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L.C. Veiga-Castelli[1], J.C. Rosa e Silva[2], J. Meola[1,2], R.A. Ferriani[2], M. Yoshimoto[3], S.A. Santos[1], J.A. Squire[3] and L. Martelli[1]

[1]Departamento de Genética, [2]Departamento de Ginecologia e Obstetrícia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil
[3]NCIC Clinical Trials Group, Department of Pathology and Molecular Medicine, Queen's University, Kingston, ON, Canada

Abstract

Endometriosis is a complex and multifactorial disease. Chromosomal imbalance screening in endometriotic tissue can be used to detect hot-spot regions in the search for a possible genetic marker for endometriosis. The objective of the present study was to detect chromosomal imbalances by comparative genomic hybridization (CGH) in ectopic tissue samples from ovarian endometriomas and eutopic tissue from the same patients. We evaluated 10 ovarian endometriotic tissues and 10 eutopic endometrial tissues by metaphase CGH. CGH was prepared with normal and test DNA enzymatically digested, ligated to adaptors and amplified by PCR. A second PCR was performed for DNA labeling. Equal amounts of both normal and test-labeled DNA were hybridized in human normal metaphases. The Isis FISH Imaging System V 5.0 software was used for chromosome analysis. In both eutopic and ectopic groups, 4/10 samples presented chromosomal alterations, mainly chromosomal gains. CGH identified 11q12.3-q13.1, 17p11.1-p12, 17q25.3-qter, and 19p as critical regions. Genomic imbalances in 11q, 17p, 17q, and 19p were detected in normal eutopic and/or ectopic endometrium from women with ovarian endometriosis. These regions contain genes such as *POLR2G*, *MXRA7* and *UBA52* involved in biological processes that may lead to the establishment and maintenance of endometriotic implants. This genomic imbalance may affect genes in which dysregulation impacts both eutopic and ectopic endometrium.

Key words: Endometrioma; Endometriosis; Comparative genomic hybridization; Chromosomal imbalances

Introduction

Endometriosis is a common benign gynecological disorder affecting about 10% of all women (1). The ectopic tissue is histologically identical to the eutopic tissue, differing in functional and biochemical aspects (2,3). Endometriosis is characterized by dysmenorrhea, dyspaneuria, pelvic pain, and infertility or even absence of symptoms (4). Moreover, endometriosis suffers malignant transformation at a frequency of 0.7 to 1% (5).

Its etiology has not been clarified; however, the most popular theory proposed to explain it is Sampson's implantation theory, which postulates that the endometrial cells exfoliated during menstruation suffer reflux through the uterine tubes, adhere to and proliferate at ectopic sites (6). On the other hand, considering retrograde menstruation as a nearly universal phenomenon, it is difficult to explain why only a fraction of women develops endometriosis (7). Some studies suggest a multidimensional etiology including hereditary, hormonal and immunological factors (8-10).

No recurrent chromosomal alteration has been identified in endometriotic tissue by karyotyping analysis, probably due to the limitations regarding cell culture (5). Comparative genomic hybridization (CGH) allows a genome-wide screening of chromosome imbalances without prior knowledge of genomic regions of interest and independent of the availability of metaphase cells from the samples to be investigated, since genomic DNA is required from the cells to be analyzed. Gogusev et al. (11) examined by CGH 18 endometriotic lesions (6 peritoneal and 2 umbilical nodules,

Correspondence: L.C. Veiga-Castelli, Departamento de Genética, FMRP, USP, Av. Bandeirantes, 3900, 14049-900 Ribeirão Preto, SP, Brasil. Fax: +55-16-3602-4910. E-mail: luciana.veigacastelli@gmail.com

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and 10 ovarian endometriomas) and recurrent alterations in gene copy number were found in 15/18 cases (83%). Genomic losses in chromosomes 1p and 22q were detected in 50% of the cases. Other common losses were observed at 5p, 6q, 7p, 9q, 16, and 17q and gains were found at 1q, 6q, 7q, and 17q. The authors also evaluated by CGH a human endometriosis-derived permanent cell line FbEM-1 from both a peritoneal implant and an ovarian endometrioma from a patient with endometriosis and observed that gains were more common than losses since a significant increase in DNA sequence copy number was detected at 1q, 5p, 6p, and 17q (12).

