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Plasminogen activator inhibitor-1 4G/5G polymorphism in infertile women with and without endometriosis

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Abstract

Objective. To evaluate *PAI-1* genotypes in a group of infertile women with or without endometriosis and control subjects. *Design.* Case–control study. *Setting*. Human Reproduction Center of Medicina do ABC Faculty. *Populatio*n. One hundred and forty infertile women with endometriosis, 64 women with idiopathic infertility and 148 fertile women as control subjects. *Methods.* The *PAI-1* 4G/5G polymorphism was identified by restriction fragment length polymorphism–polymerase chain reaction. *Main outcome measures.* Genotype distribution and allele frequency of the 4G/5G polymorphism of the *PAI-1* gene. *Results.* The frequencies of genotypes 4G/4G, 4G/5G and 5G/5G of the *PAI-1* gene in the infertile women with endometriosis were 38.6, 37.1 and 24.3%, respectively, and in the control group 24.3, 33.8 and 41.9%, respectively (p=0.003). When the infertile women with endometriosis were divided according to their endometriosis stage, genotypes 4G/4G, 4G/5G and 5G/5G were identified, respectively, in 36.7, 32.9 and 30.4% of the patients with minimal/mild endometriosis (p=0.102) and in 41.0, 42.6 and 16.4% of the patients with moderate/severe endometriosis (p=0.001); in the women with idiopathic infertility, these genotypes were found at a frequency of 29.7, 34.3 and 36%, respectively (p=0.637). *Conclusion.* The data suggest that, in Brazilian women, the *PAI-1* 4G/5G polymorphism may be associated with a risk of endometriosis-associated infertility.

Abbreviations: CI, confidence interval; EDTA, ethylenediamine tetraacetic acid; G, guanine; $MgCl_2$, magnesium chloride; OR, odds ratio; PA, plasminogen activator; PAI-1ag, plasminogen activator inhibitor-1 antigen; *PAI-1* gene, plasminogen activator inhibitor-1 gene; RFLP–PCR, restriction fragment length polymorphism–polymerase chain reaction; SERPIN, serine protease inhibitor; tPA, tissue-type plasminogen activator.

Introduction

Endometriosis is a common gynecological disease, defined as the growth of endometrial tissue outside the uterine cavity, that often results in a vast array of gynecological problems, including dyspareunia, dysmenorrhea, pelvic pain and infertility (1,2). Previous studies have revealed a great number of genetic markers related to the immune, neuroendocrine and reproductive function and of gene interactions, indicating an association between the development of endometriosis and genetic polymorphisms (3–5).

The fibrinolytic system includes a broad spectrum of proteolytic enzymes with physiological and pathophysiological functions such as fibrinolysis, tissue remodeling, tumor invasion, and also participation in the reproductive process (6,7). The plasminogen activator inhibitor-1 (*PAI-1*) gene, a member of the serine protease inhibitor family, is a main regulator of the endogenous fibrinolytic system. It inhibits the

fibrinolytic activity of the tissue-type plasminogen activator (tPA), which produces active plasmin from plasminogen, which then cleaves the fibrin (8). An altered proteolytic status has been suggested to be involved as a key factor in the development of endometriosis (9).

There is evidence of a modified fibrinolytic activity in the eutopic endometrium of women with endometriosis that could result in endometrial fragments with a high potential to adhesion onto the peritoneal lining, degradation of the extracellular matrix components, invasive growth by pericellular proteolysis and cell migration into the surrounding tissue (9).

Changes in PAI-1 biosynthesis are usually preceded by changes in its gene transcription (10), such as those produced by the guanine (G) insertion/deletion polymorphism in the promoter region of the *PAI-1* gene, named 4G/5G, described by Dawson et al. (11). *In vitro* studies suggest that the 4G allele has a greater activity than the 5G allele, since the 5G allele contains an additional binding site for a DNA-binding protein that acts as a transcriptional repressor (12).

Thus, the objective of this study was to evaluate *PAI-1* genotypes and their relations with the susceptibility to infertility and/or endometriosis.

