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Deregulation of *LOXL1* and *HTRA1* Gene Expression in Endometriosis

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Abstract

Endometriosis is a gynecologic disease characterized by the presence of endometrial tissue outside the uterine cavity. Although 15% of the female population in reproductive age is affected by endometriosis, its pathogenesis remains unclear. According to the most accepted pathogenesis hypothesis, endometrial fragments from the menstrual phase are transported through the uterine tubes to the peritoneal cavity, where they undergo implantation and growth, invading adjacent tissues. However, the establishment of the disease requires that endometrial cells present molecular characteristics favoring the onset and progression of ectopic implantation. In this investigation, we analyzed the differential gene expression profiles of peritoneal and ovarian endometriotic lesions compared to the endometrial tissue of nonaffected women using rapid subtraction hybridization (RaSH). In our study, this method was applied to samples of endometriotic lesions from affected women and to biopsies of endometrium of healthy women without endometriosis, where we could identify 126 deregulated genes. To evaluate the expression of genes found by RaSH method, we measured *LOXL1*, *HTRA1*, and *SPARC* genes by real-time polymerase chain reaction. Significant different expression was obtained for *HTRA1* and *LOXL1*, upregulated in the ectopic endometrium, suggesting that these genes are involved in the physiopathology of endometriosis and may favor the viability of endometrial cells at ectopic sites.

Keywords

endometriosis, endometrium, gene expression, *LOXL1*, *HTRA1*, RaSH

Introduction

Endometriosis is a common chronic disease and although it affects approximately 15% of the female population of reproductive age, its pathogenesis remains poorly understood.[1-6]

Accordingly to the most accepted etiology hypothesis for endometriosis, endometrial fragments containing viable cells desquamated during the menstrual phase can be transported through the uterine tubes to the peritoneal cavity, where they implant, grow, and invade the tissues of adjacent organs.[7] However, some molecular characteristics seem to favor the onset and progression of ectopic implantation and may explain why only some women develop the disease.[8,9] Several studies have demonstrated that the main molecular differences between women with and without endometriosis are related to the processes involved in apoptosis, cell adhesion, angiogenesis, estrogen biosynthesis, immune system function, as well as growth factors and metalloproteinases activity.[10-15]

Current studies have been directed at the investigation of genes with higher or lower expression in endometriotic lesion. Thus, several methodologies have been applied for specific gene screening, such as microarrays, comparative genomic hybridization (CGH), serial analysis of gene expression (SAGE), and subtraction hybridization (SH).[16-22] To identify genes conferring susceptibility to the disease, some authors attempted to clarify differences in gene expression between eutopic and ectopic tissue from the same patient.[22-24] Other groups studied women with and without endometriosis, one of which had investigated how differential gene expression between endometriotic tissue and normal eutopic endometrium could participate in the pathogenesis of the disease.[21,25]

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Considering this, the objective of the current study was to identify other genes with differential expression in endometriotic (peritoneal and ovarian) lesions compared to the endometrial tissue of nonaffected women and relate them to molecular processes that might contribute to the establishment, maintenance, and progression of endometriosis.

Materials and Methods

The study was performed in the Laboratory of Human Molecular Genetics and Cytogenetics of the Department of Genetics, School of Medicine of Ribeirao Preto, University of Sao Paulo and was approved by the Institutional Research Ethics Committee and by the National Research Ethics Committee (process no 11736/2004). Written informed consent was obtained from each patient.

