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GENERAL GYNECOLOGY

Evaluation of the frequency of G-765C polymorphism in the promoter region of the *COX-2* gene and its correlation with the expression of this gene in the endometrium of women with endometriosis

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Abstract

Objective To evaluate the frequency of polymorphism G-765C (rs20417) of the *COX-2* gene and the expression of this gene in the endometrium of women with endometriosis.

Study design This is a case–control study of 365 women with endometriosis (251 infertile and 114 fertile) submitted to laparoscopy/laparotomy with histological confirmation of endometriosis. The control group was composed of 522 fertile women without endometriosis. Of these, 37 patients from the endometriosis group and 47 from the control group were submitted to biopsy of the endometrium for analysis of the expression of the *COX-2* gene. The genotypes were determined using analysis by High-Resolution Melt. Gene expression was measured by qRT-PCR with TaqMan methodology using the GAPDH gene as normalizer of the reactions.

Results The distribution of the genotypes and alleles in the group of fertile women with moderate/severe endometriosis showed a statistically significant difference, demonstrating association of the ancestral allele, −765G, with increased risk of endometriosis ($p = 0.028$; OR 0.53; CI 0.32–0.90). The mean expression of the *COX-2* gene (mRNA PTGS2) in the group of women with endometriosis was statistically higher compared to the control group (3.85 versus 2.84, $p = 0.028$).

Conclusion The present study identified that in Brazilian women the presence of the ancestral allele, −765G, of the *COX-2* gene is associated with an increased risk for development of moderate/severe endometriosis associated with fertility, and that the eutopic endometrium of women with endometriosis showed increased expression of *COX-2* when compared to the control group.

Keywords Endometriosis · Infertility · Polymorphism · Gene expression · *COX-2* gene

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Introduction

Endometriosis is a chronic inflammation that represents one of the most common benign gynecological diseases. It is a steroid-dependent condition in which tissue histologically similar to the endometrium with glands and stroma grows outside the uterine cavity by implanting itself in other tissues and organs such as Fallopian tubes, ovaries, peritoneum, colon, rectovaginal region, and bladder, causing dysmenorrhea, pelvic pain, and infertility. It affects between 3 and 10 % of women in their reproductive phase and 20–50 % show alterations in fertility [1–3].

Despite the high prevalence of endometriosis, the exact mechanisms of its pathogenesis in causing infertility are varied. But it is known that there is a multifactorial mechanism involving anatomy, immunology, genetics, and environmental factors [4–6].

The COX-2 enzyme, also known as prostaglandin synthetase-2 (PTGS2), was isolated in 1976 and is the key in prostaglandin biosynthesis, starting from Arachidonic Acid. There are two classes of prostaglandins: COX-1 that is constitutive and is involved in the production of prostaglandins for cell maintenance functions, and the mitogen-inducible COX-2, associated with biological events such as lesion, inflammation, and proliferation. COX-2 is not

detectable in most normal tissues, and becomes abundant in activated macrophages and other cells at inflammation sites [7–9].

The COX-2 enzyme and prostaglandins have been associated with various reproductive tract diseases, including carcinomas, menorrhagia, dysmenorrhea, and endometriosis [10]. It is codified by the *PTGS2* gene, also known as *COX-2*, discovered in 1991, has approximately 8.3 kb distributed among 10 exons, and is located in the long arm of chromosome 1 (1q25.2–q25.3) [8].

Transcription of the *COX-2* gene is rapidly activated by growth factors and oncogenes with an important role in the inflammatory process and in tumorigenesis, by promoting angiogenesis, cellular proliferation, and apoptosis inhibition. It is vital in diseases of an invasive character such as endometriosis and cancer [11, 12].

A polymorphism located in the promoter region of the *COX-2* gene leads to the exchange of G (guanine) to C (cytosine) in position −765 of the gene (rs20417), causing decreased activity in vitro and consequently, reducing the formation of prostaglandins [13].

Genetic alterations in the promoter region of the *COX-2* gene can lead to important defects in gene activation and transcription, affecting the synthesis of prostaglandins. In this way, the objective of the present study was to evaluate the frequency of polymorphism G-765C (rs20417) of the *COX-2* gene, and correlate this polymorphism with the expression of the gene in the endometrium of women with endometriosis.

