CCDC22 gene polymorphism is associated with advanced stages of endometriosis in a sample of Brazilian women

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Abstract

Purpose Based on the assumption that genetic factors are involved in the etiology of endometriosis, this study aimed to investigate the possibility of rs498679 (TLR4 gene), rs1799964 (TNF- α gene), rs3024496 (IL-10 gene), and rs2294021 (CCDC22 gene) polymorphisms being associated with the occurrence of this disease in a sample of Brazilian women.

Methods We conducted a case-control study with 100 women with histological confirmation of endometriosis (endometriosis group) and 100 women submitted to laparoscopy for benign disorders, in which the absence of endometriosis was confirmed (control group). All samples were genotyped by real-time PCR technique for rs498679, rs1799964, rs3024496, and rs2294021 polymorphisms.

Results No significant difference was observed in genotypic or allelic frequencies between control and endometriosis groups for rs498679 (TLR4 gene), rs1799964 (TNF- α gene), rs3024496 (IL-10 gene), neither when comparing endometriosis subgroups (I–II versus III–IV). On the other hand, significant difference between stages I–II and III–IV of the disease

was found in genotypic and allelic frequencies for the rs2294021 (CCDC22 gene) SNP ($p = 0.048$ and $p = 0.017$, respectively).

Conclusion Our results suggest that the rs2294021 (CCDC22 gene) polymorphism could be associated with increased susceptibility to endometriosis in Brazilian women when the allele C is present. In order to clarify this result, further studies should be conducted on a larger population.

Keywords Endometriosis · Polymorphism · Brazilian population · CCDC22 · Significant association

Introduction

Endometriosis is a chronic disease present in 10% of women of reproductive age, and is characterized by endometrial tissue found outside the uterus. Clinical manifestations include infertility, chronic pelvic pain, dysmenorrhea, and dyspareunia [1–6]. Although the etiology of the disease is still unknown, it has been hypothesized to involve retrograde menstruation through the fallopian tubes during menses [7, 8]. Furthermore, it has been suggested that changes in the proper functioning of the immune system may lead to the implantation of ectopic endometrial cells and the development of this disease [3, 9].

Toll-like receptors (TLRs) are members of a large family of proteins that are expressed in several types of immune cells that participate in the detection of specific pathogen-associated molecular patterns (PAPs), which regulate the activation of both innate and adaptive immunities [10–13]. In endometriosis, TLR stimulation results in the production of pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α), which promotes the proliferation and adhesion of endometrial cells [4, 14]. Furthermore, it has been previously reported that endometriotic lesions show a significant increase

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in TLR4 expression when compared to eutopic endometrium, suggesting that TLR4 may be involved in the pathogenesis of endometriosis [15]. The A896G polymorphism (rs4986790) in *TLR4* can also affect the extracellular domain of the receptor and result in persistent inflammation, and has been associated with an increased risk of several diseases, including endometriosis [16, 17]. Furthermore, TNF- α polymorphisms may also interfere with TNF- α gene expression. In fact, the rs1799964 (T1031C) polymorphism in *TNF- α* has been associated with advanced stages of endometriosis [18–26].

On the other hand, interleukin-10 (IL-10) is an important immunoregulatory cytokine that promotes the Th2 response [27–29]. Studies have shown that IL-10 levels were higher in the peritoneal fluid of patients with endometriosis [30, 31]. Polymorphisms in *IL-10* have been described in its regulatory region, which can interfere in its expression and translation [32]. One of these polymorphisms is rs3024496, which has been associated with an increased risk for diseases such as gastric cancer [33] and visceral leishmaniasis [34].

CCDC22 is another important gene that regulates the inflammatory response by participating in the activation of NF- κ B, which is a master regulator of the immune system [35, 36]. The polymorphism rs2294021 in *CCDC22* may interfere in its expression and has been previously associated with an increased risk for idiopathic recurrent miscarriage [35]. Furthermore, this polymorphism is in close proximity to *FOXP3* and may be involved in its function. *FOXP3* is an important regulator of the immune response and is essential for the differentiation and suppression of regulatory T cells (Treg) [37]. The presence of Treg cells has been demonstrated to inhibit the recruitment of immune cells while also increasing the amount of surviving ectopic endometrial tissue, which may be involved in the pathogenesis of endometriosis. Thus, this polymorphism may be more relevant to the function of *FOXP3* [36–38].