Samples

All samples were collected from women selected according to the following inclusion criteria: age between 18 and 40 years, regular menstrual cycles (25-35 days), and at the early proliferative phase (5-8 day) of the menstrual cycle. The stages of the menstrual cycles were confirmed by histological analysis and the date of last menstruation. None of the women had used any hormonal contraceptives during the last 3 months, depot medroxyprogesterone during the last 9 months, or gonadotropin-releasing hormone (GnRH) analogues during the last 6 months. For patients with endometriosis, the disease stage was determined according to the American Society for Reproductive Medicine Classification of Endometriosis, and none of them had received any previous treatment for the disease.[26]

The samples were classified into 2 groups: group I consisted of 11 endometriotic lesions, obtained from 11 different patients with chronic pelvic pain and infertility diagnosed with endometriosis by laparoscopy and confirmed by histology. Group II (control) consisted of 11 samples of eutopic endometrium biopsies obtained with a Novak curette from 11 different healthy women without endometriosis who underwent laparoscopy for tube ligation. Among the 11 ectopic endometrium biopsy specimens, 5 were ovarian lesions at stages III (1 sample) and IV (4 samples), and 6 were peritoneal lesions at stages II (2), III (2), and IV (2).

After collection, the samples were treated with the Tissue-Tek OCT compound cryopreserver (Sakura Finetek USA Inc, Torrance, California) and immediately stored in a freezer at −80°C.

Extraction of Total RNA

The samples were washed 3 times with 1 mL of phosphate-buffered saline (PBS; 8.50 g/L NaCl, 1.11 g/L Na_2HPO_4, 2.81 g/L $Na_2HPO_4.12H_2O$, and 0.20 g/L KH_2PO_4, pH 7.0) to remove the cryopreserver. Next, total RNA was extracted from the samples (approximately 50 mg of tissue each) using TRIZOL reagent (Invitrogen Life Technologies, Paisley, United Kingdom) according to the manufacturer's instructions and stored at −80°C until processing.

Rapid SH

The rapid subtraction hybridization (RaSH) protocol described by Jiang et al [27] was applied to the RNA extracted from the endometriotic lesions of women with endometriosis and from the eutopic endometrium of women without the disease. The protocol was carried out in duplicate, with both groups serving once as testers and once as drivers.

Complementary DNA Synthesis

In all, 6 samples from the control group and 6 samples from women with endometriosis (3 peritoneal lesions and 3 ovarian lesions) were selected at random for complementary DNA (cDNA) synthesis for RaSH protocol. The 6 samples from each group were mixed, generating 2 pools containing 25 μg total of RNA each. Double-stranded cDNA was obtained using 200 U of the enzyme SuperScript II Reverse Transcriptase (Invitrogen Life Technologies) and 0.5 μg oligo(dT) $_{12\text{-}18}$ for synthesis of the first strand and 10 U *Escherichia coli* DNA ligase (Invitrogen Life Technologies) and 40U *E coli* DNA polymerase I (Invitrogen Life Technologies) for the synthesis of the second strand. The quality of cDNA synthesis was then assessed by polymerase chain reaction (PCR) with amplification of the β-actin gene.

Enzymatic Digestion and Adaptor Binding

The cDNAs were incubated with 20 U of *MboI* (Invitrogen Life Technologies) for 1 hour at 37°C, and the fragments were extracted with phenol/chloroform and precipitated with ethanol. The fragments were bound to the adaptors XDPN-12 (5′GATCTCTCGAGT3′) and XDPN-14 (5′CTGATCACTC GAGA3 ′) using 8 μL of ligase 5× buffer. Lastly, a binding reaction was initiated by the addition of 9 U of T4 ligase (Invitrogen Life Technologies) and the mixture was incubated at 14°C overnight.

Polymerase Chain Reaction and Product Purification

The samples obtained from the previous reactions were diluted in LoTE (Invitrogen Life Technologies) to a final volume of 100 μL and amplified by PCR containing 6 μL of the XDPN-18 primer (5′CTGATCACTCGAGAGATC3′), complementing the adaptors.