Patients and methods

Patients

This is a case–control study that included between May 2013 and July 2014, 365 women with endometriosis [251 infertile (mean age 32.8 ± 2.7 years, mean BMI 24.13) and 114 fertile (mean age 32.7 ± 3.9 years, mean BMI 23.49)], from the endometriosis outpatient clinic of the Faculdade de Medicina do ABC. Considering the group of infertile patients with endometriosis, 37.5 % (94/251) had minimal/mild endometriosis and 62.5 % (157/251) had moderate/severe endometriosis. Considering the group of fertile patients with endometriosis, 48.2 % (55/114) had minimal/mild endometriosis and 51.8 % (59/114) moderate/severe endometriosis.

Study inclusion criteria were women aged ≤37 years, with no use of any type of hormonal therapy for at least 3 months, submitted to laparoscopy/laparotomia to confirm diagnosis of endometriosis. The definitive diagnosis of the disease was confirmed by the histological study of the lesions and staging of the disease established as per the norms of the American Association for Reproductive Medicine (ASRM, 1997) [14]. All laparoscopic surgeries were performed by the same medical group, as well as all patients after surgery did not receive additional treatment, being told to try spontaneous pregnancy within 12 months.

All patients were followed up to verify the reproductive status and confirm pregnancy, 31 women (8.5 %) got pregnant spontaneously; 83 women (22.7 %) through assisted reproduction techniques; 161 women (44.1 %) underwent assisted reproduction treatment but failed to get pregnant and 90 women (24.7 %) were not pregnant and did not further treatment.

Exclusion criteria were women whose partners had severe male factors associated with infertility, women with endometrial polyps, hydrosalpinges, adenomyosis and submucosal and intramural myomas.

The control group was composed of 522 fertile women without endometriosis (mean age 30.7 ± 3.6 years, mean BMI 24.82), submitted to tubal ligation, from the Family Planning Outpatient Clinic of the Faculdade de Medicina do ABC.

Of these patients, 37 women of the endometriosis group and 47 women from the control group were submitted to endometrial biopsy for analysis of gene expression, and were selected for being in the late luteal phase of the menstrual cycle (around the 20th to 22nd day).

The cause of infertility was investigated in accordance with the propedeutics for infertile couples: serology tests, hormonal and biochemical profile, imaging tests, investigation of genetic and/or immunological anomalies, hysterosalpingography, hysteroscopy, laparoscopy, and semen analysis. Patients with endometriosis who were not able to conceive spontaneously or with the use of assisted reproduction treatments (ART) within a period of 1 year after laparoscopy were considered infertile.

Clinical data, samples of peripheral blood and endometrial biopsy were collected after explanation of the objectives of the study and signing of the Informed Consent Form, approved by the Local Research Ethics Committee.

DNA extraction

Five milliliters of peripheral blood was collected by peripheral venipuncture in tubes containing EDTA. The genomic DNA was extracted from the lymphocytes as per the Lahiri and Nurnberger protocol [15].

Biopsy of the endometrium and extraction of RNA

Samples of endometrium were collected during the luteal phase of the menstrual cycle (21 ± 2 days) using an aspiration cannula (Pipelle®, Prodimed, France). The luteal phase was chosen in that it comprises the window of

implantation period, that period is the most critical step in the reproduction process, is the biological phenomenon in which the blastocyst is closely connected to the endometrial surface.

The fragments of the biopsies were totally immersed in a cryoprotective solution of RNA (RNA holder, BioAgency, Brazil) and subsequently kept at a temperature of −80 °C.

RNA extraction of the RNA from the endometrium biopsies was done with Qiazol Lysis Reagent, according to the manufacturer's instructions (Qiagen®, Turnberry Lane, CA, USA).

Genotyping of polymorphism G-765C (rs20417)

Genotypes were determined using the sequences of primers forward (5′-ACGCATCAGGGAGAGAAATG-3′) and reverse (3′-AACCTTACTCGCCCCAGTCT-5′), and analyzed by High-Resolution Melt (HRM). The Polymerase Chain Reaction (PCR) was performed using the Type-it HRM PCR Kit (Qiagen®, Turnberry Lane, CA, USA) with 100 ng of DNA per reaction and Rotor Gene 6000 equipment (Corbett Research, Mortlake, Australia).

PCR conditions were those recommended by the manufacturer: 40 cycles at 95° denaturation (10 s), 60 °C (30 s), and 65 °C annealing/extension (95 min). The melting curve was obtained by successive rises in temperature until loss of fluorescence due to DNA denaturation. The primary advantages of melting curves are related to the possibility of confirming the sequences of high-specificity PCR products, since each product presents its own melting temperature and the identification of different specific or non-specific elements in the PCR product. To standardize the analysis, we used sequential samples with known identity, and thus, we established the melting curves for the different genotypes. The level of confidence adopted was higher than 98 % and all the reactions (samples, controls, and blanks) were performed in triplicate. Only those that amplified at the same Ct (cycle threshold) were considered.