We hypothesize that changes in the immune system are involved in the pathogenesis of endometriosis. Therefore, the aim of this study was to analyze for the presence of SNPs in the pro-inflammatory genes *TLR4* (rs498679) and *TNF- α* (rs1799964) as well as in immune-regulatory genes *IL-10* (rs3024496) and *CCDC22* (rs2294021) in a sample population consisting of Brazilian women. These SNPs were then analyzed for any associations with endometriosis.

Material and methods

Two hundred women were recruited between February to December 2015 at the Endometriosis Centre, Clinics Hospital, São Paulo, Brazil for this case-control study. All the participants responded to a questionnaire about their medical history and then signed an informed consent form approved by the Ethics Committee of the institution (protocol

534714/14). All research performed was ethical and in compliance with the Declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013).

The women were divided into two groups: the endometriosis group (EDT) and the control group (CT). The CT group included women that were 18–50 years old, who underwent laparoscopic surgery for uterine fibroids ($n = 54$), diagnostic laparoscopy owing to pelvic pain or infertility with no lesions ($n = 20$), tubal ligation ($n = 13$), ovarian teratoma ($n = 11$), or hydrosalpinx ($n = 2$) when the absence of endometriosis was confirmed. The EDT group consisted of patients that were 18–50 years old, who submitted to laparoscopic surgery for the evaluation of pelvic pain and/or infertility, and with histological confirmation of endometriosis and classification of the disease in stages I to IV according to the Revised American Society for Reproductive Medicine [39]. In order to compare possible genetic differences relative to the severity of the disease, we grouped together patients in stages I–II (minimal/mild) and III–IV (moderate/severe) of the disease.

For both groups, the exclusion criteria included a lack of pelvic findings of inflammatory disease, gynecological cancer, or adenomyosis.

Genotyping

Using the salting-out protocol [40], DNA was extracted from 5 ml of peripheral blood samples collected in EDTA tube. The four polymorphisms were genotyped via real-time PCR.

All real-time PCR reactions were performed on a StepOne real-time PCR system (Applied Biosystems) according to the manufacturer's instructions and were prepared by using a TaqMan® Universal PCR Master Mix, TaqMan® SNP genotyping assay mix, DNA-free water, and 20 ng of genomic DNA in a final volume of 10 μ L per well of the plate. Primers and probes for this assay are commercially available (Assay ID: C_11722238_20 for rs4986790, C_7514871_10 for rs1799964, C_15983645_10 for rs3024496, and C_16185829_20 for rs2294021). Then, the genotype of each individual was determined in an allelic discrimination graph (VIC on abscissa, FAM on ordinate).

Statistical analysis

Hardy-Weinberg equilibrium was calculated for each SNP that was evaluated. The Student's t test was used to compare means in continuous variables and the Fisher's exact test and odds ratio (OR) with a confidence interval (IC) of 95% were used for categorical variables. Statistical analyses were performed using the software GraphPad InStat version 3.10, and p value <0.05 was considered statistically significant.

Table 1 Clinical features of patients included per group

	Endometriosis (<i>n</i> = 100)	Control (<i>n</i> = 100)	<i>p</i>
Age (years)	39.12 ± 6.1	40.3 ± 10.2	0.32 ^a
Weight (kg)	68.8 ± 12.6	68.85 ± 13.61	0.99 ^a
BMI (kg/m ²)	27.28 ± 5.1	26.23 ± 5.3	0.22 ^a
Infertility, <i>n</i> (%)	6 (6.0)	26 (26.0)	0.002 ^b

^a Student's *t* test^b Chi-square test

Results

Clinical evaluations showed that the CT and EDT groups were very similar regarding age (40.3 versus 39.1 years) and body mass index (27.3 versus 26.4 kg/m²); however, as expected, patients with the disease had a higher incidence of infertility (26.0 versus 6.0%; *p* = 0.002) when compared to the control group (Table 1). 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%). Of the patients, 22 and 78% had endometriosis at stages I–II and III–IV, respectively. Peritoneal endometriosis, endometrioma, and deep endometriosis were present in 20, 4, and 76% of the patients, respectively.