Tester Digestion and SH

A 10-μg aliquot of the tester sample was digested with 20 U of the restriction enzyme *Xho*I (Gibco BRL, Invitrogen, Gaithersburg, California), the fragments obtained were diluted with 30 μL of LoTE, and 100 ng of the digestion product were mixed with 5 μg of the driver sample. The hybridization reaction was carried out by adding 16.6 μL of hybridization buffer

(0.5 mol/L NaCl, 50 mmol/L Tris/HCl, 0.2% sodium dodecyl sulfate (SDS), and 40% formamide) to the mixture. After denaturation at 100°C for 5 minutes, the solution was incubated at 42°C for 48 hours. Next, 3 μL of the hybridization product were added to the solution containing 0.5 μL of the pZErO-1 plasmid (1 μg/μL) predigested with the restriction enzyme *Xho*I and the mixture was incubated at 16°C for 3 hours for tester−tester binding to the plasmid.

Bacterial Transformation and Generation of the cDNA Libraries

In total, 2 μL of the recombinant plasmid were transformed into One Shot Top 10 Electrocomp *E coli* (Invitrogen Life Technologies) by electroporation (2.5 V, 25 μFD, 200 OHMS; GenePulser, Bio-Rad Laboratories, Hercules, California).

Colony and Bioinformatics Analysis

All bacterial colonies were analyzed by PCR using the M13 forward (5′CATTTTGCTGCCGGTC3′) and M13 reverse (5′CAGGAAACAGCTATGACC3′) primers for identifying which were presented an insert. The sequences of these clones were determined with MegaBace 1000 (Amersham Biosciences, Piscataway, New Jersey) and DYEnamic ET Dye Terminator Sequencing Kit (Amersham Biosciences).

The sequences obtained were analyzed and stored in the data bank of the Laboratory of Molecular Genetics and Bioinformatics (LGMB) at *Fundação Hemocentro* (University of Sao Paulo) for later analysis with the Generic EST Annotation Pipeline (Geap) software, developed in the same laboratory, according to the following standard values: phred cutoff of 0.09, quality value of 100 pb, minmatch 10, minscore 20, and BLAST ($1e^{-30}$).

The sequences showing at least 90% identity with those deposited in the GenBank data bank of the National Institute of Health (http://www.ncbi.nlm.nih.gov/Genbank) were selected for our data bank. Once the genes referring to these sequences were identified, they were classified in terms of their function in biological processes according to the information obtained from the public access sites Gene Ontology (http://fatigo.bioinfo.cnio.es) and National Center for Biotechnology Information—NCBI (http://www.ncbi.nlm.nih.gov). To assess the differential gene expression obtained by comparative hybridization among the tissues studied, we selected 3 genes for real-time PCR validation (*HTRA1, LOXL1*, and *SPARC*) categorized into processes believed to be part of the physiopathology of endometriosis.

Validation by Real-Time PCR

Data validation by real-time PCR was applied to all the 22 samples (N = 11 for each group). Of each group, 6 of the samples were used initially in the RaSH pool and 5 were newly selected samples.

For cDNA synthesis, 1 μg of each total RNA sample was reversely transcribed using the High Capacity cDNA Reversion Transcription Kit (Applied Biosystems, Warrington, UK), according to the manufacturer's instructions. After synthesis, each sample was diluted 1:50 in diethylpyrocarbonate (DEPC) water. The expression of the 3 selected genes was quantified by real-time PCR using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). The TaqMan Gene Expression Assays system (Applied Biosystems) was used according to the manufacturer's instructions. The probes and primers for the *LOXL1* (Hs00173746_m1), *HTRA1* (Hs00170197_m1), *SPARC* (Hs00277762_m1), and *GAPDH* (Hs99999905_m1, endogenous control) genes were obtained using the Assay-on-Demand Gene Expression Products (Applied Biosystems).

The reaction conditions were 10 μL TaqMan Universal PCR Master Mix (2×; Applied Biosystems), 1 μL of TaqMan Gene Expression Assay Mix (20×; Applied Biosystems), and 9 μL of diluted cDNA at a dilution of 1:50 in a final volume of 20 μL for each reaction. The reactions were performed at 95°C for 10 minutes, at 95°C for 15 seconds, and at 60°C for 1 minute in 40 cycles.