Materials and methods

One hundred and forty infertile women with endometriosis (mean age 34.4±4.1 years) from the Endometriosis Outpatient Clinic of the Human Reproduction Center of Medicina do ABC Faculty, Santo André, Brazil were studied. Women with endometriosis diagnosed by laparoscopy were selected and classified according to the American Society for Reproductive Medicine (13), with histological confirmation of the disease. In the endometriosis group, the stage of the disease was found to be minimal/mild (stage I and II) in 79 women (56.4%) and moderate/severe (stage III and IV) in 61 women (43.6%). Sixty-four women with idiopathic infertility (mean age 35.9±5.0 years) were also selected at the Human Reproduction Center of Medicina do ABC Faculty. For the control group, 148 fertile women (mean age 39.7 ± 3.2 years) were recruited especially for this study among a group of patients having tubal ligation at the Family Planning Outpatient Clinic of Medicina do ABC Faculty. In all of them, absence of endometriosis was confirmed.

The cause of infertility was investigated according to the minimum propedeutic procedure for infertile couples: hormone and biochemistry profile, testing for sexually transmitted diseases, imaging examinations, investigation of genetic and/or immunological abnormalities, semen analysis of the partner, hysterosalpingography, hysteroscopy and laparoscopy (performed in all women up to 36 years old and also in patients over 36 years old whenever there were symptoms or abnormalities on imaging examinations). If none of these examinations revealed an abnormality, infertility was considered idiopathic. Women with endometriosis who did not achieve pregnancy after at least six natural or induced cycles following laparoscopy were considered infertile. All women whose partner had any male factors associated with infertility were excluded.

Clinical data and peripheral blood samples were collected only after explaining the objectives of the study, and all participants signed an informed consent form, as approved by the Research Ethics Committee of the ABC School of Medicine.

Peripheral blood was collected from each patient and control subject into an EDTA-containing test tube. Genomic DNA was extracted from peripheral blood lymphocytes using an Illustra blood genomicPrep Mini Spin Kit (GE Healthcare Life Sciences, Buckinghamshire, UK), according to the manufacturer's instructions.

Molecular analysis of the *PAI-1* gene 4G/5G polymorphism (rs1799889) was performed according to the protocol of Karadeniz et al. (14). The primers used were as follows: 5′-CCAACAGAGGACTCTTGGTCT-3′ (forward) and 5′-CACAGAGAGAGTCTGGCCACGT-3′ (reverse). The PCR was carried out in a final volume of 25 μl, containing 1× buffer, 2.5 mM of $MgCl_2$, 0.1 mM of each deoxynucleoside triphosphate, 50 nM of each primer, 1 U Taq polymerase (Invitrogen, Carlsbad, CA, USA) and 200 ng of DNA. The cycling conditions comprised a denaturation step at 95°C for 10 min, followed by 35 amplification cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 45 s, and a final extension at 72°C for 7 min. The 100 bp PCR products were analysed for restriction fragment length polymorphism (RFLP) by using 5 U of *Bsl*I restriction enzyme at 55°C for 15 min (Fermentas Life Science Inc., Baltimore, MD, USA). The fragments, a single one of 99 bp for the 4G allele and two fragments of 77 and 22 bp, respectively, for the 5G allele, were visualized in 2.5% agarose gel stained with ethidium bromide, under ultraviolet light.

Statistical analyses were carried out using SPSS for Windows 11.0 (SPSS Inc., Chicago, IL, USA). The χ^2 test was used to detect differences in allele and genotype frequencies between patients and control subjects. The odds ratio (OR) was used to measure the strength of the association between the frequencies of *PAI-1* genotypes and endometriosis and/or infertility. All *p*-values were two-tailed, and 95% confidence intervals (CIs) were calculated. A p-value <0.05 was considered significant.

Results

The results are summarized in Table 1. The 4G/4G, 4G/5G and 5G/5G genotype frequencies of the *PAI-1* 4G/5G polymorphism in the infertile women with endometriosis were 38.6 (54 of 140), 37.1 (52 of 140) and 24.3% (34 of 140), respectively, and 24.3 (36 of 148), 33.8 (50 of 148) and 41.0%

Table 1. Genotypes and allele frequencies of the *PAI-1* 4G/5G polymorphism in infertile patients with endometriosis, idiopathic infertile patients and a control group.

