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GENETICS

Polymorphisms of *ICAM-1* and *IL-6* genes related to endometriosis in a sample of Brazilian women

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Abstract
Purpose This study investigated the possibility of K469E (rs5498) and G241R (rs1799969) polymorphisms, in *ICAM-1* gene, and G634C (rs1800796), in *IL-6* gene, being associated with the occurrence of endometriosis in a sample of Brazilian women.
Methods We genotyped 200 women (100 in control group and 100 in endometriosis group) by PCR-RFLP technique for G634C, K469E, and G241R polymorphisms.
Results No significant difference was observed in genotypic frequency between control and endometriosis groups for G634C and K469E polymorphisms ($p = 0.61$ and $p = 0.22$, respectively). In addition, no significant difference between stages I-II and III-IV of the disease was found for both SNPs ($p = 0.63$ and $p = 0.24$, respectively). All individuals were wild homozygotes for G241R polymorphism.
Conclusion This study suggests that polymorphisms K469E, G241R, and G634C are not associated with increased susceptibility to endometriosis in Brazilian women.

Keywords Endometriosis · Intercellular adhesion molecule-1 · Interleukin-6 · Polymorphism · Brazilian population

Capsule This study suggests that polymorphisms K469E, G241R, and G634C are not associated with increased susceptibility to endometriosis in Brazilian women.

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Introduction

Endometriosis is a chronic disease characterized by ectopic endometrial tissue, usually found in the pelvic cavity, ovaries, and uterine ligaments. This condition is present in 10 % of women in reproductive age, and it can manifest as chronic pelvic pain, dysmenorrhea, dyspareunia, and infertility [1, 2]. The hypothesis most widely accepted for the development of the disease is the retrograde menstruation through fallopian tubes during menses; however, the factors that determine the implantation and proliferation of endometrial cells outside the uterine cavity are still unknown [3, 4]. Genetic studies suggest a possible influence of SNPs (single-nucleotide polymorphisms) in genes encoding cytokines involved in the immune process in the development of endometriosis [5–7].

ICAM-1 gene (intercellular adhesion molecule-1), located on chromosome 19 (19p13), comprises seven exons and encodes a transmembrane glycoprotein with the same name, which belongs to the family of adhesion immunoglobulins (Ig) [6, 8, 9]. The expression of ICAM-1 occurs on the surface of various immunocompetent cells, such as endothelial cells, T-lymphocytes and fibroblasts [1, 10]. Its increased expression enhances the immune response by recruiting inflammatory cells [1, 11]; on the other hand, its reduction can interfere the adherence of immune cells, hindering proper immune response [6].

Two polymorphisms were identified in the coding region of *ICAM-1*: G241R and K469E. The G241R (rs1799969), mapped in exon 4, is characterized by the substitution from a guanine (G) to an adenine (A), resulting in the change of a glycine (GGG) to an arginine (AGG) at position 241 of the protein at the third Ig domain [6, 9, 11]. The variation in protein can affect the function of ICAM-1 [5] reducing the adhesion involved in the inflammatory response [6].

The SNP K469E (rs5498) is located in exon 6 [6] and consists of the exchange of a nucleotide from A to G at codon 496, promoting the variation of a lysine (AAG) for a glutamic acid (GAG) [9, 11]. The amino acid change alters the fifth Ig domain and interferes both the binding of ICAM-1 to lymphocytes LFA-1 and Mac-1 to leukocytes [12, 13].

IL-6 gene (Interleukin-6), located on chromosome 7 (7p21), encodes a cytokine that is secreted by various cells, such as B and T lymphocytes, macrophages, monocytes, fibroblasts, keratinocytes, and endothelial cells [14, 15]. It is responsible for regulating physiological and pathogenic processes, modulating the inflammatory response, cell maturation, and differentiation [16].

In several studies, the presence of this pro-inflammatory cytokine in serum and peritoneal fluid has been associated with endometriosis [15, 17]. The G634C variation (rs1800796), which comprises the exchange of a guanine for a cytosine in the promoter region of the IL-6 gene, may interfere with the expression of IL-6, and therefore, it has been studied as a possible pathogenic mechanism of endometriosis [18].