To calibrate the assay, we used 1 sample obtained from eutopic endometrium of a healthy woman without endometriosis, which was not used in the control group. For all real-time PCR assays, we considered slope curves superior or equal to −3.40, which mean minimum sequence amplification efficiency of 96.8%. Thus, the expression of each candidate gene was calculated according to the $2^{-\Delta\Delta CT}$ formula described previously by Livak and Schmittgen for each sample, and the values were used for statistical analysis.[28] The *GAPDH* gene and calibrator sample were used as normalizers for the $2^{-\Delta\Delta CT}$ calculus.

Statistical Analysis

The gene expression data were compared by the nonparametric Wilcoxon rank sum test for 2 independent samples at the 95% confidence level using the STATDISK 9.1 software.[29]

Results

Rapid SH

After the construction of cDNA libraries, 165 sequences were obtained and 145 were selected as being of good quality according to the criteria described above. These sequences were then categorized according to their participation in biological processes. Of these 145 sequences, 126 were correlated to processes that may contribute to the establishment and development of endometriosis (Table 1).

Validation by Real-Time PCR

The gene expression data obtained by real-time PCR for the 3 genes analyzed are presented in Figure 1. The Wilcoxon rank sum test showed that the expression of *SPARC* did not differ significantly between the groups of women with and without

Table 1. Genes Differentially Expressed in Endometriotic Lesions Obtained by RaSH and Categorized According to Gene Ontology Terms

Nucleic acid metabolic process	*CRABP2*, *ATP5L*, *RARSL*, *TH1L*, *ATP5A1*, *UBE1*, *TRIM28*, *SOX17*, *ENO1*, *XRCC5*, *PCBP1*, *ID2*, *RNPS1*, *LMO4 SMARCE1 DARS PABPC1 PCBP2 FOSB IMPDH2 SFRS3*, *SP1*, *NAT14*, *TIGD1*, *HNRPA1*, *PRDM7*
Cellular metabolic process	*P4HB*, *PGK1*, *CCT8*, *PPIB*, *LDHA*, *PSAP*, *ACSL5*, *TCP1*, *SEC11L1*, *LCMT1*, *GANAB*, *UQCRQ*, *TIGD1*, *HNRPA1*, *PRDM7*, *LNPEP*
Cell differentiation	*TXNIP*, *HSPA5*, *ACTG1*, *TP53I3*, *RTN4*, *HSPB1*, *DDAH2*, *NR2F2*, *EIF4G2*, *NGFRAP1*, *TXNL1*, *TPT1*, *TTC3*, *IFITM3*, *SERPINB6*
Cell communication	*RHOC*, *CAP1*, *GNB2L1*, *GNAS*, *SPARC*, *PLP2*, *CANT1*, *FKBP1A*, *STC1*, *ECM,1 ID1*, *CALM2*, *NENF*, *TMEM101*
Cellular biosynthetic process	*ATP5L RARSL ATP5A1 PIGT EEF2 DARS EIF3S12 IMPDH2 HBB PIGG EIF1 ACTB HSD3B2*
Cell death	*CFL1 HSPA5 PIGT TP53I3 RTN4 HSPB1 CUL3*, *DDAH2*, *NGFRAP1*, *TXNL1*, *TPT1*, *HEBP2*
Cell proliferation	*ITGB1*, *CD81*, *CUL3*, *ILK*, *CAPNS1*, *CD3E*, *HTRA1*, *SAT1*, *LOXL1*, *LGI1*
Cytoskeleton biogenesis	*CAP1*, *CFL1*, *ACTG1*, *TMSB10*, *TMSB4X*, *GSN*, *STX5A*, *DYNLL1*, *SNAP23*
Cell cycle process	*CCT7 EIF4G2 FOSB PSMD13 PEBP1 CDC27*
Tissue development	*CRABP2*, *SPARC*, *ATP2A2*, *TAGLN*, *LOXL1*
Cell adhesion	*ITGB1*, *ILK*, *LMO4*, *ATP2A2*
Immune system process	*CD81*, *CD3E*, *HLA-DRA*, *HEPH*
Blood vessel development	*MMP2*, *NR2F2*, *ID1*
Steroid metabolic process	*FDX1*, *HSD3B2*
Collagen catabolic process	*MMP7*

Abbreviation: RaSH, rapid subtraction hybridization.