All the SNPs evaluated in this Brazilian sample population were in Hardy-Weinberg equilibrium (rs4986790, *p* = 0.44; rs1799964, *p* = 0.08; rs3024496, *p* = 0.74; rs2294021, *p* = 0.79), and the results of the genotypic and allelic frequencies obtained for these four SNPs are described in Tables 2, 3, 4, and 5. For the rs4986790, rs1799964, and rs3024496 polymorphisms, no significant differences were found in genotypic or allelic frequencies between the groups, nor when comparing between the endometriosis subgroups (I–II versus III–IV). On the other hand, for the rs2294021 SNP, a significant difference was found in genotypic and allelic frequencies between the EDT subgroups: the CC genotype (wild-type) was

observed more frequently in patients with endometriosis stages III–IV, whereas the TT genotype was more present in patients with endometriosis I–II (OR = 0.18; IC 95% = 0.03–0.9; *p* = 0.048); the C allele was observed in 29.5 and 50% of the patients with stages I–II and III–IV (*p* = 0.017) endometriosis, respectively. These results are shown in Table 5.

Discussion

In this study, we hypothesized that genetic factors are involved in the etiology of endometriosis; therefore, the aim of this study was to evaluate the genetic predisposition to the development of this illness with regards to the presence of four polymorphisms: rs3024496, rs1799964, rs3024496, and rs2294021.

For rs4986790 in *TLR4*, our results showed that the AA genotype was predominant in both the patient and control (92 and 90%, respectively) groups, and the AG genotype was present in a lower number of subjects (8 and 10% for the EDT and CT group, respectively). In both groups, we were unable to identify individuals with the GG genotype. The distribution of the genotypic frequencies was very similar to those observed by Latha et al. [17]. In their study, an Indian sample population of women (*n* = 400) was analyzed, which consisted of patients with endometriosis (*n* = 200) and healthy individuals (*n* = 200). Their cohort consisted of 81% patients and 94.5% control subjects with the AA genotype, 15.5% patients and 5.5% control subjects with the AG genotype, and 3.5% patients with the GG genotype. However, inconsistent with our results, these authors found a significant association between the presence of the G allele with the development of the disease (*p* < 0.0001), because the AG and GG genotypes were found in a higher percentage of patients than control subjects. Some factors may be responsible for this difference, such as the constitution of the population, since the Brazilian population consists of Europeans, Amerindians, and Africans, and thus is more diverse and

Table 2 Polymorphisms rs4986790 (*TLR4* gene): genotypic and allelic frequencies in the control versus endometriosis groups (EDT) and by stage of the disease

TLR4 genotype	Controls (%)	EDT (%)	OR (95% CI)	<i>p</i> ^a	TLR4 allele	Controls (%)	EDT (%)	<i>p</i>
AA	90 (90.0)	92 (92.0)	1.00		A	190 (95.0)	192 (96.0)	
AG	10 (10.0)	8 (8.0)	0.78 (0.29–2.07)	0.80				0.81
GG	0 (0.0)	0 (0.0)	N/A	N/A	G	10 (5.0)	8 (4.0)	
EDT STAGE	I–II	III–IV	OR (95% CI)	<i>p</i> ^a	EDT STAGE	I–II	III–IV	<i>p</i>
AA	20 (90.9)	72 (92.3)	1.00		A	42 (95.4)	150 (96.2)	
AG	2 (9.10)	6 (7.7)	0.83 (0.15–4.45)	1.0				1.0
GG	0 (0.0)	0 (0.0)	N/A	N/A	G	2 (4.6)	6 (3.8)	