The products of PCR were sequenced using an ABI 3500 × L Genetic Analyzer equipment (Applied Biosystems—Life Technologies) and BigDye Terminator v3.1 Cycle Sequencing (Applied Biosystems—Life Technologies) sequencing kit. The sequences were analyzed with Geneious 3.6.1 software. The curves were used as references for the analysis of unknown samples.

Expression of *COX-2* (*PTGS2*)

The cDNA was synthesized from 100 ng of total RNA using the QuantiTect Reverse Transcription Kit, according to the manufacturer's instructions (Qiagen®, Turnberry Lane, CA, USA).

The expressions of *COX-2* (*PTGS2* mRNA) and *GAPDH* were measured by qRT-PCR, based on the TaqMan methodology, using Step-One Real-Time PCR System equipment (Life Technologies®, Foster City, CA, USA). The PCR conditions were those recommended by the manufacturer: initial denaturation at 50 °C (2 min) followed by 40 cycles of denaturation at 95 °C (15 s) and annealing/extension at 60 °C (1 min).

The quantity of cDNA *COX-2* (*PTGS2*) in the control group was compared to the respective quantities in patients with endometriosis. The G*APDH* gene was used as normalizer of the reactions. The results were analyzed by the $\Delta\Delta$Ct method [16].

Statistical analysis

Considering the sample calculation based on the polymorphism frequency in the database NBCI (National Center for Biotechnology Information), analyses were performed in SPSS for Windows 11.0 software (SPSS, Inc., Chicago, IL, USA). For the comparison of the quantitative variables, Student's t test was used, with results expressed in mean and standard deviation. For the comparison of qualitative variables, the χ^2 (Chi-squared) test was used. The level of significance considered was smaller than 0.05.

Results

The distribution of genotypes and alleles of G-765C polymorphism of the *COX-2* gene in infertile women with endometriosis, fertile women with endometriosis, and in the controls, as well as the Hardy–Weinberg equilibrium is summarized in Table 1.

When analyzing the frequencies of the genotypes and alleles among the groups of fertile and infertile women with endometriosis relative to the control group, we found no statistically significant difference.

When we divided the groups of fertile and infertile women with endometriosis according to the degree of the disease between minimal/mild (grades I and II) and moderate/severe (grades III and IV), we found no statistically significant difference between genotypes and alleles in the group of infertile women with endometriosis compared to the control group. However, when analyzing the fertile endometriosis group, we found a statistically significant difference, $p = 0.028$; OR 0.53; CI 0.32-0.90, showing that the ancestral allele, −765G, may be a risk factor for moderate/severe endometriosis in this group of patients.

The mean expression of the *COX-2* gene (*PTGS2* mRNA) in the eutopic endometrium of women with endometriosis and in the fertile controls is described in Table 2.

Table 1 Genotype and allele frequency of polymorphism G-765C (rs20417) of the *COX2* gene

COX2 SNP	Population studied	Sample power (%)	*N*	Genotypes			p^a	Alleles		p^a	OR (95 % CI)	HWE
				n (%) GG	*n* (%) GC	*n* (%) CC		*n* (%) G	*n* (%) C			
rs20417	Endometriosis in Infertile patients	0.972	251	161 (64.1)	75 (29.9)	15 (6.0)	0.202	397 (79.1)	105 (20.9)	0.074	0.79 (0.61–1.02)	0.308
	Minimal/mild endometriosis		94	60 (63.8)	31 (33.0)	03 (3.2)	0.147	151 (80.3)	37 (19.7)	0.126	0.73 (0.49–1.07)	0.916
	Moderate/severe endometriosis		157	101 (64.3)	44 (28.0)	12 (7.7)	0.460	246 (78.3)	68 (21.7)	0.228	0.82 (0.61–1.11)	0.093
	Endometriosis in Fertile patients	0.871	114	75 (65.8)	30 (26.3)	09 (7.9)	0.384	180 (79.0)	48 (21.0)	0.217	0.79 (0.56–1.12)	0.084
	Minimal/mild endometriosis		55	29 (52.7)	22 (40.0)	04 (7.3)	0.474	80 (72.7)	30 (27.3)	0.718	1.11 (0.72–1.73)	0.998
	Moderate/severe endometriosis		59	46 (78.0)	08 (13.5)	05 (8.5)	**0.01**	100 (84.7)	18 (15.3)	**0.028**	0.53 (0.32–0.90)	0.001
	Controls		522	307 (58.8)	167 (32.0)	48 (9.2)		781 (75.0)	263 (25.0)			0.002