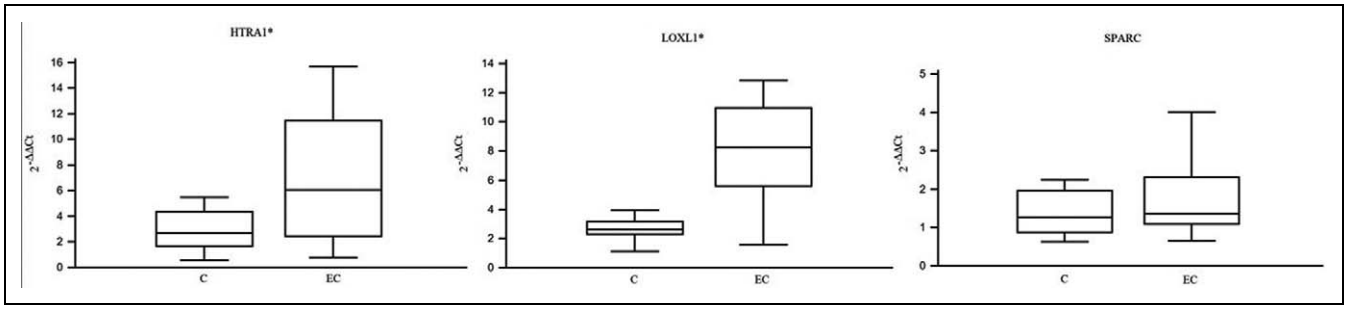

Figure 1. Gene expression quantification obtained by real-time polymerase chain reaction (PCR) for *HTRA1, LOXL1*, and *SPARC*. The vertical axis represents the mean value of gene expression based on $2^{-\Delta\Delta CT}$. On the horizontal axis: C represents samples of eutopic endometrial tissue from women without endometriosis (control group, n = 11) and EC represents samples of endometriosis lesions (affected group, n = 11). *SPARC* did not differ significantly between the groups of women with and without endometriosis. *HTRA1** and *LOXL1** had significant statistical difference between these groups at $P < .05$*.

endometriosis, whereas the expression of the *HTRA1* and *LOXL1* genes differed significantly between these groups ($P < .05$). We did not detect any association between the expression of the genes studied and the stage of endometriosis and besides there was no difference in the expression of these genes between ovarian and peritoneal lesions.

Discussion

Subtraction hybridization is one of the well known and potentially faster methods that has been used in genetics research for identifying differentially expressed genes associated with a particular disease.[30-34] Compared to other methodologies, like gene microarrays, it is stated that both have good efficacy to reveal sequences of genes with altered expression.[32]

In 2000, Jiang and colleagues described some improvements in SH method, which included significant simplification in the process of cDNA subtraction, without loss of efficiency, and reduction in the experiment's costs. The RaSH, as it was termed, results in the identification of a high proportion of differentially expressed sequences, including known genes and those not described in current DNA databases, which potentially regulate complex biological processes.[27]

In the current study, RaSH was applied generating 2 libraries from the subtraction of cDNA molecules between the groups of women with and without endometriosis. We evaluated the cDNA sets present in these libraries to discover those genes with deregulated expression in endometriotic lesions. This method allowed us to identify sequences of genes with differential expression between the 2 groups, among which we selected *HTRA1, LOXL1*, and *SPARC* for real-time PCR validation.