^a Chi-square test

N/A data not available

Table 3 Polymorphisms rs1799964 (*TNF-α* gene): genotypic and allelic frequencies in the control versus endometriosis groups (EDT) and by stage of the disease

TNF- α genotype	Controls (%)	EDT (%)	OR (95% CI)	p^a	TNF- α allele	Controls (%)	EDT (%)	p
CC	5 (5.0)	4 (4.0)	1.00		C	50 (25.0)	43(21.5)	
CT	40 (40.0)	35 (35.0)	1.09 (0.27–4.39)	1.0	T	150 (75.0)	157 (78.5)	0.47
TT	55 (55.0)	61 (61.0)	1.38 (0.35–5.42)	0.73				
EDT STAGE	I–II	III–IV	OR (95% CI)	p^a	EDT STAGE	I–II	III–IV	p
CC	2 (9.1)	2 (2.6)	1.00		C	12 (27.3)	31 (19.9)	
CT	8 (36.4)	27 (34.6)	3.3 (0.40–27.9)	0.26	T	32 (72.7)	125 (80.1)	0.30
TT	12 (54.5)	49 (62.8)	4.08 (0.52–32.0)	0.20				

^a Chi-square test

different from the Indian population, which may result in the differences of genotype frequencies observed.

Furthermore, there have been many controversial reports for the rs1799964 polymorphism in *TNF-α*. Abutorabi et al. [6] studied the rs1799964 polymorphism in a sample of Iranian women ($n = 130$) with ($n = 65$) and without ($n = 65$) endometriosis and without the disease ($n = 65$). Their result showed an increased frequency of the CC genotype in women with the disease when compared with those without ($p = 0.001$). No significant differences were found between the patients with different stages of the disease. They concluded that the presence of the T allele in the promoter region of *TNF-α* could be associated with a decreased risk of endometriosis, suggesting a protective role in the disease progression. Likewise, Lee et al. [26] evaluated the same polymorphism in 494 Korean women, 246 patients with endometriosis and 248 without. They found a significant association of the CC genotype with advanced stages of endometriosis ($p = 0.002$). On the other hand, Asghar et al. [41] studied this polymorphism in a sample cohort consisting of 315 Japanese women, 130 patients with endometriosis and 185 without. They suggest that the same polymorphism may play some protective role from the progression of endometriosis when the C allele is present, since they found a lower frequency of this allele in patients in stage IV of the disease ($p = 0.04$). However, in

our study, we did not find any significant differences in the frequencies of this *TNF-α* polymorphism between the EDT and CT groups, nor did we find any differences when we grouped individuals by EDT stages. These discrepant results could also be explained by the different genetic composition of these distinct populations.

Some studies suggest that IL-10 is associated with the development of endometriosis. It is possible that the exchange of nucleotides in the promoter region of this gene could be associated with the susceptibility of endometriosis, since it is already known that IL-10 levels are increased in the peritoneal fluid and serum of patients with this disease [5, 30, 31]. Furthermore, previous studies with other genetic markers showed a significant association with risk of endometriosis. Fan et al. [29] and Malutan et al. [42] demonstrated that the C allele of IL-10 at position −592 is more common in patients with endometriosis.

As for the rs3024496 polymorphism in *IL-10*, our results showed that the AA genotype was predominant in both the patient and control groups (92 and 90%, respectively), and the AG genotype was present in a lower number of subjects (8 and 10% for the EDT and CT groups, respectively). In both groups, we were unable to identify individuals with the GG genotype. This distribution of genotypic frequencies was similar to that observed by Latha et al. [17], where, of the 400 Indian women that

Table 4 Polymorphisms rs3024496 (*IL-10* gene): genotypic and allelic frequencies in the control versus endometriosis groups (EDT) and by stage of the disease