This methodology was also applied to the evaluation of genetic alterations in ovary carcinomas arising within endometriosis (13). Chromosome aberrations were observed in the three cases of ovary carcinomas, but no alteration was detected in the endometriotic tissue. It was concluded that these altered regions might contain tumor suppressor genes or oncogenes responsible for the malignant transformation of endometriosis.

Chromosomal imbalance screening in endometriotic tissue can be extremely informative in the search for a possible genetic marker for the disease. Moreover, the literature regarding CGH and endometriosis evaluated chromosomal imbalances only in the endometriotic lesions. The objective of the present study was to detect chromosomal imbalance by CGH in ectopic tissue samples from ovarian endometriomas and eutopic tissue from the same patients, and, consequently, to evaluate gains and/or losses of chromosomal regions, which might be involved in the developmental process of the disease.

Material and Methods

The study was conducted at the Laboratory of Molecular Cytogenetics, Department of Genetics, School of Medicine of Ribeirão Preto (FMRP-USP), Brazil, in association with the Department of Gynecology and Obstetrics (Human Reproduction Division) of the same institution. The project was approved by the Research Ethics Committee of the University Hospital, FMRP-USP, protocol #11736/2004. Written informed consent was obtained from each patient.

Samples

Twenty samples were collected from 10 patients according to the following inclusion criteria: women aged 24 to 45 years, with regular menstrual cycles and no history of any hormonal therapy during the last 6 months before collection, and samples obtained during the proliferative phase of the menstrual cycle (1st to 12th day). All patients were referred to our service by the Endoscopy and Pelvic Pain Ambulatory and the Infertility Ambulatory of the University Hospital, FMRP-USP, due to pelvic pain (7 cases) or infertility (N = 3; patients 2, 8, 9). None of the patients had received treatment for endometriosis.

The biopsies were collected by laparoscopy during the proliferative phase of the menstrual cycle and submitted to histopathological analysis. The samples were divided into two groups: a) eutopic group (EET) consisting of histologically normal endometrial biopsies collected with a Novak curette from 10 patients with a diagnosis of ovarian endometrioma, and b) ectopic group (OET) consisting of 10 ovarian endometrioma samples from the same patients. The samples were placed in Tissue-Teck® O.C.T. Compound cryopreserver (Sakura Finetek USA, Inc., USA) immediately after collection, incubated for 15 min in liquid nitrogen, and then stored at -80°C.

The endometriosis stage was determined according to the classification of the American Society for Reproductive Medicine (14). The stages of the cycles were confirmed by histological examination and the date of the last menstruation prior to the procedure.

Comparative genomic hybridization

The protocol for DNA amplification and labeling by single cell comparative genomic hybridization was applied to all samples (each eutopic or ectopic endometrial DNA) and to control DNAs as previously described, with minor modifications (15). Briefly, normal female genomic DNA (cat. #G1521) was purchased from Promega (Fisher Scientific, Ltd., Canada) and used as control. Briefly, *Mse*I restriction endonuclease digestion was performed in One-Phor-All-Buffer-Plus (New England Biolabs, Canada) for 3 h at 37°C. Primers were annealed by adding the Lib1-primer (5'-AGT GGG ATT CCT GCT GTC AGT-3') and ddMse-primer (5'-TAA CTG ACA GCdd-3'). Annealing was started at 65°C (also serving to inactivate the restriction enzyme before ligation) and the temperature was reduced to 15°C with a ramp of 1°C/min. At 15°C, 1 µL ATP (10 mM) and 1 µL T4-DNA-Ligase (5 units; Boehringer Mannheim, Germany) were added, and primers and DNA fragments were ligated overnight. For primary amplification, 40 µL consisting of expand long template and buffer 1 (Boehringer Mannheim) and dNTPs (10 mM) were added to the 10-µL ligation reaction volume. The PTC-200 thermal cycler (MJ Research) was programmed for a) 68°C for 3 min, b) 15 cycles of 94°C for 40 s, 57°C for 30 s and 68°C for 1 min and 30 s (with an additional second for each cycle), c) 8 cycles of 94°C for 40 s, 57°C for 30 s (with an additional second for each cycle) and 68°C for 1 min and 45 s (with an additional second for each cycle), d) 22 cycles of 94°C for 40 s, 65°C for 30 s and 68°C for 1 min and 53 s (with an additional second for each cycle), and e) 1 cycle of 68°C for 3 min and 40 s. A second amplification was performed and DNAs were then individually and differentially labeled using 2 µL of the primary PCR and 60 mM Tris-SO_4, pH 9.1, 10 mM $(NH_4)SO_4$, 1.5 mM $MgSO_4$, 2 U of Elongase, 1.4 µM oligonucleotide LIB1, 330 µM dGTP, dATP, and dCTP, 290 µM dTTP, and 40 µM digoxigenin 11-dUTP (for the control DNA) or biotin 16-dUTP (for the ectopic and eutopic DNA). Amplification