HTRA1 Gene Expression

The *HTRA1* gene encodes an enzyme belonging to the serine-protease family produced in large amounts by the endometrium

in the proliferative phase of the menstrual cycle. Moreover, this gene is also upregulated in the secretory phase in both endometrial glands and decidual cells, suggesting that the expression of this gene may be important for the endometrial preparation of embryo implantation.[35-37] *HTRA1* also interacts with some factors related to cell proliferation such as insulin growth factors (IGFs), which act on several tissues, stimulating cell division and differentiation.[38] Its activity is modulated by inhibitory molecules, the Insulin-Like Growth Factor Binding-Proteins (IGFBPs), which bind to IGFs and control the availability of these growth factors.[39] However, there are enzymes such as HTRA1 that may cleave these IGFBPs, thus increasing the levels of bioactive IGFs.[38,40,41] Once free to act, the IGFs may promote cell growth, an effect compatible with the findings in endometriotic lesions.[42] In addition, IGF-I may influence the ability of ectopic survival of endometrial cells by inhibiting apoptosis.[43] Considering that real-time PCR analysis revealed an increased expression of this gene in the ectopic implants, we suggest that these events may be related to the onset of endometriotic lesions. Furthermore, a previous study of our group demonstrated low expression of *IGFBP1* in endometriotic lesions compared to eutopic endometrium from the same patients.[22] The results together argue in favor of a pathway involving IGFBP1 and regulative molecules, like HTRA1, that may trigger endometriosis onset.

LOXL1 Gene Expression

The *LOXL1* gene encodes an essential amino oxidase for the biogenesis of connective tissue that catalyzes the first steps in the formation of crossed covalent bonds between collagen and elastin fibers on the extracellular matrix (ECM). This is essential for the homeostasis of various tissues, including female pelvic organs, mainly during pregnancy and delivery.[44-46] In human endometrium, LOXL1 expression has been shown to be downregulated during the window of implantation, which suggests that deregulation of this gene could also have a role at this critical time.[47] Because real-time PCR was able to detect a higher expression of the gene in the tissues from patients with endometriosis, we suggest that this alteration may increase the biogenesis of the connective tissue, facilitating the establishment of endometriotic lesions. Thus, the high expression of *LOXL1* may contribute to the maintenance of endometrial cells outside the uterine cavity by permitting them to keep bound and in contact with other tissues. In addition, considering that endometriosis has characteristics similar to those of neoplastic processes such as clonal proliferation and a tendency to metastasis and invade tissues, another interesting fact is that *LOXL1* is highly expressed in metastatic breast cancer, in agreement with our real-time PCR results of endometriotic tissues.[48] Additionally, overexpression of other *LOXL* family members (*LOXL2, LOXL3, LOXL4*) are not just implicated in invasive metastatic phenotype in breast cancer cell lines but also in the progression of several tumor types.[49-52]

In addition, Flores et al, using microarrays, identified *LOXL1* transcripts from messenger RNA (mRNA) isolated from peripheral blood lymphocytes of patients with endometriosis compared to women without the disease, suggesting that this gene is deregulated in endometriosis patients.[20] *LOXL1* have also been shown to be deregulated in rat endometriosis model as well as human endometriotic lesions when it was compared to eutopic endometrial tissue.[53-55]

SPARC Gene Expression

The *SPARC* gene codes for a glycoprotein associated with the ECM that binds to a large number of molecules stimulating changes in cell format, influencing the synthesis of ECM elements and modulating cell−matrix interactions.[56] It has also been demonstrated that *SPARC* reinforces the signaling system of Transforming Growth Factor Beta (TGF-β) a cytokine responsible for the activation of the synthesis of ECM components such as collagen.[57,58] *SPARC* also performs angiogenic activity by interaction with VEGF, and moreover studies showed that *SPARC* increases invasive potential and cellular motility in certain tumors.[59-62] Thus, upregulation of this gene would facilitate endometrial cells implantation at ectopic sites by the production of ECM molecules and may favor the invasion of these cells into adjacent tissues.