Population studied	*n*	*PAI-1* genotypes							4G4G + 4G5G		Alleles					
		4G4G	Percentage	4G5G	Percentage	5G5G	Percentage	*p*-Value[a]	*p*-Value[b]	*p*-Value	4G	Percentage	5G	Percentage	*p*-Value[a]	*p*-Value[b]	Odds ratio (95% CI)
Endometriosis-associated infertility patients	140	54	38.6	52	37.1	34	24.3	0.003	0.202	0.013	160	57.1	120	48.9	0.0002	0.068	0.93 (0.67–1.30)
Minimal/mild endometriosis	79	29	36.7	26	32.9	24	30.4	0.102	0.647	0.070	84	53.2	74	46.8	0.019	0.348	0.62 (0.42–0.91)
Moderate/severe endometriosis	61	25	41.0	26	42.6	10	16.4	0.001	0.045	0.025	76	62.3	46	37.7	0.0001	0.020	0.42 (0.28–0.65)
Idiopathic infertile patients	64	19	29.7	22	34.3	23	36.0	0.637	–	0.556	60	46.9	68	53.1	0.33	–	0.79 (0.52–1.21)
Control subjects	148	36	24.3	50	33.8	62	41.9	–	–	–	122	41.2	174	58.8	–	–	–

Note: CI, confidence interval. Minimal/mild endometriosis vs. moderate/severe endometriosis, p=0.149 (genotype) and 0.158 (alleles).
[a] p-Value vs. control subjects; [b] p-value vs. idiopathic infertility patients.

(62 of 148) in the control group (p=0.003). When the infertile women with endometriosis were divided according to endometriosis stage, the 4G/4G, 4G/5G and 5G/5G genotypes presented frequencies of 36.7 (29 of 79), 32.9 (26 of 79) and 30.4% (24 of 79) among women with minimal/mild endometriosis (p=0.102), and 41.0 (25 of 61), 42.6 (26 of 61) and 16.4% (10 of 61) in the women with moderate/severe endometriosis (p=0.001). Among the infertile women without endometriosis, genotypes 4G/4G, 4G/5G and 5G/5G were observed in 29.7 (19 of 64), 34.3 (22 of 64) and 36.0% (23 of 64), respectively (p=0.637; Table 1).

Regarding the allele distribution, allele 4G was present in 57.1% of the infertile women with endometriosis, 46.9% of the infertile women without endometriosis and 41.2% of the control subjects, whereas allele 5G was present in 48.9, 53.1 and 58.8%, respectively, in the patients with endometriosis (p=0.0002, OR=0.93, 95% CI=0.67–1.30), infertile women without endometriosis (p=0.330, OR=0.79, 95% CI=0.52–1.21) and control subjects. Of the women with minimal/mild endometriosis, 53.2% presented allele 4G and 46.8% presented allele 5G (p=0.019, OR=0.62, 95% CI=0.42–0.91). Regarding the women with moderate/severe endometriosis, the frequencies of alleles 4G and 5G were 62.3 and 37.7%, respectively (p=0.0001, OR=0.42, 95% CI=0.28–0.65; Table 1).

The power of the test was 0.99 (α=0.01) for the endometriosis-associated infertility group.

Discussion

Plasminogen activator inhibitor-1 is the main inhibitor of plasminogen activator and thus of fibrinolysis. Homozygosity for the 4G allele of the *PAI-1* gene is associated with increased transcription of the *PAI-1* gene, resulting in an enhanced gene expression (15). Individuals who are homozygous for the 4G allele have the highest, heterozygotes an intermediate and 5G homozygotes the lowest plasma PAI-1 concentrations (16). High *PAI-1* expression is associated with inhibition of the conversion of plasminogen to plasmin and subsequent hypofibrinolysis.

Bedaiwy et al. (17) studied the *PAI-1* 4G/5G polymorphism in 75 women with endometriosis and 43 control subjects. They found genotype 4G/4G in 69% of the women with endometriosis and in only 12% of the control group, while 5G/5G was present in 3% of the women with endometriosis and in 56% of the control subjects. The authors concluded that persistence of fibrin matrix could support the initiation of endometriotic lesions in the peritoneal cavity, explaining why some women with retrograde menstruation develop endometriosis and others do not.

Ramón et al. (18) studied the *PAI-1* 4G/5G polymorphism in 170 patients with endometriosis and 219 control subjects and analyzed its influence on *PAI-1* expression in

Table 2. Frequency of the *PAI-1* 4G/5G polymorphism associated with endometriosis in different population studies.