conditions were: 1 cycle of 94°C for 1 min, 60°C for 30 s, 68°C for 2 min; 10 cycles of 94°C for 30 s, 60°C for 30 s and 68°C for 2 min with 20 additional seconds for cycle. Before using the labeled DNA (2 µg), primers were removed by *Tru*I (Fermentas, Canada) digestion.

Equal amounts (8 µg) of each labeled endometriotic DNA and a normal female genomic DNA control were co-precipitated with Human Cot-1 DNA (1 mg/mL). Each labeled probe DNA was resuspended in 12 µL hybridization mixture consisting of 100% formamide and 30% dextran sulfate/4X SSC. After denaturation at 75°C for 5 min, each labeled DNA probe was co-hybridized to normal human metaphase spreads prepared by a phytohemagglutinin-stimulated peripheral blood lymphocyte culture previously denatured at 70°C for 2 min. Hybridization was carried out at 37°C for 72 h. The slides were washed three times at 45°C for 5 min each in 50% formamide/2X SSC, followed by three washes at 45°C in 0.1% SDS/0.1X SSC and three washes at 45°C in 2X SSC. Biotinylated DNA sequences were visualized with fluorescein isothyocyanate (FITC)-conjugated avidin and digoxigenin-labeled sequences were detected using anti-digoxigenin-rhodamine. Chromosome preparations were counterstained with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich Co., USA).

Digital image analysis

The metaphases were examined using an epifluorescence microscope Axioskop 2 Plus (Zeiss, Germany), with images captured and analyzed by Isis FISH Imaging System V 5.0 (Metasystem Gmbh, Germany). For each sample, 25 non-overlapping images were captured, and the data from 10 representations of each chromosome were combined. Gains and losses of chromosomes or chromosomal regions were detected on the basis of the ratio profiles deviating from the green to red balance value of 1.0 within 0.8-1.2 limits. The threshold values were determined according to previous reports (11,13). The centromeric and heterochromatic regions and the short arm of acrocentric chromosomes were not included in the interpretation of gains and losses. Chromosome Y was excluded from the analysis because the reference and test DNAs were from females.

Results

Twenty endometrial tissue samples consisting of ten eutopic endometrial tissue samples and ten ovarian endometriomas were analyzed by CGH. We detected chromosomal alterations in both groups, which are summarized in Table 1. Alterations were detected in 4/10 samples from the ectopic group and also in 4/10 samples from the eutopic group. The endometriomas presented losses at 9p (one case) and gains at 1p (1 case), 11q (1 case), 16q (1 case), 17p (2 cases), 18p (1 case), 19p (3 cases), 19q (2 cases), 20q (1 case), 22q (1 case), and Xq (1 case). The alterations observed in the eutopic group were gains at 1p (2 cases), 11q (2 cases), 16q (1 case), 17p (1 case), 17q (2 cases), 19p (4 cases), 19q (3 cases), 20q (1 case), and 22q (3 cases). Figure 1 illustrates all the alterations found in the ectopic and eutopic groups. Chromosomal regions 1p33-pter, 11q12-q13.1, 17p11.1-p12, 17q25-qter, 19, 20q11.23-qter, and 22q11.2-qter presented significant alterations.

Comparative analysis showed 5 patients (1, 2, 5, 6, and 8) without alterations in either tissue (OET/EET). One patient presented alteration only in the ectopic tissue (OET9), as a gain at 17p. One patient presented alteration only in the eutopic tissue (EET3), as gains at 19p and 22q. Chromosomal alterations were observed in both tissues in 3 patients (OET4/EET4, OET7/EET7, and OET10/EET10). All the alterations observed in the eutopic tissue (gain at 16q, 19p, and 19q) from patient 4 were also found in the ectopic tissue, in which gain at Xq was also observed.