Thus, based on the hypothesis that immune and inflammatory changes are involved in the pathogenesis of endometriosis and that the frequency of SNPs may be specific to each population, the aim of this study was to analyze, in a sample of Brazilian women, the association of SNPs G241R and K496E (*ICAM-1* gene) and G634C (*IL-6* gene) with endometriosis.

Material and methods

This was a case-control study conducted with 200 women divided in two groups: endometriosis group (EDT) and control group (CT) recruited from February to December 2015 at Endometriosis Centre, Clinics Hospital, São Paulo, Brazil. EDT group consisted of patients aged 18–50 years submitted to laparoscopic surgery for evaluation of pelvic pain and/or infertility, with histological confirmation and classification of the disease according to Revised American Society for Reproductive Medicine (1996) in stages I to IV (20). CT group included women aged 18–50 years, who underwent surgery for uterine fibroids ($n = 54$), diagnostic laparoscopy due to pelvic pain or infertility with no lesions ($n = 20$), tubal ligation ($n = 13$), ovarian teratoma ($n = 11$), hydrosalpinx ($n = 2$), when the absence of endometriosis was confirmed. For both groups, exclusion criteria were no pelvic findings of inflammatory disease, gynecological cancer, or adenomyosis.

All individuals responded a questionnaire with medical history, infertility, surgical history, pain symptoms, and drugs. All participants signed an informed consent form approved by the Ethics Committee of the Clinics Hospital from University of São Paulo Medical School (protocol 534714/14). Patients with endometriosis were subclassified in stages I to IV [19]. We grouped together patients in stages I-II (minimal/mild) and III-IV (moderate/severe), in order to compare a possible genetic difference relate with the severity of the disease.

The sample size needed for the study (68 patients for each group) was calculated based on the assumption that the frequency of G241R on controls was 6 and 23 % on endometriosis groups, based in the study Aghajanpour [1], with an α-value of 0.05 and a β-value of 0.80.

Genotyping

DNA was extracted from 5 ml of peripheral blood samples collected in EDTA tube, using salting-out protocol [20]. Polymorphisms of the *ICAM-1* and *IL-6* genes were determined by PCR-RFLP technique.

DNA amplification was performed through a single PCR reaction in a total volume of 24 μl containing 100 ng of genomic DNA, 5 mM dNTPs (Invitrogen Life Technologies), 5 mM of each primer (Invitrogen Life Technologies), 37.5 mM $MgCl_2$, 1U Taq polymerase (Sinapse Inc), 25 × buffer and 1.0 μl DMSO. Thermocycling was performed in a thermocycler Veriti (Applied Biosystems) with conditions of initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing for 1 min (temperature varied according to the SNP) and extension at 72 °C for 2 min, and final extension at 72 °C for 10 min.

For polymorphisms of *ICAM-1* gene, primers were designed using the Primer3 v0.4.0 program (http://bioinfo.ut.ee/primer3-0.4.0/). The primers used for IL-6 were based on Chae et al. [8].

For K469E polymorphism, the primers used were 5′-GGAACCCATTGCCCGAGC-3′ (forward) and 5′-GCGGTGAGGATTGCATTAGGTC-3′ (reverse) with annealing temperature 55 °C. For G241R polymorphism, the primers used were 5′-ATCCCTGTCTGCTCACACCT-3′ (forward) and 5′-GAAGGAGTCGTTGCCATAGG-3′ (reverse) and annealing temperature 57 °C. For G634C polymorphism, the primers used were 5′-GAGACGCCTTGAAG TAACTG-3′ (forward) and 5′-AACCAAAGATGTTC TGAACTGA-3′ (reverse) and annealing temperature 52 °C.

Table 1 Restriction enzymes used for K469E, G241, and G634C SNPs and DNA fragments sizes for each genotype

Polymorphism Enzyme		K469E (A/G) BstUI	G241R (G/A) BsrGI	G634C (C/G) BsrBI
Genotype	Wild homozygote (pb)	225	208	122, 60
	Heterozygous (pb)	225, 136, 89	208, 114, 94	182, 122, 60
	Variant homozygote (pb)	136, 89	114, 94	182

PCR products were subjected to digestion using restriction enzymes and then placed in greenhouse at 37 °C overnight. To confirm the digestion, the products were subjected to agarose gel electrophoresis (3 %). The possible fragments results from the digestion of each polymorphism are shown in Table 1.

