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Aberrant *FOXP3* gene expression in eutopic and ectopic endometrium of infertile women with endometriosis

ORIGINAL

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Abstract

Background: Immunological theories suggest that changes in the immune system could prevent the ability to eliminate the endometrium of the pelvic cavity. 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 and recent studies have associated the *FOXP3* gene with homeostasis of the immune system and the development of autoimmune diseases. We aimed to evaluate the expression of *FOXP3* gene in both eutopic and ectopic endometrium of infertile women with endometriosis and controls.

Methods: A case-control study was performed comprising 25 infertile women with endometriosis and 44 fertile women without endometriosis. *FOXP3* and *GAPDH* expression was measured by mRNA using quantitative reverse transcription polymerase chain reaction (qRT-PCR) based on *TaqMan* methodology. The Mann-Whitney test was used to compare the values between the groups.

Results: The results disclosed that mean expression of *FOXP3* in eutopic endometrium of endometriosis group was significantly higher when compared to the control group (p=0,008), regardless the stage of the disease. Considering the samples of the ectopic endometrium, *FOXP3* expression was also significantly higher in endometriosis group compared to the control group (p=0,004), regardless the stage of the disease.

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Conclusion: The results of this study point to an association between the expression of *FOXP3* and the genesis/progression of endometriosis

Keyword: Autoimmunity; Endometrium; Endometriosis; Infertility; *FOXP3* gene.

Introduction

Endometriosis is a common gynecological disease, defined as the growth of endometrial tissue outside the uterine cavity that often results in dyspaurenia, dysmenorrhea, pelvic pain and infertility [1,2]. It affects 3-10% of women in their reproductive years and 20-50% of women with infertility [2,3].

Numerous hypotheses have been put forward to explain the presence of ectopic endometrial tissue and stroma. Immunological theories suggest that changes in the immune system could prevent the ability to eliminate the endometrium of the pelvic cavity [4,5]. 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 [6,7]. The immune cells which are likely to play roles in this destruction, including cells such as macrophages, natural killer [NK] and cytotoxic T-cells must be tightly regulated in order to ensure that the immune response is specific to sloughed endometrial fragments and not the intact uterine tissue. The cells which are almost certainly the key regulators of this response are a distinct population of T-cells, known as regulatory T-cells, called Tregs [8,9].

Recent studies have associated the *FOXP3* gene (Gene ID: 50943, Xp11.23) with homeostasis of the immune system and the development of autoimmune diseases [8,10,11]. 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 [8,12-14].

The human endometrium is a tissue with high cell turnover and marked cyclical remodeling under the influence of ovarian steroid hormones. 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 [15]. An alteration in Treg lymphocyte infiltration generally disrupts the immunological equilibrium [16]. The increase in the number of Treg cells depends upon estrogen levels, and estrogens are also responsible for the increase in the immune suppressive potential of these cells [17]. 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 [16,18].

Considering the complex cellular and molecular mechanisms involved in endometriosis formation and progression, and that immune regulators are likely to play crucial roles in diseases within which numerous immune factors appear to be highly disturbed, we aimed to verify a possible relationship between *FOXP3* expression and the development/progression of endometriosis.

Methods

The Subjects: Among the patients of the Human Reproduction and Genetics Center of the Faculdade de Medicina do ABC, Santo André, Brazil, 25 infertile women with endometriosis (mean age 33.9±3.3y) were selected to take part in this study. They were

diagnosed with endometriosis by laparoscopy and classified according to the American Society for Reproductive Medicine (19), with obligatory histological confirmation of the disease. Patients with acute or chronic disorders, especially autoimmune diseases, were excluded. In this group, minimal/mild (stage I and II) endometriosis was found in 15 cases (60.0%), and moderate/severe (stage III and IV) endometriosis in 10 cases (40.0%).