Table 1. Chromosomal aberrations detected by comparative genomic hybridization in ectopic and eutopic tissues from 10 endometriosis patients.

Ectopic		Eutopic	
OET1	ND	EET1	ND
OET2	ND	EET2	ND
OET3	ND	EET3	+19p, +22q
OET4	+16q, +19p, +19q, +Xq	EET4	+16q, +19p, +19q
OET5	ND	EET5	ND
OET6	ND	EET6	ND
OET7	+1p, -9p, +11q, +17p, +18p, +19p, +19q, +20q, +22q	EET7	+1p, +11q, +17q, +19p, +19q, +22q
OET8	ND	EET8	ND
OET9	+17p	EET9	ND
OET10	+19p	EET10	+1p, +11q, +17p, +17q, +19p, +19q, +20q, +22q

- = loss; + = gain; ND = not detected; OET = ovarian endometrioma tissue; EET = eutopic endometrium tissue from the same patient.

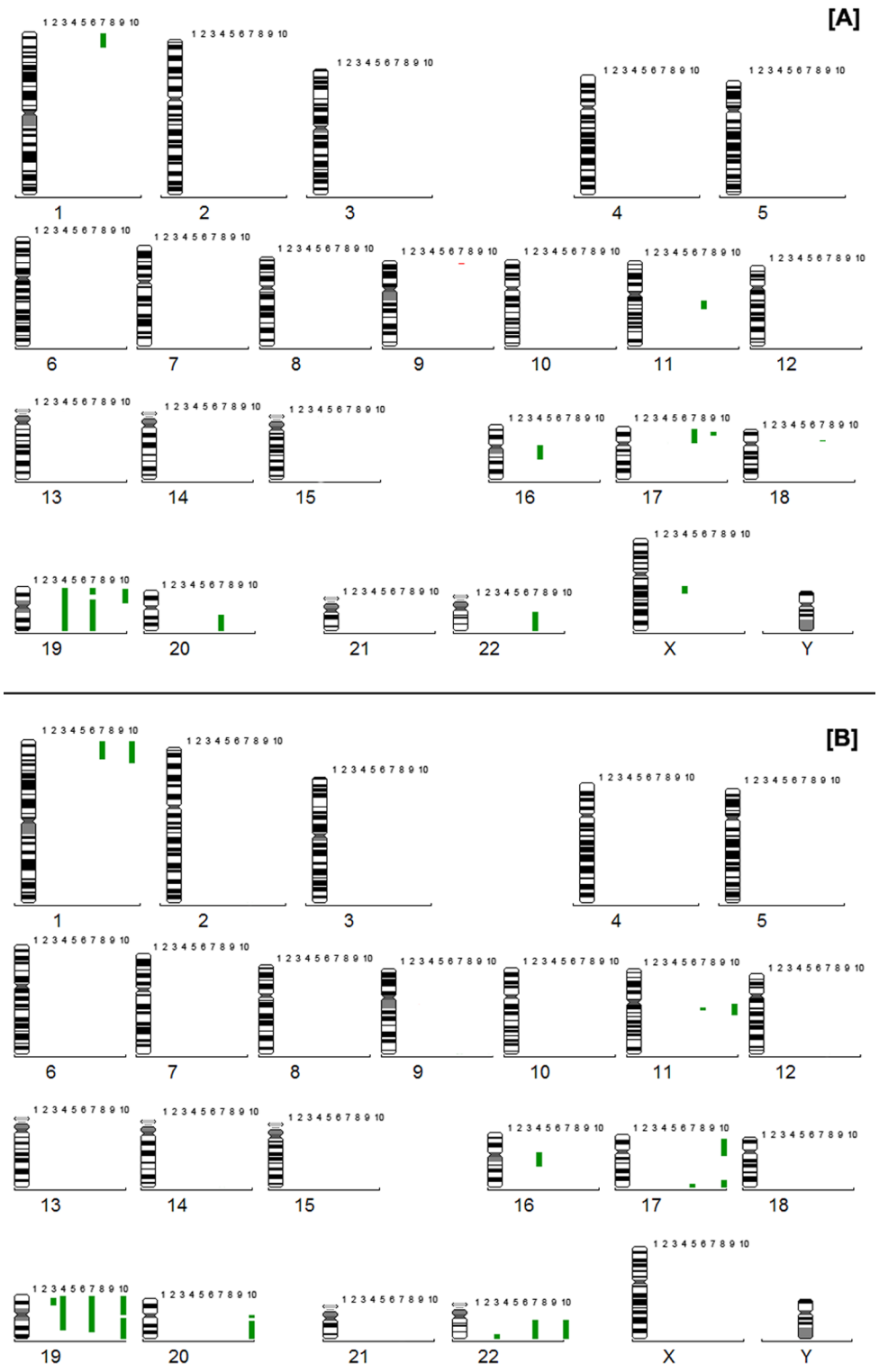

Figure 1. Chromosomal aberrations detected by comparative genomic hybridization in our samples. *A*, Losses and gains in 10 ectopic ovarian endometrioma samples. *B*, Losses and gains in 10 eutopic tissue samples from endometriosis patients. The numbers 1 to 10 at the top of each panel refer to patient identification. Losses in red and gains in green.

Patient 7 presented gains at 1p, 11q, 19p, 19q, and 22q in both tissues, but gains were observed at 17q only in the eutopic tissue and gains at 17p, 18p and 20q, as well as losses at 9p, were reported only in the ectopic tissue. Patient 10 presented gain at 19p in both tissues; however, gains at 1p, 11q, 17p, 17q, 19q, 20q, and 22q were found only in the eutopic tissue. The increased number of chromosomal alterations in patients 4, 7, and 10 did not show any correlation with symptom severity or a poor prognosis. None of the patients presented ovarian tumors within a 3-year follow-up.

The 11q, 17p, and 17q regions were selected as critical regions because they involve non-centromeric regions (or any other repetitive sequence region) and were detected in more than one sample. Some aberrations were previously described in normal tissues at 1p, 19, and 22, G-C-rich regions known to produce false-positive results by CGH (13). Nevertheless, we also considered 19p as a critical region because, differently from regions 1p and 22, it was maintained in the 3 cases of evolution (samples 4, 7, and 10) both in the eutopic and ectopic tissue, indicating a clonal origin.

Discussion