Statistical analysis

For each SNP evaluated, Hardy-Weinberg equilibrium was calculated. The *T* Student test was used to compare means in continuous variables, and chi-square coefficient ($\chi 2$) and OR (odds ratio) with confidence interval (IC) of 95 % were used to categorical variables. For all tests, the significant level was considered 5 %. Statistical analyses were performed using the software BioEstat 4 (http://bioestat.software.informer.com/4.0/).

Results

Clinical evaluation

Control and endometriosis groups were similar in relation to age (39.1 versus 40.3 years) and body mass index (27.3 versus 26.2 kg/m^2). There was a higher incidence of infertility in women with endometriosis compared to control group (26.0 versus 6.0 %; $p = 0.002$), as expected. Patients with endometriosis reported dysmenorrhea (73.0 %), chronic pelvic pain (67.0 %), deep dyspareunia (59.0 %), cyclic intestinal pain (28.0 %), and cyclic urinary pain (17.0 %).

Genetic analysis

All samples of 200 women (100 in the CT group and 100 in the EDT group) were genotyped for the three SNPs and results of genotypic and allelic frequencies are described in Table 2. All of the SPNs are in Hardy-Weinberg equilibrium in the population studied (G634C $p = 0.53$; K469E $p = 0.08$; G241R $p = 1.00$). Fragments observed after the restriction enzyme digestion are showed in Fig. 1.

ICAM-1 gene

No significant differences were found in genotypic frequencies of the SNP K469E between groups. Of the 100 patients with endometriosis, 40 exhibited wild homozygous genotype (AA), 50 were heterozygous (AG), and 10 displayed the variant in homozygous (GG). In the CT group, it was observed homozygous AA in 48 women, heterozygous GA in 38, and homozygotes GG in 14.

Table 2 Polymorphisms K469E and G634C: genotype and allelic frequencies in the control versus endometriosis groups (EDT) and by stage of the disease (20)

	N	K469E *N* (%) AA	AG	GG	p^a	Allele *N* (%) A	G	*p*
Controls	100	48 (48.0)	38 (38.0)	14 (14.0)	0.22	134 (67.0)	66 (33.0)	0.75
EDT	100	40 (40.0)	50 (50.0)	10 (10.0)		130 (65.0)	70 (35.0)	
EDT stage								
I-II	23	6 (26.1)	15 (65.2)	2 (8.7)	0.24	27 (58.6)	19 (41.3)	1.00
III-IV	77	34 (44.2)	35 (45.5)	8 (10.4)		103 (66.8)	51 (33.1)	
	N	G634C N (%) GG	GC	CC	p^a	Allele N (%) G	C	*p*
Controls	100	72 (72.0)	25 (25.0)	3 (3.0)	0.61	169 (84.5)	31 (15.5)	0.38
EDT	100	78 (78.0)	20 (20.0)	2 (2.0)		176 (88.0)	24 (12.0)	
EDT stage								
I-II	23	18 (78.3)	4 (17.4)	1 (4.3)	0.63	40 (86.9)	6 (13.1)	0.99
III-IV	77	60 (77.9)	16 (20.8)	1 (1.3)		136 (88.0)	18 (12.0)	