Considering the characteristics of these patients such as mean age, duration of infertility, body mass index (Kg/m^2), value of CA125 (miliUI/mL), value of FSH (miliUI/mL), LH (miliUI/mL), progesterone (ng/mL), prolactin (ng/mL), presence of chronic pelvic pain, tubal obstruction and male factor infertility compared to the endometriosis stage are described in **Table 1**. The surgical indication for all patients was a complaint of infertility.

Table 1: Characteristics of studied infertile patients with endometriosis.

Characteristics	Minimal/Mild	Moderate/Severe	P-value
N	15	10	
Age (Years)	33.4 (3.58)	34.44 (3.57)	0.090
Duration of Infertility (Years)	4.58 (3.84)	4.11 (3.25)	0.278
BMI (Kg/m^2)	23.67 (2.26)	25.46 (3.26)	0.006
CA125 (miliUI/mL)	34.35 (32.98)	51.98 (36.86)	<0.001
FSH (miliUI/mL)	6.03 (1.89)	6 (1.88)	0.478
LH (miliUI/mL)	6.69 (10.55)	5.06 (4.67)	0.088
Progesterone (ng/mL)	10.38 (6.73)	9.13 (10.18)	0.146
Prolactine (ng/mL)	16.49 (6.04)	16.37 (8.26)	0.456
Presence of Pevic Pain	10/15 (66%)	5/10 (50%)	0.680
Tubal Obstruction	4/15 (26%)	4/10 (40%)	0.791
Male Factor	2/15 (13%)	4/10 (40%)	0.292

BMI–Body Mass Index | CA125-Cancer Antigen 125 | FSH–Follicle-stimulating hormone | LH-Luteinizing Hormone

Values expressed as mean (standard deviation).

A control group of 44 fertile women (mean age 35.3 ± 4.6 y) was selected especially at the Family Planning Outpatient Clinic of the Faculdade de Medicina do ABC among a group submitted to tubal ligation. In all of them, absence of endometriosis was confirmed by inspection of the pelvic cavity during the laparoscopy. None of these women had any personal and/or familial history of autoimmune diseases.

Moreover, all women (cases and controls) had regular menstrual cycles (25-35 days) and did not use any hormone therapy for at least three months before surgery.

The investigation into the cause of infertility included a hormonal and biochemical profile, testing for sexually transmitted diseases, imaging examinations, investigation of genetic and/or immunological abnormalities, hysterosalpingography, hysteroscopy and laparoscopy, and semen analysis of the partner. Patients with endometriosis who did not achieve pregnancy, at least, after twelve natural or induced cycles following laparoscopy were considered infertile.

Clinical data, peripheral blood and endometrial biopsies samples were collected only after explai-

ning the objectives of the study and obtaining signed informed consent, as approved by the Faculdade de Medicina do ABC Research Ethics Committee (No.095/2009).

Biopsies: Samples were collected at the luteal phase of the menstrual cycle (21 ± 2 days). Eutopic endometrium samples were collected with the aspiration cannula (Pipelle®, Prodimed, France) and typical endometriotic lesions only of the peritoneum (ectopic endometrium) were collected during laparoscopy/laparotomy. The fragments of the biopsies were immediately placed in an extender solution of RNA (RNA holder, BioAgency, Brazil) and subsequently stored in a freezer at -80°C.

RNA extraction: RNA extraction of endometrial biopsies and endometriotic lesions was carried out with Qiazol Lysis Reagent, according to the manufacturer's instructions (Qiagen®, Turnberry Lane, CA, USA).

To measure the quantity and quality (purity) of RNA extracted, the extracted sample was measured in NanoDrop 2000 spectrophotometer (Thermo Scientific, CA/USA). The quality/purity of the material was measured by the ratio 230/260 and 260/280.

cDNA synthesis: The cDNA synthesis was done from total RNA using the QuantiTect Reverse Transcription Kit, according to the manufacturer's instructions (Qiagen®, Valencia, California, United States).

***FOXP3* gene expression:** The expression of *FOXP3* (ID: 50943 - Hs01085834_m1) and *GAPDH* (ID 2597-Hs99999905_m1) genes was measured by qRT-PCR, based on the *TaqMan* methodology using the equipment StepOne Real-Time PCR System (Life Technologies®, Foster City, CA, EUA).