IL-10 genotype	Controls (%)	EDT (%)	OR (95% CI)	p^a	IL-10 allele	Controls (%)	EDT (%)	p
CC	16 (16.0)	10 (10.0)	1.00		C	76 (38.0)	68 (34.0)	
CT	44 (44.0)	48 (48.0)	1.4 (0.71–4.25)	0.27	T	124 (62.0)	132 (66.0)	0.46
TT	40 (40.0)	42 (42.0)	1.68 (0.68–4.13)	0.27				
EDT STAGE	I–II	III–IV	OR (95% CI)	p^a	EDT STAGE	I–II	III–IV	p
CC	2 (9.1)	8 (10.3)	1.00		C	15 (34.1)	53 (34.0)	
CT	11 (50.0)	37 (47.4)	0.84 (0.15–4.55)	1.0	T	29 (65.9)	103 (66.0)	1.0
TT	9 (40.9)	33 (42.3)	0.91 (0.16–5.10)	1.0				

^a Chi-square test

Table 5 Polymorphisms rs2294021 (*CCDC22* gene): genotypic and allelic frequencies in the control versus endometriosis groups (EDT) and by stage of the disease

CCDC22 genotype	Controls (%)	EDT (%)	OR (95% CI)	<i>p</i> ^a	CCDC2 allele	Controls (%)	EDT (%)	<i>p</i>
CC	24 (24.0)	22 (22.0)	1.00		C	98 (49.0)	92 (46.0)	
CT	50 (50.0)	48 (48.0)	1.04 (0.51–2.11)	1.0	T	102 (51.0)	108 (54.0)	0.61
TT	26 (26.0)	30 (30.0)	1.26 (0.57–2.75)	0.69				
EDT STAGE	I–II	III–IV	OR (95% CI)	<i>p</i> ^a	EDT STAGE	I–II	III–IV	<i>p</i>
CC	2 (9.1)	19 (24.3)	1.00		C	13 (29.5)	78 (50.0)	
CT	9 (40.9)	40 (51.4)	0.46 (0.09–2.38)	0.48	T	31 (70.5)	78 (50.0)	0.017
TT	11 (50.0)	19 (24.3)	0.18 (0.03–0.93)	0.048				

^a Chi-square test

were analyzed, 81 and 94.5% of the patients and control subjects, respectively, had the AA genotype; 15.5 and 5.5% of the patients and control subjects, respectively, had the AG genotype; and 3.5% of the patients had the GG genotype. However, inconsistent with our results, these authors found a significant association between the presence of the G allele and the development of the disease ($p < 0.0001$). Some factors may be responsible for this difference, such as the constitution of the population, since the Brazilian population consists of Europeans, Amerindians, and Africans, and thus is more diverse and different from the Indian population, which may result in the differences of genotype frequencies observed. Even if this were the case, studies with different populations should be encouraged to elucidate any doubt about the possible influence of this SNP.

Finally, with regard to the rs2294021 polymorphism in *CCDC22*, we found no significant differences in the genotypic frequencies when we compared the CT and EDT groups, but we found a significant association between the genotypic ($p = 0.048$) and allelic frequencies ($p = 0.017$) when analyzing the EDT subgroups. We found that the T/T genotype could represent a protective effect for patients with advanced stages of endometriosis. This finding is very relevant, since we were unable to find any other studies in the literature that evaluated the risk of endometriosis related to this polymorphism. In our Brazilian sample of patients with endometriosis, 78 women had stage III–IV endometriosis, and 22 had stage I–II endometriosis, both of which could impair our subgroup analysis. Further studies are required to confirm this association using a more balanced sample.

In conclusion, this study was the first to analyze for possible associations of these polymorphisms with endometriosis in a Brazilian sample population. The presence of the rs3024496, rs1799964, and rs3024496 polymorphisms were not associated with endometriosis. The presence of the C allele in rs2294021 of *CCDC22* was associated with advanced stages of endometriosis, which could therefore be a risk factor for the development of the disease. Further studies should be

conducted to clarify the available results and to determine the genetic susceptibility to endometriosis.

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Compliance with ethical standards

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

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