[a] Chi-square test

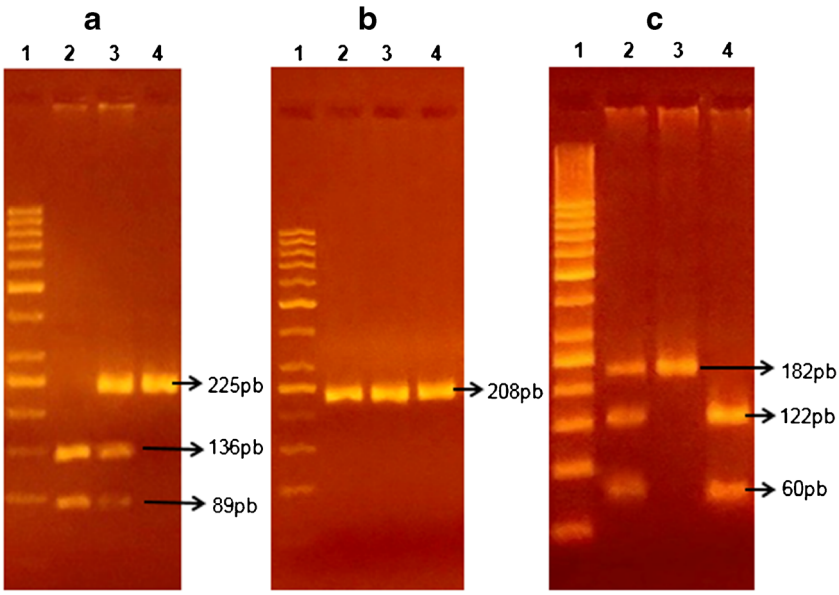

Fig. 1 The fragments observed after PCR-RFLP: **a** SNP K469E: *line 1* ladder 50 pb; *line 2* variant homozygous (fragments 136 and 89 pb); *line 3* heterozygous (fragments 225, 136, and 89 pb); *line 4* wild homozygous (fragment 225 pb). **b** SNP G241R: *line 1* ladder (50 pb); *lines 2*, *3*, and *4* wild homozygous (fragment 208 pb). **c** SNP C634G: *line 1* ladder (50 pb); *line 2* heterozygous (fragments 182, 122, and 60 pb); *line 3* variant homozygous (fragment 182 pb); *line 4* wild homozygous (fragments 122 and 60 pb)

Only the homozygous genotype GG was observed for G241R in both groups; therefore, no statistical calculation was performed.

IL-6 gene

The genotypic frequencies found in the group with endometriosis for the SNP G634C were 78 % homozygous GG, 20 % heterozygous GC, and 2 % homozygous CC. In the CT group, 72 % of women presented homozygous GG, 25 were heterozygous GC, and 3 homozygous CC. No significant differences were observed between groups.

Stage and location of endometriosis

Frequencies of SNPs K469E and G634C did not show statistical significance when comparing endometriosis subgroups (I-II versus III-IV).

Table 3 Genotypic frequencies of polymorphisms of *ICAM-1* and *IL-6* genes in different populations

	Controls Genotype (%)				Endometriosis Genotype (%)				p^a	Population	Reference
	AA	AG	GG	*n*	AA	AG	GG	*n*			
	48 (48.0)	38 (38.0)	14 (14.0)	100	40 (40.0)	50 (50.0)	10 (10.0)	100	0.22	Brazilian	This study
	130 (37.0)	164 (46.7)	57 (16.2)	351	163 (41.8)	172 (44.1)	55 (14.1)	390	0.38	Korean	Chae et al. [8]
K469E	22 (40.7)	25 (46.3)	7 (12.9)	54	16 (34.0)	24 (52.0)	6 (13.0)	46	na	Italian	Viganò et al. [6]
(ICAM-1)	65 (38.9)	74 (44.3)	28 (16.8)	167	49 (39.8)	51 (41.5)	23 (18.7)	123	0.86	Japanese	Yamashita et al. [21]
	91 (38.6)	116 (49.2)	29 (12.3)	236	72 (35.6)	93 (46.0)	37 (18.3)	202	0.21	Japanese	Kitawaki et al. [14]
	Genotype (%)				Genotype (%)				p^a	Population	Reference
	GG	GA	AA	*n*	GG	GA	AA	*n*			
	100 (100.0)	0 (0.0)	0 (0.0)	100	100 (100.0)	0 (0.0)	0 (0.0)	100	–	Brazilian	This study
	351 (100.0)	0 (0.0)	0 (0.0)	351	390 (100.0)	0 (0.0)	0 (0.0)	390	0.72	Korean	Chae et al. [8]
G241R	165 (94.3)	10 (5.7)	0 (0.0)	175	168 (89.3)	18 (9.6)	2 (1.1)	188	0.06	Italian	Viganò et al.[6]
(ICAM-1)	169 (99.4)	1 (0.6)	0 (0.0)	170	121 (100.0)	0 (0.0)	0 (0.0)	121	-	Japanese	Yamashita et al. [21]
	108 (90.0)	9 (7.5)	2 (2.5)	119	52 (62.0)	25 (29.7)	7 (8.3)	84	<0.0001	Iranian	Aghajanpour et al. [1]
	Genotype (%)				Genotype (%)				p^a	Population	Reference
	GG	GC	CC	*n*	GG	GC	CC	*n*			
	72 (72.0)	25 (25.0)	3 (3.0)	100	78 (78.0)	20 (20.0)	2 (2.0)	100	0.61	Brazilian	This study
G634C	18 (5.1)	129 (36.8)	204 (58.1)	351	24 (6.2)	148 (37.9)	218 (55.9)	390	0.75	Korean	Chae et al. [8]
(IL-6)	164 (69.5)	62 (26.3)	10 (4.2)	236	133 (65.8)	55 (27.2)	14 (6.9)	202	0.42	Japanese	Kitawaki et al. [14]