PCR reactions were processed to a final volume of 25 µL, containing 12.5 µL of 2X *TaqMan* Universal PCR Master Mix (Life Technologies ®, Foster City, CA, USA), 1.25 µL *TaqMan* assay (20x), 1µl of sample cDNA, and 10.25 µL of RNAse-free water. The PCR conditions were those recommended by the manufacturer: initial denaturation at 50° C (2 min) to initiate enzyme activity AmpErase UNG, 95 °C (10 min) to initiate the activity of the enzyme AmpliTaq Gold DNA Polymerase, followed by 40 cycles of denaturation at 95° C (15 sec) and annealing/extension at 60 ° C (1 min).

The amount of *FOXP3* mRNA from the control group was compared to the respective amounts of patients with endometriosis. The *GAPDH* gene was used as normalizer of the reactions. The results were analyzed by ΔΔCt method.[20]

Statistical analyses: Statistical analysis was performed using Stata 11.0. For comparison of quantitative variables such as age, duration of infertility, body mass index, value of CA125, amount of FSH, LH, progesterone and prolactin, t test was used, with results expressed as mean and standard deviation. To compare qualitative variables between groups such as the presence of chronic pelvic pain, presence of tubal and male factor infertility, compared to the staging of endometriosis, chi-square test was used.

The comparison between the values of *FOXP3* mRNA in the control group and the group of patients with endometriosis was performed using the Mann-Whitney. Due to non-normal distribution of the *FOXP3* expression, we chose to describe the data by percentile values (25, 50 and 75) and the mean and confidence interval of 95%. A p-value <0.05 was considered statistically significant.

Results and Discussion

The average of *FOXP3* expression found in both eutopic and ectopic endometrium of infertile women with endometriosis and controls were shown in **Table 2**.

Considering the endometrium of the women studied, the mean expression of *FOXP3* was significantly higher in the endometrium of endometriosis group than in control group (p=0.008), even when the expression of *FOXP3* was analyzed in endome-

Table 2: Values of central tendency and dispersion of FOXP3 expression found in infertile women with endometriosis and controls.

Studied Group	Eutopic Endometrium (n=25)		Ectopic Endometrium (n=25)	
	FOXP3 expression P50 (P25 – P75) Mean (IC95%)	p*	*FOXP3* expression P50 (P25 – P75) Mean (IC95%)	p*
Endometriosis	0,54 (0,34 – 1,04) 2,57 (0,51 ; 4,64)	0,008	1,54 (1,03 – 2,77) 40,32 (-2,83 ; 83,47)	0,004
Minimal/Mild (n=15)	0,54 (0,34 – 1,04) 2,26 (0,25 ; 4,27)	0,007	1,71 (1,08 – 11,08) 42,11 (-13,92 ; 98,15)	0,003
Moderate/Severe (n=10)	0,46 (0,23 – 2,06) 3,05 (-1,80 ; 7,90)	0,002	1,29 (0,46 – 2,35) 37,62 (-44,56 ; 119,80)	0,007
Control Group (n=44)	0,99 (0,55 – 1,65)		1,48 (0,99 ; 1,98)	
P50, P25, P75: Percentile 50, 25 and 75.			IC (95%): Confidence Interval of 95%.	
* Compared to Control Group				

triosis minimal/mild and moderate/severe separately. Regarding the samples of peritoneal endometrium (ectopic), *FOXP3* expression was significantly higher in endometriosis group compared to the control group (p=0.004), even when the expression of *FOXP3* was analyzed in endometriosis minimal/mild and moderate/severe separately.

The Treg cells comprise two distinct populations: natural Tregs (nTreg), which originate in the thymus and are specific to self-antigens presented by epithelial thymic cells, and induced Tregs, which are generated back into the periphery from T CD4+ (Tconv) cells through antigenic stimulation in the presence of TGFβ and foreign antigens [21]. Both populations of Treg lymphocytes express *FOXP3*, a transcription factor critical for homeostasis and the suppressor function of Tregs [10-12].

