

Analysis of *FOXP3* polymorphisms in infertile women with and without endometriosis

Gustavo M. André, M.D., Caio P. Barbosa, M.D., Ph.D., Juliana S. Teles, M.D., Fábila L. Vilarino, M.D., M.Sc., Denise M. Christofolini, Ph.D., and Bianca Bianco, Ph.D.

Division of Pathological Gynecology and Human Reproduction, Department of Gynecology and Obstetrics, Faculdade de Medicina do ABC, Santo André, Brazil

Objective: To evaluate *FOXP3* polymorphisms (rs3761549, rs3761548, rs2232368, rs2232366, and rs2280883) in a group of infertile women with and without endometriosis and controls.

Design: Case control study.

Setting: Human Reproduction Outpatient Clinic of Faculdade de Medicina do ABC.

Patient(s): The study groups were 177 infertile women with endometriosis, 71 women with idiopathic infertility, and 171 fertile women as controls.

Intervention(s): The *FOXP3* polymorphisms were identified by *TaqMan* polymerase chain reaction (PCR). The results were analyzed statistically.

Main Outcome Measure(s): Genotype distribution, allele frequency, and haplotype analysis of the *FOXP3* polymorphisms.

Result(s): Single-marker analysis revealed that *FOXP3* rs3761549 was significantly associated with endometriosis. In the infertile group without endometriosis, single-marker analysis revealed statistical difference for rs2280883 and rs2232368 *FOXP3* polymorphisms. No associations were found with rs3761548 and rs2232366 either for endometriosis-related infertility group or idiopathic infertility group. Haplotype analysis of five *FOXP3* polymorphisms identified a haplotype CTTGA associated with endometriosis and ACTAG associated with idiopathic infertility.

Conclusion(s): This is the first study to report an association between *FOXP3* polymorphisms and endometriosis and/or infertility. These findings require replication in other populations but suggest that the *FOXP3* polymorphisms can be associated with risk of idiopathic infertility (rs2280883 and rs2232368) and endometriosis (rs3761549) in Brazilian women. (*Fertil Steril*® 2011;95:2223–7. ©2011 by American Society for Reproductive Medicine.)

Key Words: Autoimmunity, endometriosis, infertility, *FOXP3* gene, polymorphism

Endometriosis is a common gynecological disease, defined as the growth of endometrial tissue outside the uterine cavity that often results in dyspareunia, dysmenorrhea, pelvic pain, and infertility (1). Several studies have revealed many genetic markers related to the immune, neuroendocrine, and reproductive function among patients with endometriosis indicating associations between the development of endometriosis and genetic polymorphisms (2, 3).

Immunologic theories suggest that changes in the immune system could prevent the ability to eliminate the endometrium of the pelvic cavity (4). In women with endometriosis is possible that changes in immunity mediated by T cells facilitate the implantation of endometrial fragments or cells in ectopic locations (5). The immune cells that

are likely to play roles in this destruction, including macrophages, natural killer (NK), and cytotoxic T cells, must be tightly regulated to ensure that the immune response is specific to sloughed endometrial fragments and not the intact uterine tissue. The cells that are almost certainly the key regulators of this response are a distinct population of T cells known as regulatory T cells called Tregs (6).

Besides the polymorphisms in genes as *PTPN22* (3), *VDR* (7), *CTLA4* (8), and *FCRL3* (unpublished data), already studied by this research group, recent studies have also associated the *FOXP3* gene (gene ID: 50943, Xp11.23) with homeostasis of the immune system and the development of autoimmune diseases (9, 10). The *FOXP3* gene is primarily expressed in CD4⁺ CD25⁺ Tregs in normal physiological conditions. It encodes FOXP3 protein, which regulates the activation of T cell, and functions as a transcriptional repressor and down-regulates cytokine production in T cells (11, 12). Polymorphisms of the *FOXP3* gene may change FOXP3 functionally or quantitatively, therefore leading to the lack of functional CD4⁺ CD25⁺ Tregs, resulting in autoimmune diseases (13).

In the present study we hypothesized that *FOXP3* polymorphisms might be involved in the pathogenesis of endometriosis and/or infertility. We examined five single nucleotide polymorphisms (SNPs) in the *FOXP3* gene (rs3761549, rs3761548, rs2232368, rs2232366, and rs2280883) with endometriosis-related infertility patients and

Received November 3, 2010; revised January 28, 2011; accepted March 9, 2011; published online April 9, 2011.