A different theory for each of the three types of endometriosis has been proposed (16-18), and it has been discussed that endometriosis has a genetic component and that acquired chromosome-specific aberrations could be involved in its pathogenesis. There are no reports regarding genomic alterations detected by metaphase CGH when comparing ovarian endometriomas and eutopic endometrium. We evaluated 10 ovarian endometrioma samples (ectopic tissue) and 10 eutopic samples from the same patients by CGH. The selection of paired samples is mandatory to minimize the individual genomic variability in the women analyzed. Considering the diversity of the Brazilian population, our control was the patient herself (eutopic tissue) in order to exclude individual genetic variation (polymorphisms and copy number variations) with no phenotypic correlation.

Alterations involving mainly gains of chromosomal regions were observed in both tissues. Some regions were excluded from CGH analysis, such as centromeric regions and heterochromatic repeat regions such as the p-arms of the acrocentric chromosomes, which cannot be evaluated because they are blocked by the unlabeled Cot-1 DNA in the hybridization. Telomeric regions were excluded because the absolute green and red fluorescence intensities gradually decrease at telomeres. Also, because some alterations were previously detected in normal tissues at 1p, 16p, 19, and 22, these G-C-rich regions known to produce false-positive results by CGH were excluded from analysis (13,19-22) except for region 19p that was identified in the present study as the only imbalance of clonal origin, which is maintained in the three cases of evolution. We detected alterations in 4/10 ovarian endometrioma samples (40%) and, differently from other reports (11,12), we observed gains mainly at 1p, 11q, 16q, 17p, 18p, 19p, 19q, 20q, 22q, and Xq, and losses at 9p. The literature is unclear about the genetics of endometriosis and there are few studies using CGH. There are reports of both total absence of aberrations detected by CGH in all endometriosis samples (13,23) and of gains and losses at chromosomal regions in 15/18 endometriotic samples (83%), mainly losses at 1p32-36, 5p, 6q, 7p14-p22ter, and 22q12.3-qter and gains at 6q, and 17q (11).