SNP Single Nucleotide Polymorphism, OR Odds Ratio, CI Confidence Interval, HWE Hardy–Weinberg Equilibrium

Bold font means *p* value is statistically significant ($p < 0.05$)

[a] *p* value of allele frequency versus control group

Considering the expression of *COX-2* (*PTGS2* mRNA) in the group of women with endometriosis, the mean expression of *COX-2* was statistically greater when compared to the control group with $p = 0.028$.

When the expression of *COX-2* was analyzed according to the stage of endometriosis, the group with minimal/mild endometriosis compared to the control group showed statistically greater expression of *COX-2* ($p = 0.029$). For the moderate/severe endometriosis group, there was no statistically significant difference when compared to the control group ($p = 0.909$).

Statistical analysis showed that the group of endometriosis associated with infertility was in Hardy–Weinberg equilibrium, regardless of the stage of the disease, while the group of endometriosis associated with fertility showed disequilibrium relative to the moderate/severe stage group ($p = 0.001$), but both groups had a sample power superior to 0.50 (Table 1).

Discussion

Single nucleotide polymorphisms (SNPs) are common in the human genome and frequently occur in specific genes involved in the genesis and predisposition to human diseases; SNPs located in exon regions may alter protein function and alterations in the regulating regions of the gene may affect their expression, and thus, their protein levels [17, 18].

COX-2 G-765C is a functional polymorphism located in position 765 bp to the front of the transcription initiation site. It changes the location of transcription activation within the promoter region of the gene to the position between 766 and 761 bp, leading to a reduction of transcription activity [13, 19].

Based on these findings, we hypothesized that polymorphism G-765C located in the region that promoted gene *COX-2* (rs20417) may result in altered function, activity, and expression of the gene, and consequently, cause impact on the synthesis of prostaglandins and pathogenesis of endometriosis.

Salazar et al. [20] studied the said polymorphism by RFLP PCR (restriction fragment length polymorphism) in 106 infertile Chilean women submitted to assisted reproduction techniques with failures in embryo implantation and 80 fertile controls. The results demonstrated a statistically significant difference in the distribution of the genotypes and alleles, suggesting an important role of the polymorphic allele, −765C, in embryo implantation failure. This study excluded only women with polycystic ovarian syndrome (PCOS), diabetes, and dyslipidemias, which was different from the present study that used groups composed exclusively of women with endometriosis.

Table 2 Expression of the *COX2* gene in eutopic endometrium

Groups	*N*	Mean of *COX2* Expression (dp)	*p* value[a]	Variation
Endometriosis	37	3.85 (9.18)	**0.028**	−0.189 to 2.215
Minimal/Mild	17	4.28 (10.51)	**0.029**	−0.359 to 3.243
Moderate/Severe	20	2.39 (4.44)	0.909	−1.590 to 0.702
Control	43	2.84 (4.55)		

Bold font means *p* value is statistically significant ($p < 0.5$)

[a] *p* value of mean expression versus control group

Kim et al. [21] carried out a study in Korea with 268 women with endometriosis in moderate/severe stages of the disease and 242 control women without endometriosis. The genotypes were analyzed by RFLP PCR, and a positive association was found between polymorphism and protection against endometriosis. The polymorphic allele −765C was found in 15.9 % in the control group versus 1.9 % in the case group.

The present study corroborates the aforementioned study since we identified that the normal allele, −765G, may be a risk factor for moderate/severe endometriosis. We noted a 78 % frequency of the normal GG genotype in the moderate/severe fertile endometriosis group versus 58.8 % in the control group ($p = 0.01$).

In 2002, Papafili et al. first identified polymorphism G-765C (rs20417) in 173 individuals submitted to myocardial revascularization surgery. They reported that the *COX-2* gene has an important regulating role in the production of prostanoids associated with trauma and inflammation, and stated that the presence of the polymorphic allele, −765C, reveals a significantly lower expression of about 30 % when compared to the ancestral allele, −765G [13].

Based on these findings by Papafili et al. in 2002, we can suggest that the presence of the mutation confers protection, since it reduces the production of prostaglandins, decreases local inflammation, and consequently protects against the development of diseases of an inflammatory character such as endometriosis. Consequently, the presence of the ancestral allele, −765G, is a risk factor for endometriosis, since it causes an increased production of prostaglandins.