G.M.A. has nothing to disclose. C.P.B. has nothing to disclose. J.S.T. has nothing to disclose. F.L.V. has nothing to disclose. D.M.C. has nothing to disclose. B.B. has nothing to disclose.

Supported by FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo) to Juliana Souto Teles, a Scientific Initiation scholarship (FAPESP 2009/01960-0) and by grants 2009/09980-2.

Reprint requests: Caio P. Barbosa, M.D., Ph.D., Faculdade de Medicina do ABC, Avenida Príncipe de Gales, 821, Santo André/SP, Brasil, CEP: 09060-650 (E-mail: caiopb@uol.com.br).

idiopathic infertile patients and assessed the association of genotype and allele frequencies between them.

MATERIALS AND METHODS

Patients

Among the patients of the Human Reproduction Service of the Faculdade de Medicina do ABC, 177 patients with endometriosis-associated infertility (mean age, 35.1 ± 3.7 years) diagnosed by laparoscopy and classified by histologic criteria according to the American Society for Reproductive Medicine (14) and 71 idiopathic infertile women (mean age, 35.7 ± 4.9 years) were selected. Women with acute or chronic medical conditions, especially autoimmune diseases, were excluded. In the endometriosis group, 45.2% (80/177) had minimal/mild endometriosis and 54.8% (97/177) had moderate/severe endometriosis. To compose the control group, 171 fertile women (mean age, 40.7 ± 4.8 years) who had undergone tubal ligation, which confirmed the absence of endometriosis, were selected from the Family Planning Outpatient Clinic of the Faculdade de Medicina do ABC.

The cause of infertility was investigated in infertile couples: hormonal and biochemistry profile, serum testing, sexual transmitted disease investigation, imaging examinations, investigation of genetic and/or immunologic abnormalities, hysterosalpingography, hysteroscopy, laparoscopy (performed in all women up to 36 years old and in patients more than 36 years old, when there were symptoms or imaging examination abnormalities), and seminal analysis. Patients 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 involved masculine factors with the infertility were excluded of the study.

Clinical data and peripheral blood samples were collected only after explaining the objectives of the study and obtaining a signed informed consent form, as approved by the local Research Ethics Committee.

DNA Extraction

Peripheral blood was collected from each patient and control in an ethylenediaminetetraacetic acid (EDTA)-containing tube. Genomic DNA was extracted from lymphocytes of peripheral blood according to Lahiri and Numberger (15).

FOXP3 Genotyping

Detection of *FOXP3* polymorphisms, rs3761548 (A-3279C) and rs3761549 (C-2383T) in the promoter region of the gene, rs2232368 (G-20A) in the intron 1 region, rs2232366 (G+87T) in the intron 5 region, and rs2280883 (A+459G) in the intron 9 region, were performed using *TaqMan* polymerase chain reaction (PCR). *TaqMan* primers and probes were commercially available and provided by Applied Biosystems (Foster City, CA). Assays were performed with *TaqMan* Universal Master Mix with 50 ng of DNA per reaction. The PCR conditions were provided by the manufacturer: 40 cycles of 95°C denaturation (15 seconds), 60°C anneal/extension (1 minute).

Statistical Analysis

Statistical analyses were carried out using SPSS for Windows 16.0 (SPSS, Inc., Chicago, IL). The χ^2 test was used to detect differences in allele and genotype frequencies between patients and controls, to calculate the power of the test, and to estimate the Hardy-Weinberg equilibrium. Statistical significant values were corrected for the number of comparisons performed by Bonferroni method. The odds ratio (OR) was used to measure the strength of the association between the frequencies of *FOXP3* genotypes and endometriosis and/or infertility. All *P* values were two-tailed, and 95% confidence intervals (CIs) were calculated. The association between the combined genotypes of *FOXP3* polymorphisms and risk of endometriosis and infertility were also evaluated by the haplotype studies using Haploview software version 4.1 available in <http://www.hapmap.org>. A *P* value < .05 was considered statistically significant.

RESULTS

Genotype and allele frequencies of the rs3761548, rs3761549, rs2232366, rs2232368, and rs2280883 *FOXP3* polymorphisms in the infertile women with and without endometriosis and controls are

shown in Table 1. Information of SNPs and summary of single marker association analysis, considering Hardy-Weinberg equilibrium and minor allelic frequencies, in infertile women with endometriosis and idiopathic infertile women are shown in Table 2. Haplotype analysis of five *FOXP3* SNPs considering the frequency in the study population and *P* value of each haplotype are shown in Table 3.

