N (%)
Yes	7 (17.07)	19 (46.34)	15 (36.59)	41 (100)	33 (40.24)	49 (59.76)
No	13 (26.53)	29 (59.18)	7 (14.29)	49 (100)	55 (56.12)	43 (43.88)
Total	20 (22.22)	42 (53.33)	22 (24.44)	90 (100)	88 (48.89)	92 (51.11)

To date, in the available medical literature one study can be found concerning the analysis of insertion/deletion angiotensin I-converting enzyme gene polymorphism in women with endometriosis. The authors determined *ACE* gene genotype in 125 women with endometriosis and in 128 from the control group. According to the authors genotypes with insertion allele of *ACE* gene (*II, ID*) seem to predispose to the development of endometriosis in Taiwan population [16].

In this study 121 women with endometriosis and 120 from the control group were analysed. The obtained results did not demonstrate any association between *ACE I/D* gene polymorphism and the prevalence of endometriosis in women nor the stage of the disease. On the other hand, Hsieh et al., estimated polymorphism only in women with advanced stage of the disease, whereas in this study the study group included all four stages of the disease acc. to rAFS [15, 16].

Owing to very differentiated as regards localization and number of endometrial foci in the investigated women, the authors divided the study group into subgroups dependently on the number of endometrial foci. In nearly half (n=55) of the investigated women endometriosis was related to one organ (most frequently it was peritoneum or ovary), in 44 women the disease occurred in two localizations and in 25 patients endometriosis affected three or more sites. (Table III).

Statistical analysis did not demonstrate any association between the number of the foci and *ACE I/D* gene polymorphism.

Infertility is one of the main symptoms accompanying endometriosis. It has been estimated that it affects even 50% of women with this disease [3, 4]. Despite the visible association between endometriosis and infertility the mechanism of this dependence has not been recognized [23]. Ovulatory disorders, disturbed transport of gametes through uterine tubes, hormonal disorders (including LH), toxic effect of factors associated with inflammatory reaction on fetus and also on sperm and embryo implantation in endometrium are among the suggested mechanisms leading to fertility disturbances in women with endometriosis [23, 24].

To date, the association has been successfully demonstrated of several polymorphisms found in human genome with infertility of women suffering from endometriosis. The studies carried out by Lamp et al., revealed increased susceptibility to fertility disorders in patients with endometriosis when two types were found of polymorphisms of encoding gene 17β-hydroxysteroid dehydrogenase: HSD17B1 A/G SNP A allele and ESR1 longer $(TA)_n$ repeats [25]. Also Marfa et al. suggest that LHbeta G1502A polymorphism may be involved in the predisposition to minimal/mild endometriosis-associated infertility, although it is not clear if endometriosis is not only a coincidental finding along with infertility [26].

In our studies infertility was found in 59 patients. No fertility disorders were observed in the remaining 62 patients with endometriosis. The frequency of genotypes: *II, ID* and *DD* and alleles *I* and *D* of *I/D ACE* polymorphism was similar in both groups. (Table IV). Insignificant differences which were noted were not statistically significant.

The major symptoms of endometriosis and reasons for its treatment, besides infertility, include pain complaints such as: strong pain with menstrual and perimenstrual periods, dyspareunia and chronic pelvic pain [3]. The precise mechanism responsible for pain in the course of endometriosis has not been known. It seems that this pain results from both direct and indirect effect of endometrial alterations. The direct effect includes implantation of endometrial foci into normal tissues and their periodical bleeding and stimulation of fibrosis. Indirect effect includes synthesis of cytokines and other proinflammatory factors which can irritate neighbouring nerve roots and silent nociceptors [27].

In this study information about pelvic pain was obtained from 90 patients with endometriosis. In 41 (45.56%) of them the pain occurred, whereas the remaining 49 (54.44%) of women did not complain of pain. The statistical analysis showed that both *DD* genotype and *D* allele of *ACE I/D* gene polymorphism were more frequent in women with pain symptoms ($p<0.05$).

As it has been already mentioned, endometriosis is estrogen-dependent and thus its development is strictly dependent on estrogen metabolism. The level of oestriol (E2) in menstrual blood of women with endometriosis is higher compared to healthy women which suggests that this hormone is synthesized in them locally in endothelium [28]. In ectopic endometrium there is observed a disturbed level of expression of enzymes engaged in estrogen metabolism in relation to normally localized tissue, among others increased expression of aromatase, 17β-hydroxysteroid dehydrogenase (17β-HSD) type 1, 7, 12, or decreased expression of 17β-HSD type 2, which leads to excessive synthesis of oestriol [29].

The studies performed by Chakrabarty et al., on animal model with the use of rat nerve cells demonstrated that oestriol on signal pathway in which type 2 angiotensin receptor (AT_2) is engaged, stimulates growth of nociceptors. Furthermore, E2 promotes neuritogenesis only in the presence of active ACE [30]. It has been known that *DD* genotype of *ACE* gene increases this enzyme activity. The results of studies of these authors can be the base for discussion why increased level of angiotensin I convertase in blood of women is more frequently associated with pain complaints than in the patients with insertion allele.

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