In a study performed in the Brazilian population using 318 patients with breast cancer and 273 controls, genotyped by RFLP PCR, a positive association was found between the polymorphic allele, −765C, and the risk for breast cancer. These results differ from those of the present study [22].

We know that studies that evaluate the frequency of SNPs (Single Nucleotide Polymorphisms) among the Brazilian population are complex, since the Brazilian population is extremely heterogeneous as a result of extensive interethnic breeding among native Indians, European settlers, and Africans [23].

Human endometrium is a highly dynamic tissue submitted to cycles of growth, differentiation, bleeding, and regeneration during the woman's entire reproductive life. Adult populations of endometrial stem cells are considered the primary cells responsible for this remarkable regenerative capacity [24].

The endometrium is the starting point in understanding how implantation occurs and how it can fail, resulting in repeated gestational losses or infertility. Many causes of decreased uterine receptivity are acquired during a woman's life. Endometriosis is an inflammatory disease that may, in some women, lead to progesterone resistance and alterations in the expression of genes in the endometrium [25]. Increased expression of anti-apoptotic genes and decreased expression of pro-apoptotic genes have been observed in the endometrium of patients with endometriosis [26].

The high expression of *COX-2* provides positive feedback. The increased production of prostanoids leads to a greater expression of *COX-2*, resulting in cellular proliferation, apoptosis inhibition, tissue invasion, and increased activity of aromatase, which consequently leads to the formation of estrogen. These are considered important steps in the development of endometriosis [27].

Cho et al. [28] studied the gene expression of *COX-2* in 26 women with endometriosis and 21 controls. They carried out biopsies of eutopic endometrium and of ovarian tissues (endometriomas) and found an association between the increased expression of *COX-2* in women with endometriosis when compared to the control group, indicating that the *COX-2* gene may be involved in the pathophysiology and progression of the disease.

Chen et al. [29] evaluated the eutopic endometrium of Chinese patients during the secretory phase of the menstrual cycle of 40 women with endometriosis and 40 women without endometriosis as controls. The gene expression was analyzed by real-time quantitative RT-PCR and they observed increased expression of some genes, including *COX-2*, when compared to the control group.

Ota et al. (2001) evaluated the expression of *COX-2* in 35 Japanese women with endometriosis, 33 with adenomyosis, and 50 fertile controls by immunohistochemistry, and observed that during the late proliferative phase, the

expression of *COX-2* was significantly greater in comparison to the control group [30].

Wang et al. [26] evaluated the eutopic endometrium of 60 Chinese women with endometriosis and 20 fertile controls during the secretory phase of the menstrual cycle by real-time quantitative RT-PCR and observed that the mRNA level of *COX-2* was significantly greater in the endometriosis group when compared to the controls.

Corroborating all the findings previously mentioned, the present study assessed the eutopic endometrium of 37 women with endometriosis and 43 fertile controls without endometriosis in which we found a significantly greater expression of *COX-2* in the endometrium of patients with endometriosis (3.85 versus 2.84, $p = 0.028$).

Increased expression of *COX-2* implies increased synthesis of the cyclooxygenase-2 enzyme, and consequently, a greater production of prostaglandins, causing an intense inflammatory response and pain, which are characteristic symptoms of endometriosis.

To our best knowledge this is the first study to assess the frequency of genotypes and alleles frequencies compared to the COX-2 gene expression. In the present study no association was found considering genotypes and alleles frequencies and the level of COX-2 gene expression. Despite the higher number of women with the ancestral allele −765G in endometriosis group, this difference was not significant compared to the control group. Perhaps the number of individuals has been insufficient for this type of analysis, and that even with the strict criteria for selection of patients, other non-genetic factors could influence this association with endometriosis.

Studies in endometriosis have been directed at understanding the heterogeneity of the characteristic symptoms of this disease, and we believe that in the future, polymorphism G-765C (rs20417) might be used along with other polymorphisms of relevant genes as a biomarker in the diagnosis and treatment of endometriosis. However, more studies are necessary to confirm our findings.

Conclusion

The present study identified that in Brazilian women the presence of the ancestral allele, −765G, of the *COX-2* gene is associated with an increased risk for development of moderate/severe endometriosis in fertile women, and that the eutopic endometrium in women with endometriosis showed an increased expression of *COX-2* relative to the control group.

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Conflict of interest The authors declare there are no conflicts of interest.

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