Single marker analysis revealed a significant association between rs3761549 polymorphism and endometriosis-related infertility ($P=.003$, OR = 2.23, 95% CI 1.33–3.75), regardless of the stage of the disease ($P=.003$ and $P=.039$, respectively, for minimal/mild and to moderate/severe endometriosis). When we compared infertile groups with and without endometriosis there was a statistically significant difference related to the studied polymorphism frequency ($P \leq .001$), suggesting that the rs3761549 polymorphism is related to endometriosis.

Considering the rs2280883 polymorphism, no difference was found between endometriosis-related infertility and controls ($P=.296$). When we studied the patients with minimal/mild endometriosis and moderate/severe endometriosis separately, no difference was found ($P=.406$ and $P=.413$, respectively). However, a statistical difference was found between idiopathic infertility and control group ($P=.024$, OR = 1.64, 95% CI 1.09–2.49). Comparing the infertile patients with and without endometriosis, no association was found. The results point to an association between idiopathic infertility and the rs2280883 polymorphism.

Similar results were found with the rs2232368 polymorphism. No difference was found between endometriosis-related infertility and controls ($P=.383$), not even considering the endometriosis stage. Nevertheless, a statistical difference was found between idiopathic infertility and the control group ($P=.034$, OR = 1.60, 95% CI 1.06–2.41). When we compared the infertile patients with and without endometriosis, no difference was found. The results demonstrated an association between idiopathic infertility and the rs2232368 polymorphism.

Regarding rs3761548 and rs2232366 no association was found between endometriosis-related infertility and idiopathic infertility groups compared with controls ($P=.2815$ and $P=.8625$, respectively), not even considering the endometriosis stage. Also when we compare infertile groups with and without endometriosis, no association was found ($P=.417$ and $P=.371$, respectively), suggesting that both rs3761548 and rs2232366 polymorphisms were not related either to endometriosis-related infertility or to idiopathic infertility.

The power of the test showed statistical significance. It ranged from 0.80 ($\alpha = 0.05$) to 0.99 ($\alpha = 0.01$) as shown in Table 1.

Haplotype analysis of five *FOXP3* polymorphisms, rs3761549, rs3761548, rs2232368, rs2232366, and rs2280883, identified a haplotype CTTGA (haplotype frequency of 7.0% in the study population, 9.4% in patients, and 4.5% in controls) associated with endometriosis ($P=.011$, OR = 0.97, 95% CI 0.85–1.10). In contrast, the haplotype CCTGA (haplotype frequency of 58.2% in the study population, 53.2% in patients, and 63.5% in controls) was associated with a low risk of endometriosis ($P=.006$, OR = 0.86, 95% CI 0.75–0.98). These data suggest that rs3761549 (C-2383T) is the most important polymorphism associated with endometriosis (Table 3). Haplotype analysis also identified a haplotype ACTAG (haplotype frequency of 30.1% in the study population, 38.0% in patients, and 26.8% in controls) that was associated with idiopathic infertility ($P=.014$, OR = 1.36, 95% CI 1.13–1.62) (Table 3).

After Bonferroni correction, the *P* values of single markers were .015 for rs3761549 polymorphism and endometriosis-related infertility ($P=.015$ and $P=.195$, respectively, for minimal/mild and to moderate/severe endometriosis), $P=.17$ for rs2280883 polymorphism and idiopathic infertility, and $P=.12$ for rs2232368

TABLE 1Genotype and allele frequencies of the *FOXP3* polymorphisms in infertile patients with and without endometriosis and in the control group.