Study	Population studied	Conclusion of the study
Bedaiwy et al. (2006) (17)	75 women with endometriosis and 43 controls	Persistence of fibrin matrix could support the initiation of endometriotic lesions in the peritoneal cavity, explaining why some women with retrograde menstruation develop endometriosis and others do not.
Ramón et al. (2008) (18)	170 patients with endometriosis and 219 controls	The increased PAI-1ag levels observed in peritoneal fluid from patients could contribute to increase the peritoneal adhesions observed in endometriosis.
Gentilini et al. (2009) (19)	368 Italian women of reproductive age with gynecological problems and 329 normal subjects	The findings exclude a significant role of this polymorphism in endometriosis development.
Present study	140 infertile women with endometriosis, 64 women with idiopathic infertility and 148 fertile women (controls)	The findings showed that, in Brazilian women, the *PAI-1* 4G/5G polymorphism is associated with an increased risk of developing endometriosis-associated infertility.

endometrial tissue and peritoneal fluid. Their results showed a similar *PAI-1* genotype distribution in patients and control subjects. In the control subjects, the *PAI-1* levels in endometrial tissue and peritoneal fluid seemed to be associated with the *PAI-1* 4G/5G polymorphism. The authors concluded that the increased plasminogen activator inhibitor-1 antigen (PAI-1ag) levels observed in peritoneal fluid from patients could contribute to an increase of the peritoneal adhesions observed in endometriosis.

In contrast, the findings of Gentilini et al. (19), who evaluated the *PAI-1* 4G/5G polymorphism in 368 Italian women of reproductive age who underwent gynecological laparoscopy for chronic pelvic pain, infertility, ovarian cysts and myomas and 329 normal subjects, did not associate this polymorphism with the development of endometriosis.

In the present study, the genotype frequencies of the *PAI-1* 4G/5G polymorphism were significantly different in infertile women with endometriosis compared with control subjects (p=0.003). Considering the women with minimal/mild endometriosis and moderate/severe endometriosis separately, the difference was more evident in the women with advanced disease (p=0.102 and p=0.001, respectively).

Regarding the infertile women without endometriosis, no significant differences from control subjects were detected. When we compared the infertile groups with and without endometriosis, we found no significant differences between the frequencies of this polymorphism in the two groups (p=0.21). Nevertheless, separating the infertile group with endometriosis by the disease stages, while there was no significant difference (p=0.35) between minimal/mild endometriosis-associated infertility patients and patients with idiopathic infertility, there was a significant difference (p=0.02) between patients with advanced stages and the idiopathic infertile patients. This suggests that, in the Brazilian population, the *PAI-1* 4G/5G polymorphism is related to the development of endometriosis-associated infertility (Table 2). In turn, minimal/mild endometriosis might be only a coincidental finding along with infertility.

It is important to keep in mind that the present study was performed with a special group of patients who had been operated by videolaparoscopy and thereafter exposed for at least 12 months to the possibility of pregnancy, had no male factor involved in the causes of infertility and, nonetheless, did not achieve pregnancy.

Genetic studies provide one important approach to define causal pathways influencing endometriosis. The number of gene mapping studies for this disease has increased in recent years as the role of genetic factors has become more widely accepted (20). Finding genetic variants contributing to complex diseases such as endometriosis is far more difficult because the contribution of individual genes is small, many genes contribute to an individual's risk of developing the disease, and disease risk is often modified by environment. Many studies are required for both the discovery and replication steps with sufficient power to detect the small effects of any individual variants (20).

We have been trying to understand which types of endometriosis should be considered a disease and could lead to infertility and should therefore be treated according to one of the available therapeutic modalities (3–5,21,22). This is not an easy task, as even during laparoscopy doubts may persist and progress. We believe that in the future it may become possible to determine which genetic profile might lead to endometriosis, which could be of great help in establishing therapeutic management and reproductive prognosis.

In conclusion, the present study showed that, in Brazilian women, the *PAI-1* 4G/5G polymorphism is associated with an increased risk of developing endometriosis-associated infertility and might contribute to the severity of endometriosis. Furthermore, minimal/mild endometriosis could be only a

coincidental finding along with infertility. However, the relevance of this variation in endometriosis is still a matter of discussion.

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