Although real-time PCR revealed that SPARC is expressed at higher levels in the group of patients with endometriosis, we detected no statistical difference in the levels of *SPARC* expression between these 2 groups. However, Meola et al had previously detected upregulation of this gene in endometriotic implants in comparison to eutopic endometrium of patients with the disease; and despite our result in the current study, we can consider that *SPARC* may participate in the establishment and survival of endometrial cells outside the uterus, developing endometriotic lesions.[22] We believe that the possible reason for the difference between these studies is due to the origin of the samples. Although the first study compared eutopic versus ectopic endometrium of the same patients, the current study sought to investigate the contribution of genes that have different expression in ectopic endometrium of endometriosis patients versus eutopic endometrium of nonaffected women (control group) to try to reveal which genes with altered expression in patients with the disease might lead them to develop endometriotic lesions. Because our work failed to detect the difference in *SPARC* expression between the groups studied, the verification of the involvement of this gene in endometriosis physiopathology deserves further investigation.

Rapid SH

As any other method used in gene expression screening experiments, like microarrays, the results obtained by RaSH must be confirmed by real-time PCR, the gold standard confirmative technique for gene expression as it can quantify gene expression accurately and reveal potential false positives.[63]

Even though we attempted to validate RaSH results for just 3 genes (whereas the method revealed an amount of 126 different sequences), we believe that the importance of our work

resides in the fact that RaSH contributed to point a panel of candidate genes that may participate in the development and maintenance of the conditions that make endometrial implants able to survive outside its original site.

Although the majority of genes were not selected for validation, 6 of them, also revealed in the current study, had been previously studied in endometriosis: *LOXL1, PSAP, SP1, MMP2, SPARC*, and *ACTB*.[20,22,24,64-69] With exception of these genes, the other 120 sequences revealed have not been studied or related to gene expression in endometriosis until now. Based on the analysis of differentially expressed sequences by women with and without endometriosis, we may state that most of the reference sequences were identified for the first time in the current study and can be better explored in future investigations, contributing to the understanding of the pathogenesis endometriosis.

Gene Expression Variability

In relation to the interpatient variability of gene expression observed in Figure 1, we suppose it occurs because endometriosis is characterized by heterogeneity because patients present variable symptoms and endometriotic lesions have wide range of size, distribution, and sites where it can develop. So this heterogenic behavior could be extended to gene expression level.

In our study, even though we tried to keep the homogeneity of characteristics shared by women in both groups (related in samples section), we have to take into account that we investigated gene expression of endometriotic lesions implanted in different locals (peritoneum and ovary) and at different stages of the disease (II-IV). Furthermore, we can consider the individual genetic variability that naturally exists among people and besides the samples were collected from Brazilian patients which represent one of the most heterogeneous populations in the world as a result of 5 centuries of interethnic crosses between people from 3 continents: the European colonizers (represented mainly by the Portuguese); the African slaves; and the autochthonous Amerindians.[70]

Conclusion

Even though we did not validate all RaSH results, we believe that this method can potentially identify transcripts differentially expressed in the tissues, which was confirmed by the significant difference in the expression of genes *HTRA1* and *LOXL1* between the normal eutopic endometrium and endometriotic lesions. The altered expression of these genes may have generated important metabolic and signaling changes in endometrial cells, possibly leading to concomitant cell proliferation and increased biogenesis of adhesive tissue, permitting the cells to continue to be viable outside the uterine cavity. We suggest that these genes may be involved in the endometriosis physiopathology, facilitating or determining pathways that are responsible for the establishment, maintenance, and development of the disease.

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Declaration of Conflicting Interests

The author(s) declared no conflicts of interest with respect to the authorship and/or publication of this article.

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