<i>FOXP3</i> polymorphism	Population studied	No.	Genotypes			Alleles		<i>P</i> value ^a	OR (95% CI)	<i>P</i> value ^b
			n (%)	n (%)	n (%)	n (%)	n (%)			
			AA	AC	CC	A	C			
rs3761548	Endometriosis-related infertility	177	15 (8.5)	89 (50.3)	73 (41.2)	119 (33.6)	235 (66.4)	.281	0.83 (0.60–1.14)	.416
A-3279C	Idiopathic infertility	71	16 (22.5)	22 (31.0)	33 (46.5)	54 (38.0)	88 (62.0)	.086		
	Controls	171	19 (11.1)	63 (36.8)	89 (52.0)	101 (29.5)	241 (70.5)			
			CC	CT	TT	C	T			
rs3761549	Endometriosis-related infertility	177	130 (73.4)	45 (25.4)	2 (1.1)	305 (86.2)	49 (13.8)	.003 ^c	2.23 (1.33–3.75)	< .001
C-2383T	Idiopathic infertility	71	66 (93.0)	5 (7.0)	0 (0)	137 (96.5)	5 (3.5)	.245		
	Controls	171	148 (86.5)	23 (13.5)	0 (0)	319 (93.3)	23 (6.7)			
			GG	GT	TT	G	T			
rs2232366	Endometriosis-related infertility	177	0 (0)	9 (5.1)	168 (94.9)	9 (2.5)	345 (97.5)	.862	1.04 (0.41–2.64)	.371
G+87T	Idiopathic infertility	71	0 (0)	1 (1.4)	70 (98.6)	1 (0.70)	141 (99.3)	.315		
	Controls	171	0 (0)	9 (5.3)	162 (94.7)	9 (2.6)	333 (97.4)			
			GG	GA	AA	G	A			
rs2232368	Endometriosis-related infertility	177	82 (46.3)	80 (45.2)	15 (8.5)	244 (68.9)	110 (31.1)	.383	1.17 (0.85–1.62)	.175
G-20A	Idiopathic infertility	71	33 (46.5)	22 (31.0)	16 (22.5)	88 (62.0)	54 (38.0)	.034 ^d	1.60 (1.06–2.41)	
	Controls	171	94 (55.0)	59 (34.5)	18 (10.5)	247 (72.2)	95 (27.8)			
			AA	AG	GG	A	G			
rs2280883	Endometriosis-related infertility	177	82 (46.3)	80 (45.2)	15 (8.5)	244 (68.9)	110 (31.1)	.296	1.21 (0.87–1.68)	.167
A+459G	Idiopathic infertility	71	33 (46.5)	22 (31.0)	16 (22.5)	88 (62.0)	54 (38.0)	.024 ^e	1.64 (1.09–2.49)	
	Controls	171	95 (55.6)	59 (34.5)	17 (9.9)	249 (72.8)	93 (27.2)			

Note: OR = odds ratio; CI = confidence interval.

^a Versus control group.^b versus idiopathic infertile group.^c power of the test was >0.99 ($\alpha = 0.01$).^d power of the test was >0.80 ($\alpha = 0.05$).^e power of the test was >0.85 ($\alpha = 0.05$).André *FOXP3* gene polymorphisms. *Fertil Steril* 2011.

TABLE 2**SNPs and summary of single marker association analysis in infertile women with and without endometriosis.**

SNP	Endometriosis				Idiopathic infertility			
	HWE	MAF	Alleles	P value	HWE	MAF	Alleles	P value
rs3761548	0.972	0.316	C:A	.247	0.004	0.32	C:A	.068
rs3761549	0.517	0.103	C:T	.002	0.873	0.058	C:T	.169
rs2232366	1.0	0.023	T:G	.663	1.0	0.017	T:G	.291
rs2232368	0.530	0.295	G:A	.340	0.001	0.308	G:A	.026
rs2280883	0.599	0.292	A:G	.260	0.002	0.304	A:G	.018

Note: SNP = single nucleotide polymorphism; HWE = Hardy-Weinberg equilibrium; MAF = minor allele frequencies.

André FOXP3 gene polymorphisms. *Fertil Steril* 2011.

polymorphism and idiopathic infertility. Considering haplotype analysis, the *P* values were .05 for haplotype CTTGA associated with endometriosis, *P*=.03 for haplotype CCTGA that was associated with a low risk of endometriosis, and *P*=.07 for haplotype ACTAG that was associated with idiopathic infertility.

DISCUSSION

In the present study, we hypothesized that the *FOXP3* polymorphisms might be involved in the pathogenesis of endometriosis and/or infertility. We examined five SNPs on *FOXP3* in patients with endometriosis-related infertility, idiopathic infertility, and a control group and assessed the association of genotype, allele frequencies, and haplotype to the risk of endometriosis or infertility. To our knowledge this is the first study to report an association between *FOXP3* polymorphisms and endometriosis and/or infertility.