[a] Difference between groups (chi-square test)

na data not available

Discussion

Endometriosis is a disease which etiology is not fully understood and may involve several environmental and genetic factors [6]. This study aimed to demonstrate the possibility of polymorphisms present in *ICAM-1* and *IL-6* genes, being related to predisposition to disease in Brazilian woman.

The results for G634C, K469E, and G241R polymorphisms from our study and from other populations are summarized in Table 3. For G634C SNP (*IL-6* gene), there was no difference between endometriosis and control groups, which was consistent with the literature [8, 14]. Of women included on this trial, 72 % presented the wild homozygous genotype (GG), 25 % heterozygous (GC), and 3 % variant homozygous (CC), similar distribution to that observed in the Japanese population (69.5 % GG, 26.3 % GC, and 4.2 % CC) [14], but inversely from the results found in the Korean population (5.1 % GG, 36.8 % GC, and 58.1 % CC) [8].

Regarding the polymorphisms of *ICAM-1* gene, no significant difference in genotype distribution of frequencies SNPs K469E and G241R between CT and EDT groups was found. These results are consistent with those observed in the following populations: Italian [6], Korean [8], Japanese [14, 21], and Iranian [1].

Similarly to other studies [8, 21], it was performed the PCR-RFLP technique for genotyping G241R polymorphism *of ICAM-1* gene using BsrGI enzyme and none of 200 individuals included on this trial had the variant allele. In the research of Yamashita et al. [21], the same enzyme was used, and only one individual of 290 participants was heterozygous.

This polymorphism was also evaluated by two other studies using a different technique (PCR specific allele—AS-PCR) and found significant results. The first study, by Viganò et al. [6], included 363 Caucasian women and they observed an increase in the frequency of variant allele A of G241R polymorphism in the CT group compared to EDT group (2.9 versus 5.8 %; $p = 0.05$), but not in genotype frequency ($p = 0.06$). Aghajanpour et al. [1] genotyped 204 women from Northern Iran and found significant association of G241R with the presence of endometriosis ($p < 0.0001$).

In our study, we found no differences between endometriosis stages for any of the polymorphisms evaluated, similarly to other authors [14, 21]. On the other hand, Viganò et al. [6] evaluated 46 women with endometriosis (29.8 % stages I-II and 70.2 % stages III-IV) and found over three times as likely patients with severe form of the disease to carry the R241 allele (OR = 3.2; 95 % CI 1.3–7.9). Likewise, Aghanjanpour et al. [1] genotyped 84 patients with endometriosis (49.9 % stages I-II and 50.1 % stages III-IV) and found significant association of the same polymorphism with stage IV of the disease ($p < 0.0001$).

Therefore, the weakness of this study is the small number of individuals analyzed in early stages (I-II) of the disease (77 % of women included were stages III and IV) and the impaired subgroup analysis. Increasing the number of analyzed individuals in different stages of the disease could better clarify the role of these polymorphisms in the etiology of endometriosis. On the other hand, although the present study demonstrated similar results with other researches, this study was important because it is the first regarding those SNPs in Brazilian women, since our population is highly heterogeneous due to mixture of three populations (European, African, and Amerindians), and has unique characteristics, and as it is known, the frequency of polymorphisms can differ between populations. Moreover, the size of our sample is in accordance to what have been done in other studies.

In summary, K469E and G241R polymorphisms of *ICAM-1* gene, as well as G634C of *IL-6* gene, are not associated with endometriosis in this studied Brazilian population. However, from the increasing evidence for genetic mechanisms in the pathogenesis of endometriosis, the search for new genetic markers should be encouraged in order to develop a minimal invasive method for predicting the predisposition and consequently the increased risk of developing the disease in comparison to the general population, thereby taking reasonable precautions.

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Compliance with ethical standards

Conflict of interest The authors declare they have no conflicts of interests.

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