This discrepancy can be explained by the heterogeneity of the sample origin. Mhawech et al. (13) evaluated endometriotic tissue from patients with ovarian carcinomas arising within endometriosis and no chromosomal alteration was found in the endometriotic tissue. They suggested that there are no established correlations between genomic alterations and a poor prognosis, although their study was performed on only 3 patients. We also did not observe any correlation between the presence or absence of chromosomal alterations and clinical follow-up in terms of fertility and cancer. In another study, an abnormal genomic profile was observed in 10/10 samples of ovarian carcinoma. Chromosomal alterations such as losses of 6q, 9p, 10q, 21q, 22q, and gains of 8q, 9q, and 12p were found in ovarian cancer samples analyzed by BAC-array-CGH (24). Array-CGH data for 5 endometriosis samples showed alterations in different chromosomes but mainly at 1p, 3p, and 4p (25). Besides, there was a considerable diversity in the alterations among patients, since they did not share any common alteration, probably due to the heterogeneity of the samples. Using array-CGH, Wu et al. (26) observed genomic alterations both in ectopic and eutopic tissues from peritoneal and ovarian endometriosis.

In the eutopic group, 40% (4/10) presented chromosomal alterations involving gains at 1p, 11q, 17p, 17q, 19p, 19q, 20q, and 22q. These results suggest that the histologically normal endometrium from women with endometriosis can present genomic alterations, as reported previously (17). Moreover, in the majority of our samples, the alterations observed in the eutopic tissue were different from those observed in ectopic tissue. In one case (patient 4), all alterations found in eutopic tissue were also observed in endometriotic tissue, which also presented gain at Xq. In three cases (3, 7 and 10), both tissues shared some alterations. This apparent lack of homogeneity indicates multiple paths leading to endometriosis. The observation of different alterations in both tissues from the same patient challenges the applicability of Sampson's theory. According to retrograde reflux theory, it is assumed that the same alterations found in the eutopic endometrium are also found in the endometriotic tissue and that the latter should present additional alterations responsible for adhesion, cell proliferation and angiogenesis, which would differentiate it

from the eutopic tissue.

We observed that alterations at 11q12.3-q13.1, 17p11.1-p12, 17q25-qter, and 19p were the most frequent in our cohort and at least one of them (19p) appeared in four samples related to three patients. Some of these genomic alterations are critical because they contain genes related to angiogenesis, cell cycle regulation, immune response, and cell adhesion (27), which may play a role in the development, establishment and maintenance of ovarian endometriomas.

In a previous study, using the same ovarian endometrioma samples, we analyzed the differential gene expression profiles of eutopic and ectopic tissues from women with endometriosis. Genes *POLR2G* [polymerase (RNA) II (DNA directed) polypeptide G], *MXRA7* [matrix-remodeling associated 7] and *UBA52* [ubiquitin A-52 residue ribosomal protein fusion product 1] were overexpressed in the endometriotic lesions and were located in our chromosome gain regions 11q13.1, 17q25.1, and 19p13.1-p12 detected by CGH, respectively (28). These genes were categorized by their functions in biologic processes according to the Gene Ontology (GO) terms (http://www.geneontology.org): *POLR2G* plays a role in signal transduction; *UBA52* is responsible for cell migration, cell motility, cell-cell signaling, positive regulation of transcriptions, growth regulation, cell communication, and cell death, and *MXRA7* acts on tissue remodeling.

The literature continues to be unclear concerning the regions of genomic imbalances in endometriomas. It is suggested that genomic variability or results from different stages of the proliferative process may play a role. Our goal was to perform a screening investigation for identifying chromosomal imbalances that might be involved in endometrioma outcome and/or its development. Despite the time-consuming procedures related to CGH analysis, the methodology is still a suitable and efficient tool for further studies in an attempt to define candidate genes.

Genomic imbalances in 11q12.3-q13.1, 17p11.1-p12, 17q25.3-qter, and 19p were detected in eutopic and/or ectopic endometrium from women with ovarian endometriosis. These regions contain genes involved in biological processes that may lead to the establishment and maintenance of endometriotic implants. This genomic imbalance may affect genes in which dysregulation impacts both eutopic and ectopic endometrium.

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