During the normal menstrual cycle the endometrium within the uterus is widely infiltrated by immune cells. The specific activities of these immune cells are crucial for the proper course of such reproductive processes as menstruation and implantation (16). An alteration in Treg lymphocyte infiltration generally disrupts the immunologic equilibrium (17). The increase in the number of Treg cells depends on estrogen (E) levels, and Es are also responsible for the increase in the immune suppressive potential of these cells (18). Estrogen levels generally seem to be linked with a decrease in TH1 response and the absence of Treg cell fluctuation

can be linked to an immune defect arising with the development of endometriosis (17, 19).

Berbic et al. (6) showed that the density of peripheral *FOXP3*+ cells increases during the follicular phase, reaching its peak during the late proliferative phase, when serum E₂ levels are also elevated. It has been proposed that under normal conditions a preovulatory increase in *FOXP3*+ cells may be required for the induction of immune tolerance required to facilitate successful embryo implantation, should it occur (16). The failure to down-regulate *FOXP3*+ expression during the secretory phase in women with endometriosis may well be attributed to the increased presence of endometrial antigens, as well as to continuous local E production (20, 21), both of which are probably stimulating continuous *FOXP3*+ cell proliferation in endometriosis.

In contrast, primary unexplained infertility has been associated with a reduced expression of *FOXP3* messenger RNA in endometrial tissue in the midsecretory phase of the menstrual cycle (22), suggesting that impaired recruitment of Treg cells, or insufficient differentiation of uterine T cells into Treg cells, even before conception, may affect the capacity to establish pregnancy.

Surprisingly, despite the crucial roles *FOXP3* Tregs play in regulation and suppression of immune response, the *FOXP3* polymorphisms have not previously been investigated in women with endometriosis and/or infertility. Immune regulators are likely to play crucial roles in diseases, within which numerous immune factors appear to be highly disturbed. In the present study, single marker analysis revealed that *FOXP3* rs3761549 was significantly associated with endometriosis (*P*=.003), regardless of the stage of the disease, *P*=.003 and *P*=.039, respectively, for minimal/mild and moderate/severe endometriosis. Considering the infertile group without endometriosis, single marker analysis revealed a statistical difference for rs2280883 (*P*=.024) and rs2232368 (*P*=.034) *FOXP3* polymorphisms. No association was found for rs3761548 and rs2232366, either for the endometriosis-related infertility group or the idiopathic infertility group. Haplotype analysis of five *FOXP3* polymorphisms identified a haplotype CTTGA associated with endometriosis (*P*=.011) and also identified a haplotype ACTAG that was associated with idiopathic infertility (*P*=.014). After Bonferroni correction, only the rs3761549 polymorphism associated with endometriosis remains statistically significant, strengthening the association of this polymorphism with the disease.

We have been dedicated to understanding which patients could benefit from the different modalities of treating endometriosis-associated infertility, even when at laparoscopy there is still some doubt about which kind of endometriosis has to be considered a disease, and as such will progress and compromise fertility. We believe

TABLE 3

Haplotype analysis of five FOXP3 SNPs considering the frequency in the studied population and P value of each haplotype in infertile women with and without endometriosis.

Haplotype	Endometriosis		Idiopathic infertility	
	Frequency ^a	P value	Frequency ^a	P value
CCTGA	0.582	.006	0.620	.3013
ACTAG	0.275	.570	0.301	.014
CTTGA	0.070	.011	0.040	.388
ACTGA	0.280	.307	0.016	.331
CTGGA	0.021	.646	0.013	.099

^a Haplotype frequency in the general study population (patients and controls).

André FOXP3 gene polymorphisms. *Fertil Steril* 2011.

that it may become possible in the future to establish a genetic profile that can identify the development of endometriosis, which could be of great help for the clinicians in establishing the therapeutic management and reproductive prognosis of their patients. Thus, the findings of the present study, together with data from other relevant polymorphisms, may, in the future, help the reproductive outcomes of women with endometriosis.

In conclusion, our data point to a possible association of the *FOXP3* polymorphisms with endometriosis (rs3761549) and idiopathic infertility (rs2280883 and rs2232368) in Brazilian women. These findings clearly need to be replicated in an independent sample and in different populations. An association between *FOXP3* and endometriosis would strongly support the hypothesis that endometriosis is an autoimmune disease.

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