Considering the critical role of Treg cells in impairing autoimmunity and previous results relating genetic polymorphisms in the *FOXP3* gene to autoimmune diseases, we aimed to evaluate the *FOXP3* expression in both eutopic and ectopic endometrium of infertile women with endometriosis. The results disclosed that the mean expression of *FOXP3* in the eutopic endometrium of endometriosis group was significantly higher when compared to the control group, regardless the stage of the disease. Considering the samples of the ectopic endometrium, *FOXP3* expression was significantly higher compared to the control group, especially in minimal/mild disease.

Berbic et al (2010) [9] showed that the density of peripheral *FOXP3*+ cells increases during the follicular phase, reaching its peak during the late proliferative phase, when serum estradiol levels are also elevated. It has been proposed that under normal conditions a pre-ovulatory rise in *FOXP3*+ cells may be required for the induction of immune tolerance required to facilitate successful embryo implantation, should it occur [15]. The failure to down-regulate *FOXP3*+ expression during the secretory phase in women with endometriosis may well be attributed to increased presence of endometrial antigens, as well as to continuous local estrogen production [22,23] both of which are probably stimulating continuous *FOXP3*+ cell proliferation in endometriosis.

In contrast, primary unexplained infertility has been associated with reduced expression of *FOXP3* mRNA in endometrial tissue in mid-secretory phase of the menstrual cycle [24], suggesting that impaired recruitment of Treg cells, or insufficient differentiation of uterine T-cells into Treg cells even prior to conception may affect the capacity to establish pregnancy in women.

André et al (2011) [25] aimed to evaluate *FOXP3* polymorphisms (rs3761549, rs3761548, rs2232368, rs2232366 and rs2280883) in a group of infertile women with and without endometriosis and controls. The single-marker analysis revealed that *FOXP3* rs3761549 was significantly associated with endometriosis (p=0.003), regardless of the stage of the disease. Considering the infertile group without endometriosis, single-marker analysis revealed statistical difference for rs2280883 (p=0.024) and rs2232368 (p=0.034) *FOXP3* polymorphisms. No associations were found considering 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 (p=0.011) and also identified a haplotype "ACTAG" that was associated with idiopathic infertility (p=0.014). After Bonferroni correction, only the rs3761549 polymorphism associated with endometriosis remains statistically significant, strengthening the association of this polymorphism with the disease. The authors concluded that *FOXP3* polymorphisms can be associated with risk of idiopathic infertility (rs2280883 and rs2232368) and endometriosis (rs3761549) in Brazilian women.

Moreover, recently our group [26] demonstrated a cumulative effect of two genetic polymorphisms (*FOXP3* C-2383T/rs3761549 and FCRL3 C-169T/rs7528684) those were previously shown to be associated with endometriosis. The combined genotypes of FCRL3 and *FOXP3* polymorphisms showed a positive association between genotypes FCRL3TT/*FOXP3*CT, FCRL3CT/*FOXP3*CT and FCRL3CC/*FOXP3*CT and the risk of endometriosis development. Besides, a progression of the disease risk was observed according to the presence of one or two copies of risk allele FCRL3 C and only one copy of risk allele *FOXP3* T (OR=2.14, OR=3.25 and OR=6.0, respectively, for genotypes FCRL3TT/*FOXP3*CT, FCRL3CT/*FOXP3*CT and FCRL3CC/*FOXP3*CT), suggesting a possible gene-gene interaction leading to a cumulative effect on endometriosis development.

Utilizing the induced non-human primate (Papio anubis) model of endometriosis, Braundmeier et al (2012) [27] showed that, in control animals, the proportion of peripheral natural Tregs (nTregs) was reduced (P<0.05) during the mid- and late secretory stages of the menstrual cycle compared with menses. The induction of disease decreased peripheral Treg expression at early time points (P<0.05) and this remained low throughout the time course, compared with the pre-inoculatory level of an individual. *FOXP3* gene expression and Treg populations were also decreased in the eutopic endometrium (P<0.05) compared with control animals, whereas these parameters were increased in ectopic lesions (P<0.05), compared with the eutopic endometrium, suggesting that a reduction in peripheral Tregs may be a causative factor for endometriosis-associated infertility, while the increase in ectopic Treg expression may aid lesion development. Furthermore, endometriosis appears to disrupt Treg recruitment in both eutopic and ectopic endometrium. As Braundmeier et al (2012) [27], we found that, in human, *FOXP3* is aberrant expressed in both eutopic and ectopic endometrium of infertile women with endometriosis, strengthening the association of *FOXP3* and the genesis/progression of endometriosis.

Chen et al (2012) [28] aimed to investigate the pathogenesis of infertility in women with endometriosis by comparing *FoxP3*+ T regulatory cells expression in the eutopic endometrium of infertile women with endometriosis (n=27) and endometrium from healthy fertile women (n=20) in peri-implantation phase by quantitative real-time RT-PCR and *FoxP3* protein expression was assessed by immunohistochemistry. Similarly with our findings they observed that *FoxP3* mRNA expression in all infertile patients with endometriosis was significantly higher than the control group. Further analysis based on the extent

of the disease revealed that *FoxP3* mRNA expression in infertile patients with advanced endometriosis was significantly higher than the mild endometriosis group and the control group. Immunohistochemistry analysis showed predominant positive staining for *FoxP3* protein in the endometrial stroma.

Podgaec et al [29] also in 2012 evaluated CD4+CD25high*Foxp3*+ cells and IL-6, IL-10, IL-17, and TGFb in the peritoneal fluid of women with endometriosis (n=70) and controls (n=28) using flow cytometry abd RT-PCR. The results disclosed that the lymphocytes in the peritoneal fluid of women with endometriosis were higher CD4+CD25high compared to the control group. *Foxp3* expression was similarly elevated in patients with the disease compared to those without. IL-6 and TGF-b were also higher in endometriosis group and IL-10 and IL-17 showed no significant differences between the two groups. The authors concluded that peritoneal fluid of patients with endometriosis had a higher percentage of CD4+CD25high*Foxp3*+ cells and also higher levels of IL-6 and TGF-b compared to women without the disease, suggesting that CD4+CD25high*Foxp3*+ cells may play a role in the pathogenesis of endometriosis.

Since retrograde menstruation is a common phenomenon that occurs in the majority of women during their reproductive life, this fact alone is not likely to be responsible for the onset of endometriosis. It is believed that intrinsic properties of the endometrial tissue are responsible for ensuring viability, adhesion, neovascularization and establishment of ectopic lesions in women with endometriosis [30].

Endometriosis has been widely characterized as an inflammatory disease. Numerous studies have shown that peritoneal leukocytes and their inflammatory mediators exert local effects, creating a microenvironment that may facilitate the development and progression of these lesions. The immune system appears to be significantly deregulated not only at the injury site, but also in the uterine cavity of women with endometriosis [4,30].

Conclusion

It is important to point out that Tregs are responsible for both beneficial and deleterious effects. Although Tregs prevent excessive inflammatory and autoimmune responses, they also suppress necessary immunity and, therefore, also need regulations to restrict their effects.

In conclusion, our data point to an association between the expression of *FOXP3* and the genesis/progression of endometriosis.

Conflict of Interest

The authors declare no conflict of interest

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Authors' Contributions

Gustavo Andre, Caio Parente Barbosa and Bianca Bianco conceived study design. Gustavo Andre, Fernanda Mafra, Viviane Cavalcanti and Tatiana Ponce performed the data collection and analysed data. Gustavo Andre, Fernanda Mafra, Caio Parente Barbosa, Denise Maria Christofolini and Bianca Bianco interpretation the data. All authors were involved in literature search, writing the paper and had final approval of the submitted and published versions. The authors